THE FLAVIN-DEPENDENT N-HYDROXYLATING ORNITHINE MONOOXYGENASE FROM
ASPERRILLUS FUMIGATUS AND THE HEME-DEPENDENT O₂-GENERATING CHLORITE
DISMUTASE FROM DECHLOROMONAS AROMATICA: STUDIES OF OXYGEN
CONSUMPTION AND PRODUCTION BY TWO MICROBIAL ENZYMES

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THE FLAVIN-DEPENDENT N-HYDROXYLATING ORNITHINE MONOOXYGENASE FROM ASPERGILLUS FUMIGATUS AND THE HEME-DEPENDENT O₂-GENERATING CHLORITE DISMUTASE FROM DECHLOROMONAS AROMATICA: STUDIES OF OXYGEN CONSUMPTION AND PRODUCTION BY TWO MICROBIAL ENZYMES

Abstract

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The appearance of oxygen in the earth’s atmosphere approximately 2.3 billion years ago led to considerable changes in the environment. The species that inhabited earth, and wished to remain, had to adapt to an oxygen-rich atmosphere. At the biochemical level, this forced the production of enzymes and cofactors that were able to react with and bind oxygen, while limiting and eliminating the toxic side products of its reduction. Modern species contain several oxygen-reactive biomolecules that are critical for survival; the following describes an in-depth examination of two enzyme systems with evolved highly sophisticated mechanisms for the use and production of molecular oxygen, respectively. The first part of the thesis describes mechanistic studies of a member of a novel subclass of N-hydroxylating flavin-dependent monooxygenases. The enzyme, L-ornithine monooxygenase (OMO), activates oxygen to a reactive flavin hydroperoxide intermediate to hydroxylate the primary amine of L-ornithine in a critical step for the biosynthesis of iron-chelating siderophores. The second half describes the CDE (Chlorite dismutase, DyP-type peroxidase, EfeB) protein-family. This microbial protein family contains the enzyme chlorite dismutase (Cld), a recently discovered enzyme from perchlorate (ClO₄⁻) respiring bacteria. These organisms have achieved the ability to respire oxochlorates and make use of the man-made molecules for their survival. Cld is a heme-dependent enzyme that catalyzes the conversion of
chlorite (ClO$_2$), the toxic end product of perchlorate respiration, to molecular oxygen (O$_2$) and chloride (Cl$^-$). Studies to understand Cld’s detailed chemical mechanism and the protein residues essential for its function are described. The large CDE protein-family, to which Cld belongs, is described in detail with structural and sequence based analyses. Work shows that several annotated Cld genes are found in non-perchlorate respiring bacteria and likely have as yet unidentified non-dismutase functions. In the last chapter, biochemical and microbiological data are presented with an effort towards determining the biological function of a Cld homolog in the pathogenic species *Staphyloccoccus aureus*. The biological roles of both the L-ornithine monooxygenase and chlorite dismutase center around redox biochemistry, and each story will be described within the context of microbial redox cofactor acquisition, synthesis, storage, and utilization. The importance of each is described in regard to virulence in pathogenic species and the potential development of new antimicrobial targets.
To Cort, my Beloved.
## CONTENTS

Figures.................................................................................................................. viii

Tables.................................................................................................................... xxvi

Preface................................................................................................................... xxviii

Acknowledgments................................................................................................. xxxiii

Abbreviations....................................................................................................... xxxv

### Chapter 1: Siderophore Biosynthesis Requires a Flavin-Dependent Monooxygenase; a Potentially Clean Antimicrobial Target

1.1 Iron trafficking as an antimicrobial target...................................................... 1
   1.1.2 Existing antibiotics and current needs...................................................... 2
   1.1.3 Fe trafficking: major aspects................................................................. 4
   1.1.4 Possible targets.................................................................................... 7
   1.1.4.1 Siderophore biosynthesis................................................................. 7
   1.1.4.2 Other enzymatic targets................................................................. 12
   1.1.4.3 Non-enzymatic and non-siderophore targets................................. 13
   1.1.5 Outlook ............................................................................................ 14

1.2 Siderophore-associate monooxygenases....................................................... 14
   1.2.1 Significance of SMO’s for virulence in pathogenic species.................. 15

1.3 Flavin chemistry and the monooxygenase reaction...................................... 16
   1.3.1 Reductive half-reaction.................................................................... 17
   1.3.2 Oxidative half-reaction.................................................................... 18

1.3.3 Mechanistic Strategies of flavin-dependent monooxygenases................. 20
   1.3.3.1 “Cautious” flavin-dependent monooxygenase.............................. 21
   1.3.3.1.1 Reactions catalyzed and significance.................................... 21
   1.3.3.1.2 Kinetic mechanism............................................................ 21
   1.3.3.1.3 Structural features.......................................................... 23
   1.3.3.2 “Bold” flavin-dependent monooxygenases................................. 23
   1.3.3.2.1 Reactions catalyzed and significance................................ 23
   1.3.3.2.2 Kinetic mechanism............................................................ 25
   1.3.3.2.3 Structural features.......................................................... 26

1.4 Contributions from the thesis.................................................................... 27

1.5 References.................................................................................................... 27

### Chapter 2: Steady-state Kinetic Studies of a Representative Siderophore Associated Flavin Monooxygenase

2.1 Introduction................................................................................................. 35

2.2 Experimental Procedure............................................................................ 38
   2.2.1 Standard Methods, Chemicals, and Equipment................................. 38
   2.2.2 Cloning and Engineering of sidA...................................................... 39
   2.2.3. Overexpression and Purification of OMO...................................... 40
   2.2.4 Analytical Gel Filtration................................................................. 40
Chapter 3: Comprehensive Spectroscopic and Transient Kinetic Studies of a Representative Siderophore Associated Flavin Monoxygenase

3.1 Introduction
3.2 Experimental Procedures
3.2.1 Rapid Reaction Studies
3.2.2 Reactions with NADPH
3.2.3 Reactions of Reduced OMO with O$_2$
3.3 Results and Analysis
3.3.1 Rapid Kinetic Studies of OMO with NADPH
3.3.2 Rapid Kinetic Studies of the Reaction of Reduced OMO with O$_2$ in the Absence of L-Orn
3.3.3 Rapid Kinetic Studies of the Reaction of Reduced OMO with O$_2$ in the Presence of L-Orn
3.4 Discussion
3.5 Summary
3.6 Acknowledgements
3.7 References

Chapter 4: Dioxygen-Generating Chlorite Dismutase and the CDE Protein Superfamily

4.1 Introduction
4.2 Heme Peroxidases
4.2.1 Peroxidase Classifications
4.2.2 Defining Features of Plant/Fungal/Bacterial Peroxidases
4.3 Sequences, Structures, & Genetics of Superfamily Members
4.3.1 Taxonomic Origins and Gene Organization
4.3.1.1 The C-Family Proteins
4.3.1.2 The D- and E-Family Proteins
4.3.2 Sequence and Structural Relationships
4.3.2.1 Domain and Tertiary Structures
4.3.2.2 Heme Binding Domains and Active Sites – C-Family
4.3.2.3 Heme Binding Domains and Active Sites – D- and E-Families
4.4 Biochemistry of Superfamily Members
4.4.1 C-Family: O$_2$-Generating Chlorite Dismutases
4.4.1.1 Stoichiometry, Specificity, and Stability
4.4.1.2 Heme Electronic Structure
4.4.1.3 Reaction Mechanism
4.4.2 D-family: Dye Decoloring Peroxidases
4.4.3 E-Family: Tat-Transport and Involvement in Fe Metabolism
4.5 Contributions from the Thesis
4.6 References
Appendix B: Understanding How the Distal Environment Directs Reactivity in Chlorite Dismutase: Spectroscopy and Reactivity of Arg183 Mutants

B.1 Introduction ................................................................................. 288
B.2 Experimental Procedures .......................................................... 291
  B.2.1 Generation of DaCld(R183Q), DaCld(R183K), and DaCld(R183A) .... 291
  B.2.2 Protein purification .............................................................. 291
  B.2.3 Steady state kinetics of chlorite decomposition ......................... 291
  B.2.4 Catalase activity ................................................................. 292
  B.2.5 Peroxidase activity .............................................................. 292
  B.2.6 UV/visible pH titrations ....................................................... 293
  B.2.7 Resonance Raman spectroscopy ............................................ 293
  B.2.8 CO complexes of WT and mutant DaClids ............................. 294
  B.2.9 Equilibrium binding of ligands .............................................. 294
  B.2.10 Redox potentials ............................................................... 295
B.3 Results and Analysis ................................................................. 296
  B.3.1 Reactivity of DaCld R183 Mutants with Chlorite Ion ................. 296
  B.3.2 Catalase and Peroxidase Activity of DaCld R183 Mutants .......... 297
  B.3.3 DaCld(R183A) .................................................................. 300
  B.3.4 DaCld(R183Q) .................................................................. 301
  B.3.5 DaCld(R183K) .................................................................. 301
  B.3.6 Effects of Mutations on Heme Conformation .......................... 304
  B.3.7 Axial Heme Coordination and Spin State of Ferrous DaCld Mutants ... 305
  B.3.8 Proximal Heme Environment as a Function of Arg183 Mutation ...... 308
  B.3.9 Distal Heme Environment Probed with Carbonyl Complexes of WT and Mutant DaClids .................................................. 309
  B.3.10 Affinity of Ferric Mutant Cllds for Exogenous Ligands ......... 318
  B.3.11 Hydrogen C330yanide ....................................................... 319
  B.3.12 Imidazole ........................................................................ 320
  B.3.13 Anions ........................................................................... 322
  B.3.14 Reduction Potential .......................................................... 322
B.4 Discussion ................................................................................... 325
B.5 Summary and Conclusions .......................................................... 333
B.6 Acknowledgements ........................................................................ 334
B.7 References .................................................................................. 334
FIGURES

**Figure 1.1** a. Mycobacterial siderophores. The salicylate-derived aryl headgroup activated by MbtA is shown in light blue throughout. b. Reaction catalyzed by MbtA. ArCP is the aryl carrier protein to which MbtA transfers its adenylated substrate. c. Sal-AMP is the native product of MbtA. d. Various analogs to Sal-AMP were designed and tested as enzyme inhibitors and antitubercular agents.

**Figure 1.2** a. Fungal siderophores (*Aspergillus fumigatus*). The hydroxyl groups added by $N^5$-ornithine monooxygenase (OMO) are shown in light blue. b. Reaction catalyzed by $N^5$-ornithine monooxygenase, a flavin adenine dinucleotide (FAD) and NADPH dependent enzyme.

**Figure 1.3** Structure of flavin derivatives and flavin-dependent enzyme reactions catalyzed in nature.

**Figure 1.4** Reductive half-reaction associated with FAD-dependent monooxygenase. Showing the isoalloxazine ring of FAD and the nicotinamide ring of NADPH with the two electron oxidized and reduced species.

**Figure 1.5** Reaction of reduced FADH$^-$ with molecular oxygen through the radical cage intermediate, and formation of the C4a-(hydro)-peroxide intermediate. The two fates of the C4a intermediate are shown; oxidation to release HO$_2^-$ or monooxygenation with substrate. Adapted from Mattevi.

**Figure 1.6** The C4a-(hydro)-peroxide protonation states. The intermediate displays umpolung or polarity inversion and can act as a nucleophile, monooxygenase, or electrophile, Baeyer-Villiger monooxygenase.

**Figure 1.7** Cleland Diagram for “Cautious” FAD-dependent monooxygenase.

**Figure 1.8** Active site of PHBH showing the “in” and “out” conformations of the FAD ring. The FAD moieties are shown in light blue (out) and yellow (in) with the substrate para-hydroxybenzoate (pOHB) in salmon. Active site residues Tyr 201, Tyr 385 and His 72 are shown in orange. Adapted from Palfey et al.

**Figure 1.9** Cleland Diagram of “Bold” FAD-dependent monooxygenases.

**Figure 1.10** Active Site Structure of Flavin Monooxygenase from *Methylophaga* sp. Strain sK1. The isoalloxazine moiety is shown in yellow with the C4, C4a and N5 atoms labeled. NADP$^+$ is shown in light blue within H-bonding distance of N5 on the flavin. Active site residues within H-bonding distance are shown from the FAD-binding domain (orange) and NADP-binding domain (green). From Alfieri et al.

**Figure 2.1** Fusarinine biosynthesis, with initiating step catalyzed by OMO.
Figure 2.2 Protein purification. SDS gel image stained with Coomassie blue. Left to right: molecular weight marker, lysate, purified OMO. The bands on the marker correspond to 170, 130, 95, 72, 56, 43, 34, 26, and 17 kDa from top to bottom........................................44

Figure 2.3 Determination of the Molar Absorptivity of OMO. A spectrum of the purified protein was recorded (---) and showed an absorbance peak at approximately 450 nm. The so-called oxidized enzyme was denatured to remove the FAD cofactor with the addition 0.2% SDS from a 10% stock and spectra were recorded until no further changes were observed (~30 minutes). The sample was centrifuged in a microfuge filter (MWCO = 10,000, Amicon) to near dryness, and the spectrum of the filtrate (- - - -) recorded. ...............................................................45

Figure 2.4 Equilibrium Binding of NADP\(^+\). A, spectral Changes with Addition of NADP\(^+\). An oxidized sample of OMO (28 μM) was titrated with NADP\(^+\) (0 – 90 μM) in a pH 8 solution of 50 mM Tris-H\(_2\)SO\(_4\) at 25 °C and spectra were recorded. Spectra are corrected for dilution and several were removed for clarity. B, data recorded in A with increasing concentrations of NADP\(^+\) were subtracted from the initial spectra of free oxidized OMO to generate the difference spectra. The largest changes were observed at 390 and 490 nm. C, changes in absorbance at 390 nm were converted to the concentration of OMO bound to NADP\(^+\) using the value of 2.632 M\(^{-1}\)cm\(^{-1}\) for Δε390 nm. The concentration of bound enzyme was plotted versus the concentration of NADP\(^+\) and the resulting pattern was fit to the isotherm. The fit yielded \(K_d = 4.58 \pm 0.34\) μM......46

Figure 2.5 Effect of halides. Dose-response curves for bromide (●) and chloride (■) were obtained by plotting the enzyme fractional activity (\(v/v_0\)), where \(v_0\) is the initial velocity in the presence of inhibitor and \(v_0\) in the absence, as a function of the concentration of compound plotted on a log scale. The IC\(_{50}\) values for Br\(^-\) and Cl\(^-\) are 59 and 108 mM. The data were fit to a sigmoidal function of the form \(y = y_{min} + (y_{max} - y_{min})/(1+((x/IC_{50}))^H)\), where \(H\) = Hill slope, \(y\) is the fractional activity of the enzyme in the presence of halide (or inhibitor), and \(x\) is the log of the concentration of halide used.......................................47

Figure 2.6 Kinetic parameters with variable NADPH (0, 6, 9, 17, 31, 92 and 154 μM) and fixed variable O\(_2\) (q195 μM, p97 μM, u60 μM, n30 μM l20 μM) in saturating L-ornithine (13 mM) solutions of 50 mM Tris-H\(_2\)SO\(_4\)pH 8.0, 37 °C........................................50

Figure 2.7 Kinetic parameters with variable NADPH (0, 6, 9, 15, 41, 90 and 150 μM) and fixed variable O\(_2\) (▼202 μM, ▲83 μM, ◆43 μM, ■22 μM ●13 μM) in sub-saturating L-ornithine (0.6 mM) solutions of 50 mM Tris-H\(_2\)SO\(_4\)pH 8.0, 37 °C........................................51

Figure 2.8 Kinetic parameters with variable L-ornithine (0.3, 0.6, 1.0, 3.0, 5.0, and 13 mM) and fixed variable NADPH (●150 μM, ▼90 μM, ▲30 μM, ◆15 μM ■9 μM ●6 μM) in saturating O\(_2\) (200 μM) solutions of 50 mM Tris-H\(_2\)SO\(_4\)pH 8.0, 37 °C........................................52

Figure 2.9 Kinetic parameters with variable L-ornithine (0.15, 0.24, 0.6, 1.0, 3.0, 5.2, and 13 mM) and fixed variable O\(_2\) (▼201 μM, ▲87 μM, ◆38 μM, ■20 μM ●13 μM) in saturating NADPH (150 μM) solutions of 50 mM Tris-H\(_2\)SO\(_4\)pH 8.0, 37 °C........................................53

Figure 2.10 Inhibition by NADP\(^+\). A, Reciprocal plot of NADP\(^+\) product inhibition versus NADPH. Inverse rates of NADPH oxidation by OMO at varying NADPH (~0, 25, 30, 60, and 120 μM) and fixed amounts of product inhibitor NADP\(^+\) (~■0 μM, ▲150 μM, ◆300 μM, ▼600 μM, and ◆900 μM) in L-ornithine (5 mM) and air-saturated solutions of 50 mM Tris-H\(_2\)SO\(_4\), 10 μM FAD, pH 8.0, 37 °C. Inset shows slope (\(K_i/k_{cat}\)) as a function of inhibitor concentration. B, Reciprocal plot of NADP\(^+\) product inhibition versus L-ornithine. Inverse rates of NADPH oxidation by OMO at varying L-ornithine (0, 0.11, 0.22, 0.55, 1.83, 4.56, 9.13, 13.04, and 18.62 mM) and fixed amounts of product inhibitor NADP\(^+\) (~■0 μM, ▲150 μM, ◆300 μM, and ▼600 μM) in NADPH (70 μM) and air-saturated solutions of 50 mM Tris-H\(_2\)SO\(_4\), pH 8.0, 37 °C. Inset shows intercept (1/\(k_{cat}\)) as a function of inhibitor concentration. Data points represent the inverse of measured rates while the
linear fits were generated from the kinetic parameters estimated by non-linear fitting (KaleidaGraph).

**Figure 2.11** Inhibition by hydroxy-L-ornithine. A. Reciprocal plot of hydroxy-L-ornithine product inhibition versus L-ornithine. Inverse rates of NADPH oxidation by OMO at varying L-ornithine (0, 0.15, 0.30, 0.54, 1.45, 5.0, and 15.0 mM) and fixed amounts of product inhibitor hydroxy-L-ornithine (• 0 μM, ○ 200 μM, ■ 800 μM, □ 2000 μM, and ◆ 3200 μM) in NADPH (150 μM) and air-saturated solutions of 50 mM Tris, 10 μM FAD, pH 8.0, 37 °C. B. Reciprocal plot of hydroxy-L-ornithine product inhibition versus NADPH. Inverse rates of NADPH oxidation by OMO at varying NADPH (0, 20, 25, 60, 120, and 150 μM) and fixed amounts of product inhibitor hydroxy-L-ornithine (• 0 μM, ○ 150 μM, ■ 600 μM, □ 900 μM, ▼ 1500 μM, and △ 3000 μM) in sub-saturated L-ornithine (1.5 mM) and air-saturated solutions of 50 mM Tris, 10 μM FAD, pH 8.0, 37 °C. Data points represent the inverse of measured rates while the linear fits were generated from the kinetic parameters estimated by non-linear fitting (KaleidaGraph).

**Figure 2.12** Cleland diagram for kinetic mechanism of OMO.

**Figure 3.1** Reduction of OMO by NADPH. The reaction mixture contained 10 μM enzyme at 25 °C. Absorbance was measured in photomultiplier mode at 450 nm and traces were fit to the sum of two exponentials. Calculated rates were 0.58, 0.67, and 0.60 s⁻¹ at 10 (long dashed line), 100 (solid line), and 200 (short dashed line) μM NADPH, where the trace at 100 μM NADPH contained 5 mM L-ornithine.

**Figure 3.2** A, formation of the C4a-hydroperoxyflavin-NADP⁺ complex. Spectra are shown at 0.004, 0.071, 0.139, 0.307, 0.577, 0.960, and 2.355 s after mixing the NADPH-reduced enzyme with air-saturated buffer (final [O₂] = 0.130 mM) in the stopped-flow instrument. Inset, oxygen dependence of the rate of C4a-hydroperoxyflavin formation in the absence of L-Orn. C4a-hydroperoxide formation was monitored at 370 nm under pseudo-first order conditions with varying [O₂]. Values for kₜobs determined from fitting single exponentials to the data are plotted versus [O₂]. The second order rate constant determined from this plot was 2.5 x 10⁻⁵ M⁻¹s⁻¹. Reaction mixtures contained 10 μM reduced enzyme-NADP⁺ at 25 °C. B, conversion of the C4a-hydroperoxyflavin-NADP⁺ complex to oxidized FAD. Spectra at 2.63, 11.25, 18.6, 29.4, 60.3, and 142.5 s after mixing from the experiment in part A are shown.

**Figure 3.3** Reoxidation of dithionite-reduced OMO. The oxidized enzyme (●) was anaerobically reduced with approximately 2 equivalents of dithionite (▲) at 25 °C in 100 mM Tris-H₂SO₄ pH 7.4 and then exposed to air (●). A spectrum of each species was recorded immediately and repeatedly to be certain no changes occurred.

**Figure 3.4** A, formation of the C4a-hydroperoxyflavin-NADP⁺ complex in the presence of L-Orn. Spectra are shown at 0.004, 0.019, 0.034, 0.049, 0.071, 0.079, 0.081, and 0.026 s after mixing the NADPH-reduced enzyme with air-saturated buffer containing 5 mM L-Orn (final [O₂] = 0.130 mM). The reaction mixture contained ~10 μM reduced enzyme-NADP⁺ complex at 25 °C. Inset, oxygen dependence of the rate of C4a-hydroperoxyflavin formation in the presence of saturating L-Orn. The conversion of the reduced enzyme-NADP⁺ complex to the C4a-hydroperoxyflavin was monitored at 370 nm in the presence of 5mM L-Orn and varying (pseudo-first order) [O₂]. Values for kₜobs were determined from fits of single exponential curves to the data and plotted versus [O₂]. The second order rate constant determined from this plot was 1.3 x 10⁵ M⁻¹s⁻¹. B, kinetic traces illustrating the O₂ dependence of the reaction of reduced enzyme-NADP⁺ complex with O₂ in the presence of L-Orn. Absorbance traces at 370 nm show the reduced enzyme-NADP⁺ complex reacting with varying [O₂] to form the C4a-hydroperoxyflavin. Final oxygen concentrations were 0.06, 0.13, 0.3, and 0.6 mM. The kinetic trace at 450 nm illustrates the formation of the oxidized FAD that occurs as the C4a-hydroperoxyflavin disappears, a reaction that shows no dependence on [O₂]. C, dependence of the rate of C4a-hydroperoxide formation on [L-Orn] at fixed oxygen. The conversion of reduced enzyme-NADP⁺ complex to the C4a-hydroperoxyflavin was monitored at 370 nm at various (pseudo-first order) [L-Orn] at fixed/saturating O₂. Rate constants were
determined from single exponential fits to the first portion of the curves. The apparent $K_d$ and maximal $k_{obs}$ determined from this plot were 680 μM and 82 s$^{-1}$. Each reaction mixture contained 10 μM reduced enzyme-NADP$^+$ complex with 0.3mM O$_2$ at 25 °C.

**Figure 3.5** A, conversion of the C4a-hydroperoxyflavin-NADP$^+$ complex to oxidized OMO in the presence of L-Orn. 10 μM reduced enzyme-NADP$^+$ complex containing 5 mM L-Orn was mixed with air-saturated buffer (final [O$_2$] = 0.3mM at 25 °C). The C4a-hydroperoxyflavin species ($A_{max}$ = 370 nm) formed and subsequently converted to the oxidized flavin. Only the spectra showing the latter conversion are shown (recorded 0.04, 0.30, 0.50, 1.10, 2.00, and 7.00 s after mixing). B, dependence of the conversion of the C4a-hydroperoxide to oxidized FAD on L-Orn at fixed/saturating O$_2$. The conversion of the C4a-hydroperoxyflavin to the oxidized FAD shown in A was monitored at 450 nm at various (pseudo-first order) L-Orn concentrations. Values for $k_{obs}$ were determined from single exponential fits to the curves. The apparent $K_d$ and maximal $k_{obs}$ determined from this plot were 2.2 mM and 1.8 s$^{-1}$.

**Figure 3.6** A, conversion of a pre-formed C4a-hydroperoxyflavin-NADP$^+$ complex to oxidized OMO in the presence of L-Orn. 10 μM reduced enzyme-NADP$^+$ complex in the absence of L-Orn was mixed with 5 mM L-Orn in the same buffer. Spectra were shown recorded 0.161, 0.352, 0.487, 0.622, 1.050, and 7.125 s after the second mixing. B, kinetic traces showing the reaction of C4a-hydroperoxide to oxidized FAD at 450 nm. Reactions were monitored at 450 nm. Final [L-Orn] = 0.0625, 0.25, 0.5 1.25, 2.5, 5.0, and 25.0 mM. C, Dependence of the conversion of the C4a-hydroperoxide to oxidized FAD on L-Orn at fixed/saturating O$_2$. The conversion of the C4a-hydroperoxyflavin to the oxidized FAD shown in A was monitored at 450 nm at various (pseudo first order) L-Orn concentrations. Values for $k_{obs}$ were determined from single exponential fits to the curves. The apparent $K_d$ and maximal $k_{obs}$ determined from this plot were 2.3 mM and 2.5 s$^{-1}$.

**Figure 3.7** A, kinetic traces illustrating the reaction of a pre-formed C4a-hydroperoxide with variable concentrations of L-Orn. The C4a-hydroperoxide was generated by mixing 10 μM reduced enzyme-NADP$^+$ complex (in the absence of L-Orn) with air-saturated buffer (final [O$_2$] = 0.3 mM, 25 °C) and aged 5 s. It was then mixed with various L-Orn concentrations in the same buffer. The data measured at 393 nm are shown, illustrating 3 consecutive exponential processes. Final [L-Orn] for the traces shown is 1.25, 2.5, 5.0, and 25.0 mM. B, singular value decomposition of the complete set of data shown in Fig. 5A, using the rate constants fit to the three kinetic phases highlighted in A. Three constituent spectra were determined, resembling previously measured spectra for C4a-hydroperoxide (dotted line), C4a-hydroxide (solid line), and oxidized FAD (dashed line).

**Figure 3.8** Kinetic mechanism for OMO.

**Figure 4.1** Conventional classifications of heme peroxidases.

**Figure 4.2** (Top) Crystal structure of yeast cytochrome c peroxidase at 1.7 Å resolution (PDB ID: 2CYP), illustrating the primarily helical nature of the protein and the heme (in pink) binding fold. (Bottom) Active sites of representative class I, II, and III plant peroxidases. HRP (left; PDB code 1hch), LiP (middle; PDB code 11ip), and CcP (right; PDB code 1zby) are shown. The heme molecule and side chains are colored by atom, with carbons (yellow), nitrogens (dark blue), oxygens (red), and iron (orange). Adapted in part from Zubieta et al.

**Figure 4.3** The Poulos-Kraut mechanism for intermediate formation (left) and the classic 3-step peroxidase catalytic cycle (right). Fe is ligated by protoporphrin IX. The picture on the right depicts the ferric active site poised for proton removal by the distal histidine base. The same residue then acts as an acid to transfer the proton to the other peroxo oxygen, generating the
Figure 4.4 Phylogenetic tree illustrating relationships between taxonomically diverse Cld, DyP, and EfeB-family proteins. The sequences indicated by the bracket on the left are from non-perchlorate respirers, and the Cld proteins are small (<200 amino acids). The sequences from the bracket on the right are full-length Clds from perchlorate respirers and/or organisms that can degrade chlorite. Color shading indicates family membership. The sequences in lighter blue appear to be from DyP subfamily C, and in the dark blue are likely Dyp Ds, but their assignment into subgroups is not certain. The darker orange highlights EfeB proteins from DyP subfamily C, and in the dark blue are likely Dyp Ds, but their assignment into subgroups is not certain. The darker orange highlights EfeB proteins from efeUOB or efeOUB operons. The light orange indicates YfeX-like proteins, sometimes called DyP subfamily B proteins but apparently more closely related to the EfeBs. Figure generated using Phylip and Tree of Life softwares. Bootstrap values exceed 80%. Species and accession numbers clockwise, beginning with the left most bracket: (C-family) Proteobacteria (unless specified otherwise): *Nitrobacter winogradskyi*, YP_319047.1; *Pseudomonas aeruginosa*, ACL31207.1; *Cupriavidus metallidurans* CH34, ABF13199.1; *Klebsiella pneumoniae* ATCC 13884, ZP_06016699.1; *Klebsiella pneumoniae* MHG 78578, ABR80622.1; *C. Nitrospira defluvii* (Nitrospira) ACE75544.1; *Idoneella* dechlororant, CAC14884.1; *Dechloromonas agitata*, AAM92878.1; *Pseudomonas chloritidismutans*, ACA21503.1; *Dechloromonas aromatica*, YP_285781.1; *Azospira oryzae*, pdb|2VXH_A. Actinobacteria: *Thermodyptes whipplei*, NP_789669.1; *Kineococcus radiotolerans*, YP_001361337.1; *Mycoceperium tuberculosis*, NP_217192.1; *Mycoceperium smegmatis*, YP_887113.1. Halobacteria: *Aeropyrum pernix*, NP_147071.2; *Thermoplasma acidophilum*, NP_393983.1; *Sulfobolus solfataricus*, ACX92972.1; *Metallophaera sedula*, ABP94989.1; *Halobacterium* sp. NRC1, NP_280706.1; *Natronomonas pharaonis*, YP_326782.1; *Halocarcula marismortui*, YP_137518.2. Deinococcus-Thermus: *Thermus thermophilus* HB8, [1VDH_A. Firmicutes: *Listeria monocytogenes*, ZP_05290848.1; *Exiguobacterium sibiricum*, YP_001812720.1; *Staphylococcus aureus*, NP_645359.1; *Bacillus antracis*, ZP_05191341.1; *Geobacillus stearothermophilus*, pdb|1TOT. (D-family) Cyanobacteria: *Cyanotheces* sp. PCC 7822, ZP_03158031.1; *Anabaena variabilis* ATCC 29413, YP_324690.1. Planctomycetes: *Rhodopirellula baltica*, YP_03158031.1; *Rhodopirellula baltica* hutchinsonii OR74A, XP_959154.2; *YP_001521418; Geobacillus stearothermophilus*, pdb|1T0T. (E-family) Cyanobacteria: *Cyanotheces* sp. PCC 7822, ZP_03158031.1; *Anabaena variabilis* ATCC 29413, YP_324690.1. Planctomycetes: *Rhodopirellula baltica* SH 1, 865998.1; *Acaryochloris marina* MBIC11017 (Cyanobacteria), YP_001521418; *Nostoc punctiforme* PCC 73102, YP_001869150.1. Basidiomycota: *Postia placenta* Mad-698-R.,XP_002474852.1; *Laccaria bicolor* S238N-H82, XP_001876926.1; *Coprinus cinereus* okayama, XP_001840251.2; *Thanatephorus cucumeris*, BAA77283.1; *Termotomycyes albuminosus* (TAP peroxidase), IAM21606.1. Ascomycota: *Aspergillus fumigatus* At293, XP_748822.2; *Neosartorya fischeri* NRRL 181, XP_001261639.1; *Neurospora crassa* OR74A, XP_959154.2; *Sordaria macrospora*, CB56381.1. Bacteroidetes: *Cytophaga hutchinsonii*, YP_68853.1. Acidobacteria: *Acidobacterium* sp. MP5ACTX9, ZP_07065051.1; *Chryseobacterium gleum* (Bacteroidetes) ZP_07087281.1. Proteobacteria: *Enhydrobacter aerosaccus* SK60, ZP_05620844.1; *Psychrobacter* sp. PRWf-1, YP_001280117.1. Firmicutes: *Ochrobacterium anthropi* ATCC 4188, YP_001373218.1. Deinococcus-Thermus: *Deinococcus radiodurans* R1, NP_285469.1. Actinobacteria: *Amycolatopsis mediterranei* U32, YP_003767800.1; *Mycoceperium smegmatis* str. MC2 155, YP_888002.1. Acidobacteria: *Candidatus Koribacter versatilis* YP_590455.1; *Streptomyces avermitilis*, NP_824776.1; *Frankia* sp. Eu11c, ZP_06237655.1. Acidobacteria: *Solfibacter usitatus*, YP_826301.1. Proteobacteria: *Myxococcus xanthus* DK 1622, YP_629952.1; *Sorangium cellulosum*, NP_001613012.1. (YfeX-like proteins) Proteobacteria: YfeX from *Klebsiella pneumoniae*, YP_001336414.1; YfeX from *Escherichia coli* str. K-12, AAC75484.2; DyPB from *Rhodococcus jostii* Rha1 (Actinobacteria) pdb|3QNR_C. (E-family) Firmicutes: *Bacillus subtilis*, BAI87494.1; *Paenibacillus*, YP_003009762.1; *Paenibacillus curdianlyticus* YK9, ZP_07387797.1. Actinobacteria: *Thermobispora bispora*, YP_003652521.1; *Micromonaspora* sp., ZP_04607718.1; *Rhodococcus jostii* RHA1, YP_705709.1. Planctomycetes: *Gordonia bronchialis* DSM 43247YP_003272734.1. Actinobacteria: *Streptomyces hygroscopicus*, ZP_05517536.1; *Catenulispora acidiphila* DSM 44928, YP_003112540.1. Proteobacteria: *Neisseria polysaccharea*, ZP_06864806.1. Firmicutes: *Ochrobacterium anthropi* ATCC 49188, YP_001372391.1. Proteobacteria: *Yersinia frederiksenii*, ZP_04632150.1; *Pantoea* sp. aB, ZP_07378163.1; *Enterobacter* sp. 638, YP_001176277.1; *EfeB from Klebsiella pneumoniae*, YP_001347712.1; *Citrobacter yougare*, ZP_06353463.1;
**Figure 4.5** Monomer of the chlorite dismutase from *Dechloromonas aromatica* (left). Proximal residues. Superimposition of monomers from the chlorite dismutase, EfeB, and DyP families (right) illustrating the common elements of their core folds. Regions of the structures with low conservation are shown in white cartoon. Regions of structural similarity are shown in cartoon and colored according to their carbon atoms by the following scheme: 1T0T (brown), 3DTZ (cyan), 1VDH (yellow), 2WX7 (light blue), 2H1Z (salmon), and 2D3Q (orange) onto 3Q08 (green). (See Table 5.1.) The heme of 3Q08 (*DaCld*) is drawn in grey sticks and the Fe center represented as an orange sphere. This figure was adapted in part from Goblirsch et al. and generated using PyMOL.

**Figure 4.6** Some of the reported oligomerization states and monomer interfaces of CDE proteins. Individual monomers are rendered as different colored cartoons, and hemes are shown as lines. TOP ROW: (Left) Pentameric structure of *D. aromatica* Cld, viewed from the C-termini containing face. The Ca(II) ions at the monomer interfaces are shown as green spheres. (Right) Close up view of the residues coordinating calcium at the interfaces. BOTTOM ROW: comparison of monomer interfaces for 3 CDE proteins viewed from the side. (Left) Cyan and green *D. aromatica* Cld monomers align in the same orientation (shown N-termini toward the top and C-termini toward the bottom). Note that the heme-containing domains are adjacent, and that the interface consists of α-helix/b-sheet interactions on adjacent subunits. (Center) The *N. winogradskyi* Cld has its monomers likewise aligned with the N-termini and C-termini on the same side of the molecule, respectively, but the composition of the interface is different. (Right) The *E. coli* EfeB dimer is shown with the C-terminus of the blue monomer and the N-terminus of the green monomer at the top of the figure. The hemes occupy opposite corners of the molecule. Adapted from Goblirsch et al. and generated using PyMOL.

**Figure 4.7** Active site of nitrite-bound *D. aromatica* Cld. The proximal His-Glu pair is shown in green, the hydrophobic Thr/Leu/Phe triad in salmon, the Trp/His network in yellow, and the distal Arg in orange. The figure was adapted in part from Goblirsch et al. and generated using PyMOL.

**Figure 4.8** Alignment of annotated chlorite dismutases from Proteobacteria. Cartoon diagram representations of protein monomers (carbon light grey) are shown, with side-chains of active site residues shown as sticks. Residues lining the expected heme pocket that fall within strictly conserved secondary structure elements are colored by atom (carbon magenta). Note that the same residues were also identified from primary sequence alignments with Proteobacterial Clds. (Left) *G. stearothermophilus* (Firmicutes); (Center) *T. thermophilus* (Deinococcus-Thermus). (Right) *T. acidophilum* (Euryarchaeota). This figure was generated using PyMOL. Adapted from Goblirsch et al.

**Figure 4.10** Active sites of crystallographically characterized D- and E-family proteins. (Top, left to right) DyP from *Thanatephorus cucumeris*; TyrA from *Shewanella oneidensis*; EfeB from *Escherichia coli*. PDB codes are given in Table 5.1. Cartoon diagram representations of protein monomers (carbon magenta) are shown, with side-chains of proposed key active site residues shown as sticks. An Asp residue that is strictly conserved in EfeB and DyP family proteins is absent in Clds. A serine present in the distal pocket of DyP and TyrA (both DyP subfamily D proteins) is absent in the *Rhodococcus* DyP-B, EfeB, and Clds. This figure is adapted from Goblirsch et al. and generated using PyMOL.

**Figure 4.11** Overlay of Cld, DyP, and EfeB family heme environments. (Left) Key distal pocket residues. (Right) Distal residues. Drawn as stick colored by atom. Carbon coloring *D. aromatica* Cld, green; *E. coli* EfeB, light blue; *T. cucumeris* DyP, orange; *S. oneidensis* TyrA, pink. Residues
in *D. aromatica* Cld are labeled in black, while those of DyP are labeled in orange. Cld lacks a distal aspartate that is present in the other structures. DyP and TyrA distal pockets additionally contain a serine with no correlate in the other structures (S331 and S244 respectively). The heme of the *D. aromatica* Cld is drawn in grey sticks and the Fe center represented as an orange sphere. This figure was generated using PyMOL and adapted from Goblirsch et al.118

**Figure 4.12** Hemes of *D. aromatica* Cld and DyP. (Top left) Interactions of the propionates of *D. aromatica* Cld. (Top right) Interactions of the propionates of the DyP from *T. cucumeris*. (Bottom) Overlay comparing the heme orientation in *D. aromatica* Cld and DyP. Active site residues and hemes are shown as sticks and colored by atom with *D. aromatica* Cld (carbon green) and DyP (carbon orange). Regions associated with DyP are labeled in orange font. Pyrrole rings of the heme b cofactor are labeled and shown in bolded font. The hemes in *D. aromatica* Cld and DyP are related by a 180° flip along an axis through pyrrole positions D and B. This figure was generated using PyMOL. Adapted from Goblirsch et al.120

**Figure 4.13** Sequence and structure in the E-family. (Top) Partial alignment of E-family proteins (including DyP-subfamily-B proteins) from diverse species, highlighting important conserved residues. Strictly conserved acidic residues are highlighted in magenta; basic residues in green; positively charged in red; polar residues in cyan; and non-polar in yellow. In addition to the residues shown in the alignment, a glutamic acid (D(103)), a glycine (G(139)) and a phenylalanine (F(140)) are strictly conserved (residue numbers from the *R. jostii* DyP B sequence). The strictly conserved residues all derive from the C-terminal domain and are near to the heme. The highlighted tryptophan is near to the position of a strictly conserved Trp in Clds in the 3 dimensional structure. (Bottom) The positions of these conserved residues are illustrated on the *R. jostii* DyP B structure (PDB ID 3QNR). The alignment was made by ClustalW; figure by PyMOL.121-122

**Figure 4.14** Strictly conserved residues from an alignment of taxonomically diverse DyP proteins (subfamilies C and D) highlighted on the structure of the DyP from *T. cucumeris*. Alignment is by ClustalW. Sequences aligned came from DyP subfamily C: *Mycobacterium* sp. (Actinobacteria), *Cytophaga hutchinsonii* (Bacteriodites), *Myxococcus xanthus* (Deltaproteobacteria), *Cyanothece* sp. (Cyanobacteria), *Ochrobactrum anthropi* (Alphaproteobacteria), *Deinococcus radiodurans* (Deinococcus-Thermus; DyP subfamily D: *Rhodopirellula baltica* (Bacteria), *Nostoc punctiforme* (Cyanobacteria), *Aspergillus oryzae* (Ascomycota), *Thanatephorus cucumeris* (Basidiomycota). The figure was made using PyMol.123

**Figure 4.15** The O-atoms of the O₂ evolved from chlorite dismutase are chlorite-derived, with no O-atom exchange from water. (Left) Mass spectrum of gaseous products from the reaction of 85 mM Cld with 50 mM chlorite in 0.1 M sodium phosphate buffer, pH 7, made in 95% oxygen-18 enriched water and (Right) by using enriched ¹⁸O chlorite in ¹⁸OH₂. Signal assignments (*m/z*): H₂ (2), N (14), H₂O (18), N₂ (28), ¹⁶O₂ (32), ¹⁸O₂ (36). Adapted from Lee et al.126

**Figure 4.16** (Left) Representative progress of reaction curves showing the disappearance of chlorite (circles) and the evolution of O₂ (diamonds) measured in parallel reactions over time. For the data shown, [ClO₂⁻]₀ = 172 μM. Each data set was fit to the Michaelis-Menten equation: V/([E₅₀%]) = kₗ [ClO₂⁻]/([ClO₂⁻] + Kₘ). (Right) Plots of initial rates of chlorite dismutation per [heme] as a function of [ClO₂⁻], measured by monitoring ClO₂⁻ depletion (circles) and O₂ evolution (diamonds). Points are averages of 3 measurements, and error bars represent ± one standard deviation. The Michaelis–Menten equation was fit to each set of data. Adapted from Streit et al.127

**Figure 4.17** Plots showing chlorite-dependent irreversible inactivation of *D. aromatica* Cld. (Left) Values for kobs describing the exponential progress of reaction curves are plotted versus [chlorite]. Data were fit to the expression kₗ = kₗ(max) [ClO2⁻]/(Kₗ(max) + [ClO₂⁻]), yielding kₗ(max) = 1.77 min⁻¹ and kₗ = 166 μM. (Right) Plot of the residual enzymatic activity after exposure to increasing concentrations of chlorite, in the presence (triangles) and absence of flavin. Adapted from Streit et al.128
(circles) of excess/600 μM guaiacol. The turnover number, i.e., the maximal number of chlorite molecules dismutated per heme, is obtained by extrapolating a line fitted through each series of points to the x-axis. Turnover numbers are $1.7 \times 10^4$ in the absence of guaiacol and $1.3 \times 10^5$ in its presence. Adapted from Streit et al.

**Figure 4.18** UV/vis titrations demonstrate the acid and alkaline forms of *D. aromatica* Cld. (A) UV/visible titration data for ferric Cld over the pH range 6.6−9.8. Spectra are shown at pH 6.6, 6.8, 7.6, 7.9, 8.5, 8.7, 8.9, 9.5, and 9.8. Peak maxima and isosbestic points (at 350 and 470 nm) are labeled. Inset: Points correspond to absorbance at the indicated wavelengths. The solid lines are the calculated titration curves obtained from the two-component global nonlinear least-squares analysis. (B) Calculated component spectra obtained from the global analysis. Peak maxima are labeled. Inset: Speciation plot showing concentrations of the two components as a function of pH. Figure adapted from Streit et al.

**Figure 4.19** Resonance Raman spectra of *D. aromatica* Cld, indicating critical features of its electronic structure. (Left) Low-frequency rR spectra of ferrous Cld obtained with 441.6- and 406.7-nm excitation. The strong increase in relative resonance enhancement of the 222-cm$^{-1}$ band is compelling evidence for its assignment to the v(Fe−His) mode. The inset shows the 2-cm$^{-1}$ shift of this mode to higher frequency in alkaline solution. (Right) Soret-excited rR spectra of alkaline Cld (pH 10) and its isotopically labeled forms. Spectra were recorded with 406.7-nm excitation (15 mW). The samples were prepared in H$_2$O, D$_2$O, or H$_2^{18}$O solutions buffered at pH 10.0 with 50 mM Ches. The top three traces are the original spectra whose acquisition times were identical. The bottom three traces are difference spectra that were generated by 1:1 digital subtraction. The difference bands reveal isotope shifts characteristic of HS and LS heme hydroxides. Adapted from Streit et al.

**Figure 4.20** Proposed reaction mechanisms for Cl-O bond cleavage and O-O bond formation from chlorite in *D. aromatica* Cld.

**Figure 5.1** Proposed reaction mechanisms for Cl-O bond cleavage and O-O bond formation from chlorite in *DaCld*.

**Figure 5.2** Transient intermediates formed upon reaction of Cld with ~3 eq PAA at pH 6. Top: Complete spectra; bottom: visible bands shown on an expanded scale for clarity. Approximately ~15 μM *DaCld* (7.5 μM final) was mixed with 50 μM peracetic acid (25 μM final) in 0.2 M citrate-phosphate buffer at pH 6 (20 °C). The initial spectrum is shown in black and the spectrum for the intermediate formed after ~1.5 seconds in red. Intervening spectra are shown in gray. Isosbestic points are present at 350, 414, 453, and 547 nm.

**Figure 5.3** Pseudo first order rate constant for Compound I formation from the reaction of ferric *DaCld WT* with PAA, measured as a function of [PAA] in 0.2 M citrate-phosphate buffer at pH 6, 20 °C. The apparent second order rate constant $k = 1.900 \pm 0.003 \times 10^5$ M$^{-1}$s$^{-1}$.

**Figure 5.4** Conversion of Compound I to Compound ES and subsequent bleaching of the heme chromophore in 0.2 M citrate-phosphate buffer at pH 6, 20 °C. Approximately ~15 μM *DaCld WT* (7.5 μM final) was mixed with 100 μM peracetic acid (50 μM final) all in 0.2 M citrate-phosphate buffer at pH 6, 20 °C. The initial spectrum is shown in red and the spectrum for Cpd ES, formed after 8.7 seconds, is in blue. The final spectrum after 300 second is shown in green, intervening spectra are omitted for clarity.

**Figure 5.5** Species observed following reaction of a pre-formed Compound I and 2 electron eq ascorbate (pH 6). *DaCld* (10 μM, 2.5 μM final) was mixed with a slight stoichiometric excess of PAA and aged for approximately 2 s. The Compound I species that formed, shown in red, was then mixed with 2 equivalent of ascorbate (5 μM final). The blue species, resembling Compound II, forms and converts to the ferric enzyme (black). Singular value decomposition (Specfit) was used to better resolve the spectra.
Figure 5.6 Pseudo first order rate constants for the reaction of ferric DaCld WT Compound I with ascorbate (left) and for the conversion of Compound ES to the ferric species (right) as a function of [ascorbate] in 0.2 M citrate-phosphate buffer at pH 6, 20 °C. The slope of the line for the top plot yields the second order rate constant of \( k = 2.5 \pm 0.3 \times 10^6 \text{M}^{-1}\text{s}^{-1} \). The bottom plot was fit to a square hyperbola to yield a maximum rate of 9.3 ± 0.5 s\(^{-1}\) and apparent \( K_d = 3.5 \pm 1.2 \text{μM} \).

Figure 5.7 Transient intermediates formed upon reaction of Cld with ~3 eq PAA at pH 8. Approximately 15 μM DaCld (7.5 μM final) was mixed with 50 μM peracetic acid (25 μM final) in 0.2 M citrate-phosphate buffer at pH 8 (20 °C). The initial spectrum is shown in black and the spectrum for the intermediate that formed after ~300 milliseconds in blue. Intervening spectra are shown in gray. Note the isosbestic points at 350, 406, and 447 nm.

Figure 5.8 Pseudo first order rate constants for ferric DaCld WT Compound ES formation from the reaction of ferric DaCld with peracetic acid in 0.2 M citrate-phosphate buffer at pH 8, 20 °C, measured as a function of [PAA]. The second order rate constant is \( k = 1.3 \pm 0.01 \times 10^6 \text{M}^{-1}\text{s}^{-1} \).

Figure 5.9 Species observed following reaction of a pre-formed putative Compound II and 1 equivalent ascorbate (pH 8). Approximately 10 μM DaCld (2.5 μM final) was mixed with a slight stoichiometric excess of PAA at pH 8 and aged for 1 s. The resulting Compound II species (blue spectrum) was mixed with 1 equivalent of ascorbate (~2.5 μM final), which resulted in formation of the black species that resembles the ferric enzyme at pH 8.

Figure 5.10. Pseudo first order rate constants for the reaction of ferric DaCld WT Compound ES and ascorbate as a function of [ascorbate] in 0.2 M citrate-phosphate buffer at pH 8, 20 °C. The plot was fit to a square hyperbola to yield a maximum rate of \( k = 0.6 \pm 0.01 \text{s}^{-1} \) and an apparent \( K_d = 3.6 \pm 0.3 \text{μM} \).

Figure 5.11 Reactions between DaCld and peracetic acid at pH 6 and 8, the intermediates observed in stopped-flow studies are indicated in orange with corresponding maximum Soret band wavelengths.

Figure 5.12 Transient intermediates formed upon reaction of Cld with a large excess (>2800 eq) of \( \text{H}_2\text{O}_2 \) at pH 6. Approximately 7 μM DaCld (3.5 μM final) was mixed with 20 mM \( \text{H}_2\text{O}_2 \) (10 mM final) in 0.2 M citrate-phosphate buffer at pH 6 (20 °C). The initial ferric spectrum is shown in black, the putative Compound III intermediate that formed after ~3 s in orange, and the final bleached spectrum that formed after 26 s in green. Intervening spectra are shown in gray.

Figure 5.13 Pseudo first order rate constants for Compound 0 formation from the reaction of ferric DaCld WT and \( \text{H}_2\text{O}_2 \) in 0.2 M citrate-phosphate buffer at pH 6, 20 °C as a function of [\( \text{H}_2\text{O}_2 \)]. The measured second-order rate constant \( k = 9.6 \pm 0.4 \times 10^1 \text{M}^{-1}\text{s}^{-1} \).

Figure 5.14 Transient intermediates formed upon reaction of Cld with a large excess of \( \text{H}_2\text{O}_2 \) at pH 8. Approximately 7 μM DaCld (3.5 μM final) was mixed with 2 mM hydrogen peroxide (1 mM final) in 0.2 M citrate-phosphate buffer at pH 6 and 20 °C. The initial spectrum is shown in black, the putative Compound III intermediate that formed after 0.03 seconds in orange, and the final apparent Compound II-like spectrum that formed after 1.1 s in blue. Intervening spectra are omitted for clarity and singular value decomposition (Specfit) was used to better resolve the spectra.

Figure 5.15 Pseudo first order rate constants for Compound 0 formation from the reaction of ferric DaCld WT and \( \text{H}_2\text{O}_2 \) (left) and conversion to Compound II (right) as a function of [\( \text{H}_2\text{O}_2 \)] in 0.2 M citrate-phosphate buffer at pH 8, 20 °C. The slope of the line for the top plot yields the second order rate constant \( k = 1.7 \pm 0.04 \times 10^4 \text{M}^{-1}\text{s}^{-1} \). The bottom plot is fit to a square hyperbola yielding a maximum rate of \( k = 7.9 \pm 0.3 \text{s}^{-1} \) and apparent \( K_d = 1.5 \pm 0.2 \text{mM} \).
Figure 5.16 Possible reactions between DaCld and H$_2$O$_2$ at acidic and basic pH, based on known and proposed reaction pathways in other heme proteins.

Figure 5.17 Reaction of ferric DaCld R183Q with 40 mM peracetic acid 0.2 M citrate-phosphate buffer at pH 6, 20 °C. Approximately 8 μM DaCld R183Q (4 μM final) was mixed with 80 mM peracetic acid (40 mM final) all in 0.2 M citrate-phosphate buffer at pH 6, 20 °C. The ferric enzyme’s spectra (black) has a Soret peak at ~ 400 nm with charge transfer bands at ~ 500 and 635 nm rapidly converts to Compound I (red, 0.048 s) with a decrease in absorptivity in the Soret peak. The Compound I species has $\lambda_{\text{max}}$ at 397 nm with a broad charge transfer band around 630 nm. Compound I eventually decays to a bleached spectra (blue) after shifting to 401 nm. Inset shows the same spectra with a zoomed in visible region.

Figure 5.18 Pseudo first order rate constants for Compound I formation from the reaction of the ferric DaCld R183Q with peracetic acid as a function of [PAA] in 0.2 M citrate-phosphate buffer pH 6.0, 20 °C. The measured $k = 3.7 \pm 0.3 \times 10^3$ M$^{-1}$ s$^{-1}$. Rate constants were taken from exponential fits to the change in absorbance at 420 nm, associated with initial Cpd I formation, as a function of time.

Figure 5.19 Singular value decomposition of the reaction of ferric DaCld R183Q with 40 mM peracetic acid in 0.2 M citrate-phosphate buffer pH 6.0, 20 °C. The spectra correspond to each species formed upon fitting the variable absorbance traces to 3 exponentials with rates of 125, 10, and 0.7 s$^{-1}$. The first intermediate (ferric enzyme) is in black followed by red, blue and green species. Inset shows the visible region of the spectra. Data were analyzed using SpecFit.

Figure 5.20 Reaction of ferric DaCld R183Q with 40 mM peracetic acid at pH 8. Approximately 8 μM DaCld R183Q (4 μM final) was mixed with 80 mM peracetic acid (40 mM final) in 0.2 M citrate-phosphate buffer at pH 8 (20 °C). The ferric enzyme’s spectra (black) has Soret peak at ~ 400 nm with charge transfer bands at ~ 500 and 635 nm rapidly converts to Compound I (red, 0.053 s) with a decrease in absorptivity in the Soret peak. The Compound I species has $\lambda_{\text{max}}$ at 397 nm with a broad charge transfer band around 630 nm. Compound I eventually decays to a bleached spectra (blue) after shifting to ~415 nm. Inset shows the same spectra with a zoomed in visible region.

Figure 5.21 Pseudo first order rate constants for Compound I formation from the reaction of the ferric DaCld R183Q with peracetic acid as a function of [PAA] in 0.2 M citrate-phosphate buffer pH 8.0, 20 °C. The measured $k = 3.8 \pm 0.4 \times 10^3$ M$^{-1}$ s$^{-1}$. Rate constants were taken from exponential fits to the change in absorbance at 420 nm, associated with initial Cpd I formation, as a function of time.

Figure 5.22 Singular value decomposition of the reaction of ferric DaCld R183Q with 40 mM peracetic acid in 0.2 M citrate-phosphate buffer pH 8.0, 20 °C. The spectra correspond to each species formed upon fitting the variable absorbance traces to 3 exponentials with rates of 115, 7, and 0.7 s$^{-1}$. The first intermediate (ferric enzyme) is in black followed by red, blue and green species. Inset shows the visible region of the spectra. Data were analyzed using SpecFit.

Figure 5.23 Active site structure of DaCld.

Figure 6.1 UV/Visible Spectrum of WT DaCld and its Cyanide Complex at pH 7.0. The UV/Visible spectrum of DaCld WT (——) and its cyanide complex (-----) are shown in 0.2 M citrate-phosphate buffer at pH 7.0, 20 °C. Approximately 3 μM of enzyme was mixed with 100 μM KCN and spectra were recorded. The inset shows the zoomed visible region of the spectrum.

Figure 6.2 UV/Visible Spectrum of DaCld (R183Q) and its Cyanide Complex at pH 7.0. The UV/Visible spectrum of DaCld (R183Q) (——) and its cyanide complex (-----) are shown in 0.2 M citrate-phosphate buffer at pH 7.0, 20 °C. Approximately 3 μM of enzyme was mixed with 1 mM
KCN and spectra were recorded. The inset shows the zoomed visible region of the spectrum.

Figure 6.3 Representative Traces of Absorbance Versus Time for Cyanide Binding to WT DaCld. A representative trace showing the change in absorbance at 418 nm upon binding of cyanide by DaCld WT in 0.2 M citrate-phosphate buffer, pH 5.5, 20 °C. Enzyme (1 μM) was mixed with 100 μM KCN. The open circles show the measured absorbance values which are fit to a single-exponential curve, equation 8.1 in the text.

Figure 6.4 Representative Linear Fit of \( k_{obs} \) versus [KCN]. Values of \( k_{obs} \) measured from traces like those shown in Figure 3 are plotted as a function of KCN. Each point represents an average of no less than 5 measurements and the error is reported as the standard deviation of those values. Data are fit to a linear expression, as described for equation 2 in the text, where the slope of each line gives \( k_{ON} \) (M\(^{-1}\)s\(^{-1}\)) and y-intercept gives \( k_{OFF} \) (s\(^{-1}\)). These are 3.9 ± 0.1 x 10\(^4\) M\(^{-1}\)s\(^{-1}\) and 0.70 ± 0.02 s\(^{-1}\) respectively for the data shown here. Data for all pH values for WT and R183 Q are reported in Tables 6.1 and 6.2.

Figure 6.5 pH Dependence of Cyanide Association Rate Constants for WT and R183Q DaCld. The apparent rate constants for the binding of cyanide to ferric DaCld and R183Q. The change in absorbance at 418 nm was monitored for the formation of the enzyme-cyanide complex in 0.2 M citrate-phosphate buffer at 20 °C after mixing of approximately 1 μM enzyme with increasing concentrations of cyanide under pseudo-first order conditions. The apparent rates from single-exponential fits were plotted as a function of cyanide and the slope of linear fits to those data were used to determine the on rates for cyanide at various pH values. The log of the on rates are plotted here as a function of pH; wild-type (●) and R183Q (■) increase sharply with pH. Data are fit to single pK\(_a\) models for wild-type (——) and R183Q (-----) as explained in the text. Error bars represent the standard deviation from no less than three measurements.

Figure 6.6 pH Dependence of Cyanide Dissociation Rate Constants for WT and R183Q DaCld. The apparent rate constants for the binding of cyanide to ferric DaCld and R183Q. The change in absorbance at 418 nm was monitored for the formation of the enzyme-cyanide complex in 0.2 M citrate-phosphate buffer at 20 °C after mixing of approximately 1 μM enzyme with increasing concentrations of cyanide under pseudo-first order conditions. The apparent rates from single-exponential fits were plotted as a function of cyanide and the y-intercept of linear fits to those data were used to determine the off rates for cyanide at various pH values. The log of the off rates are plotted here as a function of pH; wild-type (●) and R183Q (■) increase sharply with pH. Data are fit to single pK\(_a\) models for wild-type (——) and R183Q (-----) as explained in the text. Error bars represent the standard deviation from no less than three measurements.

Figure 6.7 Comparison of pH Dependence of \( pK_d^{(equil)} \) for WT DaCld and R183Q DaCld. The data shown here are from equilibrium binding of KCN to WT and R183Q carried out previously. The log of \( K_d \) values measured from equilibrium titrations of WT (●) and R183Q (■) in 0.2 M citrate-phosphate buffer, 25 °C are plotted as function of pH and fit to a two pK\(_a\) model described in the text.

Figure 7.1 SDS-PAGE of HemQ (SaCld) Purification. Protein fractions from the two-column purification of apo-HemQ are shown from left to right: MW marker, clarified lysate, Q-Sepharose, gel filtration eluate, and MW marker. Each lane contains approximately 50 μg of total protein. Molecular weight marker values from top to bottom are: 130, 100, 70 (orange), 55, 35, and 25 (orange) kDa.

Figure 7.2 UV-Visible Spectrum of Apo- and Holo-HemQ (SaCld). The UV-visible spectrum of the as purified apo-HemQ and the heme bound holo-HemQ are shown from 350 to 700 nm. Spectra of approximately 115 μM apo-HemQ and 40 μM Holo-HemQ (concentration of assumed
monomer taken from Bradford assay) were taken in 0.1 M potassium phosphate pH 6.8 following purification. Holo-HemQ contains ~0.5 heme, as indicated from pyridine hemochrome assay, per assumed protein monomer and is indicated from the increase ratio of Soret to protein absorbance.

**Figure 7.3 UV-Visible Spectrum of Ferric and Ferrous Holo-HemQ (SaCld).** The UV-visible spectrum of ferric (---) and ferrous (—) holo-HemQ (~20 μM heme bound protein monomer) in 0.1 M potassium phosphate pH 6.8. The ferric enzyme has a Soret band at ~405 nm and visible bands at 510 and 630 nm, while the ferrous protein (produced by addition of excess dithionite solid) has a Soret at 433 nm and a visible band at 558 nm. The inset shows a the visible region of the spectra from 450-700 nm. Results are summarized and compared to other CDE family proteins in Table 7.1.

**Figure 7.4 UV-Visible pH Titration of Holo–HemQ (SaCld).** The holo-enzyme (~8 μM) was diluted in 0.1 M potassium phosphate buffer at pH 6.8, 25 °C and titrated with 1 M potassium hydroxide and 1 M hydrochloric acid to determine the changes in the Soret band as a function of pH. Spectra were collected for changes from pH 6.8-5.1 and pH 6.8-10.2 every 0.1 pH unit. Spectra are shown at pH 6.8 (-----), 5.18 (-- - -), 10.22 ( - - -), and select intermediates (lighter) for clarity. The inset shows the change in absorbance at 403 nm as a function of pH.

**Figure 7.5 Circular Dichroism of Holo- and Apo-HemQ (SaCld).** CD spectra of the as purified apo-HemQ and the heme-bound holo-HemQ were measured from 190 to 250 nm with approximately 1 μM of assumed protein monomer in 0.1 M potassium phosphate buffer pH 6.8, 25 °C. Apo-HemQ (—) has a maximum at 227 nm and holo-HemQ (-----) at 234 nm.

**Figure 7.6 Gel Filtration Chromatography and Molecular Weight Determination of Apo- and Holo-HemQ (SaCld).** A. Overlay of size exclusion chromatograms for apo-HemQ, holo-HemQ, and molecular weight standards run separately on S200-sephacryl GE Biosciences resin in 0.05 M potassium phosphate with 0.15 M NaCl at pH 7.0 and 4 °C at 0.5 mL/minute. The absorbance, expressed as mAU, was measured at 280 nm to detect total protein. The molecular weight standard peaks (-----) are from left to right thyroglobulin (670 kDa), α-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (13.5 kDa). Approximately 10 mg/mL of Apo-HemQ (---) eluted near the void volume and resulted in an estimated molecular weight of 1200 kDa and the holo-HemQ (-----) sample (~10 mg/mL with 1 equivalent heme added) eluted to give a molecular weight of 187 kDa. Inset contains a linear display of the size-exclusion chromatogram with the ratio of elution to void volume plotted as a function of the log of the molecular weight of protein standards (Ο) and fit to a line. Apo-HemQ (■) and holo-HemQ (●) are shown on where they lie on the curve.

**Figure 7.7 Heme Binding to Apo-HemQ (SaCld) by UV-Visible Spectroscopy.** Approximately 2 μM of apo-HemQ was titrated from 0-50 μM heme with a 100 μM solution (made from 0.1 mM stock in 0.1 M NaOH) in 0.1 M potassium phosphate buffer pH 6.8 at 25°C. Parallel titrations were performed without protein. Each solution was allowed to incubate several hours to permit full equilibration. The difference between the spectrum of the apo-HemQ sample and the no protein control was taken at 408 nm and plotted as a function of heme. The average of three trials was taken. The data was fit using a quadratic equation taking the protein concentration (See Materials and Methods) into consideration and results in a $K_d = 720$ (210) nM.

**Figure 7.8 Equilibrium Binding of apo-HemQ for Heme and Protoporphyrin IX Measured by Fluorescence Quenching.** A. Binding isotherm for the quenching of apo-HemQ (~5 μM) in 0.1 M potassium phosphate buffer pH 6.8 by heme. The percent of quenching observed is plotted as a function of heme added and fit to a quadratic binding equation taking into account the concentration of protein (see Materials and Methods). A $K_d = 1.68 \pm 0.16 \mu M$ was determined for heme. Inset shows quenching of apo-HemQ fluorescence by addition of heme (excitation $\lambda = 284$...
nm; emission $\lambda = 300 - 400$ nm; 5 $\mu$M in 0.1 M potassium phosphate pH 6.8, 25°C). Heme was added from 1.4-111 $\mu$M and spectra were collected after several minutes of incubation to allow equilibrium to be reached. B. Binding isotherm for protoporphyrin IX (PPIX). The binding of PPIX was monitored in the same fashion as heme using a stock solution of PPIX (1 mM) dissolved in DMSO and diluted into 0.1 M potassium phosphate pH 6.8. A $K_d = 2.21 \pm 0.1 \mu$M was determined.

**Figure 7.9** UV-Visible Spectrum of the Transient Reaction of holo-HemQ (SaCld) with Oxidants A. Peracetic Acid. Ferric holo-HemQ (~8 $\mu$M final) was mixed with an approximately 10-fold excess of peracetic acid (80 $\mu$M final) in 0.1 M potassium phosphate pH 6.8 at 25 °C. Each concentration was mixed with protein individually and was allowed to incubate several hours to permit full equilibration before recording the spectra. The difference between the free and species was taken at 423 nm and plotted as a function of cyanide. The binding isotherm was fit using a quadratic equation taking the protein concentration into consideration (see Materials and Methods) and resulted in a $K_d = 8.9 \pm 0.88$ $\mu$M. B. H$_2$O$_2$. Ferric holo-HemQ (~6 $\mu$M final) was mixed with a several fold excess of hydrogen peroxide (1 mM final) in 0.1 M potassium phosphate pH 6.8 at 25 °C. Spectra shown were recorded between 0.75 ms (——) and 200 s (- - - -). A slow bleaching of the Soret band is observed, to ~405 nm, followed by a slow bleaching of the chromophore. No clear changes in the visible region occur (inset). **Figure 7.10** Equilibrium Ligand Binding to holo-HemQ (SaCld) by UV-Visible Spectroscopy. A. Cyanide. Approximately 8 $\mu$M of holo-HemQ was titrated from 0-500 $\mu$M potassium cyanide in 0.1 M potassium phosphate buffer pH 6.8 at 25 °C. Each concentration was mixed with protein individually and was allowed to incubate several hours to permit full equilibration before recording the spectra. The difference between the free and bound species was taken at 423 nm and plotted as a function of cyanide. The binding isotherm was fit using a quadratic equation taking the protein concentration into consideration (see Materials and Methods) and resulted in a $K_d = 8.9 \pm 0.88$ $\mu$M. B. Imidazole. Approximately 8 $\mu$M of holo-HemQ was titrated from 0-10 mM imidazole in 0.1 M potassium phosphate buffer pH 6.8 at 25 °C. Each concentration was mixed with protein individually and was allowed to incubate several hours to permit full equilibration before recording the spectra. The difference between the free and bound species was taken at 418 nm and plotted as a function of imidazole. The binding isotherm was fit using equation 7.1 taking the protein concentration into consideration (see Materials and Methods) and resulted in a $K_d = 10.0 \pm 0.11$ mM. **Figure 7.11** Aerobic Growth Curves for WT, $\Delta$ hemB, and $\Delta$ hemQ Strains of *S. aureus* Newman in the Absence and Presence of Exogenous Heme. A. Absence of exogenous heme. The three strains of *S. aureus* Newman cells: wildtype (○), $\Delta$ hemB (△), and $\Delta$ hemQ (□) cells were grown and monitored at 37°C, 180 rpm, for 12 hours in 150 mL Erlenmeyer flasks. Cultures of ~50 mL tryptic soy broth (TSB) were inoculated (1:100) with saturated overnights and subsequent growth was monitored by taking OD$_{600}$ via spectrophotometer every two hours, each point is an average of duplicate or triplicate measurements. B. Presence of exogenous heme. The three strains of *S. aureus* Newman cells wildtype (○), $\Delta$ hemB (△), and $\Delta$ hemQ (□) cells were grown and monitored at 37°C, 180 rpm, for 12 hours in air-tight 50 mL falcon tubes. Cultures of ~50 mL tryptic soy broth (TSB) were inoculated (1:100) with saturated overnights and subsequent growth was monitored by taking OD$_{600}$ via spectrophotometer every two hours via syringe, each point is an average of duplicate or triplicate measurements. B. Presence of exogenous heme. The three strains of *S. aureus* Newman cells wildtype (○), $\Delta$ hemB (△), and $\Delta$ hemQ (□) cells were grown and monitored at 37°C, 180 rpm, for 12 hours in air-tight 50 mL falcon tubes. Cultures of ~50 mL tryptic soy broth (TSB) were inoculated (1:100) with saturated overnights and subsequent growth was monitored by taking OD$_{600}$ via spectrophotometer every two hours via syringe, each point is an average of duplicate or triplicate measurements.
hemB (△), and Δ hemQ (□) cells were grown and monitored at 37 °C, 180 rpm, for 12 hours in air-tight 50 mL falcon tubes. Cultures of ~50 mL tryptic soy broth (TSB) supplemented with ~ 5 μM hemin (from sterile filtered 1 mM stock in 0.1 M NaOH) were inoculated (1:100) with saturated overnights and subsequent growth was monitored by taking OD$_{600}$ via spectrophotometer every two hours via syringe, each point is an average of duplicate or triplicate measurements.  

**Figure 7.13** Anaerobic Nitrate-Respiring Growth Curves for WT, Δ hemB, and Δ hemQ Strains of *S. aureus* Newman in the Absence and Presence of Exogenous Heme. A. Absence of exogenous heme. The three strains of *S. aureus* Newman cells wildtype (○), Δ hemB (△), and Δ hemQ (□) cells were grown and monitored at 37 °C, 180 rpm, for 12 hours in air-tight 50 mL falcon tubes. Cultures of ~50 mL tryptic soy broth (TSB) supplemented with 20 mM sodium nitrate were inoculated (1:100) with saturated overnights and subsequent growth was monitored by taking OD$_{600}$ via spectrophotometer every two hours via syringe, each point is an average of duplicate or triplicate measurements. B. Presence of exogenous heme. The three strains of *S. aureus* Newman cells wildtype (○), Δ hemB (△), and Δ hemQ (□) cells were grown and monitored at 37 °C, 180 rpm, for 12 hours in air-tight 50 mL falcon tubes. Cultures of ~50 mL tryptic soy broth (TSB) supplemented with 20 mM sodium nitrate and ~ 5 μM hemin (from sterile filtered 1 mM stock in 0.1 M NaOH) were inoculated (1:100) with saturated overnights and subsequent growth was monitored by taking OD$_{600}$ via spectrophotometer every two hours via syringe, each point is an average of duplicate or triplicate measurements.

**Figure 7.14** A-E. LCMS Standard Curves of Uroorphyrin and Coproporphyrin. Each curve was generated from a serial dilution of a stock solution of each porphyrin in acetonitrile. Freshly prepared solutions stored in amber vials (to reduce light absorbption) were run immediately by the LCMS method described in the text. The peak area of three measurements was averaged and plotted as a function of porphyrin concentration. Linear fits to the data were used to determine concentrations of unknown samples.

**Figure 7.15** A-E. LCMS Standard Curves of Protoporphyrin IX and Hemin. Each curve was generated from a serial dilution of a stock solution of each porphyrin in acetonitrile. Freshly prepared solutions stored in amber vials (to reduce light absorbption) were run immediately by the LCMS method described in the text. The peak area of three measurements was averaged and plotted as a function of porphyrin concentration. Linear fits to the data were used to determine concentrations of unknown samples.

**Figure 7.16** A-E. LCMS Standard Curves of Internal Standard. The curve was generated from a serial dilution of a stock solution of each porphyrin in acetonitrile. Freshly prepared solutions stored in amber vials (to reduce light absorbption) were run immediately by the LCMS method described in the text. The peak area of three measurements was averaged and plotted as a function of porphyrin concentration. Linear fits to the data were used to determine concentrations of unknown samples.

**Figure 7.17** Porphyrin Abundance for Wild-Type, Δ hemQ, and Δ hemB *S. aureus* Newman Cells. Cell cultures (50 mL) were grown under standard conditions in tryptic soy broth (TSB) at 37 °C, 180 RPM, for approximately 16 hours before harvesting. Aerobic samples were grown in 150 mL Erlenmeyer flasks and anaerobic samples in filled 50 mL falcon tubes. Harvested cells were lysed and porphyrins extracted and analyzed by LCMS as described in the Materials and Methods sections. The bar graph represents the estimated levels of A. heme and B. coproporphyrin in nanomoles of metabolite per gram of wet cell pellet for wild type (white), Δ hemQ (dark grey) and Δ hemB (light grey) strains. Error bars are the result of standard deviations taken from analysis of three separate cell cultures.

**Figure 7.18** Prokaryotic Heme Biosynthesis. The accepted reaction pathway for heme b biosynthesis from glutamyl-tRNA showing the intermediates formed in green lettering, the proper
enzyme names in grey, and the genes associated with that function found in both gram-negative and gram-positive bacteria shown in blue

**Figure A.1** Reactions catalyzed by OMO

**Figure A.2** Assay for determining hydroxylamine-derived nitrite

**Figure A.3** (A) Reaction of reduced OMO with O\textsubscript{2} to form FAD-OOH in the presence of saturating (5 mM) l-Arg, as monitored by stopped flow UV/vis spectroscopy over time (measurement times listed). The initial spectrum is shown in red, and the final in blue. Spectra measured in between are shown in gray. See ref 17 for analogous data for l-Orn and Figure 4.4 (l-Lys). (B) Concentration dependence of \( k_{\text{obs}} \) for this conversion (fit to a single exponential at 370 nm; see Appendix C Figure A.5) in the presence of l-Orn, l-Lys, l-Arg, and l-Citr. These have measured apparent \( K_0 \) values of 310 ± 40 μM, 130 ± 30 μM, 620 ± 70 μM, and 4.4 ± 0.9 mM respectively. Associated values for \( k_{\text{FAD-OOH}} \) extrapolated from these data at saturating concentrations for each compound are listed in Table A.1

**Figure A.4** FAD-OOH formation in the presence of L-Lys. The reaction of reduced OMO (red spectrum) with O\textsubscript{2} to form FAD-OOH (blue spectrum) in the presence of 5 mM L-Lys was monitored by stopped flow UV/vis spectroscopy. Spectra monitored at the intermediate times listed on the plot are shown in grey. See Figure A.3A above for analogous data measured in the presence of L-Arg. Note the much longer time scale required to achieve maximal production of the FAD-OOH intermediate in the presence of L-Lys

**Figure A.5** Representative kinetic traces at 370 nm (symbols) showing formation of FAD-OOH in the presence of A) buffer, B) L-Orn and L-Lys, and C) L-Arg and D) L-Citr with data fit (lines) to a single exponential to obtain the rate constant \( k_{\text{obs}} \), and \( k_{\text{FAD-OOH}} \) is taken as the maximal value extrapolated as each compound reaches saturation. The data from A-D are shown on a logarithmic time scale in plot E) Each reaction contains 15-20 μM enzyme reduced with 1 eq NADPH, which is subsequently mixed with air-saturated buffer containing 5 mM of the given compound. The different time scales for FAD-OOH formation in the presence of the given compounds are illustrated by these traces

**Figure A.6** Amino acids and related compounds discussed in the text

**Figure A.7** (A) Conversion of the OMO FAD-OOH to oxidized FAD and H\textsubscript{2}O\textsubscript{2} in the presence of (5 mM) l-Arg as monitored by stopped flow. (B) Concentration dependence of \( k_{\text{obs}} \) for the conversion in (A) (fit to a single exponential at 450 nm; see Appendix C Figure A.8) in the presence of l-Orn or l-Lys. These have measured apparent \( K_0 \) values of 1.1 ± 0.09 and 1.2 ± 0.1 mM respectively. Note that l-Orn-NεOH is the major product in the presence of l-Orn, while the enzyme converts O\textsubscript{2} to H\textsubscript{2}O\textsubscript{2} in l-Arg and l-Lys. Values for \( k_{\text{FAD}} \) extrapolated from these data at saturating concentrations for each compound are listed in Table A.1

**Figure A.8** Representative kinetic traces at 450 nm (symbols) showing conversion of FAD-OOH to oxidized FAD in the presence of A) buffer and L-Arg, B) L-Orn, and C) L-Lys with data in each case fit (lines) to a single exponential to obtain the rate constant \( k_{\text{obs}} \). The data from A-C are shown on a logarithmic time scale in plot D). In each case, a FAD-OOH–NADPH\textsuperscript{+} complex reacts with substrate to form L-Orn-OH and oxidized FAD, or with non-substrate to form H\textsubscript{2}O\textsubscript{2} and oxidized FAD. Each reaction contains 15-20 μM enzyme reduced with 1 eq NADPH, which is subsequently mixed with air-saturated buffer containing 5 mM of the given compound

**Figure A.9** Effects of L-Orn, L-Arg, and L-Citr on rates of NADPH-reduction of OMO. The reaction mixture contained 10 μM enzyme and a final concentration of 15 μM NADPH at 25 °C and A) buffer and L-Orn B) 5 mM L-Arg, C) 4 mM L-Citr. The data at 450 nm (symbols) were fit
to the sum of two exponentials where the first phase accounts for the majority of the amplitude change. The rate constants measured for this phase \( (k_{\text{red}}) \) were 1.21, 1.46, 5.9 and 13.9 s\(^{-1}\) for buffer, L-Orn, L-Citr, or L-Arg, respectively. The remainder of the reaction occurs with rate constants 0.2 s\(^{-1}\), 1.2 s\(^{-1}\), and 0.8 s\(^{-1}\) in the presence of buffer, L-Arg and L-Citr respectively. The observed rate constant for this noticeably slower phase showed no dependence on NADPH, substrate, or substrate analog. These results are consistent those previously described for liver microsomal FMOs, in which the biphasic reaction was explained as being due to the reaction of two enzyme forms. \(^3\) L-Arg has the most substantial effect on \( k_{\text{red}} \). (D) Concentration dependence of \( k_{\text{red}} \) in the presence of L-Arg and L-Citr, with apparent \( K_D \) values of 0.7 ± 0.1 and 4.9 ± 0.9 mM, respectively.

Figure A.10 Pathways for l-orn production and conversion into siderophores (mito = mitochondria; cyto = cytoplasm)

Figure B.1 Active site of DaCl–nitrite complex (PDB entry 3Q08)

Figure B.2 pH-rate profiles of the R183Q mutant with chloride. Plots of log(\( k_{\text{cat}} \)) (○) and log \( k_{\text{cat}}/K_m \) (●) as a function of pH using chloride at 4 °C in citrate/phosphate buffer.

Figure 6.3 UV visible pH titration for the R183Q mutant (A) pH 6.8, 6.5, 6.1, 5.8, 5.5, 5, 3.8 (B) pH 6.8, 7.3, 7.7, 8.2, 8.7, 9.4, 10, 10.5, 10.9 and (C) pH 8.4, 9.3, 9.9, 10.3, 10.6, 10.9, 11.6, 12.1.

Figure B.4 UV/visible pH titrations for mutant R183A at (A) pH 6.8, 6.5, 6.1, 5.8, 5.5, 5, 3.8 (B) pH 6.8, 7.2, 7.5, 7.9, 8.3, 8.9, 9.5, 9.9, 10.2, 10.4, 10.6 and (C) pH 8.4, 9.3, 9.9, 10.3, 10.6, 10.9, 11.6, 12.1.

Figure B.5 UV/visible pH titrations for mutant R183K at pH 6.2, 5.8, 5.4, 4.9, 4.2, 3.8, 3.3, 2.8 (B) at pH 6.9, 7.1, 7.3, 7.5, 7.8, 7.9, 8.9, 9.4, 10, 10.5, 10.9 and (C) at pH 10.04, 10.4, 10.9, 11.2, 11.6, 11.9, 12.5, 12.9.

Figure B.6. UV–visible spectra for WT and DaCld mutants at pH 6.8 in 100 mM phosphate buffer. The spectrum of WT DaCld is represented with a thick black solid line with a Soret band at 393 nm. The R183A mutant (−−−) has a spectrum similar to that of WT with a Soret band at 391 nm. The R183Q (---) and R183K (thick gray line) mutants have Soret bands at 403 and 410 nm, respectively.

Figure B.7 Soret-excited rR spectra of Cld mutants at pH 6.8. Samples were prepared in 100 mM sodium phosphate (pH 6.8): (A) high-frequency window and (B) low-frequency window. Spectra of WT Cld at pH 6.8 and 10.0 (100 mM Ches) are included for comparison to those of the mutants.

Figure B.8. Soret-excited, high frequency window rR spectra of Cld mutants as a function of pH. Samples were prepared in 100 mM sodium phosphate, pH 6.8, 100 mM Tris/HCl pH 7.8, 100 mM Tris/HCl pH 8.8, 100 mM Mes pH 10.0 and 100 mM MES pH 5.5. Spectra were collected with 406.7 nm excitation and 14 mW at the sample. A) Cld(R183K) spectra compared to the spectrum of WT Cld at pH 10, B) Cld(R183A) spectra, and C) Cld(R183Q) spectra compared to WT Cld at pH 6.8. *indicates plasma emission lines.

Figure B.9. High frequency rR spectra of ferrous Cld(R183Q) at pH 7.8 and 10.0. Spectra were acquired with 406.7 nm excitation and 15 mW at the sample. Inset:The low frequency window of the rR spectrum of ferrous Cld(R183Q) at pH 7.8 and 10.0 obtained with 441.6 nm excitation.

Figure B.10. Resonance Raman characterization of ferrous Cld(R183A) in 100 mM sodium phosphate, pH 6.8. (A) The high frequency window rR spectrum acquired with 413.1 nm excitation and 18 mW at 8 the sample. (B) The low-frequency window of the rR spectrum of
ferrous Cld(R183A) obtained with 441.6 and 413.1 nm excitation

**Figure B.11** UV-visible spectra of ferrous Cld(R183K) as a function of pH. Samples were prepared in 100 mM sodium phosphate, pH 6.8 or 100 mM Chs pH 10.0 and reduced with sodium dithionite.

**Figure B.12** pH dependence of the high-frequency rR spectrum of ferrous DaCld(R183K). Samples were prepared in 100 mM sodium phosphate at pH 6.8, 100 mM Tris-HCl at pH 7.8, 100 mM Tris-HCl at pH 8.8, or 100 mM Chs at pH 10.0 and reduced with sodium dithionite. Spectra were acquired with 406.7 nm excitation and 18 mW power at the sample. The inset shows the pH dependence of the low-frequency window of the rR spectrum of ferrous DaCld(R183K) obtained with 441.6 nm excitation.

**Figure B.13** Fit of WT DaCld−CO rR data (pH 5.8) determine the Fe−C stretching frequency of the two conformers. Spectra were acquired with 413.1 nm excitation. Original spectrum is in black, fit peaks are in blue, and the simulated spectrum is in red. The simulated difference spectrum was generated by subtraction of the simulated spectra.

**Figure B.14** Soret-excited rR spectra of WT Cld−CO as a function of pH.

**Figure B.15.** Soret-excited rR spectra of the isotopomers of CO complexes of DaCld Arg183 mutants at pH 6.8. Spectra were acquired with 413.1 nm excitation and 2 mW power at the sample.

**Figure 6.16** vFe–C–vC–O correlation plot comparing CO complexes of WT and Arg183 Cld mutants with peroxidases and myoglobin. The data used to generate the plot are listed in Table F1 of Appendix F.

**Figure B.17** Resonance Raman spectra and fits for the low frequency data for the isotopomers of Cld(R183Q)-CO at (A) pH 6.8 and (B) pH 10.0. The original data is in black; blue indicates the component peaks and red shows the calculated fit.

**Figure B.18** Representative plots illustrating data quality and analysis for DaCld(R183A)–ligand titrations. (A) Titration of KCN at pH 7.8 in a 0.1 M citrate/phosphate buffer. Spectra were measured after addition of cyanide to final concentrations of 0, 24, 48, 163, 377, 487, 574, 770, 959, 92, 1.1, 1.3, 20, 39, and 57 mM. The initial and final spectra are presented as thick black lines, and spectra are corrected for dilution. The inset shows an expanded view of the visible region of the spectrum. (B) Difference plot generated from the data in panel A. (C) Plot of ΔAbs 386 as a function of ligand concentration. The wavelength of maximum absorbance change was used to construct a plot of ΔAbs 386nm versus [L]_T (total concentration of added ligand, HCN) and fit by least squares regression to an equilibrium isotherm of the form ΔAbs=ΔAbs^max[L]_T/(K_D+ [L]_T). The K_D in this case was determined to be 553 ± 45 μM.

**Figure B.19** pK_D vs pH profiles describing the binding of (A) KCN and (B) imidazole to WT DaCld (●), DaCld(R183Q) (▲), DaCld(R183K) (■), and DaCld(R183A) (○). Data are fit to the equations described in the text.

**Figure B.20** Titration of DaCld and potassium indigo tetrasulfonate (dye) with xanthine oxidase used in the determination of its redox potential. An anaerobic solution of 2 μM Cld and 2 μM dye in 0.1 M potassium phosphate buffer (pH 7) with 1 μM benzyl viologen and 200 μM xanthine was reduced with an appropriate amount of xanthine oxidase. The spectra shown were recorded at 0, 50, 104, 158, 216, 266, 320, and 376 min. At time zero, the dye and DaCld are fully oxidized with maxima at 393 and 592 nm, respectively (—); at 376 min (blue line), they are fully reduced with a maximum at 432 nm for DaCld. The inset shows the logarithmic plot of the Nernst equation used to determine the reduction potential. The data were normalized to the concentration of Cld, and
the wavelengths oft 432 and 416 nm were used to determine the reduction potential via the relationship \(\ln(\frac{\text{Enz}_{\text{ox}}}{\text{Enz}_{\text{red}}}) = 0.73 \times \ln(\frac{\text{Dye}_{\text{ox}}}{\text{Dye}_{\text{red}}}) - 0.88\) ........................................324

**Figure B.21** Comparison of open (top and middle) and closed (bottom) conformers hypothesized to modulate DaCld activity. The proteins are drawn as cyan cartoons with important residues depicted as sticks and elements designated by color: green for carbon, red for oxygen, blue for nitrogen, and orange for phosphorus. The iron atom is shown as a rust-colored sphere. In each case, hydrogen bonding interactions that are important for maintaining the distal arginine conformation are depicted as dotted lines. The top panel shows the active site of the Cld from *N. winogradskyi* (PDB entry 3QPI) with water as its axial ligand. This water molecule is at the center of a hydrogen bonding network that connects the iron to the distal arginine in its open conformation. The distal arginine is also hydrogen bonded to a glutamine. The middle panel shows the active site of the cyano–Cld complex from *Ca. N. defluvii* (PDB entry 3NN2). The Fe-bound cyanide ligand is connected to the distal arginine via a hydrogen bonding network containing a molecule of solvent (polyethylene glycol), again in the open conformation. The side chain is further hydrogen bonded to two backbone carbonyls. The bottom panel shows the DaCld–nitrito complex (PDB entry 3Q09). The distal arginine assumes its closed conformation. These figures were generated using PyMOL (http://www.pymol.org)........................................329
TABLES

Table 2.1 Omo Purification Scheme.................................................................44
Table 2.2 Steady-State Kinetic Parameters for Omo Measured at 37 and 25 °C..........48
Table 2.3 Variable NADPH, Fixed-Variable O₂, Saturating L-Ornithine.....................50
Table 2.4 Variable NADPH, Fixed-Variable O₂, Sub-Saturating L-Ornithine..............51
Table 2.5 Variable L-Ornithine, Fixed-Variable NADPH, Saturating O₂....................52
Table 2.6 Variable L-Ornithine, Fixed-Variable O₂, Saturating NADPH..................53
Table 2.7 Summary of Steady State Kinetic Patterns Observed for Substrate Interactions......54
Table 2.8 Summary of Product Inhibition Patterns and Inhibition Constants.............57
Table 4.1 Some Available X-Ray Crystal Structures for CDE Proteins......................102
Table 4.2 Kinetic Constants for the Steady State Reaction of Chlorite Dismutases........125
Table 4.3 Kinetic Constants for Dye-Decolorizing Peroxidases with Various Substrates.....136
Table 5.1 Steady State Kinetic Constants for the Reactions of DaCld and Peroxidases with Phenolic Cosubstrates.................................................................156
Table 5.2 UV/Visible Absorbances for Stable and Intermediate States of DaCld and Well-Characterized Heme Peroxidases.........................................................158
Table 6.1 pH Dependence of Kinetic and Equilibrium Constants for Cyanide Binding to WT DaCld.............................................................................................................191
Table 6.2 pH Dependence of Kinetic and Equilibrium Constants for Cyanide Binding to R183Q DaCld.............................................................................................................192
Table 6.3 Comparison of Cyanide Association Second Order Constants between WT and R183Q DaCld and Other Heme Proteins..........................................................196
Table 7.1 UV/Visible Bands for CDE Family Proteins.............................................215
Table 7.2 Circular Dichroism Analysis of Holo- And Apo-HemQ (SaCld) with Comparison to DaCld......................................................................................................................217
Table 7.3 Heme and Porphyrin Affinities for HemQ (SaCld) and Related Heme Binding Proteins.................................................................................................................223
Table 7.4 Peroxidase Activity of HemQ (SaCld) and Relevant Heme Proteins.............224
Table 7.5 Catalase Activity of HemQ (SaCld) and Relevant Heme Enzymes

Table 7.6 Ligand Binding Results for S. Aureus HemQ (SaCld) and D. Aromatica Cld

Table A.1 Rate Constants, Apparent Rate Constants, and Coupling Ratios Measured in the Presence of the Given Compounds

Table A.2 Fitted Rate Constants For FAD-OOH Formation ($k_{FAD-OOH}$) and Conversion to Oxidized FAD ($k_{FAD}$) and Hydroxylamine Product Detection for Various Compounds in the Absence or Presence of 5 Mm L-Orn

Table A.3 Steady State Kinetic Parameters

Table B.1 Steady State Kinetic Parameters For Wt And Mutant DaClds for their Chlorite Dismutase (Top) and Peroxidase (Bottom) Reactions At Ph 6.8 And 4 °C

Table B.2 UV-Visible Absorbance $\lambda_{max}$ Values (Nanometers) for WT And Mutant DaClds at pH 6.8 and 10

Table B.3 Comparison of Fe-Co Vibrational Frequencies for Several Heme Proteins that were used to construct Figure 6.16 in the chapter. The frequencies are reported for pH 6.8 unless otherwise indicated. The frequencies for Cld-13CO complexes are in parentheses

Table B.4 Maximal $K_d$ (Micromolar) Values for WT, R183K, R183W, and R183A with Ligands Demonstrating their Varying Affinities at pH 7

Table B.5 Redox Potentials of Several Heme Proteins
Microbial Reaction to Oxygen in the Atmosphere; the Great Oxidation Event

The evolution of life on earth is a complex and fascinating topic. Over time the organisms that inhabit it have undertaken numerous transformations, some large and obvious, others small and subtle, to survive in an ever-changing environment. The biochemical reactions and processes that make life possible have, by no means, been untouched by Nature's selective pressures. The introduction of oxygen into the earth's atmosphere is perhaps one of the greatest pressures organisms have ever faced and was a critical turning point for life on earth. It is a topic that has been well discussed and examined by many, and will likely continue to be a topic of great interest. Life on earth is believed to have originated some 3.8 billion years ago consisting primarily of heterotrophic bacteria that thrived in an environment rich in reducing equivalents like hydrogen, hydrogen sulfide, and methane using carbon dioxide and sulfate as ultimate electron acceptors. This living milieu sufficed for approximately 2 billion years until an organism emerged with the ability to harness light to extract electrons from the vast amount of earth's water to produce the waste product oxygen. This chemical reaction, performed by cyanobacteria, is absolutely necessary for the complexity of life as we know it today. This so-called "Great Oxidation Event" led to an atmosphere abundant with O₂, but this was however not all positive. When oxygen is reduced to water it goes through multiple one electron reduced intermediates known as reactive oxygen species (ROS), shown below in italics:

\[ \text{O}_2 \rightarrow \text{O}_2^* \rightarrow \text{H}_2\text{O}_2 \rightarrow \text{OH}^* \rightarrow \text{H}_2\text{O} \]

These ROS, superoxide (O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl radicals (OH*), can be detrimental to the function of proteins, lipids, and DNA and are likely the causative agents of many diseases. Organisms that had previously thrived were forced to evolve systems to harness the powers of O₂ while limiting the production of ROS, to develop ways to properly detoxify the
harmful molecules, retreat to oxygen free environments, or perish. Those that were able to evolve required the appropriate resources and development of mechanisms necessary to utilize and control this powerful molecule, as a result those species became stronger and their biochemistry more complex.

**Redox Cofactors Become Valuable Commodities**

After the “Great Oxidation Event”, changes in microbial biochemistry occurred in several ways. It is believed that oxygen added to the diversity of secondary metabolites with the introduction of thousands of new reactions.\(^1\) The greatest changes likely occurred when organisms began to respire oxygen; giving greater yields of energy than ancestral anaerobic pathways. In addition, organisms began detoxifying and removing the harmful ROS formed during aerobic respiration, bettering their chances for survival. The biomolecules needed for these processes required the ability to easily transfer electrons and carefully react with kinetically inert oxygen. Redox cofactors like flavin and heme, as well as metals like iron and copper are well suited for this and instantly became valuable commodities. Nature has since evolved complex mechanisms for the acquisition, synthesis, utilization, and/or storage of those essential redox reactive molecules. This thesis describes two examples that focus on understanding those mechanisms and how they have evolved.

The first study is of a flavin-dependent enzyme, L-ornithine monooxygenase (OMO), which activates molecular oxygen to hydroxylate its amine substrate. This enzyme has a kinetic mechanism and protein structure suited to specifically hydroxylate L-ornithine without wasting valuable electrons or producing toxic ROS, like hydrogen peroxide. The reaction is also critical for siderophore biosynthesis; siderophores are low molecular weight iron-chelating molecules critical for microbial iron acquisition and storage. The work described will illustrate how microbes have responded to increased environmental oxygen in two ways: the first by evolving chemical reactions to utilize it safely and the second by synthesizing complex secondary metabolites capable of acquiring and storing essential iron.
The second study is of the bacterial heme-dependent enzyme chlorite dismutase (Cld). These proteobacterial enzymes have recently evolved the ability to convert chlorite ($\text{ClO}_2^-$) to chloride ($\text{Cl}^-$) and oxygen ($\text{O}_2$), a means of detoxifying the end product of perchlorate ($\text{ClO}_4^-$) respiration. Fundamental aspects of the dismutase reaction will be presented and discussed with a focus on understanding how the enzyme’s active site structure dictates its function. Similar heme enzymes, like peroxidases and catalases, have evolved to detoxify and utilize ROS and biochemical comparisons to Cld will be analyzed. Additionally, non-perchlorate-respiring bacteria also have genes annotated as Cld and their function in these organisms is not believed to be dismutation, but rather is involved in heme biosynthesis. The chlorite dismutase story is a good example of how Nature continues to utilize redox cofactors and protein active sites to perform novel reactions. The acquired ability to dismutate chlorite by Cld likely imitates evolutionary change that occurred after the introduction of oxygen into the environment and this work is another good representation of how selective pressures force microbial evolution and alter an organism’s biochemistry.

**Biological Relevance of Microbial Evolution**

Microbes continue to evolve today in the changing environment just as they did billions of years ago after the “Great Oxidation Event”. The biological consequences of microbial evolution are critical to understanding and sustaining life. Microbes regulate and manage redox cofactor uptake, synthesis, storage, and utilization with mechanisms that are often distinct from humans.\textsuperscript{4-7} This is an area where a thorough understanding of these processes can help in the development of methods to combat these often pathogenic species. In addition to understanding the unique redox regulatory mechanisms of microbes, a fundamental comprehension of their evolutionary process will help to anticipate conferred antimicrobial resistance and potentially exploit weaknesses for future development. To achieve such understanding requires detailed biochemical analysis at the cellular and molecular levels. Studies on enzymes like L-ornithine monooxygenase and chlorite dismutase, presented here and elsewhere, are a critical part of this fundamental comprehension and contribute greatly to our understanding of microbial life. The
work presented lays a foundation for development of antimicrobials targeting redox cofactor uptake, synthesis, storage, and utilization a potentially clean and fatal target.

**Organization of the Thesis**

The ten chapters of this thesis highlight research focusing on aspects of each of these protein families. Each in some way highlights Nature’s response to oxygen in the environment; whether it is through the adaption of enzymes to react with and respire molecules like oxygen or perchlorate or through the regulated synthesis, acquisition, and storage of essential redox cofactors, like iron and heme. Chapter 1 introduces iron trafficking and the potential for antimicrobial development of its inhibition. This creates a biological relevance for studying a member of the biosynthetic machinery required for iron acquisition; L-ornithine monooxygenase (OMO). This chapter also introduces flavin chemistry and flavin-dependent monooxygenase enzymes. Chapter 2 is the initial characterization of L-ornithine monooxygenase from the pathogenic fungus *Aspergillus fumigatus*. It is a primarily steady-state kinetic study aimed at determining the kinetic mechanism of N-hydroxylating monooxygenases. Chapter 3 is an investigation of the spectroscopic flavin intermediates formed during the reaction and their transient kinetics that supports and agrees with the steady-state kinetic results. Appendix is included for immediate reference and interested readers, though the author of this thesis did not conduct the primary experiments. It includes further work towards understanding the activation of oxygen by OMO and how non-substrate molecules appear to play a unique role in that reaction.

Chapter 4 shifts to work on chlorite dismutase (Cld) and begins with a comprehensive review of the Chlorite dismutase, Dye decolorizing peroxidase and EfeB (CDE) protein family. It summarizes sequence and structural information of Cld’s and their relations to other heme enzymes. It also tells the relatively brief history of studies on Cld and its reaction mechanism. Appendix B and Chapters 6 and 7 pertain to studies of Cld from *Dechloromonas aromatica* (DaCld). Appendix B is included as a background reference, towards which the author of this thesis made minor contributions. It shows spectroscopic and reactivity studies of distal pocket mutations of Cld from *Dechloromonas aromatica*. The model described in the appendix is cited.
and used throughout this. Chapter 6 investigates the chemistry and reactivity of DaCld with several oxidants, including hydrogen peroxide, and reveals unique pH-dependent reactivity that provides insight into the poorly understood reaction with chlorite. The next chapter, 7, is a short study on the pH-dependent kinetics of cyanide binding to DaCld. Cyanide is an important ligand for understanding the distal pocket organization of heme enzymes and this kinetic study contributes to existing Cld and cyanide information. Chapter 8 contains biochemical and microbiological analysis of the annotated chlorite dismutase from Staphylococcus aureus. Characterization of the purified protein in correlation with studies of the genetic knockout presents a framework for determining the unknown function of this protein. Lastly, chapter 9 provides a succinct summary of the results presented in this thesis. Altogether, the thesis spans 5 years of research on two fascinating protein systems that play a significant role in prokaryotic evolution and redox biochemistry.

Preface References


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ABBREVIATIONS

5c – five coordinate
6c – six coordinate
ABTS – 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
Arg – arginine
BSA – bovine serum albumin
BVMO – Baeyer-Villiger monooxygenase
CcP – cytochrome c peroxidase
CD – circular dichroism
CDE – chlorite dismutase, DyP-type peroxidase, EfeB)
CIP – Coprinus cinereus peroxidase
Cld – chlorite dismutase
Cpd I – compound I (ferryl porphyrin pi cation radical)
Cpd II – compound II (ferrly oxo)
Cpd III – compund III (ferrous superoxide)
Cpd ES – compound ES (ferryl Trp/Try radical
CHMO – cyclohexanone monooxygenase
CPDH – coproporphyrinogen dehydrogenase
CPO – coproporphyrinogen oxidase
CT – charge transfer
3,4-DOHB – 3,4-dihydroxy benzoate
DaCld – Dechloromonas aromatica chlorite dismutase
DMSO – dimethyl sulfoxide
DNA – deoxyribonucleic acid
D-Orn – D-ornithine
DyP – dye-decolorizing peroxidase
EDTA – ethylenediaminetetraacetic acid
EPR – electron paramagnetic resonance
FAD(H) – flavin adenine dinucleotide
FADH – reduced anion flavin adenine dinucleotide
FAD-OOH – C4a flavin hydroperoxide
FAD-OH – C4a flavin hydroxide
FMN – flavin mononucleotide
FMO – microsomal flavin containing monooxygenase
Glu – glutamine
His – histidine
HRP – horseradish peroxidase
HRPC – horseradish peroxidase isoenzyme C)
HS – high spin
IS – internal standard
KatG – bacterial catalase peroxidase
L-Arg – L-arginine
Leu – leucine
LFER – linear free energy plot
LiP – lignin peroxidase
L-Lys – L-lysine
L-Orn – L-ornitine
L-Orn-OH – N\textsuperscript{5}-hydroxy-L-ornithine
LS – low spin
MALDI-TOF – matrix-assisted laser desorption ionization-time-of-flight
MnP – manganese peroxidase
MWCO – molecular weight cutoff
NADPH – reduced nicotinamide adenine dinucleotide phosphate
NADP$^+$ – oxidized nicotinamide adenine dinucleotide phosphate

 NdCl – Ca. N. defluvii chlorite dismutase

 NMO – amine hydroxylating monooxygenase

 NPRB – non perchlorate-respiring bacteria

 NRPS – non-ribosomal peptide synthase

 NwCl – N. winogradskyi chlorite dimutase

 OD – optical density

 OMO – $N^5$-L-ornithine monooxygenase

 P450 – cytochrom P450 monooxygenase

 PAA – peracetic acid

 PDB – protein data bank

 PHBH – para-hydroxy-benzoate hydroxylase

 Phe – phenylalanine

 PKS – polyketide synthase

 PMSF – phenylmethanesulfonylfluoride

 $p$OHB – para-hydroxy-benzoate

 PPIX – protoporphyrin nine

 PPO – protoporphyrinogen oxidase

 SMO – siderophore-associate monooxygenase

 PRB – perchlorate-respiring bacteria

 ROS – reactive oxygen species

 rR – resonance Raman

 SaCl – Staphylococcus aureus chlorite dismutase

 SAM – S-adenosyl-L-methionine

 SCV – small-colony variant

 SDS-PAGE – sodium dodecylsulfate polyacrylamide gel electrophoresis

 Sec – secretory pathway

 SHE – standard hydrogen electrode

 SVD – singular value decomposition
Tat – twin-arginine translocation
TIGR – the institute for genomic research
TMAU – trimethylaminuria
TSB – tryptic soy broth
TSA – tryptic soy agar
UPLC-MS – ultra-performance liquid chromatography mass spectrometry
UV/Vis – UV-visible
WT – wild-type
CHAPTER 1
SIDEROPHORE BIOSYNTHESIS REQUIRES A FLAVIN-DEPENDENT MONOOXGENASE; A POTENTIALLY CLEAN ANTIMICROBIAL TARGET

Abstract – Iron is essential for the survival of most organisms. Microbial iron acquisition depends on multiple, sometimes complex steps, many of which are not shared by higher eukaryotes. Depriving pathogenic microbes of iron is therefore a potential antimicrobial strategy. The following describes general elements of microbial iron acquisition and some current and proposed work on their medicinal inhibition. This includes inhibition of siderophore biosynthesis; a specific set of microbial reactions critical for iron acquisition and storage. One essential enzyme for this process is a flavin-dependent monooxygenase. Prior to the work presented in this thesis, these siderophore-associated monooxygenases (SMO’s) were poorly characterized and their mechanism only speculated based on enzymes of similar function and primary sequence alignments. As a background for the mechanistic study of the SMO’s, a review of flavin-dependent monooxygenases and their mechanisms is presented. Understanding the molecular steps involved in catalysis will help facilitate development of future antimicrobials aimed at iron trafficking.

1.1 Iron Trafficking as an Antimicrobial Target
The bottom of the food chain is a marvelously inventive place. The diverse and changing environments that microbes inhabit, coupled with the perils of unicellular existence, demand certain resourcefulness. Many are aware of the special challenges posed by the need to acquire iron: an absolutely essential but highly insoluble metal in most biota, and a jealously protected
one inside the human body. Microbial systems for Fe uptake and trafficking are consequently
devitably or lethal, offering a means for limiting microbial growth. The potential for medicinally
impeding Fe metabolism is a commonly, if sometimes uncritically, cited justification for in-depth
biological studies of Fe acquisition. In fact, derailing the Fe supply train, while plausible as an
antimicrobial strategy, is still largely untested in practice. The following summarizes several
components of Fe trafficking pathways that are currently being pursued as or have the potential
to serve as promising antimicrobial targets.

1.1.2 Existing antibiotics and current needs

New antimicrobial targets are needed as infectious diseases spread globally and as
resistance to available antibiotics grow.\textsuperscript{1-3} The World Health Organization cites infectious
diseases as the current number one killer of children and young adults around the globe. In the
hour that one might spend reading this thesis, over 1,500 people will die of infectious diseases,
over half of them children under five. This amounts to more than 13 million deaths a year: one in
two deaths in developing countries. Disabling illnesses are expected to add substantially to the
human and economic toll of infection.\textsuperscript{4} As recent news stories have highlighted, dangerous
diseases are increasingly transported across borders. In 2000, \textasciitilde346\% of newly identified US TB
cases originated in other countries.\textsuperscript{5} The spread of TB has been hastened by the lack of public
health surveillance for this disease and by the concurrent HIV/AIDS epidemic. Current first-line
treatments for many infectious diseases are often many decades old, and resistance to many of
them is widespread. Even newer antibiotics such as itraconazole (2001) and voriconazole (2002)
have already been compromised by resistance. \textit{Klebsiella pneumonia}, for example, is a
ubiquitous, clinically important, opportunistic Gram negative pathogen that is often associated
with infections acquired in hospital settings.\textsuperscript{6,7} In 2003, 20.6\% of \textasciitilde1,000 hospital isolates of \textit{K.
pneumoniae} were resistant to 3rd generation cephalosporins, representing a nearly 50\% increase
in nonsusceptible \textit{K. pneumoniae} between 2002 and 2003 alone.\textsuperscript{8} Not only new antibiotics, but
likely antibiotics that work in new ways (vide infra), are needed to stem the tide of infectious agents, resistant or otherwise.

Successful antibiotics from penicillin to quinine have traditionally targeted facets of microbial biochemistry that are essential for and, as much as possible, unique to the pathogen. Most existing antimicrobial strategies fall into one of four categories. The first three target enzymes involved in (1) cell-wall biosynthesis; (2) protein synthesis; and (3) nucleic acid metabolism and repair. Category (4) antimicrobials interfere with membrane integrity via, for example, the use of membrane permeable ion transporters (ionophores) that upset the cellular osmotic balance. Each of these strategies clearly aims at interrupting an essential cellular process. Of all of these, (1) has perhaps the greatest potential for pathogen specificity, at least toward bacterial pathogens, as the peptidoglycan component of the cell wall is unique to them. Each of the others has the potential for cross-reacting with human or animal host cells that depend on similar biochemical pathways, with consequent toxicity. In fact, many antibiotics in classes (2)–(3) are especially lethal to rapidly proliferating cells and consequently have anticancer activity. Interestingly, rapidly proliferating cells also have amplified iron requirements. Angiogenesis inhibition, a powerful anti-cancer strategy in principle if not in current therapeutic application, starves growing cells of critical supplies, not the least of which is iron.

In contrast to these established antimicrobial approaches, interruption of Fe trafficking is a plausible but still largely unproven means of clinically controlling pathogens. However, two classic anti-malarials provide important proof-of-concept examples, in which successful drugs target the Fe trafficking and storage mechanisms of *Plasmodium falciparum*. Quinine is a natural product quinoline found in the bark of the cinchona tree. It and its derivatives have been used as malaria remedies since the 17th century. Artemisinin is a sesquiterpene lactone with an activated peroxide, and likewise a plant product (from *Artemisia annua*). It has been in use as an antimalarial in China since at least the fourth century. Though their precise mechanisms of action are still occasionally debated, both indisputably interfere with *P. falciparum*’s unusual Fe trafficking pathway, in which hemoglobin-derived heme is taken up and stored as crystalline hemin. Direct use/storage of heme makes sense in light of the malaria parasite’s preferred habitat
inside the hemoglobin-packed human erythrocyte. Quinine and related compounds are believed to disrupt the stacking and consequently prevent crystallization of heme molecules. The Fe(II) in ferroheme can reductively cleave the artemisinin peroxide bond, generating a radical species that cross-links to and interferes with metabolism and storage of the ferriheme.

1.1.3 Fe trafficking: major whistle-stops

Artemisinin and quinine likely interfere with a metabolic endpoint (iron storage). A typical iron acquisition pathway, however, has several steps, any of which might in principle serve as a potential target. A tremendous amount of work, particularly focusing on Gram negative bacteria, has been done in this area; several relevant steps are summarized only in the most schematic form, below:

(1) **Ligand exchange** A pathogen will generally encounter iron in a chelated form in which the ligand is either a protein (e.g., the transport proteins transferrin or lactoferrin in plasma or milk, respectively) or the porphyrin ring. As a first step toward acquisition, many bacterial and fungal pathogens secrete a siderophore or hemophore that binds iron (III) or heme with high affinity. A hemophore may act in conjunction with proteolytic enzymes, acting to release heme from a protein (e.g., hemoglobin). Exchange of the iron into the pathogen derived ligand allows for its specific recognition and uptake. The human immune system already defends against this step of the trafficking pathway when infection is sensed, by mounting an “iron blockade”: the removal of transferrin-bound Fe(III) from circulation and into intracellular storage within ferritin, where it is presumed to be unavailable to extracellular pathogens. Direct usage of ferritin iron by intracellular pathogens is undescribed in vivo, but possible.

(2) **Diffusion** Once repackaged in a siderophore or hemophore, the newly speciated iron must diffuse back to the microbe. Mammalian biochemistry has again devised a countermeasure against the diffusion step, at least for catecholate-containing ferrisiderophores. As an example, the mammalian immunoprotein siderocalin, first identified as neutrophil gelatinase-associated lipocalin, picks off catecholate-dependent ferrisiderophores before their safe return to the pathogen. The ferrisiderophores are entirely encompassed by this protein, which eventually is
excreted by the host. Siderophore-binding proteins aside, it is unclear how pathogens contend with the apparent inefficiency of Fe-acquisition systems that depend on diffusion of secreted molecules back to themselves. Under conditions of Fe-stress, which are expected to be common in most pathogenic milieu, microbes can secrete extremely large amounts of siderophore, even exceeding their own mass within a relatively short period of time. The importance of iron apparently justifies the metabolic investment.

(3) Specific recognition and membrane transport Bacterial ferrisiderophores are recognized and internalized by specific, Ton-B-dependent outer membrane receptors that couple to and are driven by the electrochemical gradient of the inner membrane of Gram negative bacteria. It is generally understood that siderophores and receptors have a 1:1 relationship, at least in Gram negative bacteria, though exceptions to this rule have been identified. Heme or hemophores are likewise recognized and internalized by specific outer membrane receptors. Some organisms, notably some ancient eukaryotic protozoans, have outer membrane receptors for the direct endocytosis of human transferrin or hemoglobin. In each case, specific, high-affinity receptors at the outer membrane allow for the efficient uptake of the iron source and the rejection of other molecules. The importance of specific recognition has been demonstrated in several ways. In one dramatic example, it was shown that the siderophore mycobactin T is an avid growth promoter for Mycobacterium tuberculosis, its native producer. By contrast, mycobactin S, a siderophore from the congener species M. smegmatis, cannot be incorporated by and is moreover highly toxic to M. tuberculosis. Structural analyses showed that these large natural products were completely identical except for the handedness of one stereocenter. It has been suggested that bacteria use small structural differences in their siderophores as a means of outcompeting rivals for limited Fe. Indeed, laboratory growth media are often supplemented with siderophores or unnatural chelators that a particular species is incapable of metabolizing in order to create a simulated Fe challenge. EDTA is a well-known biocontrol additive in foods, due in part to the chelator’s avid association with Fe, which it sequesters in a form that is unavailable to nonsiderophore producing bacteria. In each case, Fe is made less bioavailable by binding it to a chelator that a particular organism cannot use.
(4) **Intracellular trafficking** Many events in the care and handling of iron after it traverses the outer membrane are less well-defined. The site and agents of iron reduction, the identities of intracellular Fe(II) transporting ligands, and the timing of Fe reduction with release from the siderophore are not well understood. Iron encounters ferritin as Fe(II), becoming oxidized as part of the mineralization process. By the same token, iron is reduced as it is mobilized from the ferritin stores back into the cytoplasm.\(^{31}\) Hence, newly incorporated Fe(III) must eventually be reduced, and internal trafficking at least at some places must involve Fe(II). In the case of Gram negative bacteria, reduction could occur in either the periplasm or cytoplasm. If reduction occurs in the cytoplasm, either the ferrisiderophore (more likely) or Fe(III) alone may cross from the periplasm through the inner membrane via an ATP-powered ABC transporter. Once in the cytoplasm, release of Fe(III) from the siderophore could occur via enzymatic cleavage particularly for very tightly bound Fe(III)/siderophore complexes. The iron from ferrienterobactin and fusarinines, for example, is released via hydrolysis of their ester backbones in the cytoplasm.\(^{32,33}\) How the Fe(III) is subsequently reduced and transported is unknown. Similarly, in organisms that directly utilize heme, opening of the porphyrin macrocycle is catalyzed in the cytoplasm by heme oxygenase.\(^{34,35}\) In that case, the released iron would have to be reduced or, if already reduced, maintained stably as Fe(II).

Alternatively, reduction and consequent release of Fe(II) from a siderophore for Gram negative species could occur in the periplasm, followed by movement through an inner membrane, GTP-dependent transporter, FeoB. Transport could occur for Fe(II) alone or in conjunction with an Fe(II) binding ligand.\(^{36,37}\) Whether in the periplasm or cytoplasm, Fe(II) chelators could have multiple roles. First, they could protect Fe(II) from rapid reoxidation. Second, the Fe(II)/chelator binding energy could help to "pay" the energy toll for reducing the ferrisiderophore iron. Ferrisiderophores can have fairly negative reduction potentials, requiring a significant driving force to reduce the bound iron. Finally, such chelators could be important for transport into or proper trafficking within the cytoplasm.\(^{37}\)

(5) **Storage** A large number of organisms, including bacteria, plants, and animals, store iron in ferritins as crystalline iron oxide (ferrihydrite). Fungi are a major exception, storing iron
instead inside intracellular siderophores. These may structurally resemble the secreted siderophores but with modifications that prevent their cellular export. Other exceptions include several eukaryotic protozoa, including *P. falciparum*, *Trypanosoma*, and *Leishmania sp.* These and other blood-feeding pathogens may take up heme directly, storing iron inside vacuoles as crystalline hemozoin. The details of iron storage in many non-ferritin-producers are not well understood.

1.1.4 Possible targets

Fe trafficking could in principle be chemically interrupted at any of the steps outlined above. A large fraction of known antibiotics and drugs in general act as enzyme inhibitors. Enzyme inhibition is amenable to established methods of rational drug design, including the development of structure-activity relationships via systematic variation of the structures of inhibitors. We will consequently consider enzymatic targets first.

1.1.4.1 Siderophore biosynthesis

Siderophore biosynthesis stands out as the central enzyme-mediated event of Fe trafficking by many species. Eliminating siderophore production would block both the release of iron from host molecules (step (1) above) and the pathogen’s mechanism for transporting Fe(III) through the outer membrane (step (3)). Many organisms depend primarily or entirely on siderophore-mediated mechanisms for both processes. As a consequence, genetic knockouts of siderophore biosynthesis genes in several organisms severely restrict growth and/or virulence. Other organisms appear to be less impaired by similar gene knockouts, reflecting their ability to compensate for the lost siderophore in other ways: e.g., production of a second siderophore, use of other molecules in the host environment for mediating Fe entry as “xenosiderophores”, uptake of heme (if available), etc.

Organisms that rely on a single siderophore for one or more essential functions are likely to be especially vulnerable to interference with siderophore biosynthesis. For example, fungi often
use variants of the same siderophore to recruit iron, transport it through the cell wall, and store it
intracellularly (step (5)). In *Mycobacteria*, water-soluble mycobactins scavenge iron from the
environment; more lipophilic mycobactin variants remain mounted in the cell wall. These may
similarly serve as an iron storage reservoir, or they may have other yet unknown biological
functions. As a consequence, certain mycobacterial and fungal species may be particularly
susceptible to chemical interruption of siderophore biosynthesis.

Pioneering work has focused on inhibition of the biosynthesis of aryl-capped
siderophores, including mycobactin T in *M. tuberculosis*. The mycobactin scaffold is assembled
by a mixture of non-ribosomal peptide synthetases (NRPSs) and polyketide syntheses (PKSs).
Multi-modular NRPS/PKS enzymes catalyze the biosynthesis of a large number of siderophores,
antibiotics, and other natural products in bacteria, fungi, and plants. “Modular” refers to the
organization of the enzymes into a series of catalytic units, each of which activates a particular
monomer and carries out a single initiation, chain elongation, or termination step. NRPSs use
amino acids to build peptidic products without an RNA template, while PKSs synthesize
dalkketides in a series of carbon condensations similar to those in fatty acid biosynthesis.

Monomers are activated for chain-incorporation by ATP-mediated adenylation (formation of
monomer-adenosine monophosphate (AMP) conjugates). Both NRPSs and PKSs use the
nucleophilic sulfhydryl at the end of a Coenzyme-A-derived pantetheinate arm as the “handle”
that grasps the monomer or growing product.

Successful inhibition strategies to date have targeted aryl acid activating enzymes
including MbtA, a NRPS that initiates assembly of the mycobactin backbone at its aryl acid
(salicylate) head group. Solution of the crystal structure of DhbE, a so-called
“standalone” adenylation domain that recognizes and activates 2,3 dihydroxybenzoate (DHB) for
incorporation into bacillibactin, set the stage for this work. As an isolated enzyme and not a
subdomain of a much larger NRPS like MbtA, detailed structural characterizations of the enzyme
and its DHB-AMP complex were feasible. The DhbE structures were used to build homology
models for MbtA, which shares 76% identity with DhbE at the active site. An added, critical
observation came from work with mechanistically related adenylation enzymes, including amino
acid tRNA synthetases. These were shown to bind their acyl-AMP intermediates 2–3 times more tightly than their carboxylic acid and ATP substrates.\textsuperscript{59}

**Figure 1.1** a. Mycobacterial siderophores. The salicylate-derived aryl headgroup activated by MbtA is shown in light blue throughout. b. Reaction catalyzed by MbtA. ArCP is the aryl carrier protein to which MbtA transfers its adenylated substrate. c. Sal-AMP is the native product of MbtA. d. Various analogs to Sal-AMP were designed and tested as enzyme inhibitors and antitubercular agents.

By analogy, the MbtA inhibitor 5′-O-[N-salicylsulfamoyl] adenosine (Sal-AMS) was designed as a catalytically inactive analog of the enzyme product salicyl-AMP, with a sulfamoyl group in place of the phosphate linker (Figure 1.1 c, d).\textsuperscript{60} The compound was evaluated for its activity against MbtA as well as the enzymes YbtE and PchD, NRPSs which adenylate salicylic acid as part of the biosynthesis of yersiniabactin (by *Yersina pestis*) and pyochelin (*Pseudomonas aeruginosa*), respectively.\textsuperscript{61} Sal-AMS was shown to be a tight-binding inhibitor, with half maximal enzyme inhibitory concentration (IC\textsubscript{50}) values ranging from 10.7 to 14.7 nM. Inhibition of MbtA and YbtE by Sal-AMS was competitive with respect to ATP and noncompetitive with salicylate, indicating that Sal-AMS and ATP bind the same enzyme form and that inhibition
occurs at the initial adenylation step. Sal-AMS was further shown to inhibit production of mycobactin by *M. tuberculosis* and yersiniabactin by *Y. pestis*, respectively. Finally, the compound inhibited the growth of both organisms under iron-limiting conditions, with inhibitory concentrations inhibiting bacterial proliferation to 1% (MIC<sub>99</sub>) equal to 2.2 µM (*M. tuberculosis*) and 51.2 µM (*Y. pestis*).

Sal-AMS itself is a modestly effective lead compound that provides an important proof-of-concept. More potent inhibitors based on Sal-AMS, with better chemical and pharmacokinetic properties, were subsequently developed in a series of structure-activity relationship studies carried out by Aldrich and coworkers. Initial work focused on the critical phosphate “linker” portion of Sal-AMP that was replaced with a series of groups of varying charge and susceptibility to hydrolysis. Stability under biological conditions is particularly important, as the 50-O-(sulfamoyl)adenosine hydrolysis product is broadly cytotoxic (i.e., via mechanisms independent of MbtA). β-ketosulfonamide linked compounds were found to be equally effective at inhibiting MbtA while more resistant to hydrolysis. Using Sal-AMS as a template, further variations in its glycosyl, aryl, and nucleobase domains were examined systematically. Only modifications at the nucleobase led to improved inhibition. The DhbE/DHB-AMP crystal structure revealed hydrophobic protein-purine interactions with poorly aligned hydrogen bonding, suggesting that modifications at the nucleobase would be well tolerated. Indeed, Sal-AMS analogs with substitutions at the purine C-2 (2-phenylamino-, phenylethyl-, and 2-phenyl-Sal-AMS, Figure 1.1) were more potent than the parent compound, with apparent inhibition constants of 0.94, 0.40, and 0.27 nM, respectively. The best compound, 2-phenyl-Sal-AMS, had 24 times the potency of the lead compound (Sal-AMS) and a high binding affinity (*K<sub>d</sub> = 0.27 pM). This compound also had substantial in vitro antitubercular activity, with MIC<sub>99</sub> equal to 39 nM.

We are taking a somewhat broader approach by focusing on the enzymes that produce hydroxamate, the chelating portion of several siderophores. This is a strategy with many practical advantages. First, while siderophore scaffolds are structurally extremely diverse, most rely on one of three bidentate chelating moieties: hydroxamic acid, catecholate, or α-hydroxycarboxylic acid. Second, the chelating portions are clearly necessary for siderophore function. Third, the
Hydroxamic acids are not the strongest known chelators but they are specifically associated with the virulent forms of several organisms. This is ascribed to the hydroxamate's ability to evade siderophore-binding siderocalin, since this immunoprotein specifically binds catecholate dependent siderophores through cation-π interactions (vide supra). Finally, the monooxygenase and acyl transferase enzymes that produce hydroxamate are so-called “tailoring enzymes” that act independently of large NRPS or PKS complexes. They are consequently much simpler to prepare, study, and structurally characterize in atomic detail.

Biosynthesis of hydroxamate-dependent siderophores typically begins with hydroxylation of L-lysine or L-ornithine at the side chain amine (see Figure 1.2b). The hydroxylamine may be subsequently formylated or acylated by a GNAT-family acyltransferase, yielding the bidentate chelator. Acylation can also modify the siderophore’s solubility, depending on the nature of the acyl -R group (see Figure 1.1a). The doubly modified amino acid building block is then woven into the siderophore backbone by NRPS-dependent or NRPS-independent pathways. The latter couple activated (phosphorylated) carboxylic acids to the α-amine of the modified lysine or ornithine, using conventional non-modular enzymes.

Such pathways were recently shown to be much more widespread than ever expected, having been identified in greater than 80 diverse bacteria. Alternatively, hydroxamate is generated by formation of an amide bond between the hydroxylated amine and a carboxylic acid via NRPS -dependent or -independent pathways, as in the biosynthesis of fusaridine-C (Figure 1.2). Finally, hydroxamate can be formed by direct hydroxylation of an amide. Though chemically more difficult due to the high pKₐ/weak nucleophilicity of the amide -NH, such a route has nonetheless been proposed for mycobactins (Figure 1.1), where hydroxylation and acylation are believed to be final biosynthetic steps. Each of these biosynthetic routes involves an amine or amide hydroxylation step catalyzed by a flavin-dependent monooxygenase. Flavin monooxygenases consist of at least four families, each with distinct chemical mechanisms and primary structural features, as discussed below. The siderophore-associated monooxygenases (SMOs) constitute their own family. In higher eukaryotes, monooxygenation is a nonessential step in amine catabolism. Hence, SMOs may be a particularly clean antimicrobial target.
1.1.4.2 Other enzymatic targets

Once retrieved by the pathogen, the iron from siderophores must be released, either by reduction to Fe(II) or by cleavage of the siderophore backbone (e.g., by a specific esterase, vide supra). These enzymatic steps are so far unexplored targets. In the case of the presumed siderophore reductase, this is likely due to the still nebulous understanding of siderophore reductases even in otherwise well-characterized organisms. Many flavin reductases are known to reduce iron and iron-chelates somewhat non-specifically in vitro. These enzymes have been classified as so-called two-component systems, in which one enzyme reduces and releases flavin and a second retrieves it for reduction chemistry.\(^{71-73}\) In the case of siderophores, the flavin reductase is believed to reduce and release flavin, which can react directly with chelated Fe(III). More recently, the \([2\text{Fe}-2\text{S}]\)-cluster containing fhuF gene product was isolated from E. coli and shown to reduce Fe(III) bound to ferrioxamine B. This enzyme is flavin-independent, and consequently represents an alternative to the two-component systems. FhuF production is upregulated via a Fur-binding promoter under conditions of iron stress.\(^{74}\) Under the same
conditions, however, a thuF knockout was not growth restricted, suggesting that E. coli at least has additional means of mobilizing iron from the several siderophores it is known to use.

1.1.4.3 Non-enzymatic and non-siderophore targets

Iron trafficking systems have many essential, nonenzymatic components. Perhaps most obvious among these are the highly specific cell-surface siderophore and hemophore receptors. The siderophore/receptor interaction has already been exploited for “Trojan Horse” drug delivery, in which an antibiotic or cytotoxic agent is chemically tethered to a siderophore and the complex is actively internalized by a specific pathogen. Targeting Fe uptake itself, a small molecule that specifically binds to and thereby blocks a siderophore or heme uptake receptor could be used to more simply block iron entry into cells. Such an approach might be expected to suffer from built-in redundancies in uptake pathways, such as the ability of an organism to use multiple siderophores, or from the ability of the organism to rapidly evolve improved receptors.

Finally, siderophore/hemophore-independent steps of the Fe-trafficking process could conceivably make good antimicrobial targets, provided they are well chosen and as pathogen-specific as possible. For example, recent work has shown that metal trafficking may limit growth of pathogens that live inside mammalian macrophages. The natural resistance-associated macrophage protein (NRAMP) family consists of the transmembrane proteins Nramp1 and Nramp2. Nramp1 is expressed exclusively in cells of the immune system. It is recruited to the membrane of the phagosome as an invading pathogen is engulfed. Mutations in Nramp1 have long been known to genetically predispose an individual to certain diseases, including leprosy and tuberculosis. It was more recently shown that the Nramp proteins are divalent cation transporters, and that Nramp1 may be involved in moving phagosomal cations back to the cytoplasm for general use. By controlling divalent cation concentrations (Fe(II), Mn(II), and possibly Zn(II)), Nramp1 may also regulate the inter-phagosomal replication of bacteria. Interestingly, some phagosomal pathogens, including the protozoa (e.g., Leishmania sp.), depend on phagosomally available Fe sources but do not produce siderophores. These organisms may
be particularly amenable to highly selective, chemical interference with their Fe metabolic pathways, provided that the phagosomal compartment can be successfully reached.\textsuperscript{80}

1.1.5 Outlook

Though microbial iron metabolism has been much studied and even co-opted in order to effect drug uptake, direct assaults on iron trafficking are few. The multiple, sometimes redundant pathways used by microbes to ensure the success of iron acquisition make this a potentially challenging area for the development of therapeutic antimicrobials. A particular cause for concern is the likelihood that, in the event that one supply train is blocked, another will be activated, or that existing genes will evolve to somehow compensate. Such concerns are common to all antimicrobial efforts, however, and should not preclude further investigations. Initial studies targeting siderophore biosynthesis suggest this could be a promising future direction.

1.2 Siderophore-Associated Monooxygenases

The explosion of gene sequencing data and the pursuit of understanding microbial iron trafficking mechanisms led to the discovery of a family of flavin-dependent enzymes involved in siderophore biosynthesis, they were deemed siderophore-associated monooxygenases or SMO’s and are part of a larger sub-family of \textit{N}-hydroxylating monooxygenases (NMO’s). The NMO family includes bacterial and fungal SMO’s and the yucca enzymes that are involved in hormone biosynthesis in plants and play a key role in development. The pathogenic fungus \textit{Aspergillus fumigatus} uses two siderophores, triacetylfusarinine and ferrichrome shown in \textbf{Figure 1.1a}, to acquire and store iron.\textsuperscript{82} The hydroxyl groups attached to the amine constitute the Fe chelating moiety of the siderophore, which is critical for virulence and cannot be synthesized without this first committed step. The reaction catalyzed by L-ornithine monooxygenase (OMO) converts the amino acid L-ornithine to hydroxy-L-ornithine, using nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen (O\textsubscript{2}) (\textbf{Figure 1.1b}); subsequent steps in the biosynthetic path include acylation and further reactions with a non-ribosomal peptide synthetase (NRPS) as
described earlier. Focusing on the enzyme’s primary sequence and its relation to other flavin-dependent enzymes, it was predicted that this family was most closely related to the Baeyer-Villiger monooxygenase (BVMO) and microsomal flavin monooxygenase (FMO) family of proteins; those described below as “bold” enzymes. As will be discussed, this contradicts the known high level of substrate specificity between SMO’s compared to the promiscuity in substrates between FMO’s and some BVMO’s. The critical nature of the gene and the unknown mechanism of reaction warrant mechanistic and structural investigation.

1.2.1 Significance of OMO for Virulence in Pathogenic Species

As stated before, the gene for a siderophore associated monooxygenase is critical for virulence among important pathogenic species like *Aspergillus fumigatus*, *Klebsiella pneumonia*, and *Mycobacteria tuberculosis*. Work in this thesis focuses on *A. fumigatus*’ gene *sidA* that codes for OMO. Genetic studies of the *sidA* knockout resulted in fungi unable to grow unless supplemented with siderophores, or were only partially restored when Fe was added to the medium. In virulence models, *sidA* deletion mutants of *A. fumigatus* were avirulent; thus the protein is essential for this process and supports the aspiration to chemically inhibit the gene product. Ultimately, the goal of the work is to understand the detailed chemical mechanism of OMO to better develop and test potential inhibitors that may one day become antifungal compounds.

1.2.2 Flavin-Dependent Monooxygenases and Control of Catalysis

Siderophore monooxygenases belong to a larger family of flavin-dependent enzymes that react with oxygen for a variety of different functions. The following introduces flavin chemistry and explains the mechanisms used by characterized flavin-dependent monooxygenases to perform efficient catalysis. This background gives a scientific context for the following three chapters focused on determining the kinetic mechanism of OMO.
1.3 Flavin Chemistry and the Monooxygenase Reaction

Flavin proteins come in all shapes and sizes and participate in a variety of critical biological functions. Those functions include dehydrogenation, electron transfer, DNA repair and many others as illustrated in Figure 1.3. The cofactor has without a doubt played a vital role in oxygen respiration and survival of many organisms in an oxygen rich atmosphere. An important aspect of flavin chemistry is that it can convert between the quinone (oxidized), semiquinone (1-electron reduced) and hydroquinone (2-electron reduction) oxidation states permitting it to perform a variety of reactions. Additionally, the 2-electron reduced flavin is able to react with oxygen, adding to its range of chemistry. Variability in the flavin structure between flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and riboflavin (Figure 1.3) as well as covalent and non-covalent interactions with its protein partner influences an enzyme’s function. The specific arrangement of amino acid residues around the flavin and the properties of those amino acids; like charge, size, polarity and hydrogen bonding capabilities among others modulate the redox potential of the cofactor and position substrates in an ideal environment for catalysis. The reaction of interest for work in this thesis is that of monooxygenation. These enzymes are classified as oxidoreductases acting on a pair of donors with NAD(P)H as one and with incorporation of one atom of oxygen into the other donor. This reaction is often divided into reductive and oxidative halves; where reduction of the flavin by NAD(P)H constitutes the first and O₂ activation and hydroxylation of substrate constitute the second. This is very risky reaction and requires a well-organized strategy to productively consume the reductive power from NAD(P)H and create a reactive oxygen species poised for hydroxylation. Without an acute mechanism, electrons are wasted and hydrogen peroxide is released. A detailed description of the reaction and the mechanistic strategies used by well-characterized flavin monooxygenase enzymes is discussed below.
1.3.1 Reductive Half Reaction

As stated before, FAD can undergo 1- or 2- electron reductions with reversible electron transfer across the N1 and N5 atoms of the isoalloxazine ring as shown in Figure 1.4. The negative charge is distributed and stabilized across this moiety, and the stability of this charge appears to have a strong effect on the redox potential of the flavin. The majority of FAD-dependent monooxygenases require NADPH for a 2-electron reduction.
1.3.2 Oxidative Half-Reaction

The ground state of oxygen has two unpaired electrons (triplet) and most organic molecules have no unpaired electrons (singlet), thus a reaction between the two would require a triplet-to-singlet conversion which is forbidden by the laws of quantum mechanics. Nature has overcome this problem by reacting through multiple single electron transfers so the number of unpaired electrons stays the same before and after each step. Reduced flavin is a very strong oxidizing agent (protein bound flavin redox potentials for $E_{\text{SQ/RED}}$ range -400 - -60 mV\textsuperscript{86} and is able to do a 1-electron oxidation of $O_2$, resulting in a superoxide anion $O_2^-$ and flavin semiquinone. This radical pair is illustrated below in Figure 1.5. Now that both molecules have one unpaired electron they are able to recombine to form the flavin hydroperoxide. The intermediate consists of a covalent bond between the C4a carbon of the flavin ring and an atom of oxygen; it is comparable to the organic peroxides used by synthetic chemists and those later discussed in this thesis with regard to reactions with heme. The peroxyflavin intermediate, herein referred to as C4a-(hydro)peroxyflavin, is believed to be protonated in water and in the absence of protein or a hydroxylatable substrate decays rapidly. The decay process is a direct result of
deprotonation at position N5 and heterolytic cleavage of carbon oxygen bond giving oxidized flavin and hydrogen peroxide.

![Diagram of FADH and related compounds]

**Figure 1.5** Reaction of reduced FADH\(^ \cdot \) with molecular oxygen through the radical cage intermediate, and formation of the C4a-(hydro)-peroxide intermediate. The two fates of the C4a intermediate are shown; oxidation to release HO\(_2\)\(^ \cdot \) or monoxygenation with substrate. Adapted from Mattevi.\(^{87}\)

Reactivity of the C4a-(hydro)peroxide varies depending on the enzyme structure and the substrate to be hydroxylated. It is able to act as either an electrophile or nucleophile depending on protonation state, where the pK\(_a\) is believed to be between 7-8 permitting protonation of deprotonation in biological systems.\(^{88}\) As illustrated below in **Figure 1.6**, the C4a-(hydro)peroxide (protonated) acts as an electrophile toward nucleophilic substrates like nitrogen- and sulfur-containing molecules. Alternatively, the C4a-peroxide (deprotonated) acts as a nucleophile toward electrophilic ketones. The flavin moiety of C4a(hydro)peroxide is extremely electron withdrawing, making the O-O bond rather polar and poised for heterolytic bond cleavage of the O-O bond giving a flavin hydroxide and hydroxylated substrate. To complete the catalytic
cycle the flavin hydroxide must undergo dehydration to reform oxidized flavin. The chemical steps the FAD-monooxygenase reaction are well understood, the challenge lies in understanding how an enzyme is able to accelerate these reactions and control reactive intermediates. Up to this date, many different flavin monooxygenases have been characterized through a variety of different biochemical methods and two mechanistic strategies have emerged. The following introduces those two classes and illustrates key facets of their “bold” or “cautious” strategies.

\[ \text{Flavin-C4a-Hydroperoxide (FAD-OOH)} \]

\[ \text{Flavin-C4a-Peroxide (FAD-OO-)} \]

**Figure 1.6** The C4a-(hydro)-peroxide Protonation States. The intermediate displays umpolung or polarity inversion and can act as a nucleophile, monoxygenase, or electrophile, Baeyer-Villiger monooxygenase. Adapted from Mattevi.\(^{87}\)

### 1.3.3 Mechanistic Strategies of Flavin-Dependent Monooxygenases

Two mechanistic classes of flavin-dependent monooxygenases, reviewed by Palfey et al, are annotated as having “Bold” or “Cautious” strategies.\(^{88}\) This classification is based on when and how the C4a-(hydro)peroxide is formed and stabilized prior to hydroxylation. All FAD-dependent monooxygenases catalyze the same type of reaction where a substrate, S, is hydroxylated to product, S-OH. There is high variation in the substrates hydroxylated and two
very different strategies are used to regulate specificity and efficiency. A combination of structural, kinetic and spectroscopic results has determined how each class of enzyme performs catalysis and those will be discussed briefly below.

1.3.3.1 “Cautious” Flavin-Dependent Monooxygenase

1.3.3.1.1 Reactions catalyzed and significance

The class of FAD-dependent monooxygenases utilizing the so-called “cautious” mechanism is composed primarily of bacterial aromatic hydroxylases. These enzymes are involved in the detoxification of soil and lignin degradation. They hydroxylate a variety of aromatic substrates and perhaps the best studied examples is para-hydroxybenzoate hydroxylase (PHBH). Another “cautious” enzyme, kynurenine monooxygenase, is found in eukaryotes. It hydroxylates the aromatic amine, kynurenine, as part of tryptophan degradation and plays a critical role in mammalian neurochemistry. Another group of eukaryotic aromatic hydroxylases are the Molecules Interacting with CasL or MICALS. These also play a critical part of mammalian neurochemistry and specifically in development; their absence or malfunction leads to motor skill defects. Although, the biological substrate is unclear, and some have argued that these enzymes are not hydroxylase rather oxidases that produce \( \text{H}_2\text{O}_2 \) as a signaling molecule, their structures are homologous to the soil bacteria enzymes. This mechanistic class of enzymes is functionally diverse, but have all been shown to some degree to share a similar mechanistic strategy. The majorities are highly specific for their nucleophilic substrates and rely on protein-substrate interactions to fulfill efficient catalysis.

1.3.3.1.2 Kinetic Mechanism

In general the monooxygenase reaction is considered to have three substrates and two products (\( \text{H}_2\text{O} \) is not necessarily considered a true product). The Cleland diagram for the “Cautious” mechanism is shown below in Figure 1.7, and in this case it is a Bi-Uni-Uni-Bi ping-pong reaction where substrate, \( S \), and NADPH binds randomly to the oxidized enzyme forming a
ternary complex. After flavin reduction, NADP⁺ disassociates and O₂ enters forming a second ternary complex, prior to formation of the reactive C4a-(hydro)peroxy intermediate and subsequent substrate hydroxylation. An oxygen-binding step has not been observed, though the reaction with O₂ is believed to occur irreversibly. Lastly, the hydroxylated product, S-OH, dissociates with H₂O regenerating the oxidized enzyme.

![Cleland Diagram for “Cautious” FAD-dependent monooxygenase](image)

**Figure 1.7** Cleland Diagram for “Cautious” FAD-dependent monooxygenase

A key feature of this strategy is that flavin reduction occurs ~10⁵ fold faster in the presence of S. It is believed that S is playing an “effector” role, since it is not required for the chemical reduction, yet increases the rate of reduction. Reduction can occur without S present, but happens very slowly. Thus, the enzyme only consumes NADPH if a hydroxylateable substrate is present and proceeds to reactivity in a “cautious” or conservative manner. The second key mechanistic feature that strongly distinguishes “Cautious” from “Bold” is that NADP⁺ leaves the ternary complex prior to O₂ activation and plays no secondary role in catalysis. Reduced flavin then reacts with O₂ to form the C4a-(hydro)peroxy intermediate with a rate constant of 3.5 x 10⁵ M⁻¹s⁻¹.
1.3.3.1.3 Structural Features

The first high-resolution crystal structure of a “cautious” monooxygenase was solved for PHBH from *Pseudomonas fluorescens.* PHBH hydroxylates the aromatic substrate para-hydroxy-benzoate (p-OHB) into 3,4-dihydroxy benzoate (3,4-DOHB). A number of other high-resolution structures have been solved since and combined with solution state kinetics details of the complex chemical reaction have been revealed. Key features of the overall structure of PHBH show that the enzyme is a dimer and each monomer is composed of 3 domains: a Rossman-fold for binding the ADP-region of FAD, a substrate binding domain, and a subunit interface domain. The striking feature of the combined structural work is that the isoalloxazine moiety of the FAD cofactor is observed in two conformations, “in” and “out” shown below Figure 1.8. The “in” conformation is observed in the oxidized enzyme not bound to substrate and shows the FAD buried in the protein near the p-OHB binding site. The “out” conformation was observed for a mutated enzyme bound to p-OHB and wild-type enzyme bound to substrate analogs. It shows the FAD rotated ~ 30° from the “in” conformation and is more exposed to solvent. Higher resolution structures, point mutations and kinetic studies have revealed that the mechanism of the enzyme involves motion of FAD from the “in” (solvent protected) state to the “out” (solvent exposed) state upon binding and deprotonation of p-OHB. In the “out” conformation FAD interacts with NADPH and is reduced to the anionic species, that swings back to the “in” state where it can react with O₂ and hydroxylate p-OHB in a solvent free environment. Thus, the kinetics of substrate binding and FAD motion from two structurally distinct sites are key elements that regulate coupled efficient catalysis in “cautious” enzymes.

1.3.3.2 “Bold” Flavin-Dependent Monooxygenase

1.3.3.2.1 Reactions catalyzed and significance

The FAD-dependent monooxygenases that compose the “bold” mechanistic class are primarily composed of the liver microsomal flavin monooxygenases (FMO’s) and Baeyer-Villiger monooxygenases (BVMO’s). The FMO’s were first discovered in porcine liver by Dan Ziegler and are similar in biological function to cytochrome P450’s. They are capable of hydroxylating a
large variety of "soft" nucleophiles, primarily sulfur- and nitrogen-containing xenobiotics and are crucial for catabolism of xenobiotic compounds much like the P450’s. Their reactivity and modes of inhibition are of interest in the development of drug efficacy. Absence of these genes leads to the rare disease known as trimethylaminuria (TMAU) or “fishy-odor syndrome”\textsuperscript{96}. The molecule trimethylamine is formed during digestion and is especially common in diets composed mainly of fish. It is then metabolized or hydroxylated by an FMO for excretion, when FMO is absent the odorous molecule persists at high levels in body fluids giving off the foul “fishy” smell. The other major enzyme families with a “bold” mechanism are the BVMO’s that are found primarily in bacteria and are used for biosynthesis of secondary metabolites like polyketides and catabolism of ketone carbon sources, like carveol\textsuperscript{97}. BVMO’s tend to display a higher level of specificity compared to FMO’s and are one example where the C4a-peroxyflavin is used as a nucleophile toward electrophillic ketone substrates. They have nevertheless evolved to share a mechanistic strategy with the more promiscuous liver FMO’s.
1.3.3.2.2 Kinetic Mechanism

The Cleland diagram below (Figure 1.9) illustrates the kinetic mechanism for the “Bold” class of enzymes. This mechanism is considered a compulsory ordered Ter Bi reaction with NADPH binding first, reducing flavin prior to O₂ activation. The reaction of reduced flavin with O₂ is considered an irreversible chemical step and once the intermediate is formed can only hydroxylate substrate or decay to oxidized flavin and hydrogen peroxide. NADP⁺ remains bound to the enzyme intermediate as the substrate, S, enters and is hydroxylated. Hydroxylated substrate is then released with water, followed by NADP⁺.

![Cleland Diagram of “Bold” FAD-dependent monooxygenases](image)

This mechanism has two key features; one is that flavin is reduced and the C4a-(hydro)peroxide intermediate is formed in the absence of substrate and the second is that NADP⁺ remains bound throughout catalysis and is the last product to leave. The mechanism has been described as a “cocked gun” or the antithesis of the lock-and-key model for enzyme active sites, since the reactive intermediate is formed and stabilized in the absence of the “key” or substrate. Also there appear to be no critical interactions between the substrate and enzyme that stabilize the transition state as is traditionally characteristic of enzymes. This mechanism has been determined mostly by steady-state and spectroscopic studies. The relations between substrates and product inhibitors, combined with the fact that the C4a-(hydro)peroxy intermediate is stable for hours only if NADP⁺ is bound support this mechanistic strategy.⁹⁸
1.3.3.2.3 Structural Features

To date no structure is available for a mammalian flavin monoxygenase, however a homolog from *Methylophaga* sp. Strain sK1 was solved and revealed key active site features that support solution kinetics. The structure is dimeric with each monomer containing two domains; FAD-binding and NADP-binding. NADP⁺ was absolutely essential in stabilizing the protein for crystallography. The active site structure shown below in Figure 1.10 reveals that NADP⁺ (light blue) forms important H-bonds within the pocket to the N5 of FAD ring and they are likely critical to shield the C4a intermediate from solvent. This analysis revealed that NADP(H) was playing two roles in catalysis: one as the source of reducing equivalents as NADPH and second as a solvent shield for the C4a-(hydro)peroxy intermediate as NADP⁺.

![Figure 1.10](image_url)

*Figure 1.10* Active Site Structure of Flavin Monoxygenase from *Methylophaga* sp. Strain sK1. The isoalloxazine moiety is shown in yellow with the C4, C4a and N5 atoms labeled. NADP⁺ is shown in light blue within H-bonding distance of N5 on the flavin. Active site residues within H-bonding distance are shown from the FAD-binding domain (orange) and NADP-binding domain (green). From Alfieri et al.⁹⁹
Structures of multiple BVMO’s have been solved with the most-studied perhaps being cyclohexanone monooxygenase (CHMO). This structure reveals similar features about the enzyme active site, primarily NADP⁺ interacting as a solvent shield. Different conformations of NADP⁺ in different structures also reveal important domain movements and that the dynamics of NADP(H) interaction are critical for efficient catalysis.

1.4 Contributions from the Thesis and Acknowledgments

The data presented in this thesis provides fundamental support for the kinetic mechanism of L-ornithine monooxygenase from A. fumigatus. Little was known about the mechanism of the entire family of SMO’s and the question of whether they used a “bold” or “cautious” type of strategy is addressed. Work using both transient and steady-state kinetics aims at understanding how the enzyme achieves catalytic efficiency and substrate specificity. The data and methods presented here lay a foundation for future research in designing and test inhibitors of this class of enzymes.

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1.5 References


CHAPTER 2
STEADY-STATE KINETIC STUDIES OF A REPRESENTATIVE SIDEROPHORE-ASSOCIATED FLAVIN MONOOXYGENASE

Abstract – The growing number of antibiotic resistant bacterial and fungal pathogens has created a need for novel targets beyond those of the traditional four types. Recent studies suggest that iron metabolism may be a promising pathway for new target discovery. Essential iron is often solubilized, transported, and sometimes even stored as small molecular weight Fe(III)-organic complexes (ferrisiderophores). Aspergillus fumigatus is a widespread fungal pathogen that depends exclusively on one class of siderophore (fusarinines) for all three functions. These siderophores chelate Fe(III) via hydroxamic acid groups. To create this moiety, amine side chains of ornithine or lysine are hydroxylated by flavin-dependent siderophore monooxygenases (SMOs) and subsequently acylated by acyltransferases. Here, the complete steady-state kinetic mechanism for a representative SMO has been determined, using heterologously expressed enzyme from the pathogenic fungus A. fumigatus. Initial steady-state kinetic analysis, including product inhibition reveals that the reaction occurs through an ordered sequential mechanism where reduction of the free enzyme by NADPH occurs first followed by binding of L-ornithine and then molecular oxygen. Alternatively, L-ornithine and O$_2$ could react randomly prior to hydroperoxyflavin formation. Following hydroperoxyflavin formation L-ornithine is hydroxylated and ordered product release occurs with hydroxy-L-ornithine and the oxidized cofactor (NADP$^+$) leaving the complex, much like the flavin-containing monooxygenases (FMOs). This preliminary work suggests this class of enzymes shares mechanistic features with the “bold“ FMO’s and BVMO’s discussed earlier, but has the distinct ability to discriminate substrates at the oxygen activation step.
2.1 Introduction

Pathogenic microbes have evolved a range of strategies for co-opting, transporting, and storing the iron they scavenge from their hosts.\textsuperscript{1,2} Central to iron trafficking in many microbes are siderophores, small molecular weight secondary metabolites that competitively chelate and repackage host iron for uptake and/or intracellular storage by the pathogen.\textsuperscript{3,4} Structural diversity in siderophores is important for their specific recognition by membrane transporters.\textsuperscript{4,5} The iron ligating portions themselves, however, are far less diverse. Siderophores most often contain one of three bidentate chelating moieties: hydroxamate, catecholate, or α-hydroxycarboxylate.

Hydroxamate-dependent siderophores have special significance in pathogenic contexts. First, they are not bound by siderocalins, mammalian immunoproteins that selectively trap and thereby remove catecholate siderophores from circulating blood.\textsuperscript{6, 7} Hydroxamates are consequently associated with virulent forms of many bacteria, although they are usually weaker iron-binders than catecholates.\textsuperscript{7–11} Second, hydroxamate siderophores are acutely important to pathogenic fungi, which are not known to make catecholate or other types of chelating moieties. Many fungi, lacking ferritin, use variants of the same hydroxamate siderophore for both uptake and subsequent intracellular trafficking and storage functions.\textsuperscript{12–14} For viability and virulence, \textit{Aspergillus} species, including deadly strains of \textit{Aspergillus fumigatus}, have been shown to depend on functional pathways for biosynthesis of ferricrocin and fusarinine, siderophores involved in extracellular iron uptake and intracellular storage. Significantly, reductive pathways for mobilizing host iron, identified in some fungi, cannot compensate for the lost siderophore in many \textit{Aspergillus} species.\textsuperscript{15–17}

Hydroxamate biosynthesis is therefore an attractive target for medicinally controlling \textit{A. fumigatus} and possibly other pathogenic species. Antifungal targets are critically needed in light of recent sharp increases in hospital-associated infections, a paucity of effective treatments, and the consequent high mortality associated with invasive forms of \textit{Aspergillus}.\textsuperscript{18–21} Iron trafficking compounds are often proposed for antimicrobial targeting in principle, but specific molecular targets have been difficult to identify in practice.\textsuperscript{22} Many pathogens, for example, have multiple iron uptake pathways; if one is inhibited, another may or may not compensate. However, recent
work demonstrated that *Mycobacterium tuberculosis* cultures are susceptible to picomolar levels of chemical inhibitors of MbtA, an enzyme catalyzing biosynthesis of the aryl cap of mycobactin siderophores.\textsuperscript{23–25} Like *Aspergillus* sp., mycobacteria have been predicted to depend on variants of the same siderophore for multiple steps in the uptake, internalization, and storage of iron and hence may be especially susceptible to chemical inhibition of mycobactin biosynthesis.

The biosynthesis of siderophores by *A. fumigatus* begins with the hydroxylation of L-ornithine (L-Orn), which is the first committed step (Figure 2.1). This reaction is catalyzed by L-ornithine monooxygenase (OMO), a flavin-adenine dinucleotide (FAD)-dependent enzyme. L-Lysine monooxygenase, the first described siderophore-associated monooxygenase (SMO), was initially identified by McDougal and Nielands\textsuperscript{26} as part of the aerobactin biosynthesis operon. More recently, it was recognized that homologs of OMO and lysine monooxygenase are found in dozens of siderophore biosynthesis gene clusters.\textsuperscript{27}

![Figure 2.1 Fusaricine biosynthesis, with initiating step catalyzed by OMO.](image)
The SMOs have only moderate global homology with the well characterized families of flavin-dependent monooxygenases, such as the hepatic microsomal flavin-containing monooxygenases (FMOs), nucleophilic Baeyer-Villiger monooxygenases (BVMOs), and aromatic hydroxylases.\textsuperscript{28–31} The FMO and BVMO families share a similar domain organization with the siderophore-associated monooxygenases and with the functionally related amine-hydroxylating YUCCA enzymes from plants.\textsuperscript{32} These all have FAD- and NADPH-binding sequence motifs (associated with Rossmann folds) near the N termini and the centers of their sequences as well as a hydrophobic region near their C termini. Phylogenetic analysis places the SMOs nearest the FMO and BVMO families but within a novel subclass.\textsuperscript{33} However, among the flavin monooxygenases, the SMOs serve chemically distinct roles, being involved in biosynthetic rather than degradative processes. They almost certainly require unique ways of promoting highly substrate-specific hydroxylations. Early work on aerobactin biosynthesis established that SMOs have remarkable specificity for their substrates (e.g. they can discriminate between L-lysine and L-ornithine, molecules that differ only by one methylene unit).\textsuperscript{34–41}

Prior work with a bacterial OMO (PvdA) by Lamb and coworkers\textsuperscript{35,42} led to the conclusion that PvdA might have mechanistic differences from the cohort of known FAD-dependent monooxygenases. This study provides a full steady-state kinetic analysis of OMO including product inhibition studies. It is a crucial first step in determining how this class of enzymes regulate catalysis.

2.2 Experimental Procedures

2.2.1 Standard Methods, Chemicals, and Equipment

All solvents and reagents were obtained from commercial sources and used without further purification unless otherwise stated. α-N-Benzoyloxycarbonyl- L-ornithine was purchased from Chem-Impex. \textit{meta}-Chloroperoxybenzoic acid (ACROS, technical grade) was dried under vacuum over P$_2$O$_5$ until m.p. $>90$ °C before use. Benzaldehyde (Fisher) was filtered through basic alumina and distilled before use. Molecular biology reagents were from New England Biosciences. An Eppendorf PCR thermocycler was used for PCRs. Spectrophotometric and
steady state-kinetic measurements were made using a Varian Cary 50 spectrophotometer equipped with a Peltier-style thermostat. Additional steady state kinetic measurements were made using a Clark-type O₂ electrode (Yellow Springs Instruments). Transient kinetics were measured using a Hi-Tech Scientific DX-2 stopped flow spectrometer with diode array or photomultiplier tube detection, as described in further detail below. All reactions were carried out in 100 mM Tris-SO₄ buffer at pH 8, unless otherwise noted. Protein concentrations were routinely measured by the Bradford assay. Ultrapure Milli-Q water was used in the preparation of all reagents. All data plots shown in the paper were produced using Kaleidagraph. Data fits by non-linear regression were produced by the same software for the steady state data and by KinetAssyst for the stopped-flow data.

### 2.2.2 Cloning and Engineering of sidA

Genomic DNA from *A. fumigatus* was received as a gift from Dr. Ute Möllmann of the Hans Knöll Institute (Jena, Germany). The sidA gene encoding OMO (accession AY819708) contains one intron that was removed for recombinant expression in *E. coli*. The exons were amplified in separate PCRs from genomic DNA. The forward primer for exon 1 (5’-GGCTCTCATATGGAATCTGTTGAACGG-3’) included an NdeI site (underlined). The reverse primer was 5’-GGTTGAAGATCTCATTCACAAAAGGCGAGTCGTCACTG-3’. Another forward primer (5’-CAGTGACGACTCGCCTTTTGTGAATGAGATCTTCAACC-3’) was utilized for exon 2; the reverse primer (5’-CTCGAGTTATTACAGCATGGCTCGTAG-3’) includes an XhoI site (underlined). A mixture of the two exons served as the DNA template in a subsequent PCR, with the intron-free fragment (1561 base pairs) as the product. The amplified fragment was digested with NdeI and XhoI and ligated into complementary sites on the pET15b plasmid (AmpR, Novagen). The resultant plasmid encodes the sidA cDNA with a thrombin-cleavable, N-terminal 6-histidine tag. The plasmid was transformed into the Rosetta 2 (DE3) *E. coli* strain (Novagen) for overexpression of the OMO protein.
2.2.3. Overexpression and Purification of OMO

Cultures were grown on an Innova shaker incubator (220 rpm) in Fernbach flasks containing LB medium (1.5 liters) supplemented with 100 μg/ml ampicillin at 30 °C. The cells were harvested after 30 h by centrifugation (6000 x g, 10 min, 4 °C). The cell pellet was resuspended in 4 ml/g pellet of column binding buffer (100 mM Tris-SO4, pH 7.4, 500 mM NaCl, and 50 mM imidazole). Cells were sonicated (Branson Ultrasonifier), and the debris was removed by centrifugation (17,000 x g, 50 min, 4 °C). The supernatant was sterile-filtered and applied to a column packed with 10 ml of Ni\(^{2+}\)-Sepharose high performance (GE Healthcare) resin pre-equilibrated with Ni\(^{2+}\) ions and washed in binding buffer. The filtered lysate was passed over the resin (4 °C), and the column was washed with 10 column volumes of binding buffer and eluted in a gradient of 0–800mM imidazole in binding buffer. The most active fractions were collected and concentrated in an Amicon Ultra-15 centrifugal filter device (30,000 molecular weight cut-off). The protein was further purified and buffer-exchanged on a 300-ml Sephacryl S-200 high resolution (GE Healthcare) gel filtration column run at 0.4 ml/min. The protein solution was then concentrated in an Amicon Ultra-15 centrifugal filter device (30,000 molecular weight cut-off) to >2 mg/ml and flash-frozen with liquid N\(_2\) for storage at -80 °C. Protein concentrations were routinely determined by the Bradford assay. Bound FAD was determined as described below.

2.2.4 Analytical Gel Filtration

Analytical gel filtration used a Sephacryl S-200 high resolution (GE Healthcare) column (26 mm x 60 cm) equilibrated with 100 mM Tris-SO\(_4\) buffer, pH 8.0. A standard curve was generated using gel filtration standard (Bio-Rad) consisting of protein aggregates (void peak), thyroglobulin (bovine; \(M_r = 670,000\)), γ-globulin (bovine; \(M_r = 158,000\)), ovalbumin (chicken; \(M_r = 44,000\)), myoglobin (horse; \(M_r = 17,000\)), and vitamin B-12 (\(M_r = 1350\)).
2.2.5 Quantification of Bound Flavin

Denaturant (0.1% SDS from 10% stock) was incubated with a sample of enzyme for which a spectrum had been measured. Spectra were taken until no changes were observed, the sample was centrifuged in a microcentrifuge filter (molecular weight cut-off = 10,000) to near dryness, and the spectrum of the eluant was recorded. The molar amount of released FAD was determined using $\lambda_{\text{max}}$ and $\varepsilon$ values for free/aqueous oxidized FAD ($\lambda_{\text{max}} = 450$ nm, $\varepsilon = 11,300$ M$^{-1}$ cm$^{-1}$).\(^{43}\)

2.2.6 Influence of Halides on the Reaction

Because the amino acid substrate was supplied as the chloride salt and the hydroxyornithine product as the dibromide (see below), the possible dependence of the measured rates on [Cl$^-$] and [Br$^-$] was determined. The initial velocity of NADPH oxidation by OMO was monitored spectrophotometrically at fixed, subsaturating concentrations of l-Orn (1 mM, $\sim$2 x $K_m$) and NADPH (75 $\mu$M) in air. Subsaturation in the substrate would permit competition between it and the halide. Halide concentrations in the reactions were varied over 160 $\mu$M to 1.0 M, and dose-response curves were generated.

2.2.7 Equilibrium Binding of NADP

The dissociation constant for NADP$^+$ to the oxidized enzyme was determined by spectroscopic titration in a pH 8 solution of 50 mM Tris-H$_2$SO$_4$ at 25 °C. Fractional equivalents of NADP$^+$ were added to enzyme. To account for the possibility of slow equilibration, spectra were recorded for several minutes until no further changes were observed. Following corrections for dilution, changes in absorbance at the spectral region of maximal difference were plotted versus [titrant] and fit to Equation 2.1.

Equation 2.1  

$$\Delta A b s_{\text{obs}} = \frac{\Delta A b s_{\text{max}}}{2 E_{\text{total}}} \left[ L_0 + E_t + K_d - \sqrt{(L_0 + E_t + K_d)^2 - 4E_t + L_0} \right]$$
Where $L_0$, $E_t$, $K_d$, and $\Delta \text{Abs}_{\text{max}}$ are the initial ligand concentration, total enzyme or protein concentration, the equilibrium dissociation constants, and the maximum change in absorbance respectively.

### 2.2.8 Steady State Kinetic Studies

All reactions were initiated with enzyme, in the absence of added FAD. Rates were referenced to the concentration of flavin-containing enzyme subunit, where the FAD titer was determined as described above in 2.2.5. Reactions were monitored continuously via the oxidation of NADPH or the consumption of $O_2$. The reactions used to determine the steady state mechanism were carried out in a pH 8 solution of 50 mM Tris-$H_2SO_4$ at 37 °C.

Oxygen consumption was measured using a Clark-type $O_2$ electrode equilibrated to 37 °C for ~1 h and calibrated to the dissolved $[O_2]$ in air-saturated water at the given atmospheric pressure. Desired $O_2$ concentrations were obtained via first mixing $O_2$ and $N_2$ gases and then equilibrating the samples to those gas concentrations by purging the enclosed headspace for 3 min, followed by a 2-min equilibration of the solution in contact with the probe (headspace gas was displaced by the probe). The oxygen probe was used to measure the actual $[O_2]$. Reaction volumes were 1.6 ml, enzyme was added via a gas-tight syringe, and continuous mixing was provided from a magnetic stirrer. Additionally, NADPH was essentially held constant throughout the reaction with the use of an enzymatic system for continuous regeneration of NADPH (11 units of glucose 6-phosphate dehydrogenase and 4 mM glucose 6-phosphate). NADPH oxidation was monitored via UV-visible spectrophotometry ($\epsilon_{\text{NADPH,340\,nm}} = 6220\,\text{cm}^{-1}\text{M}^{-1}$). The enzyme was added to a cuvette, and the spectrophotometer was zeroed at 340 nm. NADPH was added and mixed by pipetting (final volume, 160 μl). The slow NADPH oxidase activity of OMO was monitored for 10 s, and the reaction was initiated by adding a small volume of L-Orn stock (HCl salt, Sigma). Rates were determined from linear regression fits to the initial linear portion of the curves. Initial rate measurements were performed in triplicate or greater. Average values are plotted with 1 S.D. as the error bar.
Kinetic parameters were determined from plots of initial rate \( (v_i) \) versus substrate concentration fit to the Michaelis-Menten equation, \( \frac{v_i}{[E]} = \frac{k_{cat}[S]}{(K_m + [S])} \), where \([E]\) represents the concentration of FAD-bound subunits. In order to obtain the most unbiased fit results, unweighted curves were fit by nonlinear regression to the full collection of data points, with three replicates at each \([S]\).\textsuperscript{45,46} Errors reported in values for \( k_{cat} \) or \( K_m \) are sums of the least square differences between the computed and actual curves. By examining the dependence of \( k_{cat} \) and \( k_{cat}/K_m \) on the concentration of a fixed variable substrate, it can be established whether the enzyme forms that the fixed variable substrate and the variable substrate combine are reversibly or irreversibly connected in the kinetic mechanism. If \( K_m/k_{cat} \) and \( 1/k_{cat} \) values vary with \( 1/[\text{fixed variable substrate}] \), it can be concluded that there is a reversible connection. For an irreversible connection, \( 1/k_{cat} \) varies, whereas \( K_m/k_{cat} \) stays the same. This approach is analogous to traditional linearization of data through double reciprocal plots, where intersecting line patterns indicate a reversible connection between the variable and fixed variable, and parallel line patterns indicate an irreversible connection. Comparing parameters derived directly from fits to Michaelis plots avoids the errors associated with double reciprocal plots.\textsuperscript{47}

2.3 Results and Analysis

2.3.1 Protein Characterization

Soluble OMO was overexpressed in \textit{E. coli} with typical yields of 20 mg of pure protein per liter of culture (Figure 2.1 and Table 2.1). Bright yellow purified OMO was isolated with 0.6 FAD released (per subunit, as determined by Bradford assay) following incubation of the protein with 0.1% SDS. Previously isolated siderophore-associated monoxygenases, by contrast, had almost no bound FAD, consistent with the relatively low measured affinities of those SMOs for FAD: \( K_d = 26 \mu M^{41} \), 9.9 \( \mu M^{35} \), and 30 \( \mu M^{36} \). In earlier work with SMOs, 10 eq of FAD were routinely added immediately prior to activity measurements in order to increase the concentration of active enzyme\textsuperscript{34–36,38–41} possibly leading to side reactions of free FAD and NADPH.
Figure 2.2 Protein purification. SDS gel image stained with Coomassie blue. Left to right: molecular weight marker, lysate, purified OMO. The bands on the marker correspond to 170, 130, 95, 72, 56, 43, 34, 26, and 17 kDa from top to bottom.

Table 2.1

OMO PURIFICATION SCHEME

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (mL)</th>
<th>Protein (mg/mL)</th>
<th>Specific Activity (units/mg)</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clarified Lysate</td>
<td>56.0</td>
<td>32.8</td>
<td>18</td>
<td>100</td>
</tr>
<tr>
<td>Nickel Column</td>
<td>3.50</td>
<td>11.3</td>
<td>797</td>
<td>9.20</td>
</tr>
<tr>
<td>Gel Filtration</td>
<td>1.50</td>
<td>15.2</td>
<td>752</td>
<td>4.97</td>
</tr>
</tbody>
</table>

Values were determined as described under "Experimental Procedures" and reflect a typical purification from 1.5 L of cell culture.

The UV-visible spectra for free and enzyme-bound FAD were similar in appearance (Figure 2.3) although the extinction coefficient determined for the enzyme-bound FAD (12,500 M⁻¹) is larger than that for the free flavin (11,300 M⁻¹).

Analytical gel filtration (against globular protein standards) gave an estimated native molecular mass of 105 kDa, suggesting that, if globular, OMO exists as a homodimer (expressed monomer 58.9 kDa). The average specific activity over three separate purifications for OMO was 657 ± 55 nmol of NADPH min⁻¹mg⁻¹ (50mM Tris-SO₄, pH 8, 37 °C). This value is comparable with
the specific activity measured for the expressed *P. aeruginosa* ornithine monooxygenase (528 ± 8 nmol of NADPH min⁻¹ mg⁻¹, 100 mM potassium phosphate, pH 8, 24 °C).³⁵

![Figure 2.3 Determination of the Molar Absorptivity of OMO. A spectrum of the purified protein was recorded (——) and showed an absorbance peak at approximately 450 nm. The so-called oxidized enzyme was denatured to remove the FAD cofactor with the addition 0.2% SDS from a 10% stock and spectra were recorded until no further changes were observed (~30 minutes). The sample was centrifuged in a microfuge filter (MWCO = 10,000, Amicon) to near dryness, and the spectrum of the filtrate (-----) recorded.](image)

2.3.2 Dissociation Constant for NADP

The affinity of the oxidized enzyme for NADP⁺ was determined by spectrophotometric titration. Difference spectra (at 390 nm) were fit to **Equation 2.1**, yielding $K_d = 4.6 \pm 0.3 \mu$M (Figure 2.4).
Figure 2.4 Equilibrium Binding of NADP⁺. A, Spectral Changes with Addition of NADP⁺. An oxidized sample of OMO (28 μM) was titrated with NADP⁺ (0 – 90 μM) in a pH 8 solution of 50 mM Tris-H₂SO₄ at 25 °C and spectra were recorded. Spectra are corrected for dilution and several were removed for clarity. B, data recorded in A with increasing concentrations of NADP⁺ were subtracted from the initial spectra of free oxidized OMO to generate the difference spectra. The largest changes were observed at 390 and 490 nm. C, changes in absorbance at 390 nm were converted to the concentration of OMO bound to NADP⁺ using the value of 2,632 M⁻¹cm⁻¹ for Δε390 nm. The concentration of bound enzyme was plotted versus the concentration of NADP⁺ and the resulting pattern was fit to the isotherm. The fit yielded $K_d = 4.58 \pm 0.34$ μM.
2.3.3 Influence of Halides on the Reaction

Some FAD-dependent enzymes, including PvdA, are inhibited by halides. Moreover, the substrate and product were used as amino acid salts (chloride and dibromide, respectively). In order to assess the possible inhibitory effects of either Cl or Br, specific activity was measured in the presence of increasing concentrations of each (potassium salt). Dose-response curves were obtained by plotting the enzyme fractional activity ($v_i/v_0$), where $v_i$ is the initial velocity in the presence of halide and $v_0$ is velocity in its absence, as a function of log[halide] (Figure 2.5). The resulting plots are sigmoidal in shape. The inflection point of the sigmoidal curve gives the concentration of halide at which activity is reduced by 50% (IC$_{50}$). Each curve was fit to a sigmoidal function of the form $y = y_{min} + (y_{max} - y_{min})/(1 + (x/IC_{50})^H)$, where $H$ represents Hill slope. This yielded IC$_{50}$(Cl) = 61 mM; IC$_{50}$(Br) = 114 mM. Hence, neither halide is expected to have an appreciable influence on the reaction at the concentrations used in this study.

![Figure 2.5 Effect of halides. Dose-response curves for bromide (●) and chloride (■) were obtained by plotting the enzyme fractional activity ($v_i/v_0$), where $v_i$ is the initial velocity in the presence of inhibitor and $v_0$ in the absence, as a function of the concentration of compound plotted on a log scale. The IC$_{50}$ values for Br and Cl are 59 and 108 mM. The data were fit to a sigmoidal function of the form $y = y_{min} + (y_{max} - y_{min})/(1 + (x/IC_{50})^H)$, where $H = $ Hill slope, $y$ is the fractional activity of the enzyme in the presence of halide (or inhibitor), and $x$ is the log of the concentration of halide used.](image-url)
2.3.4 Steady State Kinetics of the Forward Reaction

As expected for a monooxygenase, the reaction was essentially irreversible in the forward direction. $K_m$ values for each substrate were first estimated at 25 °C from plots of the initial rate ($v/[E]$, where $[E]$ [enzyme-bound FAD]) versus [substrate] with the other two substrates at very high concentrations ($\sim 10K_m$) (Table 2.2). The very low $K_m$ values for NADPH and $O_2$ were near the measurable limits using either the UV-visible (NADPH) or polarographic ($O_2$) assays. More accurate values for these numbers (at 37 °C) were derived from replots of $1/k_{cat}$ values (determined from fits to hyperbolic plots measured at several fixed variable concentrations of a second substrate) against $1/[S]$. The x-intercept of the $1/k_{cat}$ replot is $1/K_{m,S}$ (where S represents fixed variable substrate) and the y intercept gave $1/k_{cat}$ at an extrapolated infinite concentration of $S$ (Table 2.2). Using the initially measured $K_m$, saturating concentrations for each substrate were set to be ≥10 $K_m$ in the experiments described below. Note that air-saturated pure water at 25 °C and 1 atm has an $[O_2] = 260$ μM. Nearly the same $[O_2]$ was measured for 50 mM Tris-SO$_4$ buffer, pH 8, at the same temperature.

### Table 2.2

STEADY-STATE KINETIC PARAMETERS FOR OMO MEASURED AT 37 AND 25 °C.

<table>
<thead>
<tr>
<th>Variable Substrate</th>
<th>$K_m$ (M)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Measured at 37 °C</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Ornithine</td>
<td>$4.9 \pm 0.7 \times 10^{-4}$</td>
<td>$1.7 \pm 0.1$</td>
<td>$3.4 \pm 0.5 \times 10^{3}$</td>
</tr>
<tr>
<td>NADPH</td>
<td>$4.6 \pm 0.5 \times 10^{-6}$</td>
<td>$1.9 \pm 0.4$</td>
<td>$4.2 \pm 0.5 \times 10^{6}$</td>
</tr>
<tr>
<td>$O_2$</td>
<td>$1.8 \pm 0.1 \times 10^{-5}$</td>
<td>$1.9 \pm 0.1$</td>
<td>$1.1 \pm 0.1 \times 10^{6}$</td>
</tr>
<tr>
<td><strong>Measured at 25 °C</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Ornithine</td>
<td>$5.8 \pm 0.4 \times 10^{-4}$</td>
<td>0.611</td>
<td>$1.1 \pm 0.1 \times 10^{3}$</td>
</tr>
<tr>
<td>NADPH</td>
<td>$2.6 \pm 0.6 \times 10^{-6}$</td>
<td>0.599</td>
<td>$2.3 \pm 0.5 \times 10^{5}$</td>
</tr>
<tr>
<td>$O_2$</td>
<td>$1.6 \pm 0.1 \times 10^{-5}$</td>
<td>0.614</td>
<td>$3.8 \pm 0.5 \times 10^{4}$</td>
</tr>
</tbody>
</table>
In order to determine the nature and order of substrate interactions with the enzyme, initial rates were plotted as a function of the concentration of one of the substrates at each of several fixed concentrations of the second substrate with the third substrate fixed at an essentially saturating concentration. Each of the plots was fit to the Michaelis-Menten equation (Figures 2.6-2.9). Values for \( K_m \), \( k_{cat} \), and \( K_m/k_{cat} \) (and their associated errors) from the fit to each plot are tabulated in Tables 2.3-2.6, and the conclusions are summarized here. When [NADPH] was varied and \( \text{O}_2 \) was the fixed variable ([L-Orn] was fixed at the saturating concentration of 13 mM), the resulting double reciprocal plots appeared parallel; however, the relationship among the measured values of \( K_{m,NADPH}/k_{cat} \) was difficult to determine accurately due to the low \( K_{m,NADPH} \) and the rapid initial rates (Figure 2.6 and Table 2.3). The value for \( 1/k_{cat} \) clearly increases with \( 1/\text{[O}_2\text{]} \).

When the same data were measured in the presence of a fixed, subsaturating [L-Orn] (0.6 mM), the measured values of \( K_{m,NADPH}/k_{cat} \) clearly increased with \( 1/\text{[O}_2\text{]} \) (Figure 2.7 and Table 2.4). Hence, NADPH and \( \text{O}_2 \) appeared to react with irreversibly connected forms of enzyme when L-Orn is saturating, and this changes to reversibly connected enzyme forms in the presence of subsaturating L-Orn. These results are consistent with a sequential mechanism with NADPH reacting with the enzyme (to reduce the flavin), followed by the binding of [L-Orn] and then \( \text{O}_2 \). Alternatively, L-Orn and \( \text{O}_2 \) could react randomly with the enzyme following its reaction with NADPH. In the latter case, saturation with L-Orn would enforce the NADPH, L-Orn, \( \text{O}_2 \) order and, as in the sequential case, cause the other two substrates to appear irreversibly connected.

When [L-Orn] was varied, [NADPH] was the fixed variable, and \( \text{O}_2 \) was saturating (air), the resulting values of \( K_{m,L-Orn}/k_{cat} \) decreased, and \( 1/k_{cat} \) increased with \( 1/\text{[NADPH]} \) (Figure 2.8 and Table 2.5). This indicates that the two varied substrates bind enzyme forms that are reversibly connected in the reaction. The fact that at saturating concentrations of \( \text{O}_2 \) there was not a parallel pattern suggests that \( \text{O}_2 \) is either the first or (more likely) last substrate to add or that the enzyme form to which it adds is irreversibly separated from the NADPH- and L-Orn - combining forms. Because \( K_{m,L-Orn}/k_{cat} \) also decreased with \( 1/\text{[O}_2\text{]} \) at saturating NADPH (Figure 2.9 and Table 2.6), the \( \text{O}_2\)- and L-Orn- combining enzyme forms must also be reversibly connected. Collectively, the data indicate that the substrates most likely add sequentially in the
order NADPH, L-Orn, and O₂, a conclusion further corroborated in the following chapter. Steady state patterns and conclusions are listed in Table 2.7.

![Figure 2.6](image)

**Figure 2.6** Kinetic parameters with variable NADPH (0, 6, 9, 17, 31, 92 and 154 µM) and fixed variable O₂ (▼ 195 µM, ▲ 97 µM, ◆ 60 µM, ■ 30 µM ● 20 µM) in saturating L-ornithine (13 mM) solutions of 50 mM Tris-H₂SO₄ pH 8.0, 37 °C.

<table>
<thead>
<tr>
<th>O₂ (µM)</th>
<th>Kₘ, NADPH (µM)</th>
<th>kₗ (s⁻¹)</th>
<th>Kₘ, NADPH / kₗ (µM s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>195</td>
<td>3.4 ± 0.3</td>
<td>1.80 ± 0.03</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>97</td>
<td>4.4 ± 0.2</td>
<td>1.60 ± 0.02</td>
<td>2.7 ± 0.1</td>
</tr>
<tr>
<td>60</td>
<td>3.7 ± 0.5</td>
<td>1.40 ± 0.04</td>
<td>2.6 ± 0.4</td>
</tr>
<tr>
<td>30</td>
<td>3.9 ± 0.5</td>
<td>1.20 ± 0.03</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td>20</td>
<td>1.9 ± 0.3</td>
<td>1.00 ± 0.02</td>
<td>2.0 ± 0.3</td>
</tr>
</tbody>
</table>
Figure 2.7 Kinetic parameters with variable NADPH (0, 6, 9, 15, 41, 90 and 150 µM) and fixed variable O$_2$ (▼202 µM, ▲83 µM, □43 µM, ■22 µM ●13 µM) in sub-saturating L-ornithine (0.6 mM) solutions of 50 mM Tris-H$_2$SO$_4$ pH 8.0, 37 °C.

TABLE 2.4
VARIABLE NADPH, FIXED-VARIABLE O$_2$, SUB-SATURATING L-ORNITHINE

<table>
<thead>
<tr>
<th>O$_2$ (µM)</th>
<th>$K_{m, NADPH}$ (µM)</th>
<th>$K_{cat}$ (s$^{-1}$)</th>
<th>$K_{m, NADPH}$/$K_{cat}$ (µM s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>202</td>
<td>12.9 ± 0.5</td>
<td>0.90 ± 0.01</td>
<td>14.0 ± 0.6</td>
</tr>
<tr>
<td>83</td>
<td>15.1 ± 1.2</td>
<td>0.88 ± 0.02</td>
<td>16.6 ± 1.4</td>
</tr>
<tr>
<td>43</td>
<td>13.6 ± 1.9</td>
<td>0.80 ± 0.03</td>
<td>17.0 ± 2.5</td>
</tr>
<tr>
<td>22</td>
<td>14.5 ± 2.7</td>
<td>0.71 ± 0.04</td>
<td>21.2 ± 4.1</td>
</tr>
<tr>
<td>13</td>
<td>11.9 ± 1.9</td>
<td>0.52 ± 0.02</td>
<td>22.3 ± 3.7</td>
</tr>
</tbody>
</table>
Figure 2.8 Kinetic parameters with variable L-ornithine (0.3, 0.6, 1.0, 3.0, 5.0, and 13 mM) and fixed variable NADPH (●150 µM, ▼90 µM, ▲30 µM, ◆15 µM ■9 µM ●6 µM) in saturating O₂ (200 µM) solutions of 50 mM Tris-H₂SO₄ pH 8.0, 37 °C.

<table>
<thead>
<tr>
<th>NADPH (µM)</th>
<th>Kₘ,L-orn (mM)</th>
<th>kₜₐₜ (s⁻¹)</th>
<th>Kₘ,L-orn/kₜₐₜ (mM s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>150</td>
<td>0.6 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>90</td>
<td>0.7 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>30</td>
<td>0.8 ± 0.2</td>
<td>1.4 ± 0.1</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>15</td>
<td>1.2 ± 0.4</td>
<td>1.2 ± 0.1</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>9</td>
<td>0.9 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>6</td>
<td>1.0 ± 0.3</td>
<td>0.6 ± 0.1</td>
<td>1.6 ± 0.5</td>
</tr>
</tbody>
</table>
Figure 2.9 Kinetic parameters with variable L-ornithine (0.15, 0.24, 0.6, 1.0, 3.0, 5.2, and 13 mM) and fixed variable O$_2$ (▼ 201 μM, ▲ 87 μM, ◆ 38 μM, ■ 20 μM ● 13 μM) in saturating NADPH (150 μM) solutions of 50 mM Tris-H$_2$SO$_4$ pH 8.0, 37 °C.

TABLE 2.6

<table>
<thead>
<tr>
<th>O$_2$ (μM)</th>
<th>$K_{m, \text{L-orn}}$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_{m, \text{L-orn}} / k_{cat}$ (mM s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>201</td>
<td>0.37 ± 0.05</td>
<td>1.5 ± 0.1</td>
<td>0.24 ± 0.03</td>
</tr>
<tr>
<td>87</td>
<td>0.43 ± 0.06</td>
<td>1.6 ± 0.1</td>
<td>0.28 ± 0.04</td>
</tr>
<tr>
<td>38</td>
<td>0.37 ± 0.05</td>
<td>1.3 ± 0.1</td>
<td>0.29 ± 0.04</td>
</tr>
<tr>
<td>20</td>
<td>0.32 ± 0.05</td>
<td>1.0 ± 0.1</td>
<td>0.32 ± 0.05</td>
</tr>
<tr>
<td>13</td>
<td>0.30 ± 0.04</td>
<td>0.8 ± 0.1</td>
<td>0.40 ± 0.06</td>
</tr>
</tbody>
</table>
TABLE 2.7
SUMMARY OF STEADY STATE KINETIC PATTERNS OBSERVED FOR SUBSTRATE INTERACTIONS.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Fixed-Variable</th>
<th>Fixed</th>
<th>Pattern</th>
<th>Figure</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADPH</td>
<td>O₂</td>
<td>L-ornithine – Saturating</td>
<td>Intersecting</td>
<td>2.6</td>
</tr>
<tr>
<td>NADPH</td>
<td>O₂</td>
<td>L-ornithine – Sub-saturating</td>
<td>Intersecting</td>
<td>2.11</td>
</tr>
<tr>
<td>L-ornithine</td>
<td>NADPH</td>
<td>O₂ – Saturating</td>
<td>Intersecting</td>
<td>2.8</td>
</tr>
<tr>
<td>L-ornithine</td>
<td>O₂</td>
<td>NADPH – Saturating</td>
<td>Parallel</td>
<td>2.9</td>
</tr>
</tbody>
</table>

2.3.5 Dead End and Product Inhibition

True product inhibition occurs when the product combines with the product-releasing enzyme form and backs up the forward reaction, altering the steady state concentrations of the various reactive enzyme forms. By contrast, when a product simply mimics and thereby competes with its cognate substrate, pure competitive dead end inhibition is observed. Saturation in the cognate substrate eliminates the dead end inhibitory effect. If a product acts as both a dead end and product inhibitor, it should bind to both the product-releasing and cognate substrate-binding enzyme forms. Saturation in the cognate substrate in that case should eliminate dead end but not product inhibition.

We sought first to determine whether NADP⁺ acts as a dead end inhibitor relative to NADPH, as a product inhibitor, or both. When NADP⁺ was used as a fixed variable inhibitor against NADPH ([L-Orn-OH] = 0, O₂ saturating, and L-Orn (5 mM) saturating), a pure competitive pattern was clearly observed, indicating that NADP⁺ and NADPH bind exclusively to the same enzyme form (Figure 2.10). This result is consistent with NADP⁺ being the last product to leave the enzyme and NADPH the first to add, such that both species bind to the oxidized enzyme (Figure 2.12,E_{Dox}). If a second, product-releasing enzyme form could also reversibly bind NADP⁺ (e.g. the FAD C4a-hydroxide that is converted to E-FADox by dehydration; Figure 2.12, E_{OH}), a noncompetitive pattern would have been observed.
Inhibition by NADP⁺. A, Reciprocal plot of NADP⁺ product inhibition versus NADPH. Inverse rates of NADPH oxidation by OMO at varying NADPH (~0, 25, 30, 60, and 120 μM) and fixed amounts of product inhibitor NADP⁺ (~0 μM, ▲150 μM, ●300 μM, ▼600 μM, and ◆900 μM) in L-ornithine (5 mM) and air-saturated solutions of 50 mM Tris-H₂SO₄, 10 μM FAD, pH 8.0, 37 °C. Inset shows slope ($K_{m}/k_{cat}$) as a function of inhibitor concentration. B, Reciprocal plot of NADP⁺ product inhibition versus L-ornithine. Inverse rates of NADPH oxidation by OMO at varying L-ornithine (0, 0.11, 0.22, 0.55, 1.83, 4.56, 9.13, 13.04, and 18.62 mM) and fixed amounts of product inhibitor NADP⁺ (~0 μM, ▲150 μM, ●300 μM, and ▼600 μM) in NADPH (70 μM) and air-saturated solutions of 50 mM Tris-H₂SO₄ pH 8.0, 37 °C. Inset shows intercept (1/$k_{cat}$) as a function of inhibitor concentration. Data points represent the inverse of measured rates while the linear fits were generated from the kinetic parameters estimated by non-linear fitting (KaleidaGraph).

Inhibition by L-Orn-OH is likewise purely competitive ($K_{i} = 475$ μM; Table 2.8) with the cognate L-Orn substrate ([NADP⁺ = 0, O₂ = air saturation, and [NADPH] = 10$K_{m}$] (Figure 2.11A). These measurements were carried out by monitoring spectrophotometrically for NADPH disappearance using the chemically synthesized hydroxylated L-Ornithine. Slope and intercept replots ($K_{i}/k_{cat}$ and 1/$k_{cat}$ versus [L-Orn-OH]) in this case were linear. The simplest interpretation of these results is that competition between L-Orn and L-Orn-OH occurs via a single site on the same enzyme form under the given conditions, presumably the E-FADred(NADP⁺) complex.

When L-Orn is at 3$K_{m}$ (1.5 mM), uncompetitive inhibition by L-Orn-OH against varied [NADPH] is observed (Figure 2.11B) with $K_{i} = 1400$ μM (Table 2.8). Therefore, under these conditions, L-Orn-OH binds an enzyme form that is not reversibly connected to the NADPH-binding form (i.e. neither EFADox nor the E-FADred(NADP⁺) complex). L-Orn-OH therefore appears to be able to interact with two forms of the enzyme. The higher affinity interaction (with E-
FAD_{red}(NADP^+) appears to be mostly saturated with L-Orn when [L-Orn] = 1.5 mM, and was consequently not evident in the NADPH/L-Orn-OH inhibition experiments. The lower affinity site is also not apparent in the L-Orn/L-Orn-OH experiments, possibly because the two values for $K_i$ are close in magnitude (~0.5 mM and 1.4 mM). It is unclear from these experiments which enzyme form is responsible for the lower affinity L-Orn-OH/enzyme interaction. However, because the L-Orn hydroxylation step is probably not reversible, it is unlikely that L-Orn-OH binds the product-releasing form to bring about reversal of the reaction and true product inhibition. L-Orn-OH more likely inhibits the enzyme via binding to some other enzyme form, which we propose is the C4a-hydroperoxide (see below).

**Figure 2.11** Inhibition by hydroxy-L-ornithine. A. Reciprocal plot of hydroxy-L-ornithine product inhibition versus L-ornithine. Inverse rates of NADPH oxidation by OMO at varying L-ornithine (~0, 0.15, 0.30, 0.54, 1.45, 5.0, and 15.0 mM) and fixed amounts of product inhibitor hydroxy-L-ornithine (~●0 μM, ○200 μM, ■800 μM, □2000 μM, and ◆3200 μM) in NADPH (150 μM) and air-saturated solutions of 50 mM Tris, 10 μM FAD, pH 8.0, 37 °C. B. Reciprocal plot of hydroxy-L-ornithine product inhibition versus NADPH. Inverse rates of NADPH oxidation by OMO at varying NADPH (~0, 20, 25, 30, 60, 120, and 150 μM) and fixed amounts of product inhibitor hydroxy-L-ornithine (~●0 μM, ○150 μM, ◆600 μM, □900 μM, ▽1500 μM and ■3000 μM) in sub-saturated L-ornithine (1.5 mM) and air-saturated solutions of 50 mM Tris, 10 μM FAD, pH 8.0, 37 °C. Data points represent the inverse of measured rates while the linear fits were generated from the kinetic parameters estimated by non-linear fitting (KaleidaGraph).
Figure 2.12 Cleland diagram for kinetic mechanism of OMO

### Table 2.8

**Summary of Product Inhibition Patterns and Inhibition Constants**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Product Inhibitor</th>
<th>Conditions</th>
<th>Pattern/ Mode</th>
<th>$K_i^a$ (αK_S)</th>
<th>$K_s^a$</th>
<th>Figure</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADPH</td>
<td>NADP⁺</td>
<td>6 mM L-ornithine, air O₂</td>
<td>Competitive/ Intersecting on Y-axis</td>
<td>NAᵇ</td>
<td>156.5 ± 11.8</td>
<td>2.14A</td>
</tr>
<tr>
<td>L-Orn</td>
<td>NADP⁺</td>
<td>70 μM NADPH, air O₂</td>
<td>Uncompetitive/ Parallel</td>
<td>343.6 ± 18.6</td>
<td>NA</td>
<td>2.14B</td>
</tr>
<tr>
<td>L-Orn-OH</td>
<td>L-Orn-OH</td>
<td>150 μM NADPH, air O₂</td>
<td>Competitive/ Intersecting on Y-axis</td>
<td>NA</td>
<td>474.5 ± 83.3</td>
<td>2.15A</td>
</tr>
<tr>
<td>NADPH</td>
<td>L-Orn-OH</td>
<td>1.5 mM L-ornithine, air O₂</td>
<td>Uncompetitive/ Parallel</td>
<td>1433.2 ± 36.4</td>
<td>NA</td>
<td>2.15B</td>
</tr>
</tbody>
</table>

ᵇInhibition constants for the intercepts ($K_i$) and the slopes ($K_s$) were determined by taking the horizontal intercept of linear re-plots of the intercepts and slopes against inhibitor concentration.

2.3.6 NADPH Oxidase Activity

Slow NADPH oxidase activity was observed in the absence of L-Orn. This occurred with a measured $k_{cat} = 0.90 \text{ min}^{-1}$ or $0.015 \text{ s}^{-1}$ (25 °C, pH 7.4). $K_m$ values were below the estimated threshold for which either could be measured by UV-visible or O₂-electrode methods $K_m(O_2) = 10 \mu M$; $K_m\text{NADPH} \leq 5 \mu M$ (data not shown).

2.4 Discussion

Flavin monooxygenases that catalyze the N-hydroxylation of the side chain of ornithine, lysine, or other primary amines are essential for the biosynthesis of hydroxamic acids. These acids act as bidentate chelating moieties in the siderophores used by hundreds of organisms. Although these enzymes are now known to be widespread and appear to constitute a distinct
subclass of flavin monooxygenases\textsuperscript{28}, they have undergone little mechanistic study until recently.\textsuperscript{35,42} Their encoding genes are essential for survival and/or virulence in many species of pathogenic fungi, notably deadly strains of \textit{A. fumigatus} and intracellular \textit{Histoplasma capsulatum}.\textsuperscript{15–17} Serious fungal infections, particularly in their invasive forms, are associated with high mortality rates due to a dearth of effective treatments.\textsuperscript{18} We consequently chose to examine the OMO enzyme from \textit{A. fumigatus} as a representative of its class. The larger family of \textit{N}-hydroxylating monooxygenases also includes enzymes involved in the biosynthesis of the plant hormone, auxin. Low overall sequence conservation between these enzymes and other flavin monooxygenases, their roles in biosynthetic rather than degradative processes, and their unusually acute substrate specificity suggest that they may be mechanistically or structurally distinct from either of the known families of flavin monooxygenases. We therefore undertook an extensive mechanistic study of OMO to define the place of NMOs in the larger scheme of flavin enzymes and to begin to address the feasibility of using the enzyme as an antifungal target.

OMO is indeed a flavin-dependent enzyme, although only about half of the purified enzyme contained the reactive cofactor. This appears to be a common feature of the NMO family of flavoproteins as low amounts of the cofactor has been observed elsewhere.\textsuperscript{35,41} This phenomenon is uncharacteristic of most redox cofactor dependent enzymes and the nature of flavin binding may play a role in regulation of enzyme activity.

The two mechanistic families of flavin proteins described in Chapter 1, “cautious” and “bold”, were characterized for their steady-state kinetic mechanisms in similar fashions as shown here. The most well-studied “cautious” representative, para-hydroxybenzoate hydroxylase (PHBH), was shown to follow a random Bi-Uni-Uni-Bi ping-pong like mechanism.\textsuperscript{51} The key findings showed that two ternary complexes formed; the first between oxidized flavin, NADPH and the substrate \textit{p}-hydroxybenzoate and the second between reduced flavin, \textit{p}-hydroxybenzoate, and oxygen. In addition, \textit{p}-hydroxybenzoate was found to bind to oxidized enzyme with a 1:1 ratio and with relatively high affinity. Their steady-state results agree with later transient kinetic data that confirmed this type of reaction. The “bold” mechanistic class, of which OMO appears to share some sequence homology, is best represented by the Liver Microsomal
FAD-containing monooxygenase studied by Poulsen and Ziegler.\textsuperscript{44} Their steady-state results indicate an ordered Ter Bi mechanism with oxygen activation or formation of the C4a-(hydro)peroxyflavin intermediate being an irreversible chemical step. In this mechanism NADPH adds first, followed by O\textsubscript{2}, and then the hydroxylatable substrate; of which many nitrogen- and sulfur-containing molecules have been found to act. Their key findings were that NADPH and O\textsubscript{2} interacted with reversible enzymes forms and that substrates did not appear to bind the oxidized enzyme. Additionally, they found that NADP\textsuperscript{+} was a competitive inhibitor relative to NADPH and it binds oxidized enzyme, revealing that it was the last product to leave post-catalysis.

The steady-state results reported here suggest a mechanism similar to that of the “bold” FMO’s. The reversible connection between NADPH and O\textsubscript{2} at sub-saturating L-ornithine reveals that a ternary complex likely forms between oxidized enzyme, NADPH, L-ornithine, and O\textsubscript{2} prior to formation of the reactive intermediate. This relationship becomes irreversible in the presence of saturating amounts of L-ornithine forcing and ordered Ter Bi mechanism much like FMO’s except that substrate binds prior to O\textsubscript{2}. Additionally, NADP\textsuperscript{+} showed a pattern of pure competitive inhibition with NADPH, indicating that the NADP\textsuperscript{+}-releasing and NADPH-binding enzyme forms are the same. This suggests that the hydroxylated L-ornithine leaves the enzyme complex first with water, followed by NADP\textsuperscript{+} in a similar fashion to FMO’s.

These results are almost completely in agreement with a “bold” mechanism employed by many non-specific flavin enzymes. This presents the question of why a very specific enzyme like OMO, involved in biosynthesis opposed to catabolism, would use such a mechanism. A means of regulating NADPH usage and O\textsubscript{2} activation only in the presence of substrate must be present, however it is not like that observed in PHBH where substrate binds to oxidized enzyme. To answer this question further transient kinetic experiments using flavin spectroscopy must be performed. Looking at the rates of formation of different flavin intermediates and their substrate dependencies will uncover just how this “bold” mechanism differs and how specificity is achieved. The following chapter examines these reactions using rapid stopped-flow mixing techniques.
2.5 Acknowledgements

Thank you to Timothy Wencewicz for the synthesis and characterization of hydro-L-ornithine and Prof. Marvin J. Miller for intellectual and material contributions toward the synthesis and characterization of L-Orn-OH. Thank you to Dr. Alexander Gehrke of the Hans Kno¨ll Institute (Jena, Germany) for the kind gift of A. fumigatus from which genomic DNA was extracted. Amy Zercher gave technical assistance in cloning the sidA gene. Thank you to Garrett Moraski for technical assistance and for helpful discussions and Barrie Entsch (University of New England, New South Wales, Australia) for critical reading of the manuscript. NMR facilities were provided by the Lizzadro Magnetic Resonance Research Center at the University of Notre Dame, and mass spectrometry was provided by the University of Notre Dame Mass Spectrometry and Proteomics Facility (N. Sevova, Dr. W. Boggess, and Dr. M. V. Joyce; supported by National Science Foundation Grant CHE-0741793)

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2.6 References


CHAPTER 3

COMPREHENSIVE SPECTROSCOPIC AND TRANSIENT KINETIC STUDIES OF A REPRESENTATIVE SIDEROPHORE-ASSOCIATED FLAVIN MONOOXYGENASE

Abstract – In this study, the kinetic mechanism for the heterologously expressed N\textsuperscript{5}-L-ornithine monooxygenase from the pathogenic fungus Aspergillus fumigatus has been further characterized using a combination of spectroscopic and transient kinetic methods. Analyses of these data suggest a model for catalysis in which a molecule of hydroxylatable substrate serves as an activator for the reaction of the reduced flavin and O\textsubscript{2}. The rate acceleration is only 5-fold, a mild effect of substrate on formation of the C4a-hydroperoxide that does not influence the overall rate of turnover. The effect is also observed with the bacterial ornithine monooxygenase PvdA, and suggested the possibility that other small molecules might have a similar but more potent regulatory effect. The C4a-hydroperoxide is stabilized in the absence of hydroxylatable substrate by the presence of bound NADP\textsuperscript{+} (t\textsubscript{1/2} = 33 min, 25 °C, pH 8). NADP\textsuperscript{+} therefore is a likely regulator of the reaction of O\textsubscript{2} and substrate reactivity in the siderophore-associated monooxygenases. Aside from the activating effect of the hydroxylatable substrate, the siderophore-associated monooxygenases share much of their kinetic mechanism with the “bold” family of enzymes including the hepatic microsomal flavin monooxygenases and bacterial Baeyer-Villiger monooxygenases, with which they share only moderate sequence homology and from which they are distinguished by their acute substrate specificity. The remarkable specificity of the N\textsuperscript{5}-L-ornithine monooxygenase-catalyzed reaction suggests added means of reaction control beyond those documented in related well-characterized flavoenzymes. Notably, while neither of these two related classes of proteins has been shown to form complexes with their substrates, while we demonstrated that the ornithine monooxygenase and likely the other N-hydroxylating monooxygenases do.
3.1 Introduction

Work from Chapter 2 provides a basic understanding of the steady-state kinetic behavior of L-ornithine monooxygenase from *A. fumigatus* (OMO). Those methods have provided valuable information and helped in eliminating different types of mechanistic features; including a "cautious" pattern where hydroxylatable substrate binds preferably to the oxidized enzyme and NADP⁺ leaves prior to O₂ addition. However, the limits of steady-state methods do not allow us to further understand how the rates of individual steps in the reaction mechanism are regulated. Thus, transient kinetic methods were applied with the goal of determining what factors influence each step of the reaction.

Prior work with both PHBH and Liver microsomal FMO’s showed that transient kinetic data complemented and agreed with steady-state results.¹,² The same methods developed by that pioneering work are put to use here almost twenty years later. The key intermediates one can observe on a flavin spectrum are the oxidized, reduced, and hydroperoxyflavin species. The oxidized spectrum has a maximum peak at 450 nm with a double-peaked spectrum. The reduced species is bleached and contains little to no significant features (the yellow enzymes solution becomes clear). A maximum single peak at 370 nm is characteristic of the hydroperoxyflavin. All three intermediates are distinguishable and in prior work the rates of reactions in each step have been measureable with stopped-flow techniques. In fact, the C4a-(hydro)peroxyflavin intermediate can be observed on a slower time scale with FMO’s and its decay easily monitored with half-life approaching 30 minutes.

The following presents spectroscopic characterization and transient kinetic results for OMO. The enzyme was probed for its ability to reduce flavin and for formation and decay of the C4a-(hydro)peroxyflavin intermediate. Each step was studied individually in the absence and presence of L-ornithine. Analysis and results are presented showing both the changes observed in the UV/visible spectra as well as rate constants determined for the overall reaction. This data provides support for the proposed steady-state kinetic mechanism and illuminates a key regulatory mechanism in this class of enzymes that is significant to understanding how efficient catalysis and specificity is achieved.
3.2 Experimental Procedures

3.2.1 Rapid Reaction Studies

Reduction of the enzyme by NADPH and reoxidation by \( \text{O}_2 \) in the presence/absence of substrate were studied by stopped-flow spectrophotometric techniques at 25 °C in single and sequential mixing modes. For all experiments, the enzyme was placed inside a gas-tight tonometer and made anaerobic via repeated cycles of evacuation and purging with purified and hydrated argon gas. Solutions before mixing consisted of 20–40 μM FAD-containing enzyme subunit. These were mixed with an equal volume of reactant solution prepared in the same buffer from a second syringe. For anaerobic experiments, the solution in the second syringe was equilibrated with argon via bubbling; alternatively, this solution was equilibrated with air or bubbled with a defined \( \text{N}_2/\text{O}_2 \) gas mixture.

3.2.2 Reactions with NADPH

The anaerobic reduction of OMO (10 μM enzyme plus 10–200 μM NADPH, final concentrations) was monitored at 440 nm via a photomultiplier tube detector. Additional measurements were made with 200 μM NADPH plus either 200 μM NADP\(^+\) or 5 mM (saturating) L-Orn, in order to determine their effect on the reduction rate. For subsequent experiments, reduced enzyme solutions were prepared anaerobically in a tonometer by titrimetric addition of 1 eq of NADPH via a titration syringe.

3.2.3 Reactions of Reduced OMO with \( \text{O}_2 \)

All measurements were made using diode array detection. The single mixing mode was initially used to monitor reduced enzyme after mixing with buffer that contained defined concentrations of either \( \text{O}_2 \) alone or \( \text{O}_2 \) and L-Orn in combination. Subsequently, the sequential mixing mode was used for two types of experiments. In the first, NADPH-reduced enzyme was mixed with anaerobic solutions of L-Orn (0.5 and 5 mM). Following a set delay time (≥15 ms), the reaction was mixed with aerated buffer. This type of experiment was used to determine the
binding rate of L-Orn. In the second type, NADPH-reduced enzyme and aerated buffer were mixed and incubated for 5 s in order to allow for the formation of the flavin-oxygen reaction intermediate. The resulting solution was mixed with buffers containing various [L-Orn]. Details of individual experiments, including the concentrations used, are given in “Section 3.3 Results and Analysis” (Rapid Kinetic Studies) and figure legends.

3.3 Results and Analysis

3.3.1 Rapid Kinetic Studies of OMO with NADPH

Anaerobic samples of oxidized OMO (10 μM FAD) were reduced with a large stoichiometric excess of NADPH (200 μM final concentration, also anaerobic) in the stopped-flow instrument to determine the pseudo-first order rate constant for the reduction (Figure 3.1).

![Figure 3.1 Reduction of OMO by NADPH. The reaction mixture contained 10 μM enzyme at 25 °C. Absorbance was measured in photomultiplier mode at 450 nm and traces were fit to the sum of two exponentials. Calculated rates were 0.58, 0.67, and 0.60 s⁻¹ at 10 (long dashed line), 100 (solid line), and 200 (short dashed line) μM NADPH, where the trace at 100 μM NADPH contained 5 mM L-ornithine.](image)

After a lag time that may correspond to NADPH associating with the enzyme, conversion to the reduced form was observed via the loss of absorbance due to oxidized flavin (i.e. at 450 nm). A rate constant \( k_{obs} = 0.67 \text{ s}^{-1} \) for reduction was determined by fitting a sum of two
exponentials to the curve, where the first exponential function is for the lag and the second is for the reduction. The value for reduction was very close to the turnover number for the complete reaction measured at 25 °C \( (k_{\text{cat}} = 0.6 \text{ s}^{-1}, 25 \text{ °C}; \text{Table 2.2 (bottom)}) \). The rate constant and the apparent first order character of the reaction were unchanged when lower and even slightly substoichiometric (compared with FAD) amounts of NADPH were used. The lack of a response to changes in [NADPH], even when slightly less than 1 eq is used, suggests that the \( K_d \) for oxidized enzyme/NADPH is very small relative to the [NADPH] used and can be estimated as \( \leq 1 \mu\text{M} \). The rate of reduction was also measured in the presence of a saturating amount (5 mM) of L-Orn and 100 mM NADPH. L-Orn had minimal effects on the reduction. The reaction and equation below describe the interactions.

\[
\begin{align*}
K_{d,\text{NADPH}} & \\
E_{\text{FAD}} + \text{NADPH} & \rightleftharpoons E_{\text{FAD}}^*\text{NADPH} \rightarrow E_{\text{FADH}}^*\text{NADP}^+
\end{align*}
\]

\[
k_{\text{obs}} = \frac{k_{\text{red}}[\text{NADPH}]}{(K_{d,\text{NADPH}} + [\text{NADPH}])} = k_{\text{red}}
\]

### 3.3.2 Rapid Kinetic Studies of the Reaction of Reduced OMO with O\(_2\) in the Absence of L-Orn

The formation and decay of the hydroperoxide intermediate were both monitored by stoppedflow UV-visible spectroscopy. A two-peaked spectrum with a \( \lambda \) max near 450 nm is characteristic of oxidized flavin on the enzyme. Reduction of the enzyme with 1 eq of NADPH causes the yellow sample to turn clear, leading to a relatively featureless spectrum (data not shown). Anaerobic samples of OMO (10–20 μM FADH\(^-\)) reduced with stoichiometric quantities of NADPH were mixed with buffer that had been equilibrated with various concentrations of O\(_2\). The C4a-hydroperoxide (typically monitored at 370 nm) was subsequently seen to form relatively slowly (Fig. 3.2), with a \( k_{\text{obs}} \) that varied linearly with [O\(_2\)] and passed through the origin (second order \( k = 2.5 \times 10^4 \text{ M}^{-1} \text{ s}^{-1} \); Fig. 2.8, inset). A very similar rate constant was measured for FMO (\( k = 2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1} \)) at pH 8.0 and 15 °C.\(^3\) By contrast, at 0.6 mM O\(_2\), the analogous BVMO
intermediate was observed to form within the mixing time of the stopped-flow instrument (1–2 ms).\textsuperscript{57}

**Figure 3.2** A, formation of the C4a-hydroperoxyflavin-NADP\textsuperscript{+} complex. Spectra are shown at 0.004, 0.071, 0.139, 0.307, 0.577, 0.960, and 2.355 s after mixing the NADPH-reduced enzyme with air-saturated buffer (final [O\textsubscript{2}] = 0.130 mM) in the stopped-flow instrument. Inset, oxygen dependence of the rate of C4a-hydroperoxyflavin formation in the absence of L-Orn. C4a-hydroperoxide formation was monitored at 370 nm under pseudo-first order conditions with varying [O\textsubscript{2}]. Values for \(k_{\text{obs}}\) determined from fitting single exponentials to the data are plotted versus [O\textsubscript{2}]. The second order rate constant determined from this plot was 2.5 \(\times\) 10\textsuperscript{4} M\textsuperscript{-1} s\textsuperscript{-1}. Reaction mixtures contained 10 \(\mu\)M reduced enzyme-NADP\textsuperscript{+} at 25 °C. B, conversion of the C4ahydroperoxyflavin-NADP\textsuperscript{+} complex to oxidized FAD. Spectra at 2.63, 11.25, 18.6, 29.4, 60.3, and 142.5 s after mixing from the experiment in part A are shown.

The apparent C4a-hydroperoxide converted to the oxidized flavin with an isosbestic point near 400 nm and a first order rate constant \(k_{\text{obs}}\ 0.021\ \text{s}^{-1}\) (Figure 3.2). This value is independent of [O\textsubscript{2}] and very close to the turnover number for the uncoupled reaction measured at 25 °C (\(k_{\text{cat}}\ 0.025\ \text{s}^{-1}\)). Therefore, this step appears to limit the rate of the futile cycling that would lead to
H$_2$O$_2$ production. The lifetime for the C4a-hydroperoxide in pig liver FMO has been measured at pH 7.2 and 4 °C at 100 min.\textsuperscript{3} When the OMO C4a-hydroperoxide was generated at pH 7 and 4 °C, we noted that conversion to the oxidized form was complete within 2.5 h (data not shown). Hence, the OMO intermediate can also be described as long lived. Adding excess NADP\textsuperscript{+} (150 μM or about 10$K_m$NADPH, 5 $K_d$NADP\textsuperscript{+}, measured relative to the oxidized enzyme) only slightly increased the measured half-life (data not shown). Finally, the dependence of the half-life for C4a-hydroperoxylavín/FAD conversion on bound NADP\textsuperscript{+} was examined by chemically reducing an anaerobic sample of OMO with an equivalent of dithionite. Upon re-exposure to air, the reduced enzyme appears to convert directly and swiftly to FAD\textsubscript{ox} without an observable hydroperoxide intermediate (Figure 3.3).

Figure 3.3 Reoxidation of dithionite-reduced OMO. The oxidized enzyme (■) was anaerobically reduced with approximately 2 equivalents of dithionite (▲) at 25 °C in 100 mM Tris-H$_2$SO$_4$ pH 7.4 and then exposed to air (●). A spectrum of each species was recorded immediately and repeatedly to be certain no changes occurred.

3.3.3 Rapid Kinetic Studies of the Reaction of Reduced OMO with O$_2$ in the Presence of L-Orn

The formation and decay of intermediates were monitored during the reaction of O$_2$ with OMO that had been stoichiometrically reduced with NADPH, this time in the presence of L-Orn. Mixtures with varied [L-Orn] in O$_2$-saturated buffer or varied [O$_2$] in the presence of 5 mM L-Orn,
were used. The formation of the C4a-hydroperoxide was in each case slow enough to detect but was 5-fold faster in the presence of 5 mM ornithine than without ornithine. Each time course fit well (at 370 nm) to a single exponential curve. The measured values for $k_{\text{obs}}$ showed a linear dependence on $[\text{O}_2]$ (Figure 3.4A and inset) and a hyperbolic dependence on [L-Orn], with half-maximal $k_{\text{obs}}$ occurring at $K_{d(\text{app})} = 680 \, \mu\text{M}$ ornithine (maximal $k_{\text{obs}} \, 82 \, \text{s}^{-1}$) (Figure 3.4B). This apparent $K_d$ is close to the $K_{\text{m,L-Orn}}$ measured in the steady state (580 μM at 25 °C). The second order rate constant describing the $\text{O}_2$ dependence at saturating L-Orn was $k = 1.3 \times 10^5 \, \text{M}^{-1} \, \text{s}^{-1}$ (Figure 3.4C).
**Figure 3.4** A, formation of the C4a-hydroperoxyflavin-NADP⁺ complex in the presence of L-Orn. Spectra are shown at 0.004, 0.019, 0.034, 0.049, 0.071, 0.079, 0.011, and 0.026 s after mixing the NADPH-reduced enzyme with air saturated buffer containing 5 mM L-Orn (final \([O_2] = 0.130\) mM). The reaction mixture contained ~10 μM reduced enzyme-NADP⁺ complex at 25 °C. Inset, oxygen dependence of the rate of C4a-hydroperoxyflavin formation in the presence of saturating L-Orn. The conversion of the reduced enzyme-NADP⁺ complex to the C4a-hydroperoxyflavin was monitored at 370 nm in the presence of 5 mM L-Orn and varying (pseudo-first order) \([O_2]\). Values for \(k_{\text{obs}}\) were determined from fits of single exponential curves to the data and plotted versus \([O_2]\). The second order rate constant determined from this plot was \(1.3 \times 10^5\) M⁻¹ s⁻¹. B, kinetic traces illustrating the \(O_2\) dependence of the reaction of reduced enzyme-NADP⁺ complex with \(O_2\) in the presence of L-Orn. Absorbance traces at 370 nm show the reduced enzyme-NADP⁺ complex reacting with varying \([O_2]\) to form the C4a-hydroperoxyflavin. Final oxygen concentrations were 0.06, 0.13, 0.3, and 0.6 mM. The kinetic trace at 450 nm illustrates the formation of the oxidized FAD that occurs as the C4a-hydroperoxyflavin disappears, a reaction that shows no dependence on \([O_2]\). C, dependence of the rate of C4a-hydroperoxide formation on \([L-Orn]\) at fixed oxygen. The conversion of reduced enzyme-NADP⁺ complex to the C4a-hydroperoxyflavin was monitored at 370 nm at various (pseudo-first order) \([L-Orn]\) at fixed/saturating \(O_2\). Rate constants were determined from single exponential fits to the first portion of the curves. The apparent \(K_d\) and maximal \(k_{\text{obs}}\) determined from this plot were 680 μM and 82 s⁻¹. Each reaction mixture contained 10 μM reduced enzyme-NADP⁺ complex with 0.3 mM \(O_2\) at 25 °C.
The conversion of the C4a-hydroperoxide species to the oxidized flavin (Figure 3.5A) occurred in what appeared to be three consecutive exponential phases that were most easily observed near 390 nm (time course data not shown). It was not possible to cleanly deconvolute all three phases in all of the experiments. However, in the presence of high concentrations of O$_2$ and L-Orn, the individual rates could be distinguished sufficiently that the first two rate constants could be reasonably well fit. The values obtained are reported in Figure 3.8. The initial phase probably corresponds to hydroxylation of the amine with concomitant formation of the C4a-hydroxyflavin species. This species has a λ max slightly blue-shifted relative to that of the C4a-hydroperoxide. Notably, the apparent (but not genuine) isosbestic point linking the C4a-hydroperoxide (max 370 nm) and FAD shifts from 400 to 380 nm when L-Orn is present (Figures 3.2B and 3.5A), suggesting the presence of an intermediate with a shorter wavelength max. At 0.6 mM O$_2$, the rate constant for the hydroxylation varies between 5 and 8 s$^{-1}$, depending on [L-Orn].

Figure 3.5 A, conversion of the C4a-hydroperoxyflavin-NADP$^+$ complex to oxidized OMO in the presence of L-Orn. 10 μM reduced enzyme-NADP$^+$ complex containing 5 mM L-Orn was mixed with air-saturated buffer (final [O$_2$] = 0.3 mM at 25 °C). The C4a-hydroperoxyflavin species (λ$_{max}$ = 370 nm) formed and subsequently converted to the oxidized flavin. Only the spectra showing the latter conversion are shown (recorded 0.04, 0.30, 0.50, 1.10, 2.00, and 7.00 s after mixing). B, dependence of the conversion of the C4a-hydroperoxide to oxidized FAD on L-Orn at fixed/saturating O$_2$. The conversion of the C4a-hydroperoxyflavin to the oxidized FAD shown in A was monitored at 450 nm at various (pseudo-first order) L-Orn concentrations. Values for $k_{obs}$ were determined from single exponential fits to the curves. The apparent $K_d$ and maximal $k_{obs}$ determined from this plot were 2.2 mM and 1.8 s$^{-1}$. 

74
The second phase corresponds to the dehydration of the hydroxyflavin to yield FAD. This step also appears to have some dependence on [L-Orn] and occurs at 1.5–3 s\(^{-1}\). This apparent dependence is probably caused by the similarity in the rates of hydroxylation and dehydration, making the two steps difficult to parse, particularly at lower [L-Orn]. The last phase occurs with only a very small absorbance change and consequently could not be fit accurately. This step could correspond to release of the hydroxylated product and/or the oxidized NADP cofactor. A single exponential was also fit to the 450 nm traces over the entire time course between the maximization of the C4a-hydroperoxide and FAD peaks, in an effort to quantify the dependence on [L-Orn] of the composite interaction of L-Orn with C4a-hydroperoxide. A similar fitting procedure was used for data recorded for analogous experiments with FMO\(^4\). A plot of the resulting \(k_{\text{obs}}\) values versus [L-Orn] can be fit to a hyperbolic curve with an apparent \(K_d\) of 2.2 mM (Figure 3.5B). Hence, both the rates of formation and the subsequent conversion of the C4a-hydroperoxy species to the oxidized FAD demonstrated dependence on [L-Orn]. It is noted that this \(K_d\), which is for the binding of L-Orn to the C4a-flavin hydroperoxide, is 3-fold larger than the apparent \(K_d\) for binding to reduced OMO (0.68 mM).

In order to further define the interactions of L-Orn with both the reduced enzyme and the C4a-hydroperoxide species, two types of sequential mixing stopped-flow experiments were carried out. First, reduced enzyme was mixed anaerobically with saturating (5 mM) L-Orn and allowed to age for various times before mixing with \(O_2\)-saturated buffer. If equilibration of the reduced enzyme- L-Orn complex were incomplete in the selected time delay, the subsequent reaction with \(O_2\) should not all occur at the faster, L-Orn-promoted rate. However, the rate of C4a-hydroperoxide formation was unaffected by an enzyme- L-Orn incubation time as short as 15 ms, even when a lower concentration of L-Orn (0.5 mM or \(K_m\)) was used (data not shown). Hence, the reduced enzyme and L-Orn must equilibrate very rapidly (i.e. within 15 ms).

Second, the reaction of L-Orn with a preformed C4a-hydroperoxide intermediate was probed directly (Figures 3.6 and 3.7). Reduced enzyme was mixed with air-saturated buffer, and the solution was aged until the C4a-intermediate completely formed (5 s). It was then mixed with buffer containing various concentrations of L-Orn, and the subsequent reactions were monitored.
The data appeared to be very similar to the conversion of the C4a-flavinhydroperoxide to oxidized flavin observed in the single mixing experiments described above (Figures 3.4 and 3.5), in which reduced enzyme was simultaneously mixed with L-Orn and O₂. Three consecutive exponential phases were also apparent in the data. Traces at 393 nm following formation of the C4a-hydroxide at various L-Orn concentrations are shown in Figure 3.7A. The first phase was most pronounced near 390 nm. The second phase was slower and corresponded to a large change in the absorbance near 450 nm (oxidized FAD) (as seen in Figure 3.6). The third phase again resulted in only subtle absorbance changes and most likely corresponds to product release from the oxidized enzyme. Traces at 393 nm fit well to three exponentials with rate constants identical to those identified from the single mixing data above (Figure 3.4 and Figure 3.5). Singular value decomposition of the entire data set (via SpecFit) using the measured rate constants yielded a set of three component spectra that bear remarkable similarity to those measured here for the C4a-hydroperoxide and the oxidized flavin as well as a blue-shifted spectrum typical of a C4a-flavin hydroxide (Figure 3.6B and Figure 3.7).
Figure 3.6 A, conversion of a pre-formed C4a-hydroperoxylavin-NADP⁺ complex to oxidized OMO in the presence of L-Orn. 10 μM reduced enzyme-NADP⁺ complex in the absence of L-Orn was mixed with air-saturated buffer (final [O₂] = 0.3 mM at 25 °C) and aged until the C4a-hydroperoxylavin intermediate fully formed (5s). The pre-formed intermediate was then mixed with 5 mM L-Orn in the same buffer. Spectra shown were recorded 0.161, 0.352, 0.487, 0.622, 1.050, and 7.125 s after the second mixing. B, kinetic traces showing the reaction of C4a-hydroperoxide to oxidized FAD at 450 nm. Reactions were monitored at 450 nm. Final [L-Orn] = 0.0625, 0.25, 0.5, 1.25, 2.5, 5.0, and 25.0 mM. C, Dependence of the conversion of the C4a-hydroperoxylavin to oxidized FAD shown in A was monitored at 450 nm at various (pseudo first order) L-Orn concentrations. Values for k_{obs} were determined from single exponential fits to the curves. The apparent K_d and maximal k_{obs} determined from this plot were 2.3 mM and 2.5 s⁻¹.
Figure 3.7 A, kinetic traces illustrating the reaction of a pre-formed C4a-hydroperoxide with variable concentrations of L-Orn. The C4a-hydroperoxide was generated by mixing 10 μM reduced enzyme-NADP⁺ complex (in the absence of L-Orn) with air-saturated buffer (final [O₂] = 0.3 mM, 25 °C) and aged 5 s. It was then mixed with various L-Orn concentrations in the same buffer. The data measured at 393 nm are shown, illustrating 3 consecutive exponential processes. Final [L-Orn] for the traces shown is 1.25, 2.5, 5.0, and 25.0 mM. B, singular value decomposition of the complete set of data shown in Fig. 5A, using the rate constants fit to the three kinetic phases highlighted in A. Three constituent spectra were determined, resembling previously measured spectra for C4a-hydroperoxide (dotted line), C4a-hydroxide (solid line), and oxidized FAD (dashed line).

The rate constants for the hydroxylation and dehydration steps, obtained with this double mixing experiment and in the single mixing experiments described above, both were dependent on [L-Orn]. Single exponentials were fit independently over the full time course of these experiments at 450 nm, where \( k_{obs} \) depended hyperbolically on [L-Orn] (apparent \( K_d \) 2.1 mM; Figure 3.6, B and C). The apparent \( K_d \) for the C4a-hydroperoxide and L-Orn measured in both this experiment and the analogous single mixing experiment in which L-Orn and O₂ were added to the reduced enzyme concurrently are nearly the same. These results suggest that the hydroxylation reaction in either case is the same, occurring after the binding of L-Orn to the C4a-hydroperoxide.

In further support of this conclusion, experiments in which L-Orn and O₂ were independently presented to the reduced enzyme showed that either the \( E_{red}\cdot L-Orn \) complex or the reduced enzyme alone is capable of reacting with O₂. These two pathways yield the C4a-hydroperoxide at rates equal to 1) \( k[E_{red}\cdot L-Orn][O₂] \) and 2) \( k'[E_{red}][O₂] \), where \( k/k' \) is ~5. Given the
apparent $K_d$ for $E_{red}\cdot L$-Orn of 0.68 mM, $[E_{red}\cdot L$-Orn] = 1470 M$^{-1}$[$E_{red}$][L-Orn]. The ratio of rate 1 to rate 2 is therefore equal to [L-Orn] (7350 M$^{-1}$). This suggests that the two rates are equal and their associated pathways are equally populated at [L-Orn] 140 μM or when ~20% of the reduced enzyme is occupied by L-Orn (according to the measured $K_d$). The L-Orn facilitated pathway gains importance proportionally at concentrations above 140 μM. For example, at $K_{m, L$-Orn} = 0.5 mM, the L-Orn pathway would be preferred ~3.7:1. This suggests that the pathway in which L-Orn associates with the reduced enzyme and facilitates O$_2$ activation is preferentially in use over much of the L-Orn concentration range probed in this paper.
Figure 3.8 Kinetic Mechanism for OMO
3.4 Discussion

All known flavin monooxygenases activate dioxygen to produce the substrate-hydroxylating species, a flavin C4a-hydroperoxide (or peroxide) adduct. The reactive species must then be directed toward the desired substrate and at the same time protected from releasing H$_2$O$_2$ to form oxidized flavin. The two major classes of well characterized flavin monooxygenases differ according to how they achieve the regulatory functions that avoid releasing H$_2$O$_2$. In the aromatic hydroxylases, of which para-hydroxybenzoate hydroxylase is the best studied example,\textsuperscript{5} regulation occurs at the level of flavin reduction. Rapid reduction occurs only following the binding and deprotonation of the substrate, an event that triggers a protein conformational change that brings FAD and NADPH into an optimal position for hydride transfer. Thus, unless a substrate is present, the flavin is not effectively reduced, and reaction with O$_2$ cannot occur. Additional movements of the flavin enclose it and the substrate into a solvent-protected pocket, where the C4a-hydroperoxide can safely form and react with substrate.

By contrast, the hepatic FMO’s and bacterial BVMO’s regulate their reactivity at the level of the C4a-hydroperoxide itself, which when formed, is quite stable and releases H$_2$O$_2$ only very slowly in the protein’s interior.\textsuperscript{3-4,6-8} Such a mechanism suits the biological role of the FMO’s in hydroxylating and thereby increasing the aqueous solubility of xenobiotic compounds. Consistent with this role, these enzymes hydroxylate a broad variety of nitrogen- and sulfur-containing nucleophiles and even halides.\textsuperscript{9} The reactive species is understood to sit in a solvent-protected pocket that is able to exclude charged (and therefore endogenous) metabolites in favor of a broad spectrum of neutral xenobiotics. By contrast, SMOs are generally known to have acute substrate specificity, even distinguishing L-ornithine from L-lysine despite the fact that the two differ by just one methylene group.\textsuperscript{10,11} It therefore came as a surprise that OMO shared so many mechanistic features with the decidedly omnivorous FMO’s. OMO, like the bacterial PvdA\textsuperscript{12} and the FMO/BVMO enzymes, showed no substrate-dependent enhancement in the rate of FAD reduction, ruling out a possible substrate-gated regulatory mechanism of the kind identified in para-hydroxybenzoate hydroxylase. Furthermore, retention of NADP on the enzyme following reduction stabilizes the C4a-hydroperoxide intermediate in the hepatic FMO’s and the BVMO’s.\textsuperscript{3-}
and, as shown here, also in OMO. A quasi-stable hydroperoxide was observed only in the presence of NADP (Figure 3.2). In the absence of hydroxylatable substrate, decomposition of this species to form oxidized FAD and H\textsubscript{2}O\textsubscript{2} is rate limiting and slow in all of these enzymes. Exposure of dithionite-reduced FMO\textsuperscript{56} or OMO to air resulted in the rapid production of oxidized FAD without observable intermediates (Figure 3.3).

Both steady state and transient studies of OMO also indicated important differences in the way it and the FMO or BVMO enzymes interact with their hydroxylatable substrates. Steady state kinetic data for OMO showed a pattern of substrate interactions suggesting ordered binding of NADPH, L-Orn, and O\textsubscript{2}. This is different from what was observed or would be predicted for either the FMO-like or aromatic hydroxylases and initially appeared to indicate a unique mechanism. Transient kinetic and product inhibition data subsequently shed light on these results. First, as in PvdA, it was noted that the rate of formation of the C4a-hydroperoxide is regulated by the presence of substrate.\textsuperscript{12} The effect is mild, however, particularly when compared with the 105-fold enhancement in the rate of FAD reduction in the presence of substrate observed for para-hydroxybenzoate hydroxylase.\textsuperscript{1} Studies of the reaction between E-FAD\textsubscript{red}-(NADP) and O\textsubscript{2} showed that the second order rate constant increased roughly 5-fold in the presence of saturating L-Orn. However, because formation of the C4a-hydroperoxide is not the rate limiting step in catalysis (see Figure 3.8), enhancing the rate of this step has no effect on the rate of turnover. It is therefore unclear whether the observed rate acceleration serves a biological role. The dependence of the rate constant for formation of the C4a-hydroperoxide on [L-Orn] was hyperbolic with an apparent \(K_d\) of 0.6 mM, very similar to the measured \(K_i\) (0.5 mM) for L-Orn/L-Orn-OH inhibition (see below) as well as \(K_{m,L-Orn}\) (0.5 mM). This similarity suggests that each measured quantity is due to the same interaction between protein and L-Orn. In the case of \(K_{m}\) identity with the apparent \(K_d\) and \(K_i\) could be observed if L-Orn achieves equilibrium with E-FAD\textsubscript{red}-(NADP) rapidly. A rapid equilibrium was indeed identified via double mixing kinetic experiments.

Evidence for the interaction of a second enzyme form with L-Orn likewise came from both transient kinetics and product inhibition. The reaction of a preformed C4a-hydroperoxide with L-
Orn resulted in generation of hydroxylated product, demonstrating that the intermediate formed in the absence of L-Orn is catalytically competent. Moreover, the intermediate reacted with L-Orn with identical kinetics whether L-Orn was present prior to C4a-hydroperoxide formation or was added to the preformed intermediate. In either case, the reaction demonstrated a saturable dependence on [L-Orn] \( (K_{d(app)} \, 2.1 \, \text{mM}) \). The conversion of the C4a-hydroperoxide to the hydroxide and then to oxidized flavin occurred via a series of single exponential processes that were identical in each type of experiment. These observations, coupled with the fast off-rate for L-Orn from the FAD_{red}(NADP) complex, suggest that with low concentrations of L-Orn present, the molecule that is hydroxylated most likely adds to the enzyme in its C4a-hydroperoxide form.

Further evidence for the interaction of L-Orn with both FAD_{red}(NADP) and a second enzyme form came from product inhibition. The N\(^5\)-hydroxyornithine product of the reaction was shown to act as a competitive inhibitor with L-Orn, indicating that the two vie for the same reduced, NADP-bound enzyme form. By contrast, at higher but non-saturating concentrations of L-Orn, L-Orn-OH is uncompetitive with NADPH, suggesting that a second enzyme form can also bind L-Orn-OH. This form is presumably the C4a-hydroperoxide. The \( K_i \) for L-Orn-OH and this enzyme form is 1.6 mM, very similar to the measured \( K_{d(app)} \) of 2.1 mM cited above, suggesting that these two constants indeed describe the interaction of the same enzyme form with L-Orn/L-Orn-OH. Notably, the affinity of the substrate/product for this form is somewhat less than for the reduced enzyme.

A simple analysis of the relative rates of the L-Orn-promoted and -unpromoted pathways for C4a-hydroperoxide formation suggests that the former pathway is preferred at [L-Orn] 140 \( \mu \)M, which includes all of the concentration range probed in this study. The ambient concentrations of L-Orn inside the cytosol of \( A. \ fumigatus \) are not known, although ornithine is produced exclusively in the mitochondrion and actively transported into the cytosol, where siderophore biosynthesis takes place. It is possible that concentrations in this range could be reached, particularly if ornithine transport occurred in response to iron stress. It is unclear whether the L-Orn binding sites on the FAD_{red}(NADP) and C4a-hydroperoxide species are physically in the same or different locations. The kinetic and spectroscopic data are consistent.
with either two physically independent binding sites for L-Orn, one allosteric and the other
catalytic, or a single binding site that has differing L-Orn affinities depending on whether the
enzyme is in its FAD_{red}-(NADP) or C4a-hydroperoxide (NADP) form. If the former description is
true, then it is possible that other effectors that could stimulate C4a-hydroperoxide formation are
available in the cell. If the latter is more accurate, then the reaction with L-Orn and O_{2} is best
described as random. The observed dependences of both the rates of C4a-hydroperoxide
formation and conversion to FAD on [L-Orn] are consistent with either description. Deciphering
these two possibilities will be the subject of future work.

By the same token, the physical mechanism by which L-Orn accelerates C4a-
hydroperoxide formation is unknown. It is possible that binding of the positively charged L-Orn
could lower the barrier for the formation of the flavin semiquinone/ superoxide radical pair.\textsuperscript{13} This
would most likely require the regulatory and catalytic binding sites to be physically one and the
same. It is also possible that L-Orn could dynamically induce a structural change that would
promote access of the reduced flavin to O_{2} or that would bring the C4a position closer to an
enzyme or NADP-supplied positive charge. Interestingly, the turnover rates of FMOs have long
been known to be accelerated by allosteric effectors, including octylamine.\textsuperscript{8} The nature of the
effector interaction with substrate is unknown, but because it influences the turnover rate, it would
need to have its effect on a rate-limiting step (i.e. either hydroxyflavin dehydration or NADP
release at the end of each turnover). Studies of recombinant, chimeric forms of FMOs indicated
that the substrate- and octylamine-binding portions of the FMO enzymes are probably remote
from one another.\textsuperscript{14}

The ability of the C4a-hydroperoxide to form a measurable complex with its substrate is
unusual. It has been demonstrated that the FMOs in their C4a-hydroperoxide forms do not
reversibly form complexes with their hydroxylation substrates but instead react by a second order
mechanism.\textsuperscript{4,15} For OMO, saturation in the plot of $k_{obs}$ versus [L-Orn], measured under pseudo-
first order conditions for the reaction of the C4a-hydroperoxide and L-Orn, indicates that a
complex does indeed form. Moreover, this enzyme form is subject to inhibition via the binding of
L-Orn-OH. The difference in pK_{a} for the amine substrate (~10) and hydroxylamine product (~5)
indicates that the latter has a neutral side chain, whereas the former is positively charge inhibitor with a $K_i$ similar to the substrate $K_d$ (for either L-Orn binding interaction) suggests either that the substrate is deprotonated in the active site or that the side chain charge is not relevant to the binding interaction. Future work will focus on understanding the interaction of substrate-like molecules with the two L-Orn-binding enzyme forms. Such an understanding is essential for exploring possible inhibition strategies as well as for understanding the remarkable specificity of OMO and its homologs for their substrates.

3.5 Summary

The OMO from *A. fumigatus* is a representative *N*-hydroxylating monooxygenase involved in siderophore biosynthesis. We have shown that it forms a quasi-stable C4a-hydroperoxide solely in the presence of bound NADP$^+$. The formation of this species is modestly accelerated by the interaction of the reduced enzyme with a molecule of substrate. Although the effect is easily detected, it is probably insufficient to explain how OMO achieves its remarkable substrate selectivity or how it regulates the reaction with $O_2$. The effect could be more pronounced in other NMOs. On the other hand, in contrast to FMOs, the C4a-hydroperoxide of OMO appears to bind L-Orn reversibly, potentially offering a means of screening for appropriate substrates.

Further work on understanding the substrate specificity of L-ornithine monooxygenase was undertaken in the DuBois laboratory. Using a variety of substrate mimics and measuring their effect on the rates of formation and decay of the C4a-(hyro)peroxide intermediate further information was acquired. That work is supplied in Appendix A, and is not part of this primary thesis. Work in Appendix A was mostly conducted by others and the author of this thesis made minor contributions, however it has been included for the reader’s interest and immediate reference.
3.6 Acknowledgements

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3.7 References

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CHAPTER 4
DIOXYGEN-GENERATING CHLORITE DISMUTASES AND THE CDE PROTEIN
SUPERFAMILY

Abstract – The versatility of catalytic reactions performed by heme cofactors warrants it the title of Nature’s Swiss-army knife. The relationship between heme and its protein environment dictates the specific functions it can perform. Prokaryotes contain a variety of heme-dependent enzymes that take advantage of the cofactor’s catalytic versatility that greatly contributes to metabolic adaptation in these organisms. The organisms that exist in seemingly toxic and dangerous environments often have unique genetic and biochemical attributes that allow them to survive under these harsh conditions. It therefore makes sense that these organisms use heme and heme proteins to perform complicated oxidation-reduction chemistry when needed. Recently evolved classes of microbial heme enzymes, chlorite dismutases (Cld), perform a complex reaction as part of perchlorate (ClO$_4^-$) respiration. The end product of this process is the toxic molecule chlorite (ClO$_2^-$), which Cld dismutates to a chloride ion (Cl$^-$) and molecular oxygen (O$_2$). This chapter introduces chlorite dismutase and provides a structural and sequence-based analysis that indicates a link between Cld proteins and two other distinct enzyme families. Like chapter 1, which introduced siderophores and gave a brief review of flavin monooxygenase studies, it attempts to introduce the Cld protein family and presents prior mechanistic work that sought to understand the unique reaction. The goal of this chapter is to provide a context for results reported in later chapters. It also reveals that several annotated ClDs are found in non-perchlorate respiring bacteria where their true function is unknown, which is the subject of chapter 7.
4.1 Introduction

The catalytic diversity of heme proteins is vast – a result of the flexible, evolvable nature of the heme-protein combination. In the post-genomic age, novel families of heme proteins will likely continue to be identified, relationships among known families will become better defined, and biological roles for the proteins will be more precisely delineated. The CDE (Chlorite dismutases, Dye-decolorizing peroxidases and EfeB-like proteins) superfamily is a recently identified group of bacterial, archaeal, and fungal heme b-binding proteins consisting of 3 subfamilies, all sharing a highly similar monomer structure and grouped according to primary sequence similarities. Structural, genetic, and catalytic descriptions are emerging for subfamily members, many of which continue to be annotated or functionally described as heme peroxidases though their chemical and biological functions may not be clear.

All three protein families began to draw increasing attention in the early 2000s. Bacterial DyPs were the first to be studied in much detail, in the mid-1990s. These heme b containing proteins are named for their ability to catalyze the H$_2$O$_2$-dependent oxidation of chemically intransigent anthraquinone dyes, a reaction of some biotechnological importance. They are distinct on a sequence level from canonical plant, fungal, and animal peroxidases and from bacterial catalase-peroxidases. Hence, they have been assigned to their own family of peroxidases.

The Clds began to be investigated at roughly the same time. These proteins catalyze a biologically unusual O-O bond joining mechanism; efficient O-O bond formation is observed elsewhere only in the O$_2$ evolving reaction from photosystem II. Chlorite (ClO$_2^-$) is the substrate and Cl$^-$ and O$_2$ are the products of the Cld-mediated reaction, which again occurs at a heme b-containing active site. This unusual reaction is associated with an equally unusual biological process: perchlorate (ClO$_4^-$) respiration. Several species of Proteobacteria have been identified that couple the sequential reductions:

\[ \text{ClO}_4^- + 2H^+ \rightarrow \text{ClO}_3^- + H_2O \]
\[ \text{ClO}_3^- + 2 H^+ \rightarrow \text{ClO}_2^- + H_2O \]
to respiratory energy generation via electron transport and the production of an electrochemical proton gradient.\textsuperscript{7, 9-16, 16-20} The molybdopterin-containing enzyme catalyzing these two reductions, perchlorate reductase, bears clear evolutionary, structural, and functional relationships to the more widely distributed respiratory nitrate reductases. The evolutionary origins of the ClDs are less clear, as the proteins do not have close sequence relationships to peroxidases or other known heme proteins. All organisms that respire perchlorate must have a copy of the \textit{cld} gene in order to survive, as the ClO\textsubscript{2}\textsuperscript{-} that would otherwise accumulate would quickly become toxic. ClO\textsubscript{4}\textsuperscript{-}, ClO\textsubscript{3}\textsuperscript{-}, and ClO\textsubscript{2}\textsuperscript{-} are all toxic and primarily man-made. Why perchlorate respiration first evolved remains unclear. Notably, ClD-family proteins have been annotated in 13 bacterial and 2 archaeal phyla – most members of which have no known or expected perchlorate or chlorite metabolism. An understanding of the roles of these ClD-family proteins is only beginning to emerge.

Finally, the bacterial EfeB-like proteins (COG 2837) recently became of note for two reasons. First, they were identified as members of a 3-gene operon regulated by the ferric uptake regulator (Fur) in \textit{Escherichia coli}. This operon has been shown to be upregulated under acidic and hypoxic conditions in which Fe(II) is supplied as an iron source, and under general iron-deficiency. Genetic work has identified a role for EfeB in recovering iron out of ingested heme.\textsuperscript{21-25} A similar operon is found in many Gram-negative and Gram-positive organisms. Second, the EfeB protein from \textit{E. coli}, also known as YcdB, was identified as unique for being preceded by a twin-arginine transport (Tat) sequence.\textsuperscript{26} An \textit{N}-terminal Tat sequence earmarks a protein for export to the periplasm or cell exterior in its folded state. Such sequences have been associated with metal- or organic cofactor-containing proteins that acquire their cofactor in the cytoplasm, possibly during translation. YcdB/EfeB was the first known heme-containing protein to have a Tat sequence, though its biological function is undetermined.

Because the chlorite dismutases have been studied in the most detail thus far and they are the focus of much of the following work, their background will be emphasized throughout this chapter. However, comparisons among all 3 families will be made as the current state of knowledge about each is summarized. This background information and sequence/structure analysis is critical for setting the stage for work explained in subsequent chapters and will act as a
reference point for much of that work.

4.2 Heme Peroxidases

All 3 subfamilies of the CDE structural superfamily have been described at various times as peroxidases or catalases. It therefore makes sense to begin to understand them in the context of the well-characterized heme peroxidases, with which they all share some similarities. This context will be very briefly summarized here.

4.2.1 Peroxidase Classifications

The peroxidases are an extremely vast group of enzymes, spanning >7000 sequences in 940 organisms and distributed among 11 superfamilies and about 60 subfamilies. The diversity of peroxidases within a single complex organism can be quite vast: humans possess more than 30 peroxidases; the model plant species *Arabidopsis thaliana* has about 130 diverse peroxidases in 13 families and 9 subfamilies. Peroxide-reducing enzymes can depend on a variety of cofactors or reactive side chains other than heme: vanadium (haloperoxidases), cysteine or glutathione sulfhydryls (alkylhydro peroxidases, thiol peroxidases, peroxiredoxins), NADH (NADH peroxidases), and manganese (Mn catalases).

The majority of all known peroxidases contain heme. Among these, there are two major sequence groupings: the animal peroxidases and the non-animal peroxidases (*Figure 4.1*). The latter has plant, fungal, and bacterial members and the former is primarily though not exclusively animal-based. Animal peroxidases probably arose independently of the plant/fungal/bacterial peroxidase superfamily and most likely belong to a different gene family. Up to four additional sequence families of heme peroxidases are often described: catalases; di-heme cytochrome c peroxidases; the heme c-dependent haloperoxidases; and the DyP-type peroxidases. The catalases are found in bacteria, fungi, plants, and animals, have their own origins (including independence from the bifunctional bacterial catalase-peroxidases), and constitute a super group.
of their own. The placement of the other families relative to the two major heme peroxidase groupings is less clear.

![Figure 4.1 Conventional classifications of heme peroxidases](image)

Functionally, all of these enzymes share the ability to readily reduce $\text{H}_2\text{O}_2$, in the process generating reactive species capable of oxidizing diverse organic and inorganic substrates. In plants alone, they have biologically diverse roles in lignification, suberization (cork production), hormone (auxin) catabolism, and self-defense against pathogens. Given this somewhat broad functional definition of a peroxidase, the numerous and diverse proteins falling into this class, and their often low substrate specificities, it is not surprising that the biological functions and native cosubstrates for many of these proteins are unknown.

Representatives of both the animal and particularly the plant/fungal/bacterial superfamily have been well studied structurally, electronically, and mechanistically. They serve as a touchstone for understanding much of heme protein biochemistry, including members of the CDE superfamily (with which they share important structural features), and so will be further described here. Using primary sequences and the cytochrome c peroxidase (CcP) crystal structure as a guide, Karen Welinder further classified the non-animal peroxidases into three sub-classes. Class I contains the cytoplasmic prokaryotic peroxidases, which lack cysteine bridges, $\text{Ca}^{2+}$, and carbohydrates. Cytochrome c peroxidase (CcP) and the bifunctional bacterial catalase peroxidase (KatG) are two of the best studied examples of this family. Class II contains the
extracellular, glycosylated fungal peroxidases, including *Coprinus cinereus* peroxidase (CIP). Finally, Class III peroxidases are typically plant-derived and glycosylated, including possibly the best characterized peroxidase, horseradish peroxidase (HRP).

### 4.2.2 Defining Features of Plant/Fungal/Bacterial Peroxidases

Peroxidase chemistry was studied for decades prior to the availability of the first crystal structure in 1980, of the monomeric yeast cytochrome c peroxidase (CcP). This structure (Figure 4.2) illustrates several features common to the non-animal and animal peroxidases. These proteins may be monomers, dimers, or tetramers. The monomer consists of 10 alpha helices in the same orientation, with the heme bound between the second and sixth helices (B and F) from the N-terminus. The sequence between helices A-D (the distal domain) and E-J (proximal domain) suggests that a gene duplication event occurred at some point to give rise to the full length sequence. The heme b is ligated by a proximal histidine on helix F. This is hydrogen bonded to an aspartic acid from helix H. The distal pocket contains a catalytic histidine base from helix B, along with a nearby arginine. In addition to these residues, CcP contains a proximal tryptophan that undergoes 1e- oxidation as the enzyme forms its reactive intermediate (below). The CcP structure has a small β-sheet region and several loops, which are of variable length and composition in the different peroxidases.

The active site of CcP and other heme peroxidases is defined by the catalytic residues described above and depicted in Figure 4.2. The classic push-pull model of Poulos and Kraut indicates a role for each in the formation of the peroxidase reactive intermediates (Figure 4.3). The His-Asp pair lends imidazolate character to the axial ligand, supplying a stronger "push" toward cleavage of the bond on the opposite (distal) side of the porphyrin plane. The distal His (pKa < 5) serves as an active site base toward H₂O₂, removing a proton and allowing the peroxo anion to bind the ferric heme. The protonated His then acts as an acid to transfer the proton to the terminal oxygen of the bound peroxo anion, generating the leaving group water. The nearby arginine residue helps to suppress the pKa of the distal His and also polarizes the ferric peroxo species (Compound 0), promoting heterolytic bond cleavage. The arginine also hydrogen
bonds to and thereby stabilizes the resulting ferryl (Fe(IV)=O) oxygen of Compound I (Fe(IV)=O with a porphyrin cation radical) or Compound ES (Fe(IV)=O with a tryptophan radical), where CcP forms the latter.\textsuperscript{39, 40} Hence, the distal His and Arg together supply a “pull” that promotes heterolytic bond cleavage.

\textbf{Figure 4.2} (Top) Crystal structure of yeast cytochrome c peroxidase at 1.7 Å resolution (PDB ID: 2CYP), illustrating the primarily helical nature of the protein and the heme (in pink) binding fold.\textsuperscript{36} (Bottom) Active sites of representative class I, II, and III plant peroxidases. HRP (left; PDB code 1hch), LiP (middle; PDB code 1llp), and CcP (right; PDB code 1zby) are shown. The heme molecule and side chains are colored by atom, with carbons (yellow), nitrogens (dark blue), oxygens (red), and iron (orange). Adapted in part from Zubiena et al.\textsuperscript{57}
Figure 4.3 The Poulos-Kraut mechanism for intermediate formation (left) and the classic 3-step peroxidase catalytic cycle (right). Fe is ligated by protoporphyrin IX. The picture depicts the ferric active site poised for proton removal by the distal histidine base. The same residue then acts as an acid to transfer the proton to the other peroxy oxygen, generating the water leaving group. The resulting intermediate has either a porphyrin cation radical (Compound I) or a proximal tryptophan radical (Compound ES) in conjunction with a ferryl center.

The distal His base/acid has been described as the *sina qua non* of a heme peroxidase, because without it, catalysis is profoundly impaired. In mutants of horse radish peroxidase (HRP) in which this residue is substituted by a hydrophobic amino acid, the second order rate constant for formation of Compound I from the ferric enzyme and $H_2O_2$ diminishes by 5-orders of magnitude.\textsuperscript{38,41} The distal Arg is less essential, but nonetheless appears to play a role in stabilizing bound anions. Similarly, the proximal Asp and even the ligating His residues can both be substituted for other residues with typically less profound effects, provided that the heme still binds the protein.

4.3 Sequences, Structures, & Genetics of Superfamily Members

4.3.1 Taxonomic Origins and Gene Organization

Phylogenetic analysis indicates three distinct subfamilies within the CDE superfamily (Figure 4.4), but there are significant similarities in the sequences, structures, and genetic contexts of the superfamily members (discussed below). These suggest distant genetic relatedness as well as likely roles for many family members in iron metabolism.
Figure 4.4 Phylogenetic tree illustrating relationships between taxonomically diverse Cld, DyP, and EfeB-family proteins. The sequences indicated by the bracket on the left are from non-perchlorate respirers, and the Cld proteins are small (<200 amino acids). The sequences from the bracket on the right are full-length Clds from perchlorate respirers and/or organisms that can degrade chlorite. Color shading indicates family membership. The sequences in lighter blue appear to be from DyP subfamily C, and in the dark blue are likely DyP Ds, but their assignment into subgroups is not certain. The darker orange highlights EfeB proteins from *efeUOB* or *efeOUB* operons. The light orange indicates YfeX-like proteins, sometimes called DyP subfamily B proteins but apparently more closely related to the EfeBs. Figure generated using Phylip and Tree of Life softwares. Bootstrap values exceed 80%. Species and accession numbers clockwise, beginning with the left most bracket: (C-family) Proteobacteria (unless specified otherwise): *Nitro bacter winogradskyi*, YP_319047.1; *Pseudomonas aeruginosa*, ACL31207.1; *Cupriavidus metallidurans* CH34, ABF13199.1; *Klebsiella pneumoniae* ATCC 13884, ZP_0016699.1; *Klebsiella pneumoniae* MGH 78578, ZP_880622.1; C. *Nitro spira defluvii* (Nitrospirae) ACE75544.1; *I deonella dechlorator*, CAC14884.1; *Dechloromonas agitata*, AAM92878.1; *Pseudomonas chloritidum*, ACA21503.1; *Dechloromonas aromatica*, YP_285781.1; *Azospira oryzae*, pdb|2VXH_A. Actinobacteria: *Tropheryma whipplei*, NP_789669.1; *Kineococcus radiotolerans*, YP_001361337.1; *Mycobacterium tuberculosis*, NP_217192.1; *Mycobacterium smegmatis*, YP_887113.1. Halobacteriaceae: *Aeropyrum pernix*, NP_147071.2; *Thermoplasma acidiphilum*, NP_399883.1; *Sulfolobus solfataricus*, ACX92972.1; *Metallosphaera sedula*, ABP94989.1; *Halobacterium* sp. NRC1, NP_280706.1; *Natronomonas pharaonis*, YP_326782.1; *Halocar lula marismortui*, YP_137518.2. Deinococcus-Thermus: *Thermus thermophilus* HB8, |1VDH_A. Firmicutes: *Listeria monocytogenes*, ZP_05290848.1; *Exiguobacterium stibiricum*, YP_001812720.1; *Staphylococcus aureus*, NP_645359.1; *Bacillus anthracis*, ZP_00191341.1; *Geobacillus stea rothermophilus*, pdb|1TO. (D-family) Cyanobacteria: *Cyanobacterium* sp. PCC 7822, ZP_03158031.1; *Anabaena variabilis* ATCC 29413, YP_324690.1. Planctomycetes: *Rhodopseudom nula baltica* SH 1, 865998.1; *Acaryochloris marina* MBIC11017 (Cyanobacteria), YP_001521418; *Nostoc punctiforme* PCC 73102, YP_001869150.1. Basidiomycota: *Postia placenta* Mad-698-R., XP_002474852.1; *Laccaria bicolor* S238N-H82, XP_001876926.1; *Coprinopsis cinerea* okayama, XP_001840251.1; *Thanatephorus cucumeris*, BAA77283.1; *Termimyces albuminosus* (TAP peroxidase), AAM21606.1. Ascomycota: *Aspergillus fumigatus* Af293, XP_748822.2; *Neosartorya fischeri* NRRL 181, XP_001261639.1; *Neurospora crassa* OR74A, XP_959154.2; *Sordaria macrospora*, CBI56381.1. Bacteriodetes: *Phylo ca curdlanolyticus*, YP_001261639.1; *Listeria monocytogenes* ATCC 49188, YP_001373218.1. Proteobacteria: *Deinococcus* sp. PRwf-1, YP_001280117.1. Firmicutes: *Enhydrobacter aerosaccus* SK60, ZP_05620844.1; *Psychrobacter* sp. PRwf-1, YP_001280117.1. Firmicutes: *Ochrobacterium anthropi* ATCC 49188, YP_001373218.1. Deinococcus-Thermus: *Deinococcus radiodurans* R1, NP_285469.1. Actinobacteria: *Am cortylobacter mediterranei* U32, YP_003767800.1; *Mycobacterium smegmatis* str. MC2 155, YP_888002.1. Acidobacteria: *Candidatus Koribacter variabilis* YP_590455.1; *Streptomyces avermitilis*, NP_824776.1; *Frankia* sp. Eul1c, YP_06237655.1. Acidobacteria: *Solibacter usitatus*, YP_826301.1. Proteobacteria: *Mxococcus xanthus* DK 1622, YP_629952.1; *Sorangium cel lulosum*, YP_001613012.1. (YfeX-like proteins) Proteobacteria: YfeX from *Klebsiella pneumoniae*, YP_001336414.1; YfeX from *Escherichia coli* strain K-12, AAC75484.2; DypB from *Rhodococcus jostii* Rha1 (Actinobacteria) pdb|3QR1C. (E-family) Firmicutes: *Bacillus subtilis*, BA187494.1; *Paenibacillus*, YP_003009762.1; *Paenibacillus curdianolyticus* YK9, ZP_07387797.1. Actinobacteria: *Thermobispora bispora*, YP_003652521.1; *Micromonospora sp.* ZP_4607718.1; *Rhodococcus jostii* RHA1, YP_705709.1. Planctomycetes: *Gordonia bronchialis* DSM 43247YP_003273734.1. Actinobacteria: *Streptomyces hygroscopicus*, ZP_05517536.1; *Catenulispora acidiphila* DSM 44928, YP_003112540.1. Proteobacteria: *Neisseria polysaccharea*, ZP_06864806.1. Firmicutes: *Ochrobactrum anthropi* ATCC 49188, YP_001372391.1. Proteobacteria: *Yersinia frederiksenii*, YP_4632150.1; *Pantoea* sp. aB, ZP_07378163.1; *Enterobacter* sp. 638, YP_001176277.1; EfeB from *Klebsiella pneumoniae*, YP_001334712.1; *Citrobacter youngae*, ZP_06353463.1; *Escherichia coli*, YP_003233705.1; *Shigella boydii*, YP_408450.1; *Shigella dysenteriae*, ZP_03064798.1.
4.3.1.1 The C-Family Proteins

Until 2008, all known perchlorate respiring organisms were from phylum Proteobacteria. However, members of other bacterial phyla – for example, Firmicutes – were subsequently shown to reduce perchlorate, suggesting that the metabolism may exist in more organisms that originally thought.\textsuperscript{42-44} The evolution of perchlorate reduction is fascinating and complex; it is too broad of a topic to be reviewed adequately here.\textsuperscript{15, 45, 46} However, several observations about \textit{cld} genes and their association with chlorite decomposition and perchlorate reduction can be presented.

First, protein sequences falling into the Cld group come from taxonomically diverse organisms spanning 13 bacterial and two archaeal phyla, as well as one unusual representative gene in poplar trees. Interestingly, the overwhelming majority of the parent organisms are not known to respire chlorate or perchlorate and do not degrade chlorite, making the “chlorite dismutase” protein family name something of an unfortunate misnomer.\textsuperscript{47} This family is referred to as the C-family herein. In the well-studied perchlorate respirers, genetic evidence suggests that the \textit{cld} gene’s insertion into chlorate- or perchlorate-reducing gene clusters is the result of lateral gene transfer.\textsuperscript{45, 46} The \textit{cld} gene is independently regulated under its own promoter, and exhibits multiple orientations relative to the genes encoding the perchlorate reductase. The \textit{Dechloromonas aromatica} \textit{cld}, like all \textit{cld} genes in perchlorate respirers, is preceded by a SecB sequence designating it for transport to the periplasmic space in its unfolded, heme-free form.\textsuperscript{1} Notably, chlorite (which is relatively membrane-impermeable) is expected to be produced in the periplasm by periplasmic-facing perchlorate reductases ensconced in the inner membrane. None of the Proteobacterial species possessing a Cld protein known to be involved in perchlorate respiration, moreover, appears to have a second \textit{cld} gene copy.\textsuperscript{1} Gene duplication followed by divergence does not appear to have occurred in these organisms. It is possible that the first \textit{bona fide} chlorite-decomposing Cld might have been a single gene product moonlighting between ancestral and acquired functions.

Some organisms degrade chlorite but are not perchlorate respirers. These include the only eukaryote known to have a \textit{cld} sequence, the poplar tree, which was shown to reduce \textsuperscript{36}Cl-labeled perchlorate and to produce \textsuperscript{36}Cl.\textsuperscript{48} Nitrite-oxidizing organisms from the phylum Nitrospira
have been shown definitively to produce chlorite-active Clds. These metabolically flexible organisms are of biotechnological interest for their possible uses as bioremediation agents. They also help to delineate the evolution of the chlorite-decomposing activity of the cld gene product. Daims and Djinovid-Carugo et al. have noted two lineages of chlorite-decomposing Clds. One lineage is associated primarily though not completely with perchlorate respiration. It contains all Proteobacterial sequences, but also the O₂-evolving Cld from phylogenetically distant Candidatus Nitrospira defluvii. A second appears to contain only non-respirers but does have at least one documented chlorite-reactive and O₂-evolving Cld from the non-perchlorate respirer, Nitrobacter winogradskyi. This organism can reduce chlorate to generate chlorite via a respiratory nitrate reductase; it is possible that other members of this lineage do similarly. The Clds from these organisms have shorter primary sequences (<200 amino acids rather than >250 per monomer) and their tertiary structures are consequently different (see below). A similar phenomenon was noted in sequence alignments reported in Ref. 1 and reproduced in Figure 4.8 below.

Many diverse organisms possess Cld sequences that are unlikely from a biological or structural perspective (see below) to have any role in degrading chlorite. These include a variety of Gram positive and Gram negative bacteria as well as Archaea. An examination of the genetic context of some of these clds has given clues about their roles. In the Actinobacteria, the cld gene is generally located just downstream of hemG or hemH, with which it is co-operonic. These genes encode protoporphyrin oxidase and ferrochelatase, respectively, which catalyze the final two steps in heme biosynthesis. There are likewise three known cases of cld fusion to hemH in Actinobacteria. The cld gene in Firmicutes does not have the same kind of operonic structure, and is instead most often adjacent to the gene encoding acetyl-coenzyme-A:orthophosphate acetyltransferase phosphotransacetylase, a fermentative enzyme. However, a functional link to heme metabolism in a representative of each of the Actinobacteria and the Firmicutes (Mycobacterium tuberculosis and Staphylococcus aureus) was recently posited, and the gene from these organisms redubbed hemQ. The cld is located among the hem genes in Deinococci and Chloroflexi, though its function has not been explored in these phyla. In the Halobacteriacea, the cld gene always exists as a fusion with a gene having homology to isdG. The latter encodes a
heme oxygenase found in several Gram positive bacteria. Hence, the Cld proteins in these organisms appear to have something to do with heme metabolism, although their precise roles continue to be investigated.

4.3.1.2 The D- and E-Family Proteins

The DyPs and EfeBs likewise suffer from a still-evolving and sometimes misleading nomenclature, but are beginning to be better understood. Peroxibase, an online repository for information about peroxidases, lists subfamilies A-D of DyPs based on phylogenetic analysis. However, the labeling of all of these groups as DyPs is inexpedient. The EfeB proteins, for example, are listed as the DyP subfamily A, though they are almost certainly better described as an independent group. The *efeB* genes invariably exist in operons alongside *efeU* (an outer membrane ferrous iron permease) and *efeO* (a ferrous iron transporter) genes, preceded by a Fur-regulated promoter. The *efeB* gene moreover begins with a Tat translocator sequence that serves as a unique marker of this group. Studies of gene transcription and protein expression have confirmed an association between EfeB and cellular Fe status. A genetic study has linked EfeB in *E. coli* to heme metabolism. (See below.) Early work sometimes refers to EfeB as YcdB, particularly in *E. coli*.

The B subfamily of DyPs is most closely related to the EfeBs. On a phylogenetic tree containing C-family and DyP family (D-family) proteins, they emerge from the same major branch as the EfeBs (E-family) (Figure 4.4). However, the DyP-B subfamily proteins lack a signal peptide for export and consequently appear to be cytoplasmically located. They tend to be shorter (300-350 amino acids) than the Tat-transported E-family proteins (>400 amino acids), and considerably shorter than DyPs from subfamilies C or D (on the order of 440-600 amino acids). Some bacteria, for example *Rhodococci* and *E. coli*, have both a DyP-B subfamily member and an EfeB. (See also Table 4.1) In *E. coli*, the representative of the DyP-B subfamily is known as YfeX. This protein has been described as a distant paralog of EfeB, and the two have been proposed to serve similar functions in *E. coli* but in different subcellular locations. The operonic structure of *yfeX* is more complex and variable than that of *efeB*. It is likewise not preceded by a
Fur box. In short, the functional relationships between these DyP-B proteins and either the D- or the E-families are unclear. However, on a sequence level, the DyP-Bs appear to be better grouped with the E-family (Figure 4.4).

### TABLE 4.1

<table>
<thead>
<tr>
<th>PDB ID</th>
<th>Source</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>3Q08</td>
<td><em>Dechloromonas aromatica</em></td>
<td>Nitrite bound Cld from a PRBa</td>
<td>58</td>
</tr>
<tr>
<td>2VXH</td>
<td><em>Azospira oryzae</em></td>
<td>SCN-bound Cld from a PRB</td>
<td>53</td>
</tr>
<tr>
<td>3NN2</td>
<td><em>Candidatus Nitrosospira defluvii</em></td>
<td>Cyanide-bound chlorite-reactive Cld from an evolutionarily distant NPRBa</td>
<td>52</td>
</tr>
<tr>
<td>3QPI</td>
<td><em>Nitrobacter winogradskyi</em></td>
<td>Water-bound short dimeric Cld from a Proteobacterial NPRB</td>
<td>44</td>
</tr>
<tr>
<td>1T0T</td>
<td><em>Geobacillus stearothermophilus</em></td>
<td>Heme-free Cld from NPRB (firmicutes)</td>
<td>94</td>
</tr>
<tr>
<td>1VDH</td>
<td><em>Thermus thermophilus HB8</em></td>
<td>Heme-free Cld from NPRB (firmicutes)</td>
<td>59</td>
</tr>
<tr>
<td>3DTZ</td>
<td><em>Thermo acidophilium</em></td>
<td>Heme-free Cld from NPR-Archaean (Euryarchaeota)</td>
<td>95</td>
</tr>
<tr>
<td>2D3Q</td>
<td><em>Thanatephorus cucumeris</em></td>
<td>Dye-decolorizing peroxidase, D subfamily (monomeric)</td>
<td>55</td>
</tr>
<tr>
<td>2IIZ, 2HAG</td>
<td><em>Shewanella oneidensis</em></td>
<td>Prephenate dehydrogenase (TyrA); DyP D subfamily (dimeric)</td>
<td>56</td>
</tr>
<tr>
<td>2GVK</td>
<td><em>Bacteroides thetaiotaomicron</em></td>
<td>DyP, D subfamily (hexameric)</td>
<td>56</td>
</tr>
<tr>
<td>3QNR, 3QNS</td>
<td><em>Rhodococcus jostii</em></td>
<td>DyP, B subfamily; homologous to YfeX (<em>E. coli</em>)</td>
<td>63</td>
</tr>
<tr>
<td>2WX7, 2WX6, 3O72</td>
<td><em>Escherichia coli</em></td>
<td>Proposed deferrochelatase (EfeB) with and without protoporphyrin IX bound</td>
<td>54, 25</td>
</tr>
</tbody>
</table>

The C and D subfamilies of DyPs emerge on a single branch of the phylogenetic tree. They are consequently most closely related and to one another and most unequivocally classified as core members of the D-family. The Dyp-D-subfamily proteins appear to come primarily from fungi and the DyP-C-subfamily from bacteria (Table 4.1). Biologically, it is expected that they may serve roles in lignin degradation much like the Class II peroxidases (above). Hence, they have attracted the interest of the biotechnology industry.
4.3.2 Sequence and Structural Relationships

4.3.2.1 Domain and Tertiary Structures

Though their primary sequence similarities can dip below 10% in various pairwise comparisons, the C-, D-, and E-family proteins clearly share a fold (Figure 4.5). This fold has been described as ferridoxin-like. Each of the two domains consists of 3 α-helices and 4 β-strands, where the latter constitute a “ceiling” over the distal side of the porphyrin plane. Such a fold is unique in heme biochemistry. The superimposition of C, D, and E family monomers (Figure 4.6) illustrates both the core fold and the variable loops that filigree it.44, 51-57

The monomer has a pseudo two-fold axis at its center linking the two domains, though only one domain (at the C-terminal half) in each case binds porphyrin. The monomer from D. aromatica Cld is one of the most compact and is pictured in Figure 4.5 for reference. A subset of the Clds – mainly those that derive from Proteobacteria that are not perchlorate respirers – exhibits a second type of monomer structure. This is depicted in the center of the bottom row in Figure 4.6. As described above, the primary sequences of the monomers are shorter (<200 amino acids) than those for the majority of known C-, D-, or E-family proteins. (See also Figure 4.8 below.) The 3-dimensional structure of the monomer is likewise smaller: about 2/3 the size of the monomers shown in Figure 4.5.44 While the C-terminal heme-binding portion appears to be intact and in fact very closely resembles its larger relatives in when the two overlaid (not shown), much of the N-terminal portion of the protein monomer is missing. Phylogenetic analysis suggests that this form of the cld gene is not ancestral, but rather would have arisen subsequent to the other, longer cld genes.44

Though the CDE monomers are structurally similar, the tertiary structures of the proteins differ substantially. The full-length C-family proteins appear to form functional homopentamers. In one case (Azospira oryzae), a Cld crystallized as a hexamer, but mass spectrometry indicated that the solution form was pentameric.53 Unique among the C-family proteins described thus far, the D. aromatica Cld crystallizes with a Ca$^{2+}$ cation at each monomer-monomer interface. A number of pentameric crystal structures for C-family proteins have been reported by structural genomics consortia (Table 4.2). Interestingly, 3 of these crystallize without heme present. The N.
*winogradskyi* structure similarly was measured on a protein for which the heme occupancy was sub-stoichiometric. This protein is currently the only structurally characterized small C-protein. Unlike the full length Clds, it forms a dimer rather than a pentamer. The subunit interface moreover involves different portions of the monomers. The biological implications of the pentameric and dimeric structures are unclear. However, the amount of surface area at the interfaces in each case is consistent with a true solution-state oligomer.

**Figure 4.5** Monomer of the chlorite dismutase from *Dechloromonas aromatica* (left). Proximal residues. Superimposition of monomers from the chlorite dismutase, EfeB, and DyP families (right) illustrating the common elements of their core folds. Regions of the structures with low conservation are shown in white cartoon. Regions of structural similarity are shown in cartoon and colored according to their carbon atoms by the following scheme: 1T0T (brown), 3DTZ (cyan), 1VDH (yellow), 2WX7 (light blue), 2IIZ (salmon), and 2D3Q (orange) onto 3Q08 (green). (See Table 4.1) The heme of 3Q08 (DaCld) is drawn in grey sticks and the Fe center represented as an orange sphere. This figure was adapted in part from Goblirsch et al. and generated using PyMOL.
In contrast to the C-family proteins, the E-family proteins are all “full length” (370-440 amino acids) but nonetheless crystallize as dimers. Furthermore, the orientation of the two monomers relative to one another differs from what is observed in the C-family. The monomers of pentameric C-family proteins are arranged with their N-terminals on the same side of the oligomer, such that the heme-containing domains are all adjacent. By contrast, in the crystallographically characterized EfeB from *E. coli*, the N-terminus of one monomer is adjacent to the C-terminus of the next, such that the bound porphyrins occupy opposite ends of the protein (**Figure 4.7**).
Finally, the crystallographically characterized DyP proteins have been reported as monomers, such as the paradigmatic DyP from *Thanatephorus cucumeris* Dec 1, or dimers, such as the TyrA from *Shewnella oneidensis*. The *Bacteriodes thetaioamicron* structure has been described as hexameric. The significance of the multiple oligomeric states assumed by DyPs and the unusual oligomerization states of the C-family proteins are both still poorly understood.

*Figure 4.7* Active site of nitrite-bound *D. aromatica* Cld. The proximal His-Glu pair is shown in green, the hydrophobic Thr/Leu/Phe triad in salmon, the Trp/His network in yellow, and the distal Arg in orange. The figure was adapted in part from Goblirsch et al.58 and generated using PyMOL.

### 4.3.2.2 Heme Binding Domains and Active Sites – C-Family

A variety of Cld protein crystal structures are now available (Table 4.2) from perchlorate-respiring bacteria (PRB), non-perchlorate respiring bacteria (NPRB), and even one Archaeaen. These structures detail several common features of an unusual heme binding pocket.

The *D. aromatica* structure is a good representative of the group.58 The roof of the distal pocket consists of β-strands and the proximal floor of α-helices (*Figure 4.5*). In the proximal pocket, a histidine is hydrogen bonded to an aspartic acid (*Figure 4.7*). The heme propionic acids point toward three tryptophan residues that also lie just at or below the porphyrin plane, one of
which (Trp (227)) \( \pi \)-stacks with a histidine. Above the porphyrin plane, three hydrophobic residues (Thr, Leu, Phe) and an arginine form a sterically confined pocket. The arginine (183) hydrogen bonds to the Fe-bound nitrite ligand at pH 9. The same ligand rotates slightly at pH 6.5, forming a bridge between the bound nitrite and a molecule of the anionic buffer (not shown). Each of these residues has a possible role in promoting \( \text{O}_2 \) evolution from the Cld.

An alignment of the closely related Proteobacterial C-family proteins (Figure 4.8) indicates that all of these residues are strictly conserved among the full-length sequences from PRB. They moreover derive from secondary structural elements that are themselves conserved even beyond the Proteobacteria. The use of secondary sequence alignments has enabled a more reliable prediction of the active site structure of uncharacterized proteins from a primary sequence alignment.\(^1\) *N. defluvii*, which contains a highly active Cld enzyme, is a nitrifying (i.e., nitrite oxidizing) member of phylum Nitrospirae. It is not a known perchlorate reducer, and the role of chlorite decomposition in the organism is not well understood. Equivalent active site residues are conserved between *N. defluvii* and the Proteobacterial PRBs, except for two of the potential radical sites: Trp (227) is a Phe in this organism, and His (224) is Asn (not shown).\(^{47, 52}\)

Four structures for ClDs with known \( \text{O}_2 \)-evolving functions are now available, from *D. aromatica*,\(^{56}\) *A. oryzae*,\(^{53}\) *N. defluvii*,\(^{47, 52}\) and *N. winogradskyi*.\(^{44}\) The crystal structure of the *N. defluvii* Cld shows that it shares an overall monomer structure (root mean squared deviation (RMSD) = 1.00 Å), common active site architecture, and most of its active site residues with the *D. aromatica* Cld.\(^{16}\) The *A. oryzae* monomer structure is very similar to the *D. aromatica* Cld (RMSD = 0.62 Å), and it is 98% identical at the primary sequence level. A major difference lies in the placement of a 13-residue loop (218-230) containing several active site residues in the the *D. aromatica* Cld structure (Glu(220), His(224), Trp(227), (Figure 4.7) that could not be modeled in the *A. oryzae* electron density. The *N. winogradskyi* monomer structure is very similar to the *D. aromatica* monomer, though it lacks an N-terminal \( \alpha \)-helix and 4 C-terminal helices. Its distal Arg points away from the heme plane and in the opposite direction from the propionic acid side chains. Instead of an anion or imidazole, as are present in the other structures, the heme iron in this Cld binds a water molecule. This water hydrogen bonds to a second water molecule, which in
turn hydrogen bonds to the distal Arg.

Strict conservation holds for three of the distal pocket residues in the Proteobacterial non-perchlorate respiring bacteria (NPRB), with the fourth, Thr (198 in DaCld), being an Asn in some sequences. On the proximal side, as in N. defluvii, the Trp and His (227, 224 respectively in D. aromatica Cld) are not conserved in the Proteobacterial non-respirers. A Thr (231) residue that coordinates a Ca$^{2+}$ cation at the subunit-subunit interface of D. aromatica Cld is strictly conserved across all the PRB but appears to be an Arg in the non-respirers (Figure 4.8). In sum, though the proteobacterial non-respirers are not expected to have been subjected to chlorite-based selection pressure, they all possess a C-family protein with an active site architecture containing many structural elements postulated to be essential for chlorite detoxification. (See below.) It is therefore possible that these C-family proteins could indeed be capable of catalyzing chlorite dismutation to some extent.

By contrast, when phylogenetically diverse C-family proteins are compared, the degree of observed sequence conservation is relatively low. The heme-coordinating His residue (170 in D. aromatica Cld) is strictly conserved. Other residues immediately above and below the porphyrin plane are not. A strictly conserved Pro residue (148 in D. aromatica Cld) is located in the β-strand adjacent to the two strands that form the roof of the distal pocket. It likely plays a structural role in the protein fold. Additionally, two of the three active site Trp residues in D. aromatica Cld are strictly (155) and strongly (156) conserved, respectively. The position of the strictly conserved Trp (155) appears to be important, as the indole ring is sandwiched between the alkyl chains of a strictly conserved Arg residue (163 in D. aromatica Cld) and a strongly conserved Lys (151 in D. aromatica). The latter hydrogen bonds to one of the heme propionic side chains in N. winogradskyi Cld. Additionally, two hydrophobic residues near the distal heme face (Phe and Val) and an acidic residue (Glu in most phyla, but Asp in Proteobacteria) are all strongly conserved (196, 181, 195 in D. aromatica Cld respectively). The Glu/Asp forms a hydrogen bond to the strongly conserved Trp. Finally, a strongly conserved Leu (191 in D. aromatica Cld) lies at the center of the monomer-monomer interface in the pentameric Clds. The homologous residue in
Figure 4.8 Alignment of annotated chlorite dismutases from Proteobacteria. (Pages 110–111)
<table>
<thead>
<tr>
<th>Organism</th>
<th>A2</th>
<th>B1</th>
<th>B2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dechloromonas aromatica</td>
<td>LKEME LV</td>
<td>TPTIP</td>
<td>LYSTHGLD</td>
</tr>
<tr>
<td>Ideonella dechloratans</td>
<td>LKEME LV</td>
<td>TPTLFV</td>
<td>LYSTHGLD</td>
</tr>
<tr>
<td>M. magnetotacticum</td>
<td>LKEME LTV</td>
<td>TOPPLQV</td>
<td>LYSTHGLD</td>
</tr>
<tr>
<td>Dechloromonas agitata</td>
<td>LKEME LT</td>
<td>TPLTANL</td>
<td>LYSTHGLD</td>
</tr>
<tr>
<td>Ca. Nitrospira defluvii</td>
<td>TAI M</td>
<td>EQYLV</td>
<td>ALSEV</td>
</tr>
<tr>
<td>Dechlorosoma sp. KJ</td>
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<td>TPTTLA</td>
<td>YHSTGKL</td>
</tr>
<tr>
<td>Azospira oxygae</td>
<td>LKEME LV</td>
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<td>YHSTGKL</td>
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<td>Dechlorosoma bortensis</td>
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<td>YHSTGKL</td>
</tr>
<tr>
<td>Pseudomonas chloridismutans</td>
<td>LKEME LV</td>
<td>TPTTLV</td>
<td>YHSTGKL</td>
</tr>
<tr>
<td>Raistonia pickettii 123</td>
<td>LKEME LV</td>
<td>TPTTLV</td>
<td>YHSTGKL</td>
</tr>
<tr>
<td>Cupriavidus metallidurans CH34</td>
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<td>YHSTGKL</td>
</tr>
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<td>Klebsiella pneumoniae MG78578</td>
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<td>TPTTLV</td>
<td>YHSTGKL</td>
</tr>
<tr>
<td>Nitrooccus mobilis Nb-231</td>
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<td>YHSTGKL</td>
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<tr>
<td>Janthinobacterium Marseille</td>
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<td>Nitrobacter sp. Nb-311A</td>
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<td>Bradyrhizobium japonicum</td>
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<td>Pseudomonas putida</td>
<td>LKEME LV</td>
<td>TPTTLV</td>
<td>YHSTGKL</td>
</tr>
<tr>
<td>Limnobacter sp. MED105</td>
<td>LKEME LV</td>
<td>TPTTLV</td>
<td>YHSTGKL</td>
</tr>
</tbody>
</table>

It is notable that several proximal and distal pocket residues that are known/likely to be away from the porphyrin plane.

The N. winogradskyi Cld (122) points toward the distal Arg side chain (127), which itself faces away from the porphyrin plane.
can be hypothesized from known and predicted structures. There are three crystal structures of 
C-family proteins, all generated by Structural Genomics Consortia, for species from three different 
phyla that occupy branches of the phylogenetic tree beyond the Proteobacteria: Firmicutes, 
Deinococcus-Thermus, and Euryarchaeota. In each case, like *D. aromatica* Cld, the protein 
crystallized as a pentamer, though without any associated heme. The probable location of the 
heme can be discerned via the strictly conserved His residue that serves as the proximal ligand, 
and by inspection of the distinctive arrangement of a-helices and b-sheets (Figure 4.9). Distal 
and proximal residues of functional importance are more difficult to identify in the heme-less 
crystal structures as, although the main-chain overlays well with the *D. aromatica* Cld structure, 
observed side chain rotamers probably change upon heme binding. The apo-structures from 
*Geobacillus stearothermophilus* (phylum Firmicutes) and the halophilic bacterium *Thermus 
thermophilus* HB8 (phylum Deinococcus-Thermus) are essentially identical in the vicinity of the 
heme (Figure 4.9).\(^5\) The Deinococcus-Thermus *cld* lies among the Firmicutes sequences on 
the phylogenetic tree, suggesting horizontal gene transfer; indeed, it is 53% amino acid sequence 
identical to the *G. stearothermophilus* protein. Using the *G. stearothermophilus* numbering, the 
conserved axial His residue (171) defines the expected proximal pocket. The residue 
corresponding to a possible acidic hydrogen bonding partner to the axial His is unclear. Glu(220), 
the sequence equivalent that performs this role in *D. aromatica* Cld (also Glu(220)) is pointing 
away from His(171) and would need to rotate to form a hydrogen bond. There is also an Asp 
(219) residue N-terminal to the Glu which could be a potential hydrogen bonding partner to the 
axial His. This is conserved in the Firmicutes, but very variable across phyla, so it is a less likely 
partner than the much more conserved Glu. In the *Thermoplasma acidophilum* C-family protein 
(domain archaea, phylum Euryarchaeota, Figure 4.9), only the conserved Glu(195) is present in 
the proximal pocket, and it would also need to rotate into hydrogen bonding contact with the 
proximal His(146). In all three heme-less C-family structures, the two strictly conserved 
Proteobacterial C-family Trp residues that lie near the heme propionic acid side-chains are a 
Trp156 and Tyr157 (*G. stearothermophilus* numbering). The *G. stearothermophilus* and *T. 
thermophilus* HB8 distal pockets are lined exclusively with hydrophobic residues [Ile(186),
Val(199), Leu(201)], with a strictly conserved Gln(184) at the position of the *D. aromatica* Cld Arg(183). The *T. acidophilum* C-family protein distal pocket exhibits a substantially more polar environment. The active site contains three polar residues [Thr(163), Ser(161), Tyr(178)] which are strictly conserved among all Clds from Euryarchaeota. Unlike Thr(198) in the *D. aromatica* Cld (Figure 4.6), the hydroxyl group of Thr(163) points into the distal pocket. Ser(161) occupies the position of the catalytically important Arg(183) from the *D. aromatica* Cld. Thus it appears that the identity of this distal pocket residue is a key marker within phylogenetic clusters ([*Arg* in Proteobacteria, *Gln* in Firmicutes, *Ser* in Euryarchaeota]) and could be an indicator of differing C-family protein function.¹

**Figure 4.9** Active site views of crystallographically characterized apo-C family proteins illustrating possible heme environments. Cartoon diagram representations of protein monomers (carbon light grey) are shown, with side-chains of active site residues shown as sticks. Residues lining the expected heme pocket that fall within strictly conserved secondary structure elements are colored by atom (carbon magenta). Note that the same residues were also identified from primary sequence alignments with Proteobacterial Clds. (Left) *G. stearothermophilus* (Firmicutes); (Center) *T. thermophilus* (Deinococcus-Thermus). (Right) *T. acidophilum* (Euryarchaeota). This figure was generated using PyMOL. Adapted from Goblirsch et al.¹

Crystal structures are not available for representative members of most phyla of the phylogenetic tree. For the known crystal structures, primary sequence alignments do provide a reasonable indication of the residues around the heme, although ambiguities in the heme-less structures remain. For example, identification of a hydrogen bonding partner (if present) for the proximal His is uncertain. Alignment of conserved secondary structures for all structurally characterized C-family proteins (and the structurally related D- and E-family proteins) shows that
all of the residues known to line the heme pockets derive from the same set of conserved helix/sheet substructures (Figure 4.8). Mapping the actual positions assumed by sequentially conserved residues onto the secondary structural elements of the known crystal structures can help narrow down and give confidence to the likely residues defining the heme environment for structurally uncharacterized proteins of low sequence identity. Such structure-based sequence alignments indicate a conserved Ala at the position of Arg183 in the Actinobacteria, although an Arg or Lys residue is typically located one residue upstream and Asn downstream, both of which might be distal pocket candidates. A triad of potential distal hydrophobic residues is also positionally conserved in these species, though the identities of the amino acids are not.\(^1\) As sequence identity with the known crystal structures lessens, using the structures in the alignment becomes less effective, and reliance on the bootstrapping used to create the sequence alignments becomes dominant in the comparisons.

In terms of possible functions among the C-family proteins that are not known to have chlorite decomposition activity, the Proteobacterial proteins from NPRB clearly bear the closest active site resemblance to the true O\(_2\)-evolving Clds. These proteins share a substantial number of strictly conserved residues with \(D.\ aromatica\) Cld, particularly around the heme pocket, which include an Arg that aligns with Arg(183) (Figure 4.7). In keeping with this supposition, the small Cld from \(N.\ winogradskyi\), which shares most of the same active site residues including the distal Arg, catalyzes efficient O\(_2\) evolution from chlorite.\(^44\) The Actinobacteria have an Ala at the position of the distal Arg, but could have either an Arg/Lys or Asn if the alignment is shifted by a residue. The structurally characterized apo-proteins and the remaining species on the phylogenetic tree are all expected to have Gln or Ser at this position. Given the lack of any residue capable of acid/base chemistry or even of strongly polarizing a bound ligand, it would appear unlikely that these proteins would be involved in either efficient redox chemistry or the decomposition of chlorite. Indeed, only very weak peroxidase and/or catalase activities have been observed for C-family proteins studied thus far.\(^49,59,61\) These proteins could have other types of roles, as sensor/regulators, chaperones, or trafficking agents, possibly involving hydrogen-bonding from the distal pocket residue. Given the expected diversity of active site structures, it seems
reasonable that different family members may serve different biochemical and biological functions.

Finally, across the structural superfamily with the exception of the small Clds, the center of each monomer has a pseudo two-fold rotational axis, linking two highly similar domains that likely arose from gene duplication. In one domain, heme (or demetallated protoporphyrin IX, PPIX), is bound. The other is porphyrin-free. At first glance, it is not obvious why only one domain binds heme/PPIX while the other domain does not. Superimposition of the two D. aromatica Cld domains upon one another shows the two to be remarkably similar in structure. However, in the N-terminal heme-less domain the helices and β-sheet are more compact, and this is seen in all the known structures of C-, D- and E-family proteins (i.e., Clds and DyP/TyrA/EfeB; See Table 4.1 and Figure 4.5). This compactness is not a consequence of the loss of heme, as the apo-C-family protein C-terminal domains show no evidence of helix movement akin to that of the heme-less N-terminal domains of either themselves or those of the heme-containing crystal structures. Furthermore, none of the known structures has a His located equivalently to the strictly conserved proximal His ligand of the C-terminal domain. Closer inspection of the superimposed monomers reveals an extra helix formed from the protein C-terminus that is associated with the non-heme binding domain. Structurally this would impede access and sterically clash with a heme positioned equivalently to that observed in the C-terminal domains. This C-terminal α-helix is observed in all the superfamily structures except for the heme-free domain of EfeB, where the C-terminus is a disordered loop. Although EfeB lacks a structured C-terminus, it still lacks a proximal His ligand and has the same compact structure observed in all the other N-terminal domains. Overall, it appears highly unlikely that any of the N-terminal domains are competent to bind heme once fully folded.

4.3.2.3 Heme Binding Domains and Active Sites – D- and E-Families

A small number of crystal structures are available proteins described as DyPs (Table 4.1). In most cases, these proteins have been characterized for their peroxidase activities, though their broader biological roles are not clear (see below). As yet, only the E. coli EfeB protein has
been structurally characterized (Table 4.1). Structures for the DyP-B-subfamily protein from *Rhodocococcus jostii* are available; this protein is phylogenetically closer to and hence likely better classified with the EfeBs than the DyPs, although the roles of the subfamily remain unclear. (See Figure 4.2)

**Proximal and distal pockets.** Overlays of C-, D-, and E-family protein active sites (from the *T. cucumeris* DyP, *S. oneidensis* TyrA, *E. coli* EfeB, and *D. aromatica* Cld proteins) show a highly similar proximal pocket with the strictly conserved histidine being hydrogen bonded to either an aspartate or glutamate residue (Figures 4.10-4.11). The distal pocket and periphery of the porphyrin plane are surrounded by several hydrophobic residues, as in the C-family. However, the D- and E-family proteins tend to have a more polar distal pocket. Some D-family structures include a distal serine (Figure 4.10). The E-family proteins, including those from DyP subfamily B, additionally have a conserved asparagine in the distal pocket (Figure 4.11) which is involved in a hydrogen bonding network leading to the protein’s exterior. The distal pocket of these D- and E-family proteins differs most strikingly from the C-family, however, through the addition of an Asp that complements the distal Arg. The Asp-Arg pair is functionally equivalent to the His-Arg pair found in plant and fungal peroxidases, which together serve acid/base and polarizing roles toward the H$_2$O$_2$ substrate. The substitution of Asp for His in DyP is believed to be responsible for the lower-pH optimum observed for many DyP enzymes.\textsuperscript{55,62} All four residues from the B1 and B2 $\beta$-strands of Clds (Figure 4.13) also form part of the distal pocket of the EfeB/DyP/TyrA proteins. However, there are key structural differences in the architecture of these two strands in the EfeB/DyP/TyrA proteins compared to the C-family proteins that enable the accommodation of the additional Asp residue in the distal pocket. The B2 $\beta$-strand is shifted relative to the heme, altering the structural positions of the two B2 residues that form part of the distal pocket. The Phe(200) in the *D. aromatica* Cld is present in roughly the same position in all four enzyme structures (see Figure 4.10) and in the *R. jostii* DyP-B (Phe(261), not shown). Although the Ca main-chain position is spatially different and the Phe residues do not typically line up well in various primary sequence alignments, the ring position still lies in the same region of the distal pocket in all four enzymes. Unlike the side-chain of Phe(200), the Thr(198) of *D.
aromatica Cld occupies a different part of the pocket to the leucine residues at an equivalent sequence position in the EfeB/DyP/TyrA proteins (Leu331, 354, 255 respectively; Figure 4.10). The position of the distal arginine on the B1 β-strand is conserved between the EfeB/DyP/TyrA and Cld enzymes, but a bulge in the B1 β-strand of EfeB/DyP/TyrA places the other distal pocket residue from the B1 strand in a different area (D. aromatica Cld Leu185; EfeB Gly314; DyP Ser331; TyrA Ser244, Figure 4.10). The different positioning of this B1 residue, coupled with the repositioning of the B2 residue two positions N-terminal to the Phe, opens up a space such that the strictly conserved Asp residue in EfeB/DyP/TyrA (and all other D- and E-family proteins, see below) can become part of the distal pocket. The distal Asp is contributed by the L1 region in EfeB/DyP/TyrA that contains no distal pocket residues in the known C-family structures (Figure 4.13). The position of the Asp carboxylate group of EfeB/DyP/TyrA is occupied by the Leu(185) side-chain from the B1 β-strand in D. aromatica Cld. Leu, Ile and Thr are predicted from sequence alignments to be present in other Clds, and representative structures (although some are heme-less) are available in each case (Leu, D. aromatica Cld, A. oryzae Cld, N.defluvii Cld; Ile, G. stearothermophilus Cld, T. thermophilus Cld; Thr, T. acidophilum Cld). These structures show no evidence that an L1 residue will form part of the distal pocket. It is clear that the accommodation of the distal Asp through the structural alterations observed in the B1 and B2 β-strands of EfeB/DyP/TyrA compared to the known C-family protein structures will be a key distinction separating the majority, if not all, of the C-family proteins from the D/E protein families. With the presence of the L1 Asp, it is not surprising that these structurally related proteins are functionally non-identical to the Clds.
Figure 4.10 Active sites of crystallographically characterized D- and E-family proteins. (Top, left to right) DyP from Thanatephorus cucumeris; TyrA from Shewanella oneidensis; EfeB from Escherichia coli. PDB codes are given in Table 4.1. Cartoon diagram representations of protein monomers (carbon magenta) are shown, with side-chains of proposed key active site residues shown as sticks. An Asp residue that is strictly conserved in EfeB and DyP family proteins is absent in Clids. A serine present in the distal pocket of DyP and TyrA (both DyP subfamily D proteins) is absent in the Rhodococcus DyP-B, EfeB, and Clids. This figure is adapted from Goblirsch et al. and was generated using PyMOL.

Figure 4.11 Overlay of Cld, DyP, and EfeB family heme environments. (Left) Key distal pocket residues. (Right) Distal residues. Drawn as stick colored by atom. Carbon coloring D. aromatica Cld, green; E. coli EfeB, light blue; T. cucumeris DyP, orange; S. oneidensis TyrA, pink. Residues in D. aromatica Cld are labeled in black, while those of DyP are labeled in orange. Cld lacks a distal aspartate that is present in the other structures. DyP and TyrA distal pockets additionally contain a serine with no correlate in the other structures (S331 and S244 respectively). The heme of the D. aromatica Cld is drawn in grey sticks and the Fe center represented as an orange sphere. This figure was generated using PyMOL and adapted from Goblirsch et al.
Heme orientation. The D- and E-family proteins share an additional, highly significant structural deviation from those in the C-family. Although the B and D pyrrole rings (vinyl- and propionate-bearing, respectively) are in the same position in the two sets of structures, the A and C rings (vinyl- and propionate-bearing respectively) are swapped, effectively via a 180° rotation of the porphyrin around an axis through the B and D rings. This results in the porphyrin being flipped between the two families (Figure 4.12). This seemingly simple change has several repercussions for the heme propionic side chains and the distal Arg (and Asp) residues. In the Cld structures, both propionates are extended away from the porphyrin, making hydrogen bonds with two main-chain amides (Asn(117) and Tyr(118) in D. aromatica Cld, Figure 4.12). However, in the EfeB/DyP/TyrA structures, propionate D, which is spatially equivalent to the Clds’ propionate D, adopts a different conformation such that the acid group lies on the distal side of the heme, and hydrogen bonds to the distal pocket Arg. In DyP/TyrA, the distal Arg is also hydrogen bonded to the distal pocket Asp, giving an interacting triad of Asp/Arg/propionate D. In the EfeB/DyP/TyrA structures, the propionate of ring C interacts with an Asn side-chain nitrogen that lies at the kink in the proximal His-containing helix A2 (DyP, Asn(313); TyrA, Asn(229); EfeB, Asn(299) (Figure 4.12). The C propionate in TyrA is solvent-accessible at the surface, but in DyP and EfeB it is buried and interacts with arginine side chains from different parts of the structure (Arg(315) and Arg(261) respectively). The DyP propionate additionally hydrogen bonds with a Ser(174). Rotation of the heme profoundly affects the nature of the interaction of the heme with the protein surface. When in the Cld orientation, the propionic acid side chains point toward the pair of strictly conserved tryptophans. When in the DyP orientation, they become part of a more elaborate hydrogen bonding chain that in some structures (e.g., R. jostii DyP B) links the distal pocket to the protein surface and solvent.

Sequence conservation. An alignment of C-family proteins from Proteobacteria (Figure 4.8) illustrates that residues near the heme appear to be strictly conserved. However, with the exception of the proximal histidine, the active site residues are not strictly conserved across a broader selection of the numerous phyla that contain C-family proteins. The same cannot be said for the D- and E-family proteins. Far fewer well-described examples of D- and E-family
proteins are available, and those that are tend to be from less taxonomically diverse organisms than the Clds (Table 4.1). Nonetheless, alignments spanning the known diversity of D-family (C- and D-subfamily DyPs in the Peroxibase database) and E-family (Peroxibase A- and B-subfamily DyPs) proteins do clearly identify a set of strictly conserved residues in the vicinity of the heme (Figures 4.13 and 4.14). Notably, the C-family proteins are too distant from either of these groups to produce very meaningful alignments when C- and either D-/E- family proteins are compared. The same is true, though to a lesser extent, of alignments between many members of the D- and E-families. On phylogenetic trees (Figure 4.13), these proteins form discrete and measurably distant branches.

Figure 4.12 Hemes of *D. aromatica* Cld and DyP. (Top left) Interactions of the propionates of *D. aromatica* Cld. (Top right) Interactions of the propionates of the DyP from *T. cucumeris*. (Bottom) Overlay comparing the heme orientation in *D. aromatica* Cld and DyP. Active site residues and hemes are shown as sticks and colored by atom with *D. aromatica* Cld (carbon green) and DyP (carbon orange). Regions associated with DyP are labeled in orange font. Pyrrole rings of the heme b cofactor are labeled and shown in bolded font. The hemes in *D. aromatica* Cld and DyP are related by a 180° flip along an axis through pyrrole positions D and B. This figure was generated using PyMOL. Adapted from Goblirsch et al. 1
Figure 4.13 Sequence and structure in the E-family. (Top) Partial alignment of E-family proteins (including DyP-subfamily-B proteins) from diverse species, highlighting important conserved residues. Strictly conserved acidic residues are highlighted in magenta; basic residues in green; positively charged in red; polar residues in cyan; and non-polar in yellow. In addition to the residues shown in the alignment, a glutamic acid (D(103)), a glycine (G(139)) and a phenylalanine (F(140)) are strictly conserved (residue numbers from the R. jostii DyP B sequence). The strictly conserved residues all derive from the C-terminal domain and are near to the heme. The highlighted tryptophan is near to the position of a strictly conserved Trp in Clds in the 3 dimensional structure. (Bottom) The positions of these conserved residues are illustrated on the R. jostii DyP B structure (PDB ID 3QNR). The alignment was made by ClustalW; figure by PyMOL.
Alignments of diverse E-family proteins (including those also called as DyP-subfamily-B or YfeX proteins) show that 12 amino acids are strictly conserved, of which 9 are in the proximal and distal pockets and have some predictable role in chemistry (Figure 4.13). These include the distal Asp and Arg and also a distal Asn that appears to be a hallmark specifically of the E-family.\(^6\) Notably, the proximal Asp/Glu hydrogen bonding partner to the heme His ligand and the pair of Trp residues that occupied the periphery of the heme in the vicinity of the propionic acid side chains in the C family are not strictly conserved among the E-family. The D-family proteins (previously called DyP subfamilies C and D)\(^3\) are even more closely related to one another. They possess numerous strictly conserved residues distributed throughout the structure, even when proteins from a diverse set of bacteria and fungi are included in the alignment (Figure 4.14). These include the distal Arg and proximal His ligand to the heme. The distal Asp is shown as conserved on Figure 4.14, although in some sequences it is a Glu.

Figure 4.14 Strictly conserved residues from an alignment of taxonomically diverse DyP proteins (subfamilies C and D) highlighted on the structure of the DyP from \textit{T. cucumeris}. Alignment is by ClustalW. Sequences aligned came from DyP subfamily C: \textit{Mycobacterium} sp. (Actinobacteria), \textit{Cytophaga hutchinsonii} (Bacteroidetes), \textit{Mycococcus xanthus} (Deltaproteobacteria), \textit{Cyanobacteriaceae} sp. (Cyanobacteria), \textit{Ochrobactrum anthropi} (Alphaproteobacteria), \textit{Deinococcus radiodurans} (Deinococcus-Thermus; DyP subfamily D: \textit{Rhodopirellula baltica} (Bacteria), \textit{Nostoc punctiforme} (Cyanobacteria), \textit{Aspergillus oryzae} (Ascomycota), \textit{Thanatephorus cucumeris} (Basidiomycota). The figure was made using PyMol.
4.4 Biochemistry of Superfamily Members

4.4.1 C-Family: O$_2$-Generating Chlorite Dismutases

Chlorite dismutases have been isolated and/or heterologously expressed from several species of perchlorate respiring Proteobacteria. These include Dechloromonas aromatica, Dechloromonas agitata, Ideonella dechoratans, strain GR-1, and Pseudomonas chloritidismutans. They have additionally been isolated from non-Proteobacteria and non-perchlorate respirers: Candidatus Nitrospira defluvi and Nitrobacter winogradskyi, respectively (Table 4.2). These have been studied with a variety of motivations. Chlorite and perchlorate removal from fresh water via bioremediative means is of biotechnological interest. Perchlorate contamination of ground waters has been documented near sites of rocket and missile production, where perchlorate is used as a propellant. Chlorite is used in the paper and textile industries as a bulk bleach, and is itself a waste water problem. Perchlorate and chlorite have been linked to thyroid disease and anemia, due to their respective roles in interfering with thyroid hormone iodination and in heme lysis. The evolution of perchlorate respiration and the cld gene has also been of some fundamental microbiological interest, as has the unusual O-O bond joining reaction catalyzed with tremendous efficiency by some (but likely not all) Cld proteins. The diversity of species harboring a C-family protein and the likely chemical and structural variability of the proteins themselves have been discussed above. The chemistry of O-O bond formation will be addressed here, focusing on the well-characterized Cld from D. aromatica.

4.4.1.1 Stoichiometry, Specificity, and Stability

The O$_2$-evolving reaction catalyzed by the D. aromatica Cld could conceivably happen via one or two molecules of ClO$_2^-$, or by a pathway involving the incorporation of oxygen from water. A concerted pathway involving one molecule of chlorite and no intermediates is considered unlikely based on computational work. To gain better insight into the molecularity and stoichiometry of the reaction, the evolution of O$_2$ was monitored continuously via mass spectrometry. Reactions were carried out in the presence of $^{18}$OH$_2$ and Cl$^{16}$O$_2^-$, and in $^{16}$OH$_2$ with chlorite enriched in the labeled Cl$^{18}$O$_2^-$ (Figure 4.15). Each experiment was consistent with the O-atoms of O$_2$ being exclusively derived from chlorite. The absence of any exchange between
<table>
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<th>$K_M$ (μM)</th>
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<th>pH$^a$</th>
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$^a$All reported measurements made in varying concentrations (10-100 mM) of phosphate buffers.

$^b$Heterologously expressed in *E. coli.* $^c$Purified from native organism.

The water oxygens and presumed heme-chlorite-derived intermediates suggested that those intermediates must be very short lived, or that the heme was particularly inaccessible to solvent. Steady state and transient kinetics experiments have shown that the former is likely true. Because any reactive intermediates are short lived, it is highly probable that both O-atoms in the evolved O$_2$ derive from the same molecule of chlorite.

The reaction can be monitored over time most conveniently by continuous measurement of O$_2$ production, for example via Clark electrode.$^6$ The O$_2$ may also be rapidly trapped and observed by UV/visible spectroscopy, for example, via the second order reaction of O$_2$ with ferrous myoglobin. Alternatively, the production of Cl$^-$ has been observed via ion sensitive electrodes.$^7$ The continuous loss of chlorite can be monitored via UV/visible spectrophotometry, although its extinction coefficient is low. It can be discontinuously measured on enzymatically stopped reactions by iodometrically titrating the unreacted chlorite – that is, using the chlorite to oxidize I$^-$ to I$_2$, and then titrating the resulting I$_2$ colorimetrically.$^6$ Figure 4.16 shows progress of reaction curves measured via both chlorite disappearance (iodometric titration) and O$_2$ production (Clark electrode). These give identical initial rates and, when fit to exponential curves, the same
first order rate constants. Hence, chlorite decomposition and O$_2$ production are strictly kinetically coupled in the steady state.

![Figure 4.15](image)

**Figure 4.15** The O-atoms of the O$_2$ evolved from chlorite dismutase are chlorite-derived, with no O-atom exchange from water. (Left) Mass spectrum of gaseous products from the reaction of 85 nM Cld with 50 mM chlorite in 0.1 M sodium phosphate buffer, pH 7, made in 95% oxygen-18 enriched water and (Right) by using enriched $^{18}$O chlorite in $^{16}$OH$_2$. Signal assignments (m/z): H$_2$ (2), N (14), H$_2$O (18), N$_2$ (28), $^{16}$O$_2$ (32), $^{18}$O$_2$ (36). Adapted from Lee et al.\(^{77}\)

The reaction can be monitored over time most conveniently by continuous measurement of O$_2$ production, for example via Clark electrode.\(^{64}\) The O$_2$ may also be rapidly trapped and observed by UV/visible spectroscopy, for example, via the second order reaction of O$_2$ with ferrous myoglobin. Alternatively, the production of Cl$^-$ has been observed via ion sensitive electrodes.\(^7\) The continuous loss of chlorite can be monitored via UV/visible spectrophotometry, although its extinction coefficient is low. It can be discontinuously measured on enzymatically stopped reactions by iodometrically titrating the unreacted chlorite – that is, using the chlorite to oxidize I$^-$ to I$_2$, and then titrating the resulting I$_2$ colorimetrically.\(^{64}\) **Figure 4.16** shows progress of reaction curves measured via both chlorite disappearance (iodometric titration) and O$_2$ production (Clark electrode). These give identical initial rates and, when fit to exponential curves, the same first order rate constants. Hence, chlorite decomposition and O$_2$ production are strictly kinetically coupled in the steady state.
Figure 4.16 (Left) Representative progress of reaction curves showing the disappearance of chlorite (circles) and the evolution of O$_2$ (diamonds) measured in parallel reactions over time. For the data shown, [ClO$_2^-$]$\text{initial}$ = 172 $\mu$M. Each data set was fit to the Michaelis-Menten equation: 
\[ \frac{V}{[E_{\text{total}}]} = \frac{k_{\text{cat}}[\text{ClO}_2^-]}{[\text{ClO}_2^-] + K_M}. \] 
(Right) Plots of initial rates of chlorite dismutation per [heme] as a function of [ClO$_2^-$], measured by monitoring ClO$_2^-$ depletion (circles) and O$_2$ evolution (diamonds). Points are averages of 3 measurements, and error bars represent ± one standard deviation. The Michaelis–Menten equation was fit to each set of data. Adapted from Streit et al. $^{64}$

These steady state measurement techniques have been used for determining Michaelis-Menten parameters for a variety of Clds (Table 4.2 and Figure 4.16). These show $K_{M,(\text{chlorite})}$ values in the 10-100 micromolar range and values of $k_{\text{cat}}/K_M$ from $10^5$-$10^7$ M$^{-1}$s$^{-1}$. The latter are nearly in the range of diffusion control: that is, the conversion of ClO$_2^-$ to O$_2$ + Cl$^-$ is only limited by the amount of time it takes the substrate to reach the active site. A truly diffusion controlled reaction would be expected to be slowed in the presence of viscosogens, which was not observed for the $D$. aromatica catalyzed decomposition of chlorite. $^{64}$ Regardless, the reaction is remarkably fast and efficient, which is what one would expect for an enzymatic process involved in detoxification of a relatively reactive species (e.g., catalase/H$_2$O$_2$, superoxide dismutase/superoxide). This efficiency is particularly intriguing if environmental contamination by perchlorate is the impetus for its evolution. Though it is found co-deposited with some nitrates (in the Atacama desert of Chile, for example) perchlorate is not abundant in most environments. It has existed as an environmental problem for less than half a century.

Studies of the steady state reaction have further shown that the conversion of ClO$_2^-$ to O$_2$ and Cl$^-$ is stoichiometric under every condition of pH, temperature, and buffer thus far monitored. It is moreover stoichiometric in the presence of millimolar concentrations of reductants and
hypochlorite-trapping species. Reductants such as guaiacol and ABTS (2,2′-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid) are commonly used as colorimetric reagents to monitor peroxidase reactions. Both react readily with sterically accessible Compound I and Compound II intermediates as hydrogen atom (H•) donors. Monochlorodimedone likewise is frequently used in studies of hypochlorite-generating reactions for the spectrophotometric detection and quantification of hypochlorite. The reaction of D. aromatica Cld with chlorite retains its 1:1 chlorite:O₂ stoichiometry in the presence of these compounds, and moreover, does not generate any detectable oxidized or chlorinated product. This indicates that any reactive intermediates in the catalytic cycle are either short lived, sterically inaccessible to the trapping cosubstrates, or intrinsically unreactive. The latter does not seem to be the case, at least as far as the peroxidase reaction is concerned. The D. aromatica Cld is a moderately reactive peroxidase when H₂O₂ and ABTS/guaiacol are the substrates, and a fairly robust peroxidase when the oxidant is peracetic acid (H₃C(O)OOH). It therefore appears most plausible that if Compound I or Compound II like intermediates are produced during the reaction with chlorite, they must be short lived, and hence do not react with external substrates. Moreover, there is no evidence for escape of a hypochlorite intermediate from the active site. (See below and Chapter 5 for further discussion of intermediates.)

Early work with the D. aromatica Cld showed that residual chorite often remained at the end of the reaction. If the enzyme were exchanged into new buffer and exposed to a new dose of chlorite, no O₂ was produced. Truncation in the progress of reaction curves appeared to be due to irreversible inactivation of the enzyme. Chlorite-dependent inactivation of the enzyme is associated with loss of the heme chromophore, likely due to oxidative opening of the porphyrin ring. Similar mechanism-based inactivation is observed in other heme enzymes; in peroxidases, for example, exposure to H₂O₂ without a reductant leads to heme destruction. Consistent with a pseudo first order process (excess substrate, catalytic amounts of enzyme), the progress of reaction curves (Figure 4.16) can typically be fit to single-exponential curves (for example: [O₂] = [O₂]₀[1 – e⁻kt]). A series of kinetic curves were measured for variable concentrations of chlorite. A plot of kobs values versus chlorite concentration (Figure 4.17) is not linear, as it would be for a
simple second order process. Rather, the plot of $k_{\text{obs}}$ versus $[\text{ClO}_2^-]$ approaches a limiting value (Figure 4.17). If the limitation in $k_{\text{obs}}$ is attributed to chlorite-dependent enzyme inactivation, an expression for $k_{\text{obs}}$ can be written: $k_{\text{obs}} = k_{\text{inact}(\text{max})}[\text{ClO}_2^-]/(K_{\text{inact}} + [\text{ClO}_2^-])$, where $k_{\text{inact}(\text{max})}$ describes the maximal/saturating rate constant. Fitting the $k_{\text{obs}}$ versus $[\text{ClO}_2^-]$ plot to this equation yields $K_{\text{inact}} = 166 \, \mu\text{M}$ and $k_{\text{inact}(\text{max})} = 1.77 \, \text{min}^{-1}$.

![Figure 4.17 Plots showing chlorite-dependent irreversible inactivation of D. aromatica Cld.](image)

Values for $k_{\text{obs}}$ describing the exponential progress of reaction curves are plotted versus [chlorite]. Data were fit to the expression $k_{\text{obs}} = k_{\text{inact}(\text{max})}[\text{ClO}_2^-]/(K_{\text{inact}} + [\text{ClO}_2^-])$, yielding $k_{\text{inact}(\text{max})} = 1.77 \, \text{min}^{-1}$ and $K_{\text{inact}} = 166 \, \mu\text{M}$. (Right) Plot of the residual enzymatic activity after exposure to increasing concentrations of chlorite, in the presence (triangles) and absence (circles) of excess/600 $\mu\text{M}$ guaiacol. The turnover number, i.e., the maximal number of chlorite molecules dismutated per heme, is obtained by extrapolating a line fitted through each series of points to the $x$-axis. Turnover numbers are $1.7 \times 10^4$ in the absence of guaiacol and $1.3 \times 10^5$ in its presence. Adapted from Streit et al.\textsuperscript{64}

The number of turnovers (per heme) that the enzyme can catalyze before inactivation was determined using the method described by Silverman for suicide inactivation. Briefly, Cld was incubated with increasing equivalents of $\text{ClO}_2^-$, and the reaction was allowed to go to completion. The enzyme was then exchanged into fresh buffer and the remaining activity measured via the standard assay. The residual activity is the remaining activity (per mg enzyme) divided by the activity (per mg) of a sample of enzyme before incubation in $\text{ClO}_2^-$. Residual activity was plotted versus the $\text{ClO}_2^-$/heme ratio (Figure 4.17), yielding a straight line with a small amount of curvature at higher ratios. Such a deviation from linearity may be due to the ability of
accumulating chloride product to protect the enzyme from further inactivation. A line fit to the linear portion of the data allows for extrapolation to the x-axis, yielding $1.7 \times 10^4 \text{ClO}_2^-$ molecules decomposed per active site before inactivation (0% residual activity). The one-electron peroxidase substrate guaiacol was observed to increase the turnover number of the enzyme to $1.3 \times 10^5$ (per heme) under the same conditions (Figure 4.17).  

4.4.1.2 Heme Electronic Structure

The UV/vis spectrum has been measured for several Clds. These spectra share a markedly blue-shifted Soret band relative to heme peroxidases and myoglobins, providing the earliest indication that the Clds possessed a structurally distinct active site. Like many heme proteins, the Cld from *D. aromatica* has distinct pH-titratable forms. The enzyme is stable between pH 4.8 and 10.3; outside of these extremes, the heme chromophore diminishes and the heme apparently dissociates from the denatured protein. (Figure 4.18) These transitions are irreversible. Between pH 4.8 and 10.3, the enzyme is active. It has its greatest activity below a pKₐ of 6.5, with a maximal $k_{cat}/K_M = 3.2 (0.4) \times 10^7 \text{M}^{-1}\text{s}^{-1}$ at pH 5.2 ($k_{cat} = 2.0 (0.6) \times 10^5 \text{s}^{-1}$, 4 °C). Above this pKₐ, both kinetic parameters decrease 30-50 fold.  

UV/vis and rR (resonance Raman) pH titrations further indicated a pKₐ at pH 8.7. The acidic form of *D. aromatica* Cld (below this pKₐ) has its Soret maximum at 393 nm, a broad Q band envelope at 509 nm, and a charge transfer (CT) band at 648 nm. These features are consistent with a 5 coordinate high-spin (5cHS) heme. The alkaline form has a Soret maximum at 410 nm and α/β bands at 539 and 574 nm. These band positions are typical of low spin hydroxide complexes of heme proteins having proximal His ligands. Similar acid/alkaline transitions are observed in other His-ligated heme proteins. In typical histidinate-ligated peroxidases such as horseradish peroxidase (HRP), they occur with a pKₐ at pH 11-12. By contrast, in proteins with neutral His ligands including sperm whale and human myoglobin, the O₂-sensing *Sinorhizobium meliloti* FixL protein, and the bacterial heme oxygenase HmuO, the transition occurs at pH 9.6 or below.  

In addition to the Soret and visible bands, a charge transfer (CT) band at 608 nm was also observed for the alkaline form. This is indicative of the presence of a high spin Fe
component, which was likewise observed by rR.

Figure 4.18 UV/vis titrations demonstrate the acid and alkaline forms of *D. aromatica* Cld. (A) UV/visible titration data for ferric Cld over the pH range 6.6−9.8. Spectra are shown at pH 6.6, 6.8, 7.6, 7.9, 8.5, 8.7, 8.9, 9.5, and 9.8. Peak maxima and isosbestic points (at 350 and 470 nm) are labeled. Inset: Points correspond to absorbance at the indicated wavelengths. The solid lines are the calculated titration curves obtained from the two-component global nonlinear least-squares analysis. (B) Calculated component spectra obtained from the global analysis. Peak maxima are labeled. Inset: Speciation plot showing concentrations of the two components as a function of pH. Figure adapted from Streit et al.\textsuperscript{78}

The identification of the alkaline species as the ferric-hydroxy form was further confirmed via rR, using samples prepared in H\textsubscript{2}O, D\textsubscript{2}O, or H\textsubscript{2}^18O solutions buffered at pH 10.0. These spectra, Figure 4.19, showed definitively that an Fe(III)-OH bond was present at high pH. The Fe-OH stretching frequency at 514 cm\textsuperscript{-1} (in H\textsubscript{2}O) is very low relative to the analogous stretching frequencies in the alkaline forms of other heme enzymes. A low frequency indicates a weak bond, which could be due to an especially strong Fe-His bond on the other side of the porphyrin plane. However, the rR spectrum of the ferrous *D. aromatica* Cld indicated that this bond was actually quite weak. The vibrational frequency of 222 cm\textsuperscript{-1} is akin to what has been measured in myoglobin, which has a neutral histidine imidazole that is hydrogen bonded to a backbone carbonyl. This suggests that, though a proximal His-Glu hydrogen bond is in place in the *D. aromatica* Cld, it must be a weak interaction. This is in contrast to typical heme peroxidases, which have a strong Fe-His bonding interaction due to anionic/imidazolate character in the
proximal ligand.\textsuperscript{78}

Figure 4.19 Resonance Raman spectra of \textit{D. aromatica} Cld, indicating critical features of its electronic structure. (Left) Low-frequency rR spectra of ferrous Cld obtained with 441.6- and 406.7-nm excitation. The strong increase in relative resonance enhancement of the 222-cm\(^{-1}\) band is compelling evidence for its assignment to the \(\nu(\text{Fe–His})\) mode. The inset shows the 2-cm\(^{-1}\) shift of this mode to higher frequency in alkaline solution. (Right) Soret-excited rR spectra of alkaline Cld (pH 10) and its isotopically labeled forms. Spectra were recorded with 406.7-nm excitation (15 mW). The samples were prepared in H\(_2\)O, D\(_2\)O, or H\(_2\)\(^{18}\)O solutions buffered at pH 10.0 with 50 mM CHES. The top three traces are the original spectra whose acquisition times were identical. The bottom three traces are difference spectra that were generated by 1:1 digital subtraction. The difference bands reveal isotope shifts characteristic of HS and LS heme hydroxides. Adapted from Streit et al.\textsuperscript{78}

An Fe-His(neutral) heme is expected, according to the classic model of Poulos and Kraut described above, to supply a commensurately weak “push” toward bond cleavage on the distal side of the heme. Consistent with that expectation, the Fe(III)/(II) redox potential for \textit{D. aromatica} Cld was measured at -23 mV using the “method of Massey”: an enzymatically driven redox titration using xanthine oxidase to deliver electrons. The extent of reduction of a dye mediator is
measured spectrophotometrically. The Cld is significantly less oxidizing than non-animal peroxidases, which have reduction potentials that are typically more negative than -180 mV.\textsuperscript{82}

\subsection*{4.4.1.3 Reaction Mechanism}

The O\textsubscript{2}-evolving Clids catalyze both Cl-O breaking and O-O bond forming reactions. Homo- and heterolytic bond breaking processes and their reverse are fundamental to biological reactions involving O\textsubscript{2} and its aqueous reduction products. The Cl-O bond breaking process may in principle be homolytic, resulting in the formation of Compound II and the radical hypochloryl (ClO\textsuperscript{*}) leaving group (\textbf{Figure 4.20}). Or, it could be heterolytic, resulting in the formation of Compound I and the anionic hypochlorite (ClO\textsuperscript{-}). The latter process is analogous to what occurs in a heme peroxidase, in which the O-O bond of Compound 0 (Fe(III)-OOH) breaks with both electrons traveling to the leaving group (H\textsubscript{2}O).

Once the Cl-O bond breaks, the leaving group and high-valent Fe/O intermediate must recombine to form an O-O bond. The sterically confined distal pocket of \textit{D. aromatica} Cld and its homologs may serve an important role in confining the high-valent intermediate and leaving group, predisposing them toward recombination. As described above, in the \textit{D. aromatica} Cld, no side reactivity (peroxidase, peroxygenase, chlorination, \textsuperscript{16}OH\textsubscript{2}/\textsuperscript{18}OH\textsubscript{2} exchange) is observed even in the presence of large amounts of highly reactive “trapping” substrates. Moreover, the turnover number for chlorite (~17,000 s\textsuperscript{-1}heme\textsuperscript{-1}) is relatively high, indicating that the O\textsubscript{2} evolution reaction occurs with fidelity and with very infrequent loss of either reactive leaving group molecules or high-valent intermediates. Recombination of Compound II and hypochloryl would occur by a radical process; hypochlorite would act as a nucleophile to attack the electron-deficient oxygen of Compound I. These two O-O bond joining mechanisms mirror the proposed mechanisms for O-O formation from the S4 state of photosystem II.\textsuperscript{83,84} The proposed peroxychlorite product species (\textbf{Figure 4.20}) would likely be unstable, breaking down to give the O\textsubscript{2} and Cl\textsuperscript{-} products rather readily.
Attempts to observe high-valent intermediates in the *D. aromatica* Cld reaction with chlorite have so far not been fruitful. The reaction is very fast, and no intermediates are directly observed. However, the reaction with peroxides and peracids has been studied in depth as discussed in Chapter 7.

4.4.2 D-family: Dye Decoloring Peroxidases

The dye-decolorizing peroxidase (DyP) family of enzymes is named for the unique ability of its members to react with and oxidatively degrade anthraquinone dyes. These enzymes were first discovered nearly two decades ago. While a biological function has not been clearly established, they have been of particular interest to the biotechnological applications community (previously reviewed by Sugano). These DyPs or D-family proteins are distinct, structurally and mechanistically, from classic heme peroxidases that catalyze similar reactions. Lignin-degrading fungi and bacteria (primarily actinomycetes) secrete extracellular oxidases and heme peroxidases that have been useful in degrading a variety of aromatic compounds. These include synthetic dyes such as reactive blue 5 (RB5) or reactive black 5, both used in the textile industry. The first characterized and best studied lignolytic peroxidases are the Class II lignin peroxidases (LiP) and manganese peroxidases (MnP). The first characterized of these came from the fungus.

*Figure 4.20* Proposed reaction mechanisms for Cl-O bond cleavage and O-O bond formation from chlorite in *D. aromatica* Cld.
Phanerochaetes chyrosporium. The LiP not only oxidized lignin, but was able to decolorize dyes either directly or indirectly through a mediator such as veratryl alcohol. MnPs generally cannot decolorize organic dyes, but rather oxidize Mn(II) to Mn(III). Some enzymes were more recently identified which are capable of both Mn(II) and aromatic dye oxidation. These so-called versatile peroxidases appear to have separate binding sites for Mn(II) and organic substrates. The characterized D-family proteins have likewise come primarily from fungal and bacterial (including actinomycetes) sources. These include the fungus Bjerkandera adusta Dec 1 (the new name for Thanatephorus cucumeris Dec 1), the jelly fungus Auricularia auricula-judae, the cyanobacterium Anabaena sp. Strain PCC 7120, and most recently the soil actinomycete, Rhodococcus jostii RHA1. DyP enzymes from these organisms are described below. (See Table 4.3)

Bjerkandera adusta Dec 1 was noted for the ability to degrade anthraquinone dyes, notably RB5, in accord with increased levels of peroxidase activity. Anthraquinone dyes are categorized by their 3-ring aromatic core structure of 9,10-anthraquinone. Other common “azo” dyes such as those degraded by LiPs and versatile peroxidases contain a diazene (-N=N-) functional group. Anthraquinone dyes had not previously been known substrates for peroxidase enzymes; hence the heme peroxidase isolated from this species was of keen interest. This enzyme was the first and remains the most studied DyP. Homologs have subsequently inherited the same name, occasionally with the species/strain of origin indicated by a subscript (e.g., DyPBaDec1). DyPBaDec1 is an approximately 60 kDa glycoprotein with a Soret peak at 406 nm and visible bands at 510 and 640 nm. Kinetic studies showed a $k_{cat}/K_M$(RB5) of $4.8 \times 10^6$ (M$^{-1}$ s$^{-1}$) at optimal pH of 3.2 and 30 °C. This low pH optimum is unusual for heme peroxidases, which normally are active above the imidazolium/imidazole $pK_a$ of the distal base (pH ~4). Following cloning of the gene from cDNA, it was clear that no common peroxidase motifs were present. A novel peroxidase family was born.

Approximately ten years later, the first crystal structure of DyP BaDec1 was published and revealed a unique enzyme that lacked essential peroxidase residues. Importantly, the distal His base of canonical heme peroxidases was replaced with aspartic acid in the DyP. This initially explained the very low pH optimum: if this aspartic acid were acting as the base, then it would
<table>
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<th>Name</th>
<th>Organism</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_{M,(H_2O_2)}$ (μM)</th>
<th>$k_{\text{cat}}/K_{M,(H_2O_2)}$ (M$^{-1}$s$^{-1}$)</th>
<th>pH, Temp (°C)</th>
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<td>RB19</td>
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<td>Anabaena sp. strain PCC7120</td>
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<td>ABTS</td>
<td>63</td>
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<td>ND</td>
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<td>-</td>
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<td>2.4 x 10$^7$</td>
<td>2.3</td>
<td>ABTS</td>
<td>97</td>
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$^a$RB5 = reactive blue 5; $^b$ABTS = 2,2'-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid; $^c$ND = not determined.
remain in active/deprotonated form even below pH 4. Spectroscopic analysis of the reaction of DyP with peroxide revealed formation of the ferryl porphyrin cation radical, Compound I. This species formed with one equivalent of H$_2$O$_2$. Its presence was indicated by a characteristic decrease in the Soret and a broadening in the visible region with peaks at 530, 556, 615 and 644 nm. Reaction of the DyP with greater than one equivalent of H$_2$O$_2$ resulted in a similar spectrum, and was called Compound II (the one electron oxidized Compound I), though this remains to be confirmed by other spectroscopic or kinetic tools. Recent structural and ligand binding analysis has led to the proposal of a swinging aspartic acid mechanism for Compound I formation, where the Asp acts as an active site base toward H$_2$O$_2$, but must swing into the proper conformation to be close enough for deprotonation to occur.$^{62}$

With the recent surge in available genomic data, more D-family enzymes are being annotated and are beginning to be characterized. Two proposed D-family enzymes from the jelly fungus *Auricularia auricula-judae*, called AjP I and AjP II, have been characterized to a limited extent.$^{87, 88}$ The 51 and 40 kDa proteins were originally purified from the wood-colonizing jelly fungus grown in a beech wood medium. Both are heme enzymes with absorption maxima at 407 (Soret), 500 and 632 nm and have been described as reddish in color. These enzymes react with a variety of canonical and non-canonical peroxidase substrates including ABTS and the dye RB5. Similar to DyP$_{BADec1}$, they exhibit low pH optima (3.0 for RB5 and 4.5 for ABTS) with $k_{cat}/K_M$(RB5) values of 5.0 x 10$^6$ and 1.7 x 10$^7$ (M$^{-1}$s$^{-1}$) for AjP I and II, respectively. The low pH activity and stability are comparable to that found in many plant peroxidases and were attributed to the microenvironment of wood rot (approximately pH 2.5). Based on these characteristics and sequence analysis, these two peroxidases have been described as two fungal isoforms within the D-family family of enzymes.
The fungal species represent a large majority of annotated D-family peroxidases, but bacterial species are also well represented, particularly in the C-subfamily of DyPs. Bioinformatic analysis of the genome of the primitive cyanobacterium (blue-green algae) *Anabaena* sp. strain PCC 7120 revealed a putative heme-dependent peroxidase, called AnaPX. The gene product was over-expressed in *E. coli* and isolated for biochemical characterization. The enzyme purified as a brownish red solution with a Soret peak at 404 nm and visible bands near 500 and 630 nm, similar to other D-family enzymes. Additional investigation revealed that AnaPX formed a characteristic Compound I spectrum at pH 4.5 with one equivalent of H$_2$O$_2$. These enzymes showed high temperature and low pH stability, similar DyP$_{BADec1}$, and a $k_{cat}/K_M$(RB5) at a rapid $1.2 \times 10^7$ (M$^{-1}$s$^{-1}$). In an effort to increase the enzyme’s stability toward higher concentrations of H$_2$O$_2$, methionine residues surrounding active site were mutated. Results showed a few methionine mutants were indeed more stable, and had similar reactivity towards RB5. This suggested that methionine residues play a role in electron transport leading to enzyme degradation; their mutation could lead to more robust catalysts for practical applications.

The D-family homologs from the actinomycete *Rhodococcus jostii* RHA1 were recently characterized by Eltis and Bugg, in two parts. *Rhodococci* are of strong biotechnological interest, as they are capable of biodegrading a large variety of compounds, including lignin, and converting complex carbon sources into more useful building block units. Two enzymes annotated as DyP-A and DyP-B, from the Peroxibase DyP-subfamilies A and B, respectively, were characterized for their biochemical properties and possible utility in lignin degradation. Using genetic knockouts and analysis of reaction products from a lignin degrading assay, the heme-dependent DyP-B was shown to be capable of manganese-dependent lignin degradation. This is the first recombinant lignin peroxidase to be characterized. The gene product DyP-A, by contrast, showed no observable lignin peroxidase activity. As a member of the E-family, it would appear to be more likely involved in Fe uptake and/or heme metabolism. (See below.)

Further kinetic and spectroscopic investigation showed that DyP-B has a Soret band at 404 nm, a slight shoulder at 360 nm, and visible bands at 503 and 634 nm. DyP-A has a Soret near 408 nm and visible peaks at 502 and 632 nm (pH 7.5). Upon reaction with H$_2$O$_2$, DyP-B
formed a spectrum characteristic of Compound I with a decreased Soret band at 396 nm, a shoulder near 340 nm, and visible bands at 580, 613, and 648 nm. This species formed with one equivalent of H$_2$O$_2$ and had a half-life of ~9 s at pH 7.5, 25°C. The intermediate was further investigated using electron paramagnetic resonance (EPR) spectroscopy. The spectrum showed a broad isolated asymmetrical radical with unresolved hyperfine couplings centered near $g = 2.005$. This spectrum is not representative of Compound I from other heme peroxidases, and may be due to a novel positioning of the radical away from either the porphyrin or a tryptophan side chain. Alternatively, when DyP-A was mixed with an equivalent of H$_2$O$_2$ under the same conditions, an absorption spectrum similar to a Compound II species appeared, with Soret peak at 419 nm and $\alpha$ and $\beta$ bands at 557 and 528 nm.

The second-order rate constant for Compound I formation from DyP-B and H$_2$O$_2$ was $1.79 \times 10^5$ (M$^{-1}$s$^{-1}$). This is comparable to the steady-state $k_{\text{cat}}/K_M$(H$_2$O$_2$) of $2.10 \times 10^5$ (M$^{-1}$s$^{-1}$) measured with ABTS as the cosubstrate. Both DyP-A and -B were analyzed for the ability to degrade dyes, like RB5, with values of $k_{\text{cat}}/K_M$(RB5) = $1.28 \times 10^4$ (M$^{-1}$s$^{-1}$) for DyP-B and $k_{\text{cat}}/K_M$(RB5) = $1.4 \times 10^2$ (M$^{-1}$s$^{-1}$) for DyP-A. Hence, while the DyP-B appears to exhibit quite respectable reactivity as a peroxidase, even toward anthraquinone dyes, DyP-A does not.

4.4.3 E-Family: Tat-Transport and Involvement in Fe Metabolism

The members of the E-family are the first heme proteins known to be preceded by a Tat secretion peptide. This signal is necessary for export of folded proteins through a biological membrane, for example, from the cytoplasm into the periplasmic space or cell wall. They have been found in bacteria, archaea, and chloroplasts. Tat-dependent secretion is distinct from the general secretion (Sec) pathway, and it operates using different machinery. Transport of the latter proteins is in their unfolded state, and they are presumed to acquire their non-covalently bound cofactors in the compartment where folding takes place. The Tat pathway is known to be particularly important in halophilic bacteria, which live under highly saline conditions. The Bacillus subtilis E-family protein (called YwbN) is one of two proteins in this Gram positive bacterium that are secreted in a strictly Tat-dependent fashion, of a total of 69 Tat-sequence-containing proteins.
Under high salt conditions, the secretion properties of YwbN change. First, the typical two-protein Tat translocase used by YwbN acquires an additional subunit from the organism’s other Tat translocase. Second, a significant amount of Sec-dependent transport of YwbN is observed. Under low salinity conditions, mutants in the Tat-translocase or in ywbN show severe growth defects. The same defects were observed for mutants in the ywbL or ywbM genes, which are co-operonic with ywbN and homologous to E. coli efeU (Fe inner membrane permease) and efeO (periplasmic Fe transporter), respectively. Importantly, these growth defects can be completely “rescued” by addition of Fe(II)SO₄ (10 mM) to the low salt growth media for any of these mutants. The same amount of Fe(III)Cl₃ had a small growth promoting effect on the ywbM, ywbN, or Tat translocase mutants, and none at all on the ywbL mutant. These results are consistent with YwbL acting as a specific Fe(III) permease. YwbM could function as an Fe(II) or Fe(III) transporter.

What, then, of the E-family protein, YwbN? Given that it is (under most conditions) obligately Tat-translocated, it would appear that heme-binding is essential for its function. Its association with an Fe transporter and Fe(III) inner membrane permease in a Fur-regulated operon further suggests that it is directly associated with Fe uptake. One obvious possibility is that it could act as an Fe(II) peroxidase, in a manner analogous to MnPs or versatile peroxidases. The apparent divalent metal transporter YwbM/EfeO could deliver the Fe(II) to YwbN/EfeB. Such a biological role is consistent with the structure of EfeB, which has at least one clear pathway from the active site to the protein exterior involving the propionic acid side chain on the heme ring D. It would also be consistent with the known peroxidase chemistry of E- and D-family proteins.

This general model was supported by genetic studies in E. coli. All 3 protein products of the Fur-regulated efeOUB operon are required for uptake of Fe(II) under acidic, aerobic, Fe-deficient conditions. (The FeoAB Fe(II) transporter is operative under anaerobic conditions.) Based on homology, EfeU is predicted to be a 7-transmembrane domain inner membrane iron permease similar to the Ftr1p permease found in Sacchromyces cerevisiae, other fungi, and some algae. In fungi, the Ftr1p permease works together with a membrane bound multicopper oxidase (e.g., Fet3p) and secreted ferric reductases (Fre1p/Fre2p). Multicopper oxidases have 3 cupredoxin domains which together constitute the O₂-dependent, Fe(II)-oxidizing active site.
Fre1p or Fre2p are heme and flavin dependent Fe(III) NADPH-dependent reductases that reduce environmental Fe(III) to Fe(II). The Fe(II) is bound by Ftr1p and reoxidized Fet3p. Many bacteria have the Ftr1p homolog, EfeU, but only cyanobacteria have associated Fet3p-like proteins. Instead, EfeU is typically co-operonic with EfeO, an unusual periplasmic (SecB-dependent transport) protein with an N-terminal cupredoxin domain and a C-terminal peptidase M-75 domain containing a conserved HxxE divalent metal binding motif. EfeO may or may not serve a role analogous to Fet3p.  

Genetic studies in the K12 strain of *E. coli* suggested a different role for EfeB in iron uptake: removing iron from ingested heme. Generally, *E. coli* K12 strains do not have the ability to acquire their iron from heme because they lack a functional outer membrane transporter for heme. Letoffé et al. introduced the gene encoding the transporter HasR from *Serratia marcescens* to *E. coli* K12, which conferred the ability to use heme as an iron source. E. coli must therefore contain other necessary protein machinery for heme iron acquisition, including an as-yet unidentified heme oxygenase (HO) enzyme. Screening a library of plasmid transfectants for fluorescent porphyrin intermediates, they identified YfeX as a possible candidate for heme oxygenase activity. YfeX and YcdB (EfeB) are paralogs, and both were thus tested for HO activity. Cells over-expressing both proteins were shown to accumulate protoporphyrin IX, based on fluorescent and mass spectral analysis. In addition, the cells can produce mesoporphyrin IX from meso-heme, an unnatural heme analog, showing that the accumulation of deferrochelation products is not caused by an upregulation of heme biosynthesis. The purified YfeX was shown to have a high affinity for both heme and protoporphyrin IX, which appear to have the same binding site. Mutation of the proximal histidine residue of EfeB eliminated the protoporphyrin IX accumulation phenotype in complementation studies, and significantly decreased the affinity of pure YfeX for heme and protoporphyrin IX. Deferrochelation activity has not been detected in vitro suggesting a missing cofactor or possibly a different mechanism of action for the E-family. These observations have also not been fully reconciled with the prior study on *E. coli* or the work with *B. subtilis*.  

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141
4.5 Contributions from the Thesis and Acknowledgements

The C-, D- and E-families share a similar monomer structure and heme binding pocket. The groundwork laid in understanding the biochemistry of ClDs will likely be of great use in further work on the D- and E- family proteins, which to date have not received as comprehensive attention. The rest of this thesis will highlight detailed biochemical and microbiological research done on C-family proteins from *Dechloromonas aromatica* and *Staphylococcus aureus*. Specifically in regard to how critical active site residues direct reactivity in DaCld and on the reactivity and kinetics of O-O bond cleavage in the enzyme and what that can tell us about the reaction with chlorite. Lastly, an effort toward biochemical characterization and functional determination of Cld from *S. aureus* will be the first step toward understanding the role of an annotated Cld in a non-perchlorate respiring bacteria.

Material in this chapter has been published and reprinted with permission from: DuBois, J.L. and Mayfield, J.A. Dioxygen-Generating Chlorite Dismutase and the CDE Protein Superfamily. *Handbook of Porphyrin Science*. Ed. Karl M. Kadish; Kevin M. Smith; and Roger Guilard. Vol 19: Biochemistry of Tetrapyroles – Part II. World Scientific, 2012. 90: 232-277. Print. JAM contributed portions toward the text and provided editorial and research assistance, and JLD contributed toward the text and developed the final manuscript. Special thanks are given to Richard Kurker for sequence and structural alignment analysis.

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CHAPTER 5
REACTIONS OF THE O₂ EVOLVING CHLORITE DISMUTASE WITH PEROXIDE AND PERACID AND IMPLICATIONS FOR THE PROTEIN FAMILY

Abstract – In order to understand the relationships between the O–O cleaving and the highly unusual O–O bond forming reactions catalyzed by the heme-containing chlorite dismutases, the reactions of the heme-dependent enzyme chlorite dismutase from Dechloromonas aromatica (DaCld) with peroxide and peracid were studied using steady-state and transient kinetics. Rapid mixing studies of DaCld with peracetic acid (PAA) revealed a pH-dependent reaction pathway. At pH ≤ 6, heterolytic cleavage of the O–O bond of PAA cleanly yielded the ferryl porphyrin cation radical (Compound I) (second-order \( k = 1.9 \times 10^5 \text{ M}^{-1}\text{s}^{-1} \)). At alkaline pH values (pH ≥ 8), the reaction proceeds more rapidly (\( k = 1.3 \times 10^6 \text{ M}^{-1}\text{s}^{-1} \)) and the first observed intermediate is a ferryl uncoupled to a protein-based radical (Compound ES). The identity of the latter was confirmed via freeze-quench EPR. Both intermediates were examined using double-mixing stopped-flow experiments to determine their ability to react with ascorbate. A complete peroxidase reaction sequence at pH 6 required two equivalents of ascorbate and only one equivalent at pH 8, suggesting that only the ferryl can oxidize the substrate in the latter case. Though efficient peroxidase reactivity was observed with the low-pKₐ PAA in the steady state, reactions with excess hydrogen peroxide and other neutral oxidants were considerably slower, less efficient, and complicated by bleaching of the heme chromophore. The Cld members of the CDE structural superfamily appear to lack structural features necessary for efficient reactivity with \( \text{H}_2\text{O}_2 \), suggesting alternate biological roles.
5.1 Introduction

Homo- and heterolytic bond breaking and joining processes are fundamental to biological reactions involving \( \text{O}_2 \) and its aqueous reduction products.\(^1\)–\(^4\) Heterolytic O–O bond cleavage, for example, is invoked as an essential part of oxygen activation in cytochrome P450s and in peroxide activation in heme peroxidases. In the P450s, the reaction begins with a ferrous heme and \( \text{O}_2 \), followed by the sequential addition of two electrons and a proton to generate a ferric hydroperoxy [Fe(III)-OOH] species (Compound 0).\(^5\)–\(^6\) The same intermediate forms from the reaction of \( \text{H}_2\text{O}_2 \) and the ferric heme in peroxidases.\(^7\) As an essential part of the reaction mechanism in peroxidases, a residue in the distal pocket over the \( \text{H}_2\text{O}_2 \)-binding face of the heme acts as a base toward \( \text{H}_2\text{O}_2 \), deprotonating it and allowing the peroxo anion to bind the Fe(III). This residue then serves as an acid toward the terminal oxygen of the Fe(III) –OOH complex. Heterolytic cleavage of the ferric-hydroperoxy O–O bond yields the strongly electrophilic ferryl porphyrin cation radical (Compound I) and water: Fe(III)por + \( \text{H}_2\text{O}_2 \) \( \rightarrow \) [Fe(IV)=O]por\(^* \) + \( \text{H}_2\text{O} \).\(^8\)–\(^10\) Outside of heme biochemistry, analogous heterolytic bond cleavage processes have been proposed as part of \( \text{O}_2 \) activation in mononuclear and dinuclear non-heme iron enzymes, as well as the binuclear copper enzymes.\(^11\)–\(^15\)

Homolytic O–O bond cleavage processes have likewise been observed. The oxygen carrier protein myoglobin, for example, can react with \( \text{H}_2\text{O}_2 \) via a homolytic pathway, directly generating the ferryl species (Compound II) and a hydroxyl radical: Fe(III)por + \( \text{H}_2\text{O}_2 \) \( \rightarrow \) (Fe(IV)=O)por + HO\(^*\).\(^16\)–\(^18\) Some mutant peroxidases that lack a distal acid/base residue likewise favor homolytic cleavage of the peroxide O–O bond.\(^19\) Rodriguez-Lopez and coworkers, for example, observed homolytic cleavage of \( \text{H}_2\text{O}_2 \) in the distal histidine to leucine (H42L) mutant of horseradish peroxidase isoenzyme C (HRP-C). The resulting products were ferryl heme (Compound II) and a hydroxyl radical, which they proposed reacted away from the active site to produce other protein radicals.\(^19\) Finally, studies of heme model complexes in water have shown both homolytic and heterolytic bond cleavage. The type of bond cleavage and second order rates depended on the pK\(a\) of the leaving group alcohol.\(^20\)
Photosystem II catalyzes the formation of an O–O bond via proposed mechanisms that are the microscopic reverse of those described for O–O bond breakage in the heme systems.\textsuperscript{3,21} Namely, a radical mechanism has been suggested in which two oxyl radicals join—one bound to Ca\textsuperscript{2+} and the other ligated to a formally Mn\textsuperscript{IV} ion—in the oxygen evolving cluster.\textsuperscript{22–25} Alternatively, a Ca\textsuperscript{2+}-bound hydroxide nucleophilically attacks the electron deficient oxygen of the Mn\textsuperscript{V}=O in the oxygen evolving cluster in the two electron, microscopic reverse of heterolytic bond cleavage.\textsuperscript{26,27}

A related mechanism is proposed for the O–O bond joining process in heme-dependent catalases, in which a high-valent Compound I species first forms via heterolytic bond cleavage in the reaction between a molecule of H\textsubscript{2}O\textsubscript{2} and the ferric enzyme.\textsuperscript{28,29} A second molecule of H\textsubscript{2}O\textsubscript{2} then reduces the Compound I by two electrons, yielding O\textsubscript{2}, water, and ferric heme.

Chlorite dismutase (Cld, EC 1.13.11.49) is a heme-dependent enzyme that catalyzes bond breaking and forming steps analogous to those described for the peroxidases, photosystem II, and catalases above.\textsuperscript{30,31} In the conversion of a single molecule of chlorite (ClO\textsubscript{2}–) to dioxygen and chloride a Cl–O bond of ClO\textsubscript{2}–, analogous to the O–O bond of peroxide, breaks and the O–O bond of O\textsubscript{2} then forms. This is the only well validated biological reaction for efficient O–O bond formation outside of photosystem II. The proposed mechanisms for the bond breaking/forming steps are likewise analogous to those proposed above. Steady-state kinetic studies of the Cld from \textit{Dechloromonas aromatica} (DaCld) have shown that a Michaelis complex forms between the ferric DaCld and chlorite that can be modeled as an the Fe-chlorite collision complex (\textit{Figure 5.1}).\textsuperscript{32} The ligated O–Cl bond can then break by either homolytic or heterolytic processes, leading to Compound II with a chlorine monoxide radical leaving group, or Compound I and hypochlorite. The intermediate/leaving group pair then recombine via radical or nucleophilic joining mechanisms, respectively, to generate a transient peroxychloride species bound to Fe(III). This breaks down to give the O\textsubscript{2} and Cl\textsuperscript{−} products. The sterically confined distal pocket observed in the crystal structure appears to promote recombination of the high valent intermediate with the leaving group with no observable loss of intermediates and with exquisite fidelity.\textsuperscript{33} As a final and mechanistically unique alternative, release of O\textsubscript{2} and Cl\textsuperscript{−} from chlorite could occur in a single concerted step.
DaCld carries out its bond breaking/forming steps with great rapidity, efficiency, and fidelity, in spite of the challenges that O$_2$-generation presents for synthetic catalysis. While a direct study of the bond breaking/forming steps would be of keen interest, the reaction with chlorite is so fast that observation of transient intermediates by standard stopped flow techniques (~1.5 ms mixing) has not been possible. We have sought to understand the peroxidase-like chemistry of DaCld in light of the well-developed chemical, biochemical, and structural models of heme peroxidases, the chemical similarities between H$_2$O$_2$ and ClO$_2^-$ as substrates, the proposed biological role of the chlorite dismutases and their structural relatives in the large CDE superfamily to which it belongs as peroxidases, and the intimate connections between O$_2$ generating and O$_2$/H$_2$O$_2$ utilizing metalloenzymes. Reactions of DaCld with H$_2$O$_2$ and with peracetic acid (PAA, CH$_3$(CO)OOH, pK$_a$ = 8.2), alone and in the presence of common peroxidase reducing substrates, have been comprehensively studied by transient and steady-state kinetic methods. Because pH has been observed to have strong influences on both the reactivity and spectroscopy of DaCld,\textsuperscript{34} this work was carried out over a range of pHs. The results have important repercussions for understanding biological and synthetic O–O joining and lysing reactions.

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**Figure 5.1** Proposed reaction mechanisms for Cl-O bond cleavage and O-O bond formation from chlorite in DaCld.
5.2 Experimental Methods

5.2.1 Reagents

Stocks of reagent grade hydrogen peroxide (H$_2$O$_2$) (35% Acros) and peracetic acid (PAA) (Sigma-Aldrich) were freshly made and their concentrations were determined by iodometric titration. Briefly, to 100 μL of a solution of stock was added 10 μL of 12 M HCl and ~10 μL of a solution of KI (7 mM final concentration). The resulting I$_2$ that formed (yellow solution) was titrated by addition of 2–200 μL volumes of a 1 mM sodium thiosulfate standard (Titristar) in the presence of 2 μL of a starch indicator (Ricca Chemicals/1.0% w/v) until the blue color due to I$_2$ disappeared. For each mole of peroxide or peracid initially present, 2 moles of sodium thiosulfate are consumed. Reductants used were guaiacol (99%, Acros), 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) (TCI-Ace) and L-ascorbic acid (Sigma).

5.2.2 Protein preparation and analysis

Protein expression and purification were carried out as previously described for both the wild-type and R183Q mutant. Purified/concentrated solutions of enzyme were stored in 0.1 M phosphate buffer pH 6.8 at -80 °C at 5-20 mg/mL as determined by the Bradford assay (Bio-Rad). All data herein are referenced to the concentration of heme-bound protein subunit as determined by the pyridine hemochrome assay of Trumpower.

5.2.3 Chlorite dismutase steady-state assay

Continuous oxygen production from chlorite decomposition was routinely measured using a Clark-type O$_2$ electrode. The electrode was equilibrated to the desired temperature (4 °C) and calibrated using for reference the calculated O$_2$ concentration of air-saturated water at the working temperature and ambient atmospheric pressure. The specific activity of the enzyme was defined as μmoles of O$_2$ produced min$^{-1}$mg$^{-1}$ enzyme (4 °C, 0.1 M phosphate buffer pH, 6.8, 2 mM chlorite). For pH-dependent studies, the following buffers were used: 0.1 M citrate-phosphate
(pH 4-8) and 0.1 M borate-phosphate or 0.2 M glycine (pH 8-10). The pH was determined via electrode immediately prior to use and adjusted as needed.

5.2.4 Steady-state kinetics

Peroxidase activity was studied by measuring the initial rates for the oxidation of guaiacol or ABTS using hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) or peracetic acid (PAA). Reactions were monitored spectroscopically at 25 °C in a 1 cm pathlength quartz cuvette using a Varian Cary 50 spectrometer with temperature control from a Peltier cooler. Guaiacol oxidation was followed at 460 nm (\epsilon\textsubscript{470} = 26.6 mM\textsuperscript{-1} cm\textsuperscript{-1}) and ABTS oxidation at 414 nm (\epsilon\textsubscript{414} = 36 mM\textsuperscript{-1} cm\textsuperscript{-1}). Reactions were initiated by the addition of 1-2 μM DaCld. Initial rates were determined from the slope of the initial (~5% of the reaction) change in absorbance, relative to background oxidation measured in the absence of enzyme.

Catalase activity measurements were made by continuously monitoring oxygen production polarographically on a Clark-type O\textsubscript{2} electrode. Equilibration and calibration were performed as described above. Reactions were carried out in a 1.5 mL chamber in which buffer alone was equilibrated for 5 minutes under N\textsubscript{2} gas, to bring the starting O\textsubscript{2} concentration to between 0-50 μM. The probe was inserted into the solution and measurements were begun. Excess H\textsubscript{2}O\textsubscript{2} was then added via gas-tight Hamilton syringe and background reactivity was measured. Finally, 1-2 μM DaCld was added via gas-tight syringe.

5.2.5 Transient kinetics

Reactions with H\textsubscript{2}O\textsubscript{2}, PAA, and reductants were monitored using a Hi-Tech SF-61DX2 stopped-flow system. Reactions were carried out in single mixing mode by rapidly mixing the enzyme (~1-10 μM) with various concentrations of potassium cyanide. Apparent rate constants (k\textsubscript{obs}) were calculated by fitting single-exponential curves using the KinetAssyst software from Tgk Scientific. Second-order rate constants were determined under pseudo-first order conditions from
the slope and intercepts of $k_{obs}$ versus cyanide concentration. Both enzyme and ligand were dissolved in the same buffer prior to binding experiments and the pH was confirmed routinely.

Sequential-mixing stopped-flow analysis was used to determine the rates of reduction of Compounds I and II. For these, the appropriate intermediate was prepared by rapidly mixing enzyme with approximately 1 eq of PAA. This was subsequently mixed with a solution containing various concentrations of reductant (ascorbate). All measurements were carried out at 20 °C. In some instances, data sets were subjected to singular value decomposition to obtain computed spectra for reaction intermediates, using the apparent rate constants obtained as described above and kinetic models input into the SPECFIT global analysis software.

5.3 Results and Analysis

5.3.1 pH dependence of steady state and transient kinetics

The steady state reaction of DaCld with chlorite was previously studied over a wide range of pH. The steady state and transient kinetics of the DaCld reactions with $\text{H}_2\text{O}_2$ and PAA were studied here in detail and at several pH values over which the enzyme is stable (pH 6-10). The reactions with PAA change dramatically around neutral pH. Several results are presented at pH 6 and 8 (Table 5.1), as these values illustrate the behavior of DaCld at acidic and alkaline pH's, respectively.

5.3.2 Catalase activity of DaCld

The catalase reaction is posited to begin with peroxide activation followed by the two electron reduction of the intermediate via a second molecule of $\text{H}_2\text{O}_2$:

(1) $\text{Catalase-Fe(III)} + \text{H}_2\text{O}_2 \rightarrow \text{Compound I} + \text{H}_2\text{O}$

(2) $\text{Compound I} + \text{H}_2\text{O}_2 \rightarrow \text{Catalase-Fe(III)} + \text{H}_2\text{O} + \text{O}_2$

Rates of $\text{O}_2$ production from DaCld/$\text{H}_2\text{O}_2$ were measured in the presence of concentrated (20 mM) $\text{H}_2\text{O}_2$ from pH 4–10. In this pH range, the values obtained were not significantly above background/no enzyme controls, with rates of $\text{O}_2$ generation at pH 8, 9, and 10: 0.89 ± 0.10, 0.75 ± 0.02, and 1.40 ± 0.32 μmoles $\text{O}_2$ min$^{-1}$mg$^{-1}$ enzyme, respectively. For comparison, in its
reaction with ClO₂⁻, DaCld has a specific activity of \(4.7 \pm 0.3 \times 10^3\) μmoles ClO₂⁻ min⁻¹mg⁻¹ enzyme at pH 6.8, 25 °C. Typical catalases have specific activities in the range of 2-30 \(\times 10^4\) units/mg, where a unit is defined as μmoles of H₂O₂ consumed per minute in 60 mM H₂O₂ at pH 7.0 and 37 °C.

5.3.3 Steady-state kinetics of DaCld in catalysis of the reaction of H₂O₂ or peracetic acid with guaiacol

The peroxidase reaction similarly begins with peroxide activation followed by two sequential one-electron oxidations (hydrogen atom transfers) of the substrate AH:

\[
\begin{align*}
(1) & \quad \text{Peroxidase-Fe(III)} + H_2O_2 \rightarrow \text{Compound I} + H_2O \\
(2) & \quad \text{Compound I} + AH \rightarrow \text{Compound II} + A^* \\
(3) & \quad \text{Compound II} + AH \rightarrow \text{Peroxidase-Fe(III)} + A^* + H_2O
\end{align*}
\]

The steady-state reaction of DaCld with H₂O₂ and the commonly used peroxidase co-susubstrate guaiacol (2-methoxyphenol) was studied at pH 6 and 8. At both pH values, H₂O₂ is an exceedingly poor oxidant for DaCld, yielding values for \(k_{cat} \sim 10^3\) fold and \(k_{cat}/K_{M,(H2O2)} \sim 10^4-10^5\)-fold smaller than values measured for the well-studied horseradish peroxidase-C (HrP-C) at pH 7 (Table 5.1). The reaction is slightly more efficient at the higher pH [reflected in the value of \(k_{cat}/K_{M,(H2O2)}\)], though only by \(\sim 10\) fold. By contrast, PAA is a significantly better peroxidase oxidant with DaCld. Though the values of \(k_{cat}\) are similar for PAA and H₂O₂, \(k_{cat}/K_{M,(PAA)}\) is 2-3 orders of magnitude higher than \(k_{cat}/K_{M,(H2O2)}\).

The \(k_{cat}\) for the reaction with chlorite is considerably larger, likely because the reaction depends on the formation of a single enzyme-substrate complex rather than sequential reactions with three separate substrate molecules (oxidant, HA, HA) as expected for the peroxidase reaction. The magnitude of \(k_{cat}/K_{M,(oxidant)}\) is largely due to the efficiency of formation of the initial enzyme-substrate complex. Because chlorite is expected to be effectively completely deprotonated over the entire pH range studied (HClO₂ ⇌ ClO₂⁻ + H⁺; \(pK_a = 1.86\)), the observed pH-dependent behavior must be due to properties of the enzyme. Similarly, the relatively high \(pK_a\) of H₂O₂ (11.6) suggests that it is present in its neutral form throughout the pH range studied. The
slightly greater efficiency of the reaction with PAA at pH 8 relative to pH 6 may be due to increased deprotonation of the PAA (CH\(_3\)COOH \rightleftharpoons CH\(_3\)COO\(^-\) + H\(^+\); pK\(_a\) = 8.2).

5.3.4 Characterization of enzyme intermediates and kinetics of the reaction with PAA at pH 6

The ferric form of DaCld at pH 6 has a Soret maximum at 392 nm and two charge-transfer bands near 510 nm and 645 nm. Upon reaction with ≥3 eq PAA, the ferric species converts isosbestically (points at ~350, 414, 453, and 547 nm) to what appears to be, on the basis of the diminished intensity of the Soret and the positions of the visible bands, a Compound I species (Soret at 395 nm, a slight shoulder at 341 nm, α and β bands near 525 and 550 nm, and charge-transfer bands at 600 and 645 nm) (Figure 5.2, Table 5.2). A second-order rate constant (\(k_{\text{Compound I}} = 1.9 \times 10^5 \text{ M}^{-1}\text{s}^{-1}\)) for its formation was determined from the slope of the plot of \(k_{\text{obs}}\) (obtained from fits of single exponential curves to the data at 391 nm) as a function of [PAA] (Figure 5.3).
Figure 5.2 Transient intermediates formed upon reaction of Cld with ~3 eq PAA at pH 6. Top: Complete spectra; bottom: visible bands shown on an expanded scale for clarity. Approximately ~15 μM DaCld (7.5 μM final) was mixed with 50 μM peracetic acid (25 μM final) in 0.2 M citrate-phosphate buffer at pH 6 (20 °C). The initial spectrum is shown in black and the spectrum for the intermediate formed after ~1.5 seconds in red. Intervening spectra are shown in gray. Isosbestic points are present at 350, 414, 453, and 547 nm.

Figure 5.3 Pseudo first order rate constant for Compound I formation from the reaction of ferric DaCld WT with PAA, measured as a function of [PAA] in 0.2 M citrate-phosphate buffer at pH 6, 20 °C. The apparent second order rate constant $k = 1.900 \pm 0.003 \times 10^5 \text{M}^{-1}\text{s}^{-1}$. 
TABLE 5.2
UV/VISIBLE ABSORBANCES FOR STABLE AND INTERMEDIATE STATES OF DaCLD AND WELL-CHARACTERIZED HEME PEROXIDASES

<table>
<thead>
<tr>
<th>Species</th>
<th>Soret</th>
<th>CT1</th>
<th>β</th>
<th>α</th>
<th>CT2</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>DaCLD</em> Ferric pH 6</td>
<td>392 (99)</td>
<td>645 (2.2)</td>
<td>525 (6.9)</td>
<td>550</td>
<td>510 (9.7)</td>
<td></td>
</tr>
<tr>
<td><em>DaCLD</em> Compound I pH 6</td>
<td>395</td>
<td>600, 645</td>
<td>525</td>
<td>555</td>
<td>None</td>
<td>This work</td>
</tr>
<tr>
<td><em>DaCLD</em> Compound ES pH 6</td>
<td>410</td>
<td>None</td>
<td>525</td>
<td>555</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td><em>DaCLD</em> Ferric pH 8</td>
<td>396 (109)</td>
<td>645 (3.3)</td>
<td>535</td>
<td>sh.</td>
<td>510</td>
<td></td>
</tr>
<tr>
<td><em>DaCLD</em> Compound 0 pH 8</td>
<td>408</td>
<td>None</td>
<td>535</td>
<td>575</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td><em>DaCLD</em> Compound ES pH 8</td>
<td>412 (125)</td>
<td>None</td>
<td>525</td>
<td>555</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Ferric HRP</td>
<td>402.5</td>
<td>643 (3.23)</td>
<td>530 (8.4)</td>
<td>580</td>
<td>498</td>
<td></td>
</tr>
<tr>
<td>HRP Ferrous-O₂</td>
<td>417 (108)</td>
<td>None</td>
<td>544</td>
<td>580</td>
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<td></td>
</tr>
<tr>
<td>HRP Compound I</td>
<td>400</td>
<td>651, 622</td>
<td>525 − sh.</td>
<td>577 −</td>
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<td>42</td>
</tr>
<tr>
<td>HRP Compound II</td>
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<td>527 (9.5)</td>
<td>555</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Ferric DypB</td>
<td>404</td>
<td>634</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>DypB Compound I</td>
<td>397</td>
<td>648</td>
<td>580,</td>
<td></td>
<td>56</td>
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<tr>
<td>Ferric DypA</td>
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<td>632</td>
<td></td>
<td>502</td>
<td></td>
<td></td>
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<tr>
<td>DypA Compound II</td>
<td>419</td>
<td>619</td>
<td>528</td>
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<td>Ferric EfeB</td>
<td>406</td>
<td>660</td>
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<td>485</td>
<td>58</td>
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<tr>
<td>EfeB Compound I</td>
<td>414</td>
<td>603</td>
<td>530</td>
<td>555</td>
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When fewer than 5 equivalents of PAA are used, Compound I is observed to form and decay isosbestic back toward what appears to be the resting ferric form of the enzyme, though incompletely (Soret maximum near 404 nm and charge-transfer bands at 520 and 650 nm). The ferric species maximizes at ~15 seconds following mixing, and then the chromophore bleaches completely, indicating heme destruction. With ≥10 eq of PAA, Compound I decays first to what appears to be a characteristic Compound II or Compound ES species with a Soret maximum at 410 nm, α/β bands near 525 and 555 nm, and no charge-transfer bands. The rate of conversion of Compound I to Compound II/ES is independent of [PAA] (100-500 μM), occurring via a single exponential process with \( k = 0.95 \text{ s}^{-1} \) (Figure 5.4).

Compound ES, first identified in CcP, is isoelectronic with Compound I, but its UV/vis absorbance is indistinguishable from that of Compound II. In Compound ES, the ferryl species is coupled to a nearby amino acid radical (e.g., tryptophanyl), and its strongly
Figure 5.4 Conversion of Compound I to Compound ES and subsequent bleaching of the heme chromophore in 0.2 M citrate-phosphate buffer at pH 6, 20 °C. Approximately ~15 μM DaCld WT (7.5 μM final) was mixed with 100 μM peracetic acid (50 μM final) all in 0.2 M citrate-phosphate buffer at pH 6, 20 °C. The initial spectrum is shown in red and the spectrum for Cpd ES, formed after 8.7 seconds, is in blue. The final spectrum after 300 second is shown in green, intervening spectra are omitted for clarity.

absorbing Soret reflects intact aromaticity in the porphyrin ring. Here, the Compound II/ES species appears to reach its full absorptivity in a kinetic phase that is distinct from the subsequent chromophore bleaching.

To confirm the identity of the Compound I intermediate and measure its subsequent rate of reduction by peroxidase substrates, sequential mixing experiments were carried out. Compound I was generated by mixing a slight stoichiometric excess of PAA with the enzyme and aging for 2 seconds. This putative Compound I intermediate was mixed with either 1 or 2 eq of ascorbate, and the course of the reaction to form a stable 1e⁻ oxidized semidehydroascorbate was followed in the stopped-flow spectrophotometer. One electron donation from ascorbate (which can act as either a one or two electron donor) is the most commonly observed pathway in peroxidases, in which the semidehydroascorbate is believed to be stable in solution or to dimerize slowly over time.\(^{40,41}\) When 1 eq of ascorbate was added, a ferryl species without a coupled organic radical (e.g., Compound II, Compound ES) formed with a Soret maximum at 412 nm, α, β bands near 525 and 555 nm, and no charge-transfer bands (Figure 5.5, Table 5.2). The ferryl species is fairly stable, but subsequently decays via bleaching of the chromophore with a first order rate constant of 0.7 s\(^{-1}\). The time course of the Compound I/ferryl conversion fit well to
Figure 5.5 Species observed following reaction of a pre-formed Compound I and 2 electron eq ascorbate (pH 6). DaCld (10 μM, 2.5 μM final) was mixed with a slight stoichiometric excess of PAA and aged for approximately 2 s. The Compound I species that formed, shown in red, was then mixed with 2 equivalent of ascorbate (5 μM final). The blue species, resembling Compound II, forms and converts to the ferric enzyme (black). Singular value decomposition (Specfit) was used to better resolve the spectra.

Figure 5.6 Pseudo first order rate constants for the reaction of ferric DaCld WT Compound I with ascorbate (left) and for the conversion of Compound ES to the ferric species (right) as a function of [ascorbate] in 0.2 M citrate-phosphate buffer at pH 6, 20 °C. The slope of the line for the top plot yields the second order rate constant of $k = 2.5 \pm 0.3 \times 10^6 \text{M}^{-1}\text{s}^{-1}$. The bottom plot was fit to a rectangular hyperbola to yield a maximum rate of 9.3 ±0.5 s$^{-1}$ and apparent $K_d = 3.5 \pm 1.2 \mu$M.
single exponential curves at 412 nm. Apparent rate constants were also determined in the presence of ≥10 eq ascorbate (pseudo first-order conditions). The fitted rate constants depend linearly on ascorbate concentration, yielding a second order rate constant of 2.5 \( \times 10^6 \) M\(^{-1}\)s\(^{-1}\) (Figure 5.6).

Reacting Compound I with 2 eq of ascorbate resulted in the initial formation of a ferryl species which converted completely back to the resting ferric enzyme. The ferryl/ferric conversion occurs with isosbestic points at 340, 406 and 447 nm. Apparent rate constants for the ferryl/ferric conversion were determined by fitting kinetic data measured at varying [ascorbate] to single exponential curves. The resulting \( k_{\text{obs}} \) values exhibit a hyperbolic dependence on [ascorbate] with a maximum \( k_{\text{obs}} \) of 9.3 ± 0.5 s\(^{-1}\) and an apparent \( K_d = 3.5 ± 1.2 \) μM (Figure 5.6).

5.3.5 Characterization of enzyme intermediates and transient kinetics of the reaction with peracetic acid at pH 8

The ferric form of DaCl at pH 8.0 has a Soret maximum at 396 nm (slightly red shifted relative to pH 6) and two charge-transfer bands near 510 nm and 645 nm (unchanged with pH). Upon reaction with ≥3 eq of PAA, the ferric species converts directly and with isosbestic behavior (points at 350, 406, and 447 nm) to an apparent ferryl (Compound II or Compound ES) (Figure 5.7). This species has a Soret maximum at 415 nm with greater absorptivity relative to the ferric species and a slight shoulder at 350 nm. The α and β bands appear at 525 and 555 nm. These features are typical of a His-ligated Compound II, such as that observed in HRP (Table 5.2). The absorbance versus time data at 415 nm fit well to single exponential curves. Values for \( k_{\text{obs}} \) were measured under pseudo first-order conditions (≥10 eq PAA) as a function of [PAA] to determine a second order rate constant for ferryl formation at 1.3 \( \times 10^6 \) M\(^{-1}\)s\(^{-1}\) (Figure 5.8). The intermediate persists for several seconds, and then the heme chromophore bleaches. Bleaching is faster at higher concentrations of oxidant.

The ferryl species was prepared and subsequently reacted with 1 eq of reductant in a sequential mixing experiment. In the first mix, the enzyme was reacted with a slight stoichiometric excess of PAA. After aging for 1s to ensure essentially complete formation of the ferryl, a second
Figure 5.7 Transient intermediates formed upon reaction of ClD with ~3 eq PAA at pH 8. Approximately 15 μM DaClD (7.5 μM final) was mixed with 50 μM peracetic acid (25 μM final) in 0.2 M citrate-phosphate buffer at pH 8 (20 °C). The initial spectrum is shown in black and the spectrum for the intermediate that formed after ~300 milliseconds in blue. Intervening spectra are shown in gray. Note the isosbestic points at 350, 406, and 447 nm.

Figure 5.8 Pseudo first order rate constants for ferric DaClD WT Compound ES formation from the reaction of ferric DaClD with peracetic acid in 0.2 M citrate-phosphate buffer at pH 8, 20 °C, measured as a function of [PAA]. The second order rate constant is $k = 1.3 \pm 0.01 \times 10^6$ M$^{-1}$s$^{-1}$.

Mix with 1 eq of ascorbate was carried out, and spectra were subsequently measured. The ferryl species was observed to convert completely and with clear isosbestic points (350, 406, and 447 nm) to the starting ferric enzyme (Figure 5.9). A Compound ES (two oxidizing equivalents above the ferric starting material and one above Compound II) intermediate might exhibit these results if the ascorbate reacts as a one electron reductant toward the ferryl, bypassing the organic radical (which could be at a site remote from the iron). Alternatively, a single molecule of ascorbate might
be reacting as a concerted two electron reductant toward the ferryl and a radical that is uncoupled but nearby. This pathway appears unlikely given the expected 1e⁻ reactivity of ascorbate. Finally, the observed results are consistent with a Compound II intermediate being reduced by 1e⁻ to return the ferric starting material.

Figure 5.9 Species observed following reaction of a pre-formed putative Compound II and 1 equivalent ascorbate (pH 8). Approximately 10 μM DaCld (2.5 μM final) was mixed with a slight stoichiometric excess of PAA at pH 8 and aged for 1 s. The resulting Compound II species (blue spectrum) was mixed with 1 equivalent of ascorbate (~2.5 μM final), which resulted in formation of the black species that resembles the ferric enzyme at pH 8.

The same sequential reaction was then performed using pseudo first-order concentrations (≥10 eq) of ascorbate to determine the dependence of the rate of ferryl reduction on [ascorbate]. The plot of $k_{obs}$ (derived from fits of single exponential curves to data) versus [ascorbate] is hyperbolic with a maximum first order rate constant of 0.6 s⁻¹ and apparent $K_d = 3.6$ μM (Figure 5.10).

The reaction of DaCld with PAA at pH values between pH 6 and 8 was also studied. As expected, mixtures of Compound I and ferryl species were observed (data not shown). A $pK_a$ for the Compound I versus ferryl formation reactions lies between pH 6 and 9 but an exact pH value for it could not be reliably determined. A summary of the reaction with PAA at pH 6 and 8 is illustrated in Figure 5.11.
Figure 5.10. Pseudo first order rate constants for the reaction of ferric DaClID WT Compound ES and ascorbate as a function of [ascorbate] in 0.2 M citrate-phosphate buffer at pH 8, 20 °C. The plot was fit to a rectangular hyperbola to yield a maximum rate of \( k = 0.6 \pm 0.01 \) s\(^{-1}\) and an apparent \( K_d = 3.6 \pm 0.3 \) μM.

Figure 5.11 Reactions between DaClID and peracetic acid at pH 6 and 8, the intermediates observed in stopped-flow studies are indicated with corresponding maximum Soret band wavelengths.

5.3.6 Characterization of enzyme intermediates and transient kinetics of the reaction of DaClID with H\(_2\)O\(_2\) at pH 6 and 8

Relatively low concentrations of H\(_2\)O\(_2\) (10-500 eq) resulted in little interpretable change in the UV/vis spectrum of ferric DaClID. Following reaction with a large excess of H\(_2\)O\(_2\) (10 mM, i.e., 1000 eq) at pH 6, the ferric enzyme slowly converts (within 2 s) to a species with a Soret maximum at 408 nm with visible bands at 535 and 575 nm (Figure 5.12, Table 5.2). This
species, which has a spectrum reminiscent of Compound 0 (ferric-hydroperoxy complex) or Compound III (ferric-superoxide)\textsuperscript{43,44}, is observed only at higher concentrations of oxidant. Kinetic traces corresponding to its formation (at 408 nm) fit well to single exponential curves. The linear relationship between $k_{\text{obs}}$ and $[\text{H}_2\text{O}_2]$ yields a second-order rate constant of 96 M$^{-1}$s$^{-1}$ (Figure 5.13). Following its formation, the chromophore for this species converts to a verdoheme-like spectrum with a broad band at 650 nm growing in and a concomitantly bleaching Soret (Figure 5.12).\textsuperscript{45,46} Bleaching is relatively slow ($k = 0.1$ s$^{-1}$) and dependent on $[\text{H}_2\text{O}_2]$.

**Figure 5.12** Transient intermediates formed upon reaction of Cld with a large excess (>2800 eq) of H$_2$O$_2$ at pH 6. Approximately 7 μM DaCld (3.5 μM final) was mixed with 20 mM H$_2$O$_2$ (10 mM final) in 0.2 M citrate-phosphate buffer at pH 6 (20 °C). The initial ferric spectrum is shown in black, the putative Compound III intermediate formed after ~3 s in orange, and the final bleached spectrum that formed after 26 s in green. Intervening spectra are shown in gray.
Figure 5.13  Pseudo first order rate constants for Compound 0 formation from the reaction of the ferric DaCld WT with H₂O₂ in 0.2 M citrate-phosphate buffer at pH 6, 20 °C as a function of [H₂O₂]. The measured second-order rate constant, \( k = 9.6 \pm 0.4 \times 10^1 \text{M}^{-1} \text{s}^{-1} \).

Following the reaction of ferric DaCld with a large excess of H₂O₂ at pH 8, a similar Compound 0 or Compound III-like species is formed. This has a Soret maximum at 408 nm, visible bands at 535 nm and a slight shoulder at 575 nm (Figure 5.14). Formation of this species also appears to be overall second order in enzyme and H₂O₂ with a significantly higher rate constant of \( 1.7 \times 10^4 \text{M}^{-1} \text{s}^{-1} \) (Figure 5.15). This species converts directly to what appears to be a ferryl species (Soret maximum at 415 nm and \( \alpha, \beta \) bands at 525 and 555 nm). Kinetic traces for the conversion to the ferryl fit well to single exponential curves at 415 nm. A plot of associated \( k_{obs} \) values depends hyperbolically on [H₂O₂] with a \( k_{max} \) of 7.9 s⁻¹ and apparent \( K_d = 1.5 \) mM (Figure 5.15).

A hallmark of a true peroxidase is the rapid reaction of H₂O₂ with the ferric heme, which occurs with second-order rate constants on the order of \( 10^7-10^8 \text{M}^{-1} \text{s}^{-1} \). By contrast, the analogous rate constants are \( 10^2-10^3 \text{M}^{-1} \text{s}^{-1} \) for ferric (met) myoglobin. The second-order rate constants for the reactions of DaCld with H₂O₂ are much closer in magnitude to the latter: \( 1.2 \times 10^2 - 1.6 \times 10^4 \text{M}^{-1} \text{s}^{-1} \) from pH 6-8. These rate constants are also comparable to those measured for peroxidases in which the distal His has been substituted by mutation with a hydrophobic residue, yielding an active site configuration resembling that of DaCld. Removal of the distal His,
Figure 5.14 Transient intermediates formed upon reaction of Cld with a large excess of \( \text{H}_2\text{O}_2 \) at pH 8. Approximately 7 μM DaCld (3.5 μM final) was mixed with 2 mM hydrogen peroxide (1 mM final) in 0.2 M citrate-phosphate buffer at pH 6 and 20 °C. The initial spectrum is shown in black, the putative Compound III intermediate that formed after 0.03 seconds in orange, and the final apparent Compound II-like spectrum that formed after 1.1 s in blue. Intervening spectra are omitted for clarity and singular value decomposition (Specfit) was used to better resolve the spectra.

Figure 5.15 Pseudo first order rate constants for Compound 0 formation from the reaction of ferric DaCld WT and \( \text{H}_2\text{O}_2 \) (left) and conversion to Compound II (right) as a function of [\( \text{H}_2\text{O}_2 \)] 0.2 M citrate-phosphate buffer at pH 8, 20 °C.. The slope of the line for the top plot yields the second order rate constant \( k = 1.7 \pm 0.04 \times 10^4 \text{M}^{-1}\text{s}^{-1} \). The bottom plot is fit to a rectangular hyperbola yielding a maximum rate of \( k = 7.9 \pm 0.3 \text{s}^{-1} \) and apparent \( K_d = 1.5 \pm 0.2 \text{mM} \).

which acts as a base, has a profound (5 orders of magnitude) effect on the rate of Compound I formation since deprotonation of \( \text{H}_2\text{O}_2 \) and concomitant binding of the peroxy anion often limits the reaction rate.\(^8,19,49\)

The intermediates observed in the reactions of DaCld and \( \text{H}_2\text{O}_2 \) (Figure 5.16) are moreover different from those observed in true peroxidases, which react with \( \text{H}_2\text{O}_2 \) to form
Compound I. The first detectable intermediate for the reaction with DaCld resembles either a ferric-hydroperoxy (Compound 0) or a ferric-superoxy (Compound III) species, both of which have similar UV/vis spectra.\textsuperscript{19,37,50,51} Accumulation of Compound 0 would suggest that deprotonation of the H$_2$O$_2$ to form the hydroperoxy adduct, while slow relative to Compound I formation in a peroxidase, is faster than the subsequent bond cleavage steps. The putative Compound 0-like intermediate converts either to Compound II (observed at pH 8) or verdoheme (pH 6) prior to complete loss of the heme chromophore. Relatively rapid suicide inactivation of DaCld with concomitant heme destruction was previously demonstrated after only about 500 turnovers with H$_2$O$_2$.\textsuperscript{32} This observation, the exceedingly low values for both $k_{\text{cat}}$ and $k_{\text{cat}}/K_M$(H$_2$O$_2$), and the complete absence of catalase activity are all in keeping with the sluggish H$_2$O$_2$ reactivity observed in the transient state.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure5_16}
\caption{Possible reactions between DaCld and H$_2$O$_2$ at acidic and basic pH, based on known and proposed reaction pathways in other heme proteins.}
\end{figure}
5.3.7 Characterization of enzyme intermediates and transient kinetics of the reaction of DaCld R183Q with Peracetic Acid at pH 6 and 8

The resting ferric DaCld R183Q spectrum has a Soret maximum at ~ 400 nm with charge transfer bands at ~ 500 and 635 nm, the spectrum is unchanged as a function of pH as previously determined\textsuperscript{52}. Upon reaction of the mutant with several equivalents (>100) of peracetic acid at pH 6 a species similar to wild-type Compound I forms and then shifts to what may be a Compound ES species prior to bleaching as shown in Figure 5.17. The Compound I species has decreased molar absorptivity with $\lambda_{\text{max}}$ at 397 nm and broad charge transfer band around 630 nm, as shown in the red spectrum. This species appears to form following pseudo-first order kinetics with a rate constant, $k = 3.7 \pm 0.3 \times 10^3$ M$^{-1}$s$^{-1}$ (Figure 5.18). Cpd I in R183Q slowly decays to what is likely Compound III, much like that observed for wild-type upon reaction with H$_2$O$_2$. This is better observed in Figure 5.19 where singular value decomposition was used to deconvolute the spectra. Here one can see that the blue species formed after the red species has similarities to Cpd III, with shifted $\lambda_{\text{max}}$ to ~ 401 nm and $\alpha, \beta$ bands at 575 and 535 nm. The species then decays to the bleached species shown in green. The rate of conversion from Cpd I to Cpd III in this reaction appears to depend on [PAA] and ranges from 1 – 10 s$^{-1}$ (data not shown) and the species is better observed at higher concentrations of oxidant. The reaction at pH 8 is almost identical to that at pH 6 as seen in Figure 5.20; a Cpd I species forms immediately with a pseudo-first order rate constant, $k = 3.8 \pm 0.4 \times 10^3$ M$^{-1}$s$^{-1}$ (Figure 5.21) then converts to Cpd III and subsequently decays. The Cpd III species at pH 8 is slightly more blue shifted at ~415 nm and the $\alpha, \beta$ bands are not as pronounced using SVD as shown in Figure 5.22. Overall the reaction of DaCld R183Q with peracetic acid is independent of pH and forms Cpd I prior to bleaching with a relatively high second order rate constant compared to wild-type.
Figure 5.17 Reaction of ferric DaCld R183Q with 40 mM peracetic acid 0.2 M citrate-phosphate buffer at pH 6, 20 °C. Approximately 8 μM DaCld R183Q (4 μM final) was mixed with 80 mM peracetic acid (40 mM final) all in 0.2 M citrate-phosphate buffer at pH 6, 20 °C. The ferric enzyme’s spectra (black) has a Soret peak at ~ 400 nm with charge transfer bands at ~ 500 and 635 nm rapidly converts to Compound I (red, 0.048 s) with a decrease in absorptivity in the Soret peak. The Compound I species has λ\text{max} at 397 nm with a broad charge transfer band around 630 nm. Compound I eventually decays to a bleached spectra (blue) after shifting to 401 nm. Inset shows the same spectra with a zoomed in visible region.

Figure 5.18 Pseudo first order rate constants for Compound I formation from the reaction of the ferric DaCld R183Q with peracetic acid as a function of [PAA] in 0.2 M citrate-phosphate buffer pH 6.0, 20 °C. The measured $k = 3.7 \pm 0.3 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$. Rate constants were taken from exponential fits to the change in absorbance at 420 nm, associated with initial Cpd I formation, as a function of time.
Figure 5.19 Singular value decomposition of the reaction of ferric DaCld R183Q with 40 mM peracetic acid in 0.2 M citrate-phosphate buffer pH 6.0, 20 °C. The spectra correspond to each species formed upon fitting the variable absorbance traces to 3 exponentials with rates of 125, 10, and 0.7 s\(^{-1}\). The first intermediate (ferric enzyme) is in black followed by red, blue and green species. Inset shows the visible region of the spectra. Data were analyzed using SpecFit.

Figure 5.20 Reaction of ferric DaCld R183Q with 40 mM peracetic acid at pH 8. Approximately 8 μM DaCld R183Q (4 μM final) was mixed with 80 mM peracetic acid (40 mM final) in 0.2 M citrate-phosphate buffer at pH 8 (20 °C). The ferric enzyme’s spectra (black) has Soret peak at ~400 nm with charge transfer bands at ~500 and 635 nm rapidly converts to Compound I (red, 0.053 s) with a decrease in absorptivity in the Soret peak. The Compound I species has λ\(_{max}\) at 397 nm with a broad charge transfer band around 630 nm. Compound I eventually decays to a bleached spectra (blue) after shifting to ~415 nm. Inset shows the same spectra with a zoomed in visible region.
Figure 5.21 Pseudo first order rate constants for Compound I formation from the reaction of the ferric DaCld R183Q with peracetic acid as a function of [PAA] in 0.2 M citrate-phosphate buffer pH 8.0, 20 °C. The measured \( k = 3.8 \pm 0.4 \times 10^3 \, \text{M}^{-1} \, \text{s}^{-1} \). Rate constants were taken from exponential fits to the change in absorbance at 420 nm, associated with initial Cpd I formation, as a function of time.

Figure 5.22 Singular value decomposition of the reaction of ferric DaCld R183Q with 40 mM peracetic acid in 0.2 M citrate-phosphate buffer pH 8.0, 20 °C. The spectra correspond to each species formed upon fitting the variable absorbance traces to 3 exponentials with rates of 115, 7, and 0.7 s\(^{-1}\). The first intermediate (ferric enzyme) is in black followed by red, blue and green species. Inset shows the visible region of the spectra. Data were analyzed using SpecFit.
5.4 Discussion

The reactions of \( \text{DaCld} \) with \( \text{H}_2\text{O}_2 \) and peracetic acid have been comprehensively studied in order to better understand the relationships between the \( \text{O–O} \) cleaving (from \( \text{H}_2\text{O}_2 \), PAA) and the highly unusual \( \text{O–O} \) bond forming (from \( \text{ClO}_2^- \)) reactions that many proteins in the chlorite dismutase family efficiently catalyze. This work likewise addresses the potential chemical and biological functions of the diverse Cld family and the CDE structural superfamily. These proteins are frequently annotated as peroxidases based on their inclusion of a His-bound heme, though their real biological functions often remain unclear.

The results here show definitively that \( \text{DaCld} \), and by likely extension its close relatives in the Cld family, are clearly not efficient peroxidases. \( \text{DaCld} \) reacts slowly even with very high (\( \geq 1000 \)) equivalents of \( \text{H}_2\text{O}_2 \) under stopped flow conditions. It forms not Compound I, as in a peroxidase, but what appears to be a ferric-hydroperoxy adduct (Figure 5.14). Both results could be ascribed to the Clds’ lack of a distal histidine or other residue to act as an active site base, a signature structural feature of peroxidases. Similar behavior was observed for other high \( pK_a \) (\( \geq 10 \)) peroxides and peracids, several of which were examined (e.g., tert-butylperoxide, cumylperoxide) but none that reacted appreciably with \( \text{DaCld} \) (data not shown). By contrast, peracetic acid reacts rapidly with \( \text{DaCld} \), with second order rate constants that increase with pH (Figure 5.11). The relatively rapid reactions with PAA and the pH dependence of the rate constants could both reflect the more extensive deprotonation of the peracid (\( pK_a = 8.2 \)) at higher pH and the ease of complex formation of the anions with the ferric heme.

More strikingly, the intermediates formed between \( \text{DaCld} \) and PAA change dramatically with pH. At moderately acidic pH (6), the ferric species converts completely to the product of heterolytic bond cleavage, Compound I, upon reaction with 3 eq of PAA. Under pseudo first-order conditions, the reaction of the pre-formed \( \text{DaCld} \) Compound I (ferryl por\( \text{Fe}^{III} \)) with ascorbate is very rapid and overall second order (\( k = 2.6 \times 10^6 \text{ M}^{-1}\text{s}^{-1} \)), indicating that the active site readily accommodates the organic substrate much like a peroxidase. The resulting ferryl species, which is likely Compound II, converts nearly completely to the ferric starting material following binding and reaction with a second molecule of ascorbate (\( K_{d\text{apparent}} = 3.5 \text{ M} \); \( k_{\text{max}} = 9.3 \text{ s}^{-1} \)). By contrast,
the reaction between DaCld and 3 eq of PAA at alkaline pH (8) results in the complete conversion of the ferric starting material to a ferryl species without a coupled porphyrin radical. This species returns completely to the ferric starting material following reaction with 1 equivalent of ascorbate. Under pseudo first-order conditions, an apparent binding step with ascorbate with an affinity matching that at pH 6 ($K_{\text{d,apparent}} = 3.6 \ \mu M; \ k_{\text{max}} = 0.6 \ \text{s}^{-1}$), was observed, though ascorbate turnover is about an order of magnitude slower.

The pH-dependent PAA reactivity can be interpreted in one of two ways. First, the reaction mechanism may undergo a shift, from heterolytic bond cleavage at acidic pH to homolytic at alkaline. Such a shift could be supported by a structural rearrangement of the distal arginine (Arg183) side chain, which we have previously proposed goes from a heme-directed closed to a solvent-directed open conformation with a $pK_a$ near 6.6. A heme-oriented Arg183 might be expected to promote heterolytic bond cleavage by polarizing the breaking O–O bond of PAA. At the same time, the closed Arg183 would be optimally situated to stabilize an anionic acetate leaving group. The open orientation of the side chain, on the other hand, would render the heme pocket more hydrophobic, stabilizing the neutral acetyl radical and supporting homolytic O–O cleavage at higher values of pH.

The data are also consistent with heterolytic formation of Compound I at both acidic and alkaline pH. In this interpretation, the rates of Compound I formation and of its conversion to Compound ES would both necessarily accelerate with pH, making Compound ES the first observable intermediate under alkaline conditions. Migration of the radical away from the porphyrin and onto various redox amino acid side chains – typically tryptophans or tyrosines – occurs as part of the regular catalytic cycle in some peroxidases and as an unwanted side reaction in others. The DaCld crystal structure shows 3 highly conserved Trp residues along the periphery of the heme (Figure 5.23), any or all of which might provide a conduit for radical migration. To rationalize the observed reactivity of the Compound ES intermediate with a single equivalent of ascorbate, one would have to propose that the reductant concomitantly donates two electrons to two sites on the protein to retrieve the ferric form, or that the ascorbate selectively reduces the ferryl iron to the ferric, leaving the radical species behind.
The measurement of EPR spectra for the freeze-trapped intermediates affords the most straightforward means of resolving these two models. EPR showed unequivocally that the acidic intermediate does not possess an organic radical uncoupled from the ferryl, while the alkaline species does. In the latter case, the organic radical was spin-quantified at 1 per heme-containing monomer, strongly suggesting that this intermediate is best described as a Compound ES. Hence, the reactions with PAA appear to occur by heterolytic O–O cleavage at both acidic and alkaline pH, with Compound I formation and conversion to ES both accelerating with pH.

How do these results reflect on the likely mechanism for O₂ production from the native substrate, chlorite? From a thermodynamic perspective, the net two-electron reduction of chlorous acid (HClO₂) to hypochlorous acid (HClO + H₂O) under acidic conditions is strongly favorable (E° = 1.674 V versus the standard hydrogen electrode, SHE; ΔG° = -77.21 kcal/mol). E° for the two electron reduction of PAA [CH₃(CO)COOH + H⁺ ⇌ CH₃COO⁻ + H₂O] has been measured at a comparably exothermic 1.565 V vs SHE (ΔG° = -72.18 kcal/mol). From the catalyst’s side, the potentials relevant to Compound I and Compound II formation are not known for DaCld, but have been measured for the HRP-C Fe(IV)=O/ferric and Fe(IV)=O(por•⁺)/Fe(IV)=O couples at 0.96-0.99V and 0.94-0.97V (SHE), respectively (pH 6-6.5), or approximately -22 kcal/mol each. Based on these values, heterolytic (O)Cl–O⁻ bond cleavage via a peroxidase-like
catalyst is expected to be thermodynamically favorable by -33.0 kcal/mol at acidic pH, and slightly less exothermic for PAA. The heme environment in DaCld is far less oxidizing than in HRP-C, however, with a measured Fe(III)/Fe(II) reduction potential of -0.023 V (versus -0.180 to -0.280 V for typical peroxidases and -0.278 V for HRP-C). This suggests that DaCld-mediated bond heterolysis should also be less exothermic.

Homolytic bond cleavage is likely to be even less exothermic or endothermic overall. To our knowledge, measured potentials for the ClO\(_2^-\),2H\(^+\)/ClO\(^*\), H\(_2\)O and CH\(_3\)(CO)COOH, H\(^+\)/CH\(_3\)COO\(^*\), H\(_2\)O couples are not available. However, the standard potential for the related one electron reduction in aqueous solution, ClO\(_2^-\) + 1e\(^-\) + 2H\(^+\) ⇌ H\(_2\)O + ClO\(^*\), is reported as 1.115 V (\(\Delta G^\circ = -25.71\) kcal/mol). Coupling this reduction to the oxidation of the ferric heme to Fe(IV)=O (~22 kcal/mol) yields a significantly less exothermic reaction overall (\(\Delta G^\circ = -3.7\) kcal/mol). The DaCld Fe(IV)=O/Fe(III) oxidation potential is likely much more positive. Hence, we expect that DaCld-mediated homolytic bond cleavage is far less thermodynamically favorable than heterolysis.

One can likewise consider these reactions from a kinetic point of view. The importance of an active site base for rapid reactions of DaCld or peroxidases with H\(_2\)O\(_2\) has already been discussed. In DaCld or distal His mutants of peroxidases, Fe(O) species either are not observed following reaction with H\(_2\)O\(_2\) or form at dramatically (5-orders of magnitude) lower rates. The kinetics and mechanisms of O–O bond cleavage from the Fe-OOH complex have been rationalized according to the stability of the leaving groups. Results from a variety of studies using linear free energy relationship (LFER) analyses have shown that second order rate constants for RO–O(H) bond heterolysis depend linearly on the pK\(_a\) of leaving group (RO\(^-\) + H\(^+\) ⇌ ROH).\(^{20,53}\)

Leaving groups that are stronger acids (and hence relatively stable anions) lead to faster heterolytic bond scission. At some point as leaving group pK\(_a\)s increase, the downward linear trend ceases and the plot becomes roughly horizontal. Here, the competing homolytic pathway, with its neutral radical leaving group (RO\(^*\)), begins to predominate.
Heterolytic scission of the CH₃(CO)O–O(H) and OCl–O bonds results in acetate ($pK_a = 4.5$) and hypochlorite ($pK_a = 7.5$), respectively. A full LFER analysis for DaCld was not possible, due first to the protein’s lack of reactivity with high-$pK_a$ peroxides and peracids and second to the insolubility of other potentially interesting oxidants in water. However, leaving group arguments clearly point toward kinetically favorable heterolysis for PAA, and a somewhat more ambiguous situation for chlorite. A $pK_a$ of 7.5 is near the heterolytic/homolytic transition for model complex reactions carried out in aqueous solution. Where this $pK_a$ might lie for the DaCld active site is not clear. To assess the importance of the polarizing influence of R183 on O–O bond cleavage, we examined the reaction of the R183Q mutant with PAA. In the mutant, the alkylguanidinium side chain of arginine is exchanged for its far less polar, uncharged alkylamide isostere. The Q183 side chain was observed by resonance Raman spectroscopy to exist in a mixture of open and closed conformations across the entire range of pH for which the protein is catalytically active. The values for $k_{cat}$ and $k_{cat}/K_M$ are likewise independent of pH. The fact that this mutant reacts heterolytically with PAA to form Compound I/acetate at both acid and alkaline pH values suggests that even a considerably less electropositive active site can nonetheless stabilize an anionic leaving group. Hence, we predict that hypochlorite ought to form stably in the presence of Arg183, whether the side chain is oriented in the closed or open form. R183 also appears to play a role in accelerating migration of the porphyrin radical away and into the protein matrix.

What do these results suggest about the likely chemical and biological roles for other chlorite dismutases, and more distantly related members of the CDE superfamily? Little is yet known about the mechanisms or functions of many of these proteins. The conserved distal aspartic acid found in the DyP and EfeB members of this family has been proposed to serve *in lieu* of the conventional distal His base in these proteins. Critically, DaCld – and, based on structure-based sequence alignments, likely all members of the chlorite dismutase subfamily – possesses neither a distal Asp nor any other residue likely to serve as an active site base. In stark contrast with the results presented here, the EfeB and DyP subfamily proteins from *Rhodococcus jostii* RH1 (also called DypA and DypB, respectively) reacted avidly with $\text{H}_2\text{O}_2$. The EfeB-subfamily protein formed a ferryl (Compound II or ES) intermediate following reaction
with just one equivalent of H$_2$O$_2$. The DyP subfamily protein likewise appeared to convert stoichiometrically to a stable Compound I under similar conditions (pH 7.5, 25 ºC; half-life = 9 min). The latter formed with a second order rate constant $>10^5$ M$^{-1}$s$^{-1}$. In addition, the two proteins exhibit steady state constants with H$_2$O$_2$ that are much closer to what one would expect for an efficient peroxidase (Table 1). Perhaps the best studied DyP protein, from Thanatephorus cucumeris, DyP$_{Dec1}$, oxidatively decolorizes the anthraquinone dye RB5 with a $k_{cat}/K_M$(H$_2$O$_2$) of 1.0 x 10$^7$ M$^{-1}$s$^{-1}$ at a pH optimum of 3.2; this compares well to HRP’s value of 3.8 x 10$^6$ M$^{-1}$s$^{-1}$ for the same reaction, with an optimal pH of 4.0.$^{57}$ By the same token, Compound I intermediates have been proposed for the reaction of both DyP$_{Dec1}$ and the EfeB from Escherichia coli (also known as YcdB).$^{54,58}$ Hence, H$_2$O$_2$-reactivity is so weak as to be biologically unlikely for DaCld and its chlorite dismutase homologs, while the known peroxide reactivity of DyPs and EfeBs appears to be substantial. How this relates to the biological functions of these protein families is not entirely clear. The EfeB proteins have been associated by genetic studies with bacterial iron uptake.$^{59–63}$ The DyP proteins are particularly abundant in fungi, where they could serve roles in the biodegradation of lignin and other plant-derived products.$^{64–67}$ Notably, though their active sites and tertiary structures are highly similar, the Cld-family proteins exhibit a 180° flip of the heme around an axis spanning the B and D pyrrole rings relative to the proteins in the EfeB and DyP subfamilies. This rotation removes hydrogen bonding connections between the distal arginine and one of the propionic side chains, and appears to be essential for the conformational mobility of the arginine residue in the DaCld distal pocket.$^{31}$

5.5 Summary and Conclusions

The reactions of DaCld with H$_2$O$_2$ suggest that this and similar proteins are unlikely to act as functional peroxidases or catalases in vivo. This is not due to the inability of the reducing substrate to approach the reactive heme: rapid peroxidase activity with ascorbate is observed when peracetic acid is the oxidant, and the expected Compound I and II intermediates form. Rather, the lack of a distal base likely limits the ability of the enzyme to form the initial Compound 0 ferric-hydroperoxy complex. This structural feature sets DaCld and the whole chlorite dismutase

178
family in strong contrast with their DyP and EfeB structural relatives, which possess a basic distal aspartate and react readily with H\textsubscript{2}O\textsubscript{2}. Heterolytic cleavage of the bound peracid O–O bond in DaCld is exclusively observed over the full range of pH values for which the protein is stable. At weakly acidic pH, Compound I is the initially observed intermediate, while at alkaline pH (≥8), Compound ES forms an order of magnitude more quickly, though the identity of the uncoupled, protein-based radical is unclear. These results suggest that both Compound I formation and its conversion to Compound ES accelerate with increasing pH. Though rapid intermediate formation may be due to increased deprotonation of PAA to yield the anion, why increased pH accelerates the Compound I/ES conversion is unclear, although R183 appears to play some role. Analogies from the reactions with PAA suggest that the O\textsubscript{2} generating reaction with chlorite should occur preferentially via heterolytic cleavage of the Cl–O bond, followed by nucleophilic attack of a hypochlorite leaving group upon a Compound I intermediate. Such a mechanism remains to be confirmed empirically or supported theoretically.

5.6 References


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CHAPTER 6
pH DEPENDENCE OF CYANIDE BINDING TO DECHLOROMONAS AROMATICA CHLORITE DISMUTASE AND ITS DISTAL ARGinine MUTANT (R183Q)

Abstract – A pH-dependent kinetic binding study with hydrogen cyanide was performed. Prior work has shown a protein-based shift in dismutase reactivity, distal pocket polarity, and reactivity with peroxide and peracid as a function of pH in the wild-type enzyme. The proposed mechanism suggests changes associated with the lone polar distal arginine, Arg183. Cyanide binding has played an integral role in the understanding of distal heme enzyme pockets and is generally considered a surrogate for the binding of hydrogen peroxide. Association rates of both the wild-type enzyme ($DaCldWT$) and mutant R183Q ($DaCldR183Q$) were measured using rapid mixing stopped-flow techniques. Rates increase substantially with increasing pH suggesting preferred ligation of the cyanide ion as its deprotonation occurs at $pK_a \sim 9.1$. Wild-type $DaCld$ has association rates with cyanide 3 orders of magnitude larger compared to $DaCld$ R183Q across pH 5 – 8.5, suggesting a critical role for Arg183 in anion binding. Rates of dissociation also increase with increasing pH for the wild-type enzyme and $DaCld$R183Q off rates are independent of pH. The off rate increase for WT occurs with $pK_a \sim 6.7$, similar to the $pK_a$ observed from dismutase activity. The protonation event likely represents similar enzyme-dependent processes. The mechanism of cyanide binding to $DaCld$ WT and R183Q is compared to other relevant heme enzymes. Implications for the role of arginine in the poorly understood dismutase reaction are presented.
6.1 Introduction

The heme-dependent enzyme chlorite dismutase (Cld) is distinguished from other heme proteins for the unique O-O bond forming reaction it performs with substrate chlorite (ClO$_2^-$). The reaction is highly efficient with $k_{cat}/K_m$ of $3.2 \times 10^7$ M$^{-1}$s$^{-1}$, near diffusion limit.$^1$ Crystal structure analysis has shown that the enzyme contains a proximal histidine ligated to the heme iron and a hydrophobic distal pocket with a lone polar arginine residue.$^2$ The proposed mechanism for chlorite dismutation begins with formation of an enzyme-substrate complex that either directs heterolytic or homolytic cleavage of the Cl-O bond to form the ferryl-oxo species Compound I (CpdI) or Compound II (CpdII), respectively. This yields either hypochorite (ClO$^-$) or hypochloryl radical (ClO•) intermediates that recombine with Cpd I or II in a concerted manner to form an O-O bond and release Cl$^-$. It has been shown that the distal arginine residue is critical in accelerating the rate of dismutation. Replacement of this residue lowers $k_{cat}/K_m$ 3-4 orders of magnitude.$^3$ Both the presence and orientation of Arg183 appear to be vital for efficient catalysis. pH-dependent studies of the wild-type enzyme revealed a decrease in activity above pH 6.7, which appears to be caused by a reorganization of the hydrogen-bonding network in the active site pocket and conformational changes of Arg183 as indicated by spectroscopic and kinetic studies.$^4$ At pH values below 6.7, Arg183 is primarily oriented above the heme Fe and poised for reactivity, while above pH 6.7 it favors a conformation away from the heme plane. The role Arg183 plays in the catalytic mechanism of Cld has been suggested to be two fold: stabilization of the anionic substrate and intermediates and providing polarity required for the Cl-O bond breaking reaction.

Cld’s and peroxidases have many things in common: proximal histidine ligated to heme iron, distal pocket argnine, and the ability to react with small oxidants like hydrogen peroxide (H$_2$O$_2$) and ClO$_2^-$. The key distinction between the two is the lack of a distal pocket base. Classic peroxidases, like cytochrome c peroxidase (CcP) and horseradish peroxidase (HRP), contain a distal histidine residue. This residue is deprotonated across the most active range of each enzyme and its role is to remove a proton from the substrate H$_2$O$_2$ ($pK_a = 11.8$) as the enzyme cleaves the O-O bond to form CpdI. When His is absent, Cpd I formation is diminished by up to 5
orders of magnitude.\textsuperscript{5,6} The recently discovered Dye-decolorizing peroxidases (DyP’s) have an active site aspartic acid that is believed to play an analogous role to histidine. Mutation of this residue decreases peroxidase reactivity to almost undetectable levels.\textsuperscript{7} Recent studies of DyPB from \textit{Rhodococcus jostii} RHA1 show that removal of distal Asp does not diminish Cpd I formation and that the distal Arginine is more critical for peroxidase activity.\textsuperscript{8} Structural analysis of Cld’s and DyP’s has shown that these two enzyme families share a similarly organized heme fold and overall structural organization, despite relatively low primary sequence homology.\textsuperscript{9} Peroxidase activity of Cld has been observed ($k_{cat}/K_{m,H_2O_2} = 4.1 \times 10^7$ M$^{-1}$s$^{-1}$) and it is fairly inefficient compared to HRP ($k_{cat}/K_{m,H_2O_2} = 4.5 \times 10^5$ M$^{-1}$s$^{-1}$). Using the alternate oxidant peracetic acid (PAA; $pK_a = 8.2$), which does not require deprotonation above pH below 8, the peroxidase activity is about 2 orders of magnitude greater. Additionally Cpd I was observed upon reaction of Cld with PAA at pH 6 and forms relatively rapidly ($k = 1.9 \times 10^5$ M$^{-1}$s$^{-1}$). Studies of the pH-dependent behavior for this reaction show that at higher pH values (>pH 8), Cpd I is not observed on the stopped-flow time scale, rather the ferryl-oxo species coupled to a protein based radical (Cpd ES) is observed and forms with a greater rate of reaction ($k = 1.3 \times 10^6$ M$^{-1}$s$^{-1}$). If Arg183 is mutated to glutamine the rate of reaction is decreased substantially ($k = 3.7 - 3.8 \times 10^3$ M$^{-1}$s$^{-1}$) and the pH-dependent change in reaction is not observed as Cpd I is the only species formed at all pH values. While unlikely, it has not been entirely ruled out that Arg183 can act as an active site base toward some substrates. While the presence and orientation of Arg183 clearly contributes toward dismutase and peroxidase reactions it is likely that other factors such orientation of other active site residues, heme conformation, and protein dynamics may also play a significant role.

Beyond chemical reactivity, differences in heme protein behavior are abundant. The binding reaction between heme iron and hydrogen cyanide (HCN; $pK_a = 9.1$) has been a useful tool for several years. The molecule acts as a good surrogate for hydrogen peroxide and is an invaluable tool for the current understanding of heme active sites and reaction mechanisms. The most widely accepted mechanism of cyanide binding to heme proteins is that the protonated hydrocyanic acid diffuses into the protein active site where it binds to iron. The proton of the HCN-Fe complex may be removed or remain associated as part of the complex. In peroxidase
enzymes, the binding of HCN mimics the binding of H$_2$O$_2$, as both preferentially react when the distal histidine is deprotonated and is able to act as a base. Studies on peroxidases, like CcP, have shown that the formation of Cpd I and cyanide binding have similar pH-dependencies and occur with similar rates.\textsuperscript{10} Binding of H$_2$O$_2$ is thought to be rate limiting for Cpd I formation, thus studies with HCN are likely mimicking how enzymes interact with the oxidant. Ligand binding has many advantages over reactivity; first of all there is a strong observable shift in the heme Soret band upon formation of the iron-HCN complex, second the complications that arise from formation of reactive redox intermediates and the side reactions that can occur with H$_2$O$_2$ are not an issue with HCN. Due to the valuable information acquired from cyanide binding studies for many heme enzymes, we have explored how the chlorite dismutase from \textit{Dechloromonas aromatica} and its distal arginine mutant R183Q binds to cyanide and how pH effects this reaction. These studies provide further insight into the role of distal arginine and how pH modulates the enzyme’s active site configuration.

6.2 Experimental Methods

6.2.1. Ligand and buffer solutions
Potassium cyanide (Sigma) stock solutions were prepared daily in the appropriate buffers and the pH adjusted. All buffers used were made from concentrated stock of citrate and potassium phosphate (dibasic). Final buffers contained 0.2 M citrate-phosphate and were used from pH 5.5 – 8.5.

6.2.2 Protein purification and preparation
Overexpression and purification of both the wild-type (WT) and Arg183Gln (R183Q) \textit{Dechloromonas aromatica} chlorite dismutase proteins was performed as previously described with no major changes.\textsuperscript{1,3} Protein concentration was routinely determined using the method of Bradford (BioRad) and heme concentration via the pyridine-hemochrome method of
Dismutase activity was routinely monitored, as previously described, to test the competency of each enzyme stock solution.\footnote{11}

### 6.2.3 UV/Visbile (UV/Vis) Spectroscopy

Rapid kinetic measurements were taken using a Hi-Tech SF-61DX2 stopped-flow system in diode-array and photomultiplier modes. Spectra were collected from 320 – 700 nm in diode array and at ~ 420 nm with the photomultiplier setup. Temperature was held constant 20 °C using a circulating water bath (Thermo).

### 6.2.4 Reaction with HCN

Reactions were carried out under pseudo-first order conditions by rapidly mixing WT or R183Q DaCld (~ 1-10 μM final) with varied final concentrations of potassium cyanide (10 – 500 μM for WT and 0.1 – 100 mM for R183Q). Both the enzyme and ligand were diluted into the same buffer prior to binding experiments and the pH was confirmed routinely with pH electrode (Corning). Rate constants (\(k_{\text{obs}}\)) were calculated by fitting single-exponential curves of changes at ~ 420 nm versus time using the KinetAssyst software from Tgk Scientific and the following equation:

\[
\Delta Abs = \exp(-k_1 t)
\]

where \(\Delta Abs\) is the change in absorbance, \(k\) is the calculated rate constant , and \(t\) is time.

Second-order rate constants were then determined from fits of \(k_{\text{obs}}\) versus cyanide (KCN) concentration to the following equation:

\[
\text{Equation 6.2} \quad k_{\text{obs}} = k_{\text{ON}}[CN] + k_{\text{OFF}}
\]

\[
\text{Equation 6.3} \quad K_d = \frac{k_{\text{OFF}}}{k_{\text{ON}}}
\]

where \(k_{\text{ON}}\) is the rate of cyanide binding and \(k_{\text{OFF}}\) is the rate of dissociation. Kinetic values of the dissociation constants \(K_d\) were determined using equation 8.3. The linear fits were performed using Kaleidagraphe.
6.2.5 pH-Dependent Data Analysis

The pH-dependent data were fit using equations with one or two pK_a's, as previously described. In the equation, \( c \) is a pH-independent measure of the parameter \( y \) (the measured rates of association, dissociation or \( K_D \)) and \( K_{a(1)} \) and \( K_{a(2)} \) describe its dependence on the protonation state.

\[
\text{Equation 6.4} \quad \log(y) = \log\left(\frac{c}{1 + \frac{[H^+]}{K_{a(1)}}}\right)
\]

\[
\text{Equation 6.5} \quad \log(y) = \log\left(c \times \frac{1 + \frac{[H^+]}{K_{a(1)}}}{1 + \frac{[H^+]}{K_{a(2)}}}\right)
\]

6.3 Results and Analysis

6.3.1 UV/Visible Properties of WT and R183Q DaCl\(d \) and their Cyanide Complexes

The UV/visible spectra of DaCl\(d \) (WT) and its cyanide complex are shown in Figure 6.1. The heme spectrum of the free enzyme has a Soret peak at 391 nm and visible bands at 514 and 644 nm at pH 7.0. The cyanide complex shifts the Soret to 418 nm with shoulder at 360 nm. The cyanide complex also has an apparent increase in absorbtivity. The visible bands also shift to 540 nm with a slight shoulder at 565 nm. DaCl\(d \) (R183Q) has a slightly distorted spectrum compared to wild-type with the Soret at ~ 402 nm as shown in Figure 6.2. The visible bands are located at ~ 509, 530, and 635 nm. Upon addition of cyanide the Soret shifts to 418 nm with a shoulder near 360 nm, similar to the WT-CN complex. The visible bands for the R183Q-CN complex are located at ~540 nm with a very slight shoulder near 565 nm.
Figure 6.1 UV/Visible Spectrum of WT DaCld and its Cyanide Complex at pH 7.0. The UV/Visible spectrum of DaCld WT (-----) and its cyanide complex (- - - - -) are shown in 0.2 M citrate-phosphate buffer at pH 7.0, 20 °C. Approximately 3 μM of enzyme was mixed with 100 μM KCN and spectra were recorded. The inset shows the zoomed visible region of the spectrum.

Figure 6.2 UV/Visible Spectrum of DaCld (R183Q) and its Cyanide Complex at pH 7.0. The UV/Visible spectrum of DaCld (R183Q) (-----) and its cyanide complex (- - - - -) are shown in 0.2 M citrate-phosphate buffer at pH 7.0, 20 °C. Approximately 3 μM of enzyme was mixed with 1 mM KCN and spectra were recorded. The inset shows the zoomed visible region of the spectrum.
6.3.2 Kinetics of Cyanide Binding.

Binding of hydrogen cyanide \((\text{HCN} \rightleftharpoons \text{H}^+ + \text{CN}^-; pK_a = 9.1)\) to ferric \(\text{DaCl}d\) WT and R183Q from pH 5 to 8.5 is monophasic and yields good fits to single exponentials curves, as represented in Figure 6.3. Above pH 8.5 the rate of binding became too fast to observe for the wild-type enzyme using rapid-mixing techniques. Measurements at lower temperatures (4 °C) did not decrease the rate significantly to permit analysis. The apparent rates that were measured are linear with increasing concentrations of cyanide under pseudo first-order conditions, where the slope and y-intercepts give apparent rates of association \((k_{\text{ON}})\) and dissociation \((k_{\text{OFF}})\) respectively, shown in Figure 6.4. Data for each kinetic constant from pH 5.5 – 8.5 are listed in Table 6.1 and 6.2 for WT and R183Q respectively. The values for \(K_{\text{d(\text{kinetic})}}\) were obtained from \(k_{\text{ON}}\) and \(k_{\text{OFF}}\) values entered into equation 8.3.

![Figure 6.3](image-url)

**Figure 6.3** Representative Traces of Absorbance Versus Time for Cyanide Binding to WT \(\text{DaCl}d\). A representative trace shows the change in absorbance at 418 nm upon binding of cyanide by \(\text{DaCl}d\) WT in 0.2 M citrate-phosphate buffer, pH 5.5, 20 °C. Enzyme (1 µM) was mixed with 100 µM KCN. The open circles show the measured absorbance values that are fit to a single-exponential curve, equation 8.1 in the text.
Figure 6.4 Representative Linear Fit of $k_{obs}$ versus [KCN]. Values of $k_{obs}$ measured from traces like those shown in Figure 3 are plotted as a function of KCN. Data here are from values collected with 1 μM DaCld WT in 0.2 M citrate-phosphate buffer, pH 5.5, 20 °C mixed with KCN (10 - 60 μM). Each point represents an average of no less than 5 measurements and the error is reported as the standard deviation of those values. Data are fit to a linear expression, as described for equation 2 in the text, where the slope of each line gives $k_{ON}$ (M$^{-1}$s$^{-1}$) and y-intercept gives $k_{OFF}$ (s$^{-1}$). These are 3.9 ± 0.1 x 10$^4$ M$^{-1}$s$^{-1}$ and 0.70 ± 0.02 s$^{-1}$ respectively for the data shown here. Data for all pH values for WT and R183 Q are reported in Tables 6.1 and 6.2.

### Table 6.1

PH Dependence of Kinetic and Equilibrium Constants for Cyanide Binding to WT DaCld$^a$

<table>
<thead>
<tr>
<th>pH</th>
<th>$k_{ON}$ (M$^{-1}$s$^{-1}$)</th>
<th>$k_{OFF}$ (s$^{-1}$)</th>
<th>$K_d$ (kinetic) (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.5</td>
<td>1.3 ± 0.1 x 10$^4$</td>
<td>42 ± 7.0</td>
<td>3.3 ± 0.5</td>
</tr>
<tr>
<td>8.0</td>
<td>8.3 ± 0.4 x 10$^6$</td>
<td>26 ± 12.0</td>
<td>3.1 ± 1.5</td>
</tr>
<tr>
<td>7.5</td>
<td>3.9 ± 0.1 x 10$^6$</td>
<td>2.9 ± 0.8</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>7.0</td>
<td>1.3 ± 0.1 x 10$^8$</td>
<td>0.8 ± 0.6</td>
<td>0.6 ± 0.4</td>
</tr>
<tr>
<td>6.5</td>
<td>4.4 ± 0.1 x 10$^9$</td>
<td>0.8 ± 0.1</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>6.0</td>
<td>1.3 ± 0.1 x 10$^9$</td>
<td>0.7 ± 0.1</td>
<td>5.2 ± 0.4</td>
</tr>
<tr>
<td>5.5</td>
<td>3.9 ± 0.1 x 10$^4$</td>
<td>0.70 ± 0.02</td>
<td>18.9 ± 0.5</td>
</tr>
<tr>
<td>5.0</td>
<td>1.0 ± 0.1 x 10$^4$</td>
<td>0.9 ± 0.2</td>
<td>83.6 ± 15.0</td>
</tr>
</tbody>
</table>

$^a$All data collected at in 0.2 M citrate-phosphate buffer, 20 °C.
TABLE 6.2
PH DEPENDENCE OF KINETIC AND EQUILIBRIUM CONSTANTS FOR CYANIDE BINDING
TO R183Q DACLDA

<table>
<thead>
<tr>
<th>pH</th>
<th>$k_{ON}$ (M$^{-1}$s$^{-1}$)</th>
<th>$k_{OFF}$ (s$^{-1}$)</th>
<th>$K_{d(kinetic)}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.5</td>
<td>$5.7 \pm 0.1 \times 10^4$</td>
<td>$0.15 \pm 0.20$</td>
<td>$2.7 \pm 4$</td>
</tr>
<tr>
<td>8.0</td>
<td>$2.2 \pm 0.1 \times 10^4$</td>
<td>$0.36 \pm 0.18$</td>
<td>$16.4 \pm 8$</td>
</tr>
<tr>
<td>7.5</td>
<td>$8.3 \pm 0.1 \times 10^3$</td>
<td>$0.23 \pm 0.10$</td>
<td>$27.8 \pm 12$</td>
</tr>
<tr>
<td>7.0</td>
<td>$2.9 \pm 0.1 \times 10^3$</td>
<td>$0.17 \pm 0.02$</td>
<td>$60.4 \pm 6$</td>
</tr>
<tr>
<td>6.5</td>
<td>$7.0 \pm 0.1 \times 10^2$</td>
<td>$0.75 \pm 0.08$</td>
<td>$1073.1 \pm 117$</td>
</tr>
<tr>
<td>6.0</td>
<td>$2.4 \pm 0.1 \times 10^2$</td>
<td>$0.34 \pm 0.06$</td>
<td>$1388.4 \pm 267$</td>
</tr>
<tr>
<td>5.5</td>
<td>$5.4 \pm 0.2 \times 10^1$</td>
<td>$1.11 \pm 0.13$</td>
<td>$20464.1 \pm 2457$</td>
</tr>
<tr>
<td>5.0</td>
<td>$1.9 \pm 0.1 \times 10^1$</td>
<td>$0.23 \pm 0.08$</td>
<td>$12540.5 \pm 4543$</td>
</tr>
</tbody>
</table>

*aAll data collected at in 0.2 M citrate-phosphate buffer, 20 °C.

6.3.3 pH Dependence of Association Rate Constants

Association rates were determined for WT and R183Q and plotted as function of pH shown in Figure 6.5. Wild type DaCld cyanide association rates increase with pH; ranging from $1.0 \pm 0.02 \times 10^4$ M$^{-1}$s$^{-1}$ to $1.3 \pm 0.04 \times 10^7$ M$^{-1}$s$^{-1}$ at pH 5 and 8.5 respectively. The WT DaCld pH dependent curves for on rates fit to a single pK$_a$ model giving a value of $8.1 \pm 0.2$. The R183Q mutant shows very similar pH dependence for the on rates with a nearly linear increase. This occurs with much slower rates, approximately 500-fold lower values for $k_{ON}$ compared to wild-type. A slow reaction was observed at low pH with a rate constant of $1.9 \pm 0.13 \times 10^7$ M$^{-1}$s$^{-1}$ and approximately three orders of magnitude greater at pH 8.5 with $5.7 \pm 0.01 \times 10^4$ M$^{-1}$s$^{-1}$. The pH dependent single pK$_a$ model fit to R183Q association rates yields a value of $8.9 \pm 0.4$. The values obtained for both species point towards the deprotonation of HCN at pK$_a$ 9.1. It is unfortunate that data could not be obtained at higher pH, as it appears the wild-type enzyme may be plateauing slightly sooner than R183Q. This could be interpreted as hydroxide ion competing with CN$^-$ for binding to the heme iron. A pK$_a$ for this transition was previously shown to occur at pH 8.7 for the WT, but not for R183Q.$^{3,4}$ This may explain the discrepancy in these two values.
6.3.4 pH Dependence of Dissociation Rate Constants

Dissociation rates were determined for WT and R183Q and plotted as function of pH shown in Figure 6.6. The off rates for WT are unchanged up to pH 6.5, where they increase from 0.84 ± 0.1 s⁻¹ to 42 ± 7 s⁻¹ at pH 8.5. This curve fits to a two pKₐ model and gives values of 6.7 ± 0.1 and 9.3 ± 0.9. The off rates measure for R183Q appear to be unaffected by pH, having slow values (0-1 s⁻¹) at all pH’s measured and no pKₐ was determined. The pKₐ of 6.7 found here for WT is consistent with the pKₐ value obtained from steady-state reactions with chlorite, where above pH 6.7 the enzyme becomes less active.⁴ This event was first attributed to a protein based acid-base reaction, however resonance Raman spectroscopy suggests a conformational model where sampling of an “open” and “closed” structures occurs with motion of Arg183.⁵ Dissociation with CN⁻ is most likely associated with reprotonation of the Fe(III)-CN complex which likely lowers the pKₐ of CN⁻ from 9.1.
The apparent rate constants for the binding of cyanide to ferric DaCld and R183Q. The change in absorbance at 418 nm was monitored for the formation of the enzyme-cyanide complex in 0.2 M citrate-phosphate buffer at 20 °C after mixing of approximately 1 μM enzyme with increasing concentrations of cyanide under pseudo-first order conditions. The apparent rates from single-exponential fits were plotted as a function of cyanide and the y-intercept of linear fits to those data were used to determine the off rates for cyanide at various pH values. The log of the off rates are plotted here as a function of pH; wild-type (○) and R183Q (■) increase sharply with pH. Data are fit to single pKa models for wild-type (——) and R183Q (· · · · · ·) as explained in the text. Error bars represent the standard deviation from no less than three measurements.

6.3.5 Comparison of Equilibrium Cyanide Binding Constants for WT and R183Q

A comparison of the pH-dependent equilibrium binding of cyanide to WT and R183Q is shown in Figure 6.7. These data were previously acquired and the results are published, for the sake of comparison they are shown here. Both curves have essentially the same S-shape with higher affinity at higher pH. The WT enzyme has ~3 log units higher affinity compared to R183Q. It has been shown that the R183Q mutant has low affinity for anionic ligands, like azide and fluoride. The pKa values from models fit to the WT data are 6.0 ± 0.7 and 8.8 ± 0.7. Similarly, values for R183Q are 5.8 ± 0.2 and 8.6 ± 0.2. These were interpreted as being caused by the deprotonation of HCN and pKa 9.1, and each enzyme having higher affinity for CN⁻. It should also be noted that at pKa 8.7 the alkaline transition occurs in the WT enzyme, and no such transition occurs in R183Q, thus deprotonation of HCN is the more likely answer though the hydroxide ion
could compete with CN$. The lower $pK_a$ value was initially attributed to a deprotonation event on the protein, but was later thought to be the pH where HCN is the only species and CN$^-$ is not present.

Figure 6.7 Comparison of pH Dependence of log $K_d^{(equil)}$ for WT DaCld and R183Q DaCld. The data shown here are from equilibrium binding of KCN to WT and R183Q carried out previously. The log of $K_d$ values measured from equilibrium titrations of WT (●) and R183Q (■) in 0.2 M citrate-phosphate buffer, 25 °C are plotted as function of pH and fit to a two $pK_a$ model described in the text.

6.3.6 Comparison of Second Order Rate Constants for Cyanide Binding of WT and R183Q DaCld with Other Heme Enzymes

Examining kinetic values of cyanide binding in other heme-dependent enzymes with similar active site configuration can be very helpful in understanding the mechanism of this interaction. Some of those data are compiled in Table 6.3 below. The first observation with both WT and R183Q is that each enzyme apparently appears to preferentially bind CN$, rather than HCN. This is evident from the much higher affinity and the rapid on rates observed at high pH, compared to the high pH drop observed in enzyme like HRP that prefer HCN. This is expected with the lack of an active site base capable of deprotonating HCN. Histidine residues found in most peroxidases and myoglobin are thought to play this role. The preference for CN$^-$ is also observed in enzymes like EcDos and the histidine mutant of yeast CCP (H52L) which both
contain a lone distal arginine. Another feature from this data is the extremely rapid rates of association observed for WT Cld. The value at pH 7.0 is $1.3 \times 10^6$. This is one of the highest observed association rates comparable to spleen myeloperoxidase at $2.5 \times 10^6$. These rapid rates for CN association can be attributed to stabilization of the positive charge on Arg183 and Fe(III) that otherwise exist in a very non-polar active site. Rates of dissociation in WT and R183Q are comparable to most enzymes that preferentially bind HCN, except for CcP(H52L).

### TABLE 6.3

**COMPARISON OF CYANIDE ASSOCIATION SECOND ORDER CONSTANTS BETWEEN WT AND R183Q DaCld AND OTHER HEME PROTEINS**

<table>
<thead>
<tr>
<th>Protein</th>
<th>pH</th>
<th>Temp. (°C)</th>
<th>$k_{ON} \text{ (M}^{-1}\text{s}^{-1})$</th>
<th>$k_{OFF} \text{ (s}^{-1})$</th>
<th>Preferred Ligand</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DaCld</td>
<td>7.0</td>
<td>20</td>
<td>$1.3 \times 10^6$</td>
<td>0.8</td>
<td>CN⁻</td>
<td>this</td>
</tr>
<tr>
<td>DaCld (R183Q)</td>
<td>7.0</td>
<td>20</td>
<td>$7.0 \times 10^4$</td>
<td>0.17</td>
<td>CN⁻</td>
<td>work</td>
</tr>
<tr>
<td>HRP</td>
<td>7.0</td>
<td>25</td>
<td>$9.8 \times 10^4$</td>
<td>0.28</td>
<td>HCN</td>
<td>16</td>
</tr>
<tr>
<td>yeast CCP</td>
<td>7.0</td>
<td>25</td>
<td>$1.1 \times 10^5$</td>
<td>0.9</td>
<td>HCN</td>
<td>17</td>
</tr>
<tr>
<td>yeast CCP (H52L)</td>
<td>7.0</td>
<td>25</td>
<td>$7.0 \times 10^3$</td>
<td>0.15</td>
<td>CN⁻</td>
<td>14</td>
</tr>
<tr>
<td>spleen MPO</td>
<td>7.0</td>
<td>20</td>
<td>$2.5 \times 10^6$</td>
<td>0.65</td>
<td>HCN</td>
<td>18</td>
</tr>
<tr>
<td>sperm whale metMB</td>
<td>7.0</td>
<td>20</td>
<td>$2.3 \times 10^4$</td>
<td>0.0004</td>
<td>HCN</td>
<td>12</td>
</tr>
<tr>
<td>EcDosH</td>
<td>7.0</td>
<td>25</td>
<td>$7.5 \times 10^4$</td>
<td>0.0001</td>
<td>CN⁻</td>
<td>13</td>
</tr>
<tr>
<td><em>C. vinosum</em> cyt c</td>
<td>7.0</td>
<td>25</td>
<td>$2.3 \times 10^3$</td>
<td>$1.3 \times 10^{-4}$</td>
<td>CN⁻</td>
<td>19</td>
</tr>
</tbody>
</table>

### 6.4 Discussion

The most surprising result from this study is the extremely rapid rate of formation for the cyanide DaCld (WT) complex and the difference in dissociation rates at high pH between DaCld (WT) and (R183Q).

The pH-dependent equilibrium binding of CN⁻ to wild-type DaCld has been previously reported. The interaction appears to be influenced by two acid/base reactions. The ligand HCN ionizes to CN⁻ at ~pH 9.1 and the heme iron undergoes an alkaline transition at pH 8.7. It is possible that the CN⁻ and OH⁻ ligands begin to compete for Fe, however the best interpretation is that CN⁻ is now the dominant ligand since the affinity has reached a plateau rather than decreased. The second pK₈ occurs at pH 6.0. This was initially believed to be a protein based
protonation change where the distal arginine became protontated and could no longer act as a base toward HCN. Later interpretation suggested that this value was not a true pKₐ, but rather the point where HCN is the only species present.³ This likely remains the better interpretation of this data.

Results of the kinetic studies with DaCld (WT) show that association of the enzyme with CN⁻ becomes very rapid at high pH. Measurements above pH 8.5 became very fast and difficult to obtain. This is likely due to the full deprotonation of HCN at pH 9.1. This extremely rapid rate of association for DaCld (WT) (pH 7.0, 1.3 x 10⁶ M⁻¹ s⁻¹) is somewhat surprising, considering most enzymes that prefer the anionic form of the ligand have much lower k_ON values (Table 6.3). For example myoglobin, which favors binding to CN⁻, forms the complex at 3.2 x 10² M⁻¹ s⁻¹ and even the oxygen sensor EcDosH that also contains a lone distal arginine associates with CN⁻ at 7.5 x 10¹ M⁻¹ s⁻¹.¹²,¹³ DaCld (WT) is more comparable based on rates to peroxidase enzymes and their mutants. The rapid reaction is likely the result of stabilization of the enzyme active site by binding of the anionic ligand. Arginine and Fe(III) are the only charged species in a very non-polar active site, which also contains threonine, leucine and phenylalanine residues, making it a very unstable environment for charged species. A much lower energy state is achieved as an anionic ligand, CN⁻, binds and neutralizes some of the positive charge. A similar situation is observed in Ccp (H52L) where the polar histidine has been replaced with non-polar leucine.¹⁴ The peroxidase active site now contains a lone polar arginine that is also stabilized by the binding of CN⁻, with a similar rate constant of 1.7 x 10⁶ M⁻¹ s⁻¹. Another interesting aspect of this rapid reaction with CN⁻ is the ability of the ligand to diffuse through the protein matrix. It is generally believed that HCN can travel through protein more efficiently; however it appears here that CN⁻ is able to enter the DaCld (WT) active site more efficiently. The reaction between DaCld (WT) and peracetic acid at pH 8 forms Cpd ES with a rate constant of 1.3 x 10⁶ M⁻¹ s⁻¹, comparable to CN⁻ association rates at pH 8.0 of 8.3 x 10⁶ M⁻¹ s⁻¹. Similar observations are observed at lower pH, the reaction rate with protonated PAA at pH 6 is 1.9 x 10⁵ M⁻¹ s⁻¹ and with protonated HCN at pH 6 is 1.3 x 10⁵ M⁻¹ s⁻¹. This suggests that DaCld (WT) is poised to have anionic species diffuse through the protein matrix.
matrix more efficiently, as one would expect with the rapid reactivity observed with anionic chlorite (ClO$_2^-$; pKa = 1.8).

Dissociation rates for DaCld (WT) are comparable to other heme enzymes, particularly peroxidases, with rates between 0 - 1 s$^{-1}$ at neutral pH, see Table 6.3. However, at higher pH values the off rates begin to increase for DaCld (WT). It has been shown that the distal histidine of peroxidases is able to H-bond with the bound cyanide ligand and destabilizes the Fe-CN complex. Slow dissociation is observed in distal histidine mutants of CcP compared to WT, which is believed to be caused by the lack of H-bonding between the Fe(III)-CN complex the distal HIs. A conformer model has been proposed for DaCld (WT) where Arg183 is able to adopt open and closed conformations, with the guanidinium side chain over the heme plain in the closed conformation and away from iron in the open conformation. It appears pH is able to modulate the two conformations and the previously observed pKa at 6.7 may be a direct or indirect result of that. The cyanide dissociation data for DaCld (WT) gives a pKa at 6.7 where at higher pH the Fe-CN complex is destabilized and dissociates at a higher rate. This destabilization could be explained by competition of the heme iron with hydroxide ions at higher pH, although that transition occurs at higher pH. Another possibility is that Arg183 has become deprotonated, lost its positive charge and is no longer able to stabilize the complex. A positive charge would most likely stabilize the Fe(III)-CN ligand and be favored, while an H-bond would destabilize the complex favoring HCN. Arg could still H-bond to the complex if deprotonated, however this would be a destabilizing interaction. Another possibility is that as Arg183 begins to favor the open conformation and the lack of distal charge neutralization destabilizes the cyanide complex causing the observed increase in dissociation rates. The association and dissociation data support both of these models.

No alkaline transition has been observed for DaCld (R183Q) or any mutations of Arg183, and the relative affinity for anionic ligands is very low. The affinity for cyanide$^-$ is 4-fold lower for the mutant and neither azide (N$_3^-$) nor fluoride (F$^-$) has been observed to bind, compared to WT, which has high affinity for both ligands. Resonance Raman and UV/Vis data have shown that R183Q is a mixture of 5cHS and 6cHS heme, with the 6cHS species containing a molecule of
water. The rate of binding the mutants are significantly slower compared to DaCld (WT) ranging from $1.0 \times 10^1 - 5.7 \times 10^4 \text{M}^{-1}\text{s}^{-1}$ from pH 5.5 to 8.5. The fits to pH models give a similar $pK_a$ (~9.0) for the cyanide protonation and the anionic species is the preferred ligand for both R183Q and WT. Removal of Arg183 by Gln relieves the active site of significant positive charge and the resting pocket is likely more stable thus CN$^-$ binding has less effect on charge neutralization. Displacement of water would require an extra step that may not be favorable.

The dissociation rates, however, reveal an interesting effect on the Arg183 mutation. The DaCld (R183Q) data show that no pH dependence is observed for off rates of the cyanide complex. The rates are close to those observed for WT (0 - 1 s$^{-1}$) at low pH, but dramatically different at high pH. This suggests the complex is fairly stable over the entire pH range. If Gln were modeled as H-bonding to the CN complex it would be assumed that this interaction destabilizes the complex and pH has no dramatic effect on glutamine or its conformation. DaCld (R183Q) has been shown to adopt two conformations it does not appear to be dependent on pH, as no protein-based $pK_a$ value has been observed in reactivity or binding for this mutant. The most likely situation is that the DaCld (R183Q)-CN complex keeps Gln in the closed conformation and slow dissociation is caused by H-bonding between Fe(III)-CN and Gln183.

### 6.5 Summary and Conclusions

Rapid association between DaCld (WT) and CN is observed and increases as a function of pH suggesting the favored ligand is the cyanide anion. The formation of the DaCld (WT)-CN complex is highly favored due to neutralization of the positively charged Arg183 and heme iron in an unstable hydrophobic distal pocket. Cyanide dissociation rates also increase with pH, but the transition appears to occur at a lower pH (6.7). Destabilization of the DaCld (WT)-CN complex is likely caused by the absence of the charge of Arg183 either by deprotonation or adopting the out conformation. Slow cyanide association data from DaCld (R183Q) confirms in the absence of the distal Arg that binding of anionic ligands is disfavored. It also suggests that the conformation of the distal Gln is not dependent on pH, between 5 and 8.5; as it equally destabilizes the CN complex at all pH’s tested.
6.6 References


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CHAPTER 7
THE HEME-CONTAINING CHLORITE DISMUTASE OR HEMQ FROM STAPHYLOCOCCUS AUREUS AND ITS SMALL COLONY VARIANT MUTANT PHENOTYPE

Abstract – Sequence and structural analysis suggest that annotated chlorite dismutase genes found in non-perchlorate respiring bacteria have non-dismutase function. The following is an attempt to understand the function of the cld gene product from the pathogenic species Staphylococcus aureus using a combined biochemical and microbiological approach. Heterologously expressed and purified Staphylococcus aureus Cld (SaCld) is an approximately 29 kDa protein with relatively low affinity for heme (720 nM). The heme-bound protein has no dismutase activity and modest peroxidase ($k_{cat}/K_m, \text{H}_2\text{O}_2 = 4.8 \times 10^5 \text{M}^{-1}\text{s}^{-1}$) and catalase activities ($k_{cat}/K_m, \text{H}_2\text{O}_2 = 3.4 \times 10^3 \text{M}^{-1}\text{s}^{-1}$). The genetic knockout which causes a small-colony variant phenotype suggesting a link to cellular respiration. In liquid cultures poor growth of the knockout is observed and rescued by addition of heme to the media. Mutant cells produce half the amount of heme compared to wild-type and accumulate the heme biosynthesis intermediate corpoporphyrin, as measured by liquid-chromatography mass spectrometry. The results presented here suggest a possible catalytic or regulatory role for SaCld in heme b metabolism.

7.1 Introduction

Associating genes with chemical and biological functions is one of the great scientific challenges of our time. The chlorite dismutases (Clds or C-family proteins) constitute a widespread family of heme-binding proteins found in at least 13 bacterial and two archaeal phyla and for which chemical and biological roles remain unclear. The same heme fold is common to the much larger CDE structural superfamily, of which the C-family is a constituent. Heme
proteins are involved in a wide variety of important cellular processes including respiration, sensing, and multiple essential biosynthetic and biodegradative pathways. The small subgroup of Cld- or C-family proteins from which the Clds derive their name is known to play a key role in perchlorate (ClO$_4^-$) respiration, an unusual metabolic pathway found primarily in Proteobacteria including the well-studied, sequenced strain, *Dechloromonas aromatica*. The origins of perchlorate respiration remain a fascinating topic of research, primarily because the lion’s share of the earth’s perchlorate is human-made and relatively recently deposited. These perchlorate-associated Clds catalyze the stoichiometric conversion of the toxic endproduct of perchlorate respiration, chlorite (ClO$_2^-$), into harmless Cl$^-$ and O$_2$. Hence, the nature of the Cld-catalyzed reaction and its biological role in the perchlorate-respiring bacterium are both clear.

A *cld* gene, however, is found in many microbes, including important pathogenic species like *Staphylococcus aureus* that have no known ability or environmental demand to respire or detoxify oxochlorates. These genes have been observed to appear in a number of genomic contexts, potentially correlating with function. In perchlorate respiring bacteria, *cld* genes co-localize with a set of genes encoding a respiratory perchlorate reductase, though the *cld* gene is typically under separate regulation. In Actinobacteria, *cld* genes are co-operonic with *hem* genes encoding the biosynthesis of heme. Using genetic and complementary *in vitro* methods, Dailey *et al.* confirmed an association between the *cld* gene, termed *hemQ*, and the genes encoding the last two last steps of heme biosynthesis from Gram-positive bacteria (*B. subtilis* and *Mycobacterium tuberculosis*). The same gene has been shown in *B. subtilis* to have functional or regulatory links to anaerobic metabolism. Given the many links between heme and respiratory biochemistry, we hypothesized functional links between *cld* genes and both pathways, although the exact biochemical contribution of the gene product to either is yet indeterminate.

Combined structural, sequence, and experimental analyses focusing on the Clds and related proteins themselves have pointed toward structural features that are critical for various catalytic processes. This work predicts that many of the annotated Clds have activities distinct from chlorite decomposition or possibly even redox biochemistry. In particular, a catalytically important arginine (residue 183 in the Cld from *D. aromatica, DaCld*) essential for efficient chlorite
decomposition is absent from non-Proteobacterial Clds, including the Cld from *S. aureus* (*Sa*Cld or HemQ). In *Sa*Cld and all other Clds from the Firmicutes phylum, this arginine appears to be replaced by a neutral glutamine.

Here, we present a combined biochemical and genetic approach to understanding *Sa*Cld in the context of the well-characterized *Da*Cld homolog and the fascinating, diverse genetics literature describing relevant biochemical pathways. Our results confirm links between *cld*, heme biochemistry, and respiration, as the *cld* knock out appears to be a small colony variant (SCV) on solid media. Initial probes of the phenotype of the knock out and an extensive characterization of the protein suggest a non-catalytic and possibly regulatory role.

### 7.2 Experimental Methods

#### 7.2.1 Materials

Diluted stocks of reagent grade hydrogen peroxide (H$_2$O$_2$) (35%, Acros) and peracetic acid (PAA) (Sigma-Aldrich) were freshly made and their concentrations were routinely determined by iodometric titration. The one-electron reductant used for the peroxidase assay was 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) (TCI-Ace). All porphyrin standards and substrates were purchased from Frontier Scientific (Logan, UT). All solvents used were HPLC grade from Fluka Analytical.

#### 7.2.2 Cloning, Expression, and Purification of *Sa*Cld (HemQ) protein

The gene for chlorite dismutase (alternatively annotated as *cld*, *hemQ*, or *ywft*) from *Staphylococcus aureus* strain COL was obtained from TIGR. A Quick-Change PCR-mutagenesis kit (Stratagen) was used to insert an Nde1 cut site upstream of the gene using the following primer: 5’-TGTACAAAAAAGCAGGCTTCATATGAGTCAAGCAGCCGAAAC-3’ and its reverse complement. Additionally, an Nde1 cut site found in the gene itself was silently mutated using a second primer 5’-CCTAATCCCTACATATACCAGCTGTGCAAGGACGGAAC-3’ and its reverse complement. The amplification product was cloned following digestion with the
Nde1/BsrG1 restriction enzymes (Promega) into a complementarily cut pET41(a) (P<sub>T7</sub>, Kan<sup>R</sup>, Novagen) expression vector. The newly constructed expression vector was sequenced for errors and mutations at the University of Notre Dame Sequencing Facility. The correct plasmid was used to transform competent Escherichia coli Tuner (DE3) cells (Novagen) via heat shock for use in protein over expression. Cell stocks were maintained and stored as frozen glycerol (30%) stocks at -80 °C.

Overexpression of the SaCld (HemQ) protein was achieved in a manner similar to that used for chlorite dismutase from Dechloromonas aromatica.<sup>7</sup> Briefly, a freshly saturated culture of E. coli Tuner (DE3) cells containing the pET-41(a) expression vector with untagged hemQ (cld) prepared from a single colony from an LB agar (50 mg kanamycin / mL) plate was used to inoculate (1:100) 50 mL of Terrific Broth (TB). The 50 mL culture was grown overnight at 37 °C and used to inoculate 1 L of TB in a Fernbach flask. This culture was grown at 30 °C until $\text{OD}_{600nm}$ reached ~ 0.4 - 0.6 and induced at this point with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) as the temperature was lowered to 20 °C. Cells were harvested by centrifugation (Beckaman J-2 centrifuge: JA-10 rotor, 7,000 x g, 30 min, 4 °C) after 24 hours and stored at -80 °C until use.

For purification, approximately 10 grams of wet cell pellet was resuspended in 5 volumes of 0.1 M potassium phosphate buffer pH 7.4. Cells were lysed by sonication using a micro-tip fitted Branson ultrasonifier at 65% with 3 s pulses at 2 s intervals on ice in the presence of 1 mM of the protease inhibitor phenyl-methyl-sulfonate fluoride (PMSF). Cellular debris was removed by centrifugation (JA-20 rotor, 17,000 x g, 50 min, 4 °C) and the soluble fraction was retained. This fraction was dialyzed against 20 mM Tris-Cl buffer at pH 8.6, 4 °C, using 10,000 molecular weight cutoff (MWCO) dialysis tubing (Fisher) with 3 buffer exchanges with at least 3 h per cycle. The dialysate was loaded at 4 mL/min onto a pre-equilibrated (20 mM Tris-Cl pH 8.6) Sepharose-Q-by Fast Flow anion exchange column (GE) using an AKTA Prime protein purification system (GE Biosciences). The column was washed with several volumes of the loading buffer until no more absorbance was detected at 280 nm. A linear salt gradient was initially run in order to determine an approximate [NaCl] at which the protein elutes. Fractions were analyzed by UV-Vis
(A280) and SDS-PAGE to detect the presence of the 29 kDa protein monomer, which was found to elute at approximately 300 mM NaCl. Subsequent purifications used 250 and 300 mM NaCl in 20 mM Tris-Cl, pH 8.6, to wash away contaminants and elute the protein in a stepwise manner, respectively. The eluate was concentrated using a stirred-cell N2 gas pressure concentrator (Amicon) with a 10,000 MWCO YM-20 membrane. The protein (~10 mL) was loaded onto a 2.6 x 240 cm gel filtration column (S-200 Sephacryl, GE) by gravity and was run at 0.4 mL/min in 0.1 M potassium phosphate buffer pH 6.8. Fractions were collected and screened for purity using SDS-PAGE. Fractions containing pure protein were pooled and concentrated in 10,000 MWCO centrifuge concentrators (Millipore) (JA-10, 3,000 x g, 20 min, 4 °C). Aliquots of the protein were flash frozen in liquid nitrogen and stored at -80°C until needed.

7.2.3 Protein and Heme Concentration

To determine protein concentrations, the Bradford method was used (BioRad) with a standard curve generated using Bovine Serum Albumin (BSA). Final heme concentration was determined by the pyridine-hemochrome method of Trumpower using horse heart myoglobin as a standard (Sigma).

7.2.4 Reconstitution of apo-HemQ (SaClid) with heme

HemQ (SaClid) was isolated with very low equivalents (< 0.1) of heme bound per monomer. DaCld produced and purified under similar conditions, by contrast, purifies with near stoichiometric heme bound. To increase the heme content, the apo-protein was incubated at 4°C for several hours with ~2 equivalents of hemin (from 1 mM stock dissolved in 0.1 M NaOH) (Frontier Scientific) per protein monomer. A gel filtration column (S-100 Sephacryl, GE) was used to remove excess heme after incubation.
7.2.5 Molecular Weight Determination

The molecular weights of the holo and apo proteins were estimated by size exclusion chromatography using a 1.6 x 18 cm analytical gel filtration column (S-200 Sephacryl, GE) run at 0.5 mL/min using 0.05 M potassium phosphate 150 mM NaCl pH 7 at 4 °C. Molecular weight standards (BioRad) were used to make a standard curve based on elution volumes: protein aggregate (Void), thyroglobulin (670 kDa), α-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (13.5 kDa). The method assumes globular protein structures and is consequently approximate.

The monomeric molecular weight was determined by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) at the Notre Dame Mass Spectrometry Facility using a Bruker Autoflex III spectrometer.

7.2.6 Absorption Spectroscopy

All UV/visible spectra were recorded on a Varian Cary 50 Spectrometer with temperature control from a Peltier cooler.

7.2.7 UV-Visible pH Titrations

The holo-enzyme was titrated in 0.1 M potassium phosphate buffer starting at pH 6.8, 25 °C and finishing at pH 5.1 or 10.2. Each titration was conducted in a 2 mL cuvette with the protein at ~8 μM heme-containing monomer, where the heme concentration was determined via the pyridine hemochrome assay. The solution was constantly stirred, and the pH was continuously monitored using a pH meter with a glass electrode (Corning pH 430). Small volumes of HCl (1 M) or NaOH (1 M) were added to the solution and the pH was measured, after which it was analyzed by UV–visible spectroscopy. The spectra were adjusted for dilution.
7.2.8 Heme and Ligand Equilibrium Binding

A 1 mM stock of heme was made in 0.1 M NaOH and diluted to working concentrations in 0.1 M potassium phosphate buffer, pH 6.8. All other ligands were prepared in the same buffer and corrected for any pH changes. Equilibrium dissociation constants were determined via UV/visible titration (Varian Cary 50) of 5-10 µM holo (heme-bound) or apo protein with increasing concentrations of ligand (0-500 µM for HCN; 0-10 mM for imidazole; 0-50 µM for heme). Separate samples were prepared for each ligand concentration and were allowed to incubate several hours in tubes to achieve full equilibration. UV/Visible spectra were recorded for each sample and equilibrium constants determined from plots of the change in absorbance at an appropriate wavelength as a function of ligand concentration. For heme, two separate titrations were performed in the absence and presence of protein, and the difference between the two spectra was plotted as a function of heme concentration, to correct for contributions due to free heme. The data were fit to Equation 7.1, the equilibrium isotherm appropriate for weakly binding ligands:

\[
\Delta \text{Abs}_{obs} = \frac{\Delta \text{Abs}_{max}}{2E_{total}} [L_0 + E_t + K_d - \sqrt{(L_0 + E_t + K_d)^2 - 4E_t + L_0}]
\]

Where \(L_0\), \(E_t\), \(K_d\), and \(\Delta \text{Abs}_{max}\) are the initial ligand concentration, total enzyme or protein concentration, the equilibrium dissociation constants, and the maximum change in absorbance.\(^9\)

Titrations using fluorescence spectroscopy were carried out using a Jobin Yvon Horiba FluoroMax-3 fluorimeter in scanning mode. Apo-HemQ (SaCld) (5 µM) was diluted in 0.1 M potassium phosphate pH 6.8 and titrated with stocks of heme (1 mM in 0.1 M NaOH) and protoporphyrin IX (1 mM in DMSO) each diluted to 500 µM at 25 °C. The percent of fluorescence quenched was plotted as a function of ligand concentration and fit using a modified form of the equation for UV/Visible titrations, where \(\Delta \text{Abs}\) is change to % quenched.
7.2.9 Circular Dichroism (CD)

CD absorption spectra of purified holo- and apo- HemQ (SaCld) were measured using a Jasco circular dichroism spectrometer. Approximately 1 μM apo- and holo- HemQ (SaCld) was scanned from 190 – 250 nm in 0.1 M potassium phosphate buffer pH 6.8, 25 °C. The data was analyzed using two web servers, K2D3 and SOMCD, to estimate the content of alpha-helix, beta-sheet and turns/random segments.\textsuperscript{10,11}

7.2.10 Transient Kinetics

For transient kinetic measurements of the reaction of holo-HemQ (SaCld) with peracetic acid (PAA) and hydrogen peroxide, a Hi-Tech SF-61DX2 stopped-flow system in single-mixing mode was used. Briefly, ~8 μM of heme containing monomer was rapidly mixed with varying concentrations of oxidant in 0.1 M potassium phosphate buffer pH 6.8, 25 °C. The reaction was monitored for various amounts of time from 320 to 700 nm using diode array detection. Rates were determined by fitting to a single exponential curve shown in \textbf{Equation 7.2}.

\textbf{Equation 7.2} \quad \Delta \text{Abs} = \exp(-k_1 t)

where \(\Delta \text{Abs}\) is the change in absorbance, \(k_1\) is the calculated rate constant, and \(t\) is time.

Second-order rate constants were then determined from fits of \(k_{\text{obs}}\) versus cyanide (KCN) concentration to the following equation:

7.2.11 Steady State Catalytic Assays

Peroxidase activity was studied by measuring initial rates of 2,2'-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid (ABTS) oxidation using \(\text{H}_2\text{O}_2\) (0-30 mM) or PAA (0-10 mM). Reactions were monitored spectroscopically at 25 °C in a 1 cm pathlength quartz cuvette in 0.1 M potassium phosphate buffer, pH 6.8. ABTS oxidation was followed at 414 nm (\(\varepsilon_{414} = 36 \text{ mM}^{-1}\text{cm}^{-1}\)) Reagent concentrations were varied over 0.01 - 1.0, 0.5 - 30, 0.005 - 10 mM (ABTS, \(\text{H}_2\text{O}_2\), and
PAA, respectively). Reactions were initiated by the addition of 1-2 μM of heme-containing monomer. Initial rates were determined from the first 5-10% of the linear portion of the absorbance curve, relative to background oxidation measured in the absence of enzyme.

Continuous oxygen production due to chlorite decomposition was routinely measured using a Clark-type O$_2$ electrode (YSI Incorporated) with a custom-built water bath jacket for temperature control. The electrode was equilibrated to the desired temperature (25 °C) and calibrated against air-saturated Milli-Q water (Millipore) using the calculated O$_2$ concentration of air-saturated water at the working temperature and ambient atmospheric pressure as a reference. Chlorite solutions (0-20 mM) were delivered from freshly prepared stocks and continuously stirred, with reactions initiated via syringe injection of 5 μL protein (final concentration = 1-2 μM in heme-containing monomer).

Catalase activity measurements were made by continuously monitoring oxygen production polarographically on the same Clark-type O$_2$ electrode using the method of Del Río.$^{12}$ Equilibration and calibration were performed as described above. Reactions were carried out in a 1.5 mL chamber in which buffer alone was equilibrated for 5 minutes under N$_2$ gas, to bring the starting O$_2$ concentration to between 0-50 μM. The probe was inserted into the solution and measurements initiated. Varying concentrations of H$_2$O$_2$ (0-50 mM) were added via gas-tight Hamilton syringe and background reactivity was measured. Finally, 1-2 μM enzyme was added via gas-tight syringe. Initial rates were determined from the first 5-10% of the linear portion of the O$_2$ production curve, relative to background O$_2$ production measured in the absence of enzyme. All steady state kinetic parameters were obtained from plots of initial rate/[heme-containing monomer] versus [substrate] fit to the Michaelis-Menten equation.

### 7.2.12 Bacterial Culture Growth

All growth curves were measured in 50 mL of media grown on a shaker incubator (Innova) at 37 °C, 180 rpm. Most growth experiments used tryptic soy broth (TSB) medium consisting of tryptone, soytone, sodium chloride, and dibasic potassium phosphate in ultra pure
water (Millipore). Cultures were grown in 125 mL glass Erlenmeyer flasks under aerobic conditions and 50 mL plastic Falcon tubes for anaerobic conditions. All cultures were seeded 1:100 from 5 mL TSB cultures grown in 15 mL plastic falcon tubes. These were initiated from freshly streaked TSB-agar plates. Because small colony variant (SCV) strains grow more slowly, the $\Delta$ hemB and $\Delta$ hemQ(cld) plates bearing this phenotype were incubated for two days at 37 °C versus overnight (WT). Further, seed cultures for $\Delta$ hemB and $\Delta$ hemQ(cld) were used when freshly saturated, which took roughly 42 hours, and 12 hours for wildtype. For all cultures optical densities at 600 nm were measured on 0.5 mL aliquots withdrawn every two hours over a 12-hour period using a biophotometer (Eppendorf). Anaerobic cultures were sampled via sterile syringe needles both to maintain anaerobicity and to prevent contamination. No-bacteria/media controls and routine plate streaking were routinely used to check for colonies not sharing the expected SCV morphology for $\Delta$ hemB and $\Delta$ hemQ(cld). Flasks exhibiting reversion or contamination were discarded.

7.2.13 Porphyrin Metabolite Extraction and Analysis

Cells were grown as described above, pelleted via gentle centrifugation (Beckman J-10, 7,000 x g, 15 min, 4 °C), and frozen at -80 °C prior to analysis. Pellets were subsequently thawed and resuspended in ~5 mL of 1:1 1M HCl:DMSO. The suspensions were lysed by sonication on ice using a micro-tip fitted Branson ultrasonifier at 65% for 5 minutes using 3 s pulse and 2 s intervals. Cellular debris was removed by centrifugation (Beckman JA-20, 17,000 x g, 30 min, 4 °C). The soluble fraction was diluted to 50 mL with deionized water and loaded onto a washed and pre-equilibrated C18 Sep-Pak column (Waters). A colored band due to porphyrin metabolites is readily observed in the column resin and is eluted in approximately 2 mL of acetonitrile (Fluka). The solution was filtered using a 0.45 μm syringe filter (VWR) and concentrated to 200 μL by speed vac. To each sample, 0.8 μM of internal porphyrin standard (2-vinyl-4-hydroxymethyl-deuteroporphyrin IX) was added prior to analysis by ultra-performance liquid
chromatography/mass spectrometry (UPLC-MS) to control for run-to-run variability in instrument performance.

Samples were injected onto a Dionex Ultimate 3000 UPLC instrument and separation was achieved by linear gradient elution at 4 mL/min on a BDS Hypersil C18 column (particle size = 2.4 μm) (ThermoScientific) at 50°C transitioning from 100% solvent A (0.1% formic acid in water) to 100% solvent B (0.1% formic acid in methanol) over 20 min. Visible spectra were measured continuously over 390-420 nm. The UPLC was coupled to a Bruker MicroTOF-Q11 electrospray mass analyzer operating in positive ion mode. The mass spectrometer used a capillary voltage of 4500V at 180°C and the nebulizing gas was set at 6.0 mL/min. The instrument was operated and data analyzed using the Hystar software package. This instrument identifies metabolites via their exact molecular weights, which are determined with accuracies of ± 0.005 amu.

A standard mix of porphyrin metabolites and heme intermediates (Frontier Scientific) was used to determine elution times and to construct a standard curve for each metabolite via their measured extracted ion chromatogram (EIC) peak area. EIC peak areas for the metabolites in the samples were referenced to these standard curves in order to determine their concentrations. Standard curves for uroporphyrin III (M/Z = 830.23; 0.14 – 2.8 μM), coproporphyrin III (M/Z = 645.27; 0.14 – 2.8 μM), protoporphyrin IX (M/Z = 562.26; 0.18 – 3.5 μM), hemin (M/Z = 616.18; 0.6 – 7.5 μM), and the internal standard (2-vinyl-4-hydroxymethyl-deuteroporphyrin IX) (M/Z = 567.26; 0.13 – 2.6 μM) were generated from triplicate measurements. All stock solutions were made and diluted in analytical grade acetonitrile (Fluka Analytical).

7.3 Results and Analysis

7.3.1 Isolation and Characterization of Recombinant HemQ (SaCld) from S. aureus

When prepared under conditions that would yield a fully-heme-incorporated DaCld, the S. aureus HemQ (Cld) homolog purified at high yields (15 mg/L culture) (Figure 7.1) but with minimal heme bound (~0.1 - 0.2 heme molecules per protein monomer). To increase the yield of the heme-bound protein, the isolated apo-HemQ was incubated in the presence of 1-2
equivalents of hemin (from 1 mM stock in 0.1 M NaOH) for several hours at 4 °C. Higher equivalents were used in order to avoid heme precipitation and nonspecific association of heme with the protein. Following gel filtration, samples contained approximately 0.5 - 0.6 heme per monomer. The UV-visible spectra of the heme free (apo-HemQ ((SaCld)) and heme bound (holo-HemQ (SaCld)) proteins are shown in Figure 7.2 where the relative ratios of the Soret band to protein absorbance at 280 nm illustrate the weak affinity of the protein for the cofactor. Holo-HemQ (SaCld) prepared in this way was used for all subsequent measurements. Apo-HemQ (SaCld) was used for heme and porphyrin titrations. Protein concentrations herein refer to the concentration of heme-containing monomer, unless otherwise specified.

**Figure 7.1** SDS-PAGE of HemQ (SaCld) Purification. Protein fractions from the two-column purification of apo-HemQ are shown from left to right: MW marker, clarified lysate, Q-Sepharose, gel filtration eluate, and MW marker. Each lane contains approximately 50 μg of total protein. Molecular weight marker values from top to bottom are: 130, 100, 70 (orange), 55, 35, and 25 (orange) kDa.
Figure 7.2 UV-Visible Spectrum of Apo- and Holo-HemQ (SaClD). The UV-visible spectrum of the as purified apo-HemQ and the heme bound holo-HemQ are shown from 350 to 700 nm. Spectra of approximately 115 μM apo-HemQ (SaClD) and 40 μM Holo-HemQ (SaClD) (concentration of assumed monomer taken from Bradford assay) were taken in 0.1 M potassium phosphate pH 6.8 following purification. Holo-HemQ contains ~0.5 heme, as indicated from pyridine hemochrome assay, per assumed protein monomer and is indicated from the increase ratio of Soret to protein absorbance.

7.3.2 UV-Vis Spectroscopic Characterization

The Soret for holo-HemQ in 0.1 M potassium phosphate buffer pH 6.8 has a maximum at approximately 405 nm ($\epsilon_{405} = 37 \text{ mM}^{-1}\text{cm}^{-1}$), with broad charge transfer bands near 510 and 630 nm (Figure 7.3). A slight shoulder in the Soret around 350 nm may be due to a small amount of free heme. The band energies are reminiscent of those obtained for five-coordinate high spin species. The ferrous species was generated via titrimetric addition of dithionite, shifting the Soret to 433 nm and yielding a visible peak at 558 nm. Spectral peaks are summarized in Table 7.1 with comparisons to other relevant heme proteins.
Figure 7.3 UV-Visible Spectrum of Ferric and Ferrous Holo-HemQ (SaCld). The UV-visible spectrum of ferric (---) and ferrous (-----) holo-HemQ (~20 μM heme bound protein monomer) in 0.1 M potassium phosphate pH 6.8. The ferric enzyme has a Soret band at ~405 nm and visible bands at 510 and 630 nm, while the ferrous protein (produced by addition of excess dithionite solid) has a Soret at 433 nm and a visible band at 558 nm. The inset shows the visible region of the spectra from 450-700 nm. Results are summarized and compared to other CDE family proteins in Table 7.1.

7.3.3 pH Titration of Holo-HemQ (SaCld)

Many heme proteins, including DaCld and most peroxidases, undergo spectral transitions in the UV/vis with pH.\textsuperscript{14,15} At the high and low pH extremes, loss of the heme chromophore is typically associated with dissociation of the heme from the protein as it denatures. Within the range of pH over which the protein is stable, an alkaline transition is often observed, corresponding to the conversion of a 5-coordinate, typically high spin heme to a 6-coordinate, low spin ferric-hydroxy heme species. The position of the pKₐ for this transition is related to the electronic structure of the distal pocket. For heme peroxidases such as HRP (distal His/Arg), the
<table>
<thead>
<tr>
<th>Protein &amp; Organism</th>
<th>Soret (nm)</th>
<th>Visible bands (nm)</th>
<th>pH</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cld (HemQ) Fe(III) S. aureus</td>
<td>405 (350 sh(^a))</td>
<td>510, 630</td>
<td>6.8</td>
<td>This work</td>
</tr>
<tr>
<td>Cld (HemQ) Fe(II) S. aureus</td>
<td>433</td>
<td>558</td>
<td>6.8</td>
<td></td>
</tr>
<tr>
<td>Cld Fe(III) D. aromatica</td>
<td>388</td>
<td>510, 644</td>
<td>6.8</td>
<td>7</td>
</tr>
<tr>
<td>Cld Fe(II) D. aromatica</td>
<td>434</td>
<td>555, 585</td>
<td>6.8</td>
<td></td>
</tr>
<tr>
<td>Cld R183Q Fe(III) D. aromatica</td>
<td>403</td>
<td>509, 644</td>
<td>6.8</td>
<td>18</td>
</tr>
<tr>
<td>DypA R. jostii RHA1</td>
<td>408</td>
<td>502, 632</td>
<td>7.5</td>
<td></td>
</tr>
<tr>
<td>DypB R. jostii RHA1</td>
<td>404 (360 sh(^a))</td>
<td>503, 634</td>
<td>7.5</td>
<td>66</td>
</tr>
<tr>
<td>DyPT. cucumeris Dec 1</td>
<td>406</td>
<td>510, 640</td>
<td>5.0</td>
<td>40</td>
</tr>
<tr>
<td>YfeX E. coli</td>
<td>404</td>
<td>510, 640</td>
<td>8.0</td>
<td>25</td>
</tr>
<tr>
<td>HemQ M. tuberculosis</td>
<td>403</td>
<td>510 (broad)</td>
<td>8.0</td>
<td>4</td>
</tr>
<tr>
<td>EfeB(YcdB) Fe(III) E. coli</td>
<td>406</td>
<td>485, 660</td>
<td>8.0</td>
<td>67</td>
</tr>
<tr>
<td>EfeB(YcdB)Fe(II) E. coli</td>
<td>432</td>
<td>558, 662</td>
<td>8.0</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)sh = shoulder

transition occurs with a pK\(_a\) typically above 11.\(^{16,17}\) For DaCld (distal Arg183), it shifts downward to pH 8.9.\(^{14}\) The R183Q mutant of DaCld, by contrast, exhibits no alkaline transitions, and instead remains in either a high spin 5- or 6-coordinate (presumably water bound) form at all values of pH.\(^{18}\) The holo-HemQ protein was titrated with KOH and HCl and its pH-dependent UV/vis changes examined (Figure 7.4). At pH values lower than 6, the chromophore diminishes and the spectrum becomes distorted, apparently due to protein denaturation. This is a relatively high threshold pH for acid instability; DaCld denatures with a midpoint pH of 4.8 and heme peroxidases can be stable at as low as pH 3-4. At alkaline pH, no reversible transitions are observed. Rather, the Soret peak and charge transfer bands diminish and broaden irreversibly, suggestive of the loss of cofactor binding and the appearance of free heme near pH 8.0. This alkaline behavior is very similar to that identified in DaCld R183Q, in which no alkaline transition occurs and loss of heme binding begins around pH 9.
Figure 7.4 UV-Visible pH Titration of Holo–HemQ (SaCld). The holo-enzyme (≈8 μM) was diluted in 0.1 M potassium phosphate buffer at pH 6.8, 25 °C and titrated with 1 M potassium hydroxide and 1 M hydrochloric acid to determine the changes in the Soret band as a function of pH. Spectra were collected for changes from pH 6.8-5.1 and pH 6.8-10.2 every 0.1 pH unit. Spectra are shown at pH 6.8 (-- -- --), 5.18 (-- --), 10.22 (- - - -), and select intermediates (lighter) for clarity. The inset shows the change in absorbance at 403 nm as a function of pH.

7.3.4 Circular Dichroism

Both the holo- and apo- forms of HemQ (SaCld) were characterized by their circular dichroism spectra shown in Figure 7.5. The apo-protein has a peak maximum at 227 nm that shifts to 234 nm with the addition of heme. Using the K2D and SOMCD web-based deconvolution software, the percentage of secondary structure for the apo- and holo-proteins were determined. The results are summarized in Table 7.2 with a comparison to WT D. aromatica chlorite dismutase. It shows that the apo-protein contains approximately 17% alpha-helix, 30% beta-sheet and 40-50% turns or random/disorder segments. The holo-protein contains slightly less alpha-helix (5-11%), more beta-sheet (37-38%) and roughly the same amount of turns and random segments at >50%. The similarity in structural elements suggests that no major structural changes occur, however, the apparent shift in spectral peak energies and the changes in alpha helix and beta sheet percentages implies subtle structural motion upon the binding of heme as observed for ligand binding in other proteins. Additionally, when compared to the percentages generated from the crystal structure of Ywfl, a proposed chlorite dismutase
like gene from *Bacillus stearothermophilus*, the percentages mostly disagree except for the β-sheets. This structure does not have heme bound, thus is considered apo, there is a detergent molecule H-bonded to the conserved proximal histidine (H171) that may be occupying the heme site. The detergent may be stabilizing the structure and removing a lot of the disordered elements observed in the CD results.

![Figure 7.5](image.png)

**Figure 7.5** Circular Dichroism of Holo- and Apo-HemQ (SaCld). CD spectra of the as purified apo-HemQ (SaCld) and the heme-bound holo-HemQ (SaCld) were measured from 190 to 250 nm with approximately 1 μM of assumed protein monomer in 0.1 M potassium phosphate buffer pH 6.8, 25 °C. Apo-HemQ (——) has a maximum at 227 nm and holo-HemQ (- - - -) at 234 nm.

**TABLE 7.2**

CIRCULAR DICHROISM ANALYSIS OF HOLO- AND APO-HEMQ (SACLD) WITH COMPARISON TO DACLD.

<table>
<thead>
<tr>
<th>Structure</th>
<th>apo-HemQ (SaCld)</th>
<th>holo-HemQ (SaCld)</th>
<th>WT DaCld</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>k2b3 SOMCD X-Ray</td>
<td>k2b3 SOMCD k2b3 SOMCD</td>
<td>SOMCD X-Ray</td>
</tr>
<tr>
<td>α – helix</td>
<td>%    %    %</td>
<td>%    %    %</td>
<td>%    %</td>
</tr>
<tr>
<td>β – sheet</td>
<td>29   32  29</td>
<td>37   38  15</td>
<td>5      27</td>
</tr>
<tr>
<td>Turn/Random</td>
<td>54   42  32</td>
<td>52   57  46</td>
<td>22     37</td>
</tr>
</tbody>
</table>

*a* Data taken from crystal structure of ywfI from *Bacillus Stearothermophilus* (PDB 1T0T) bound to Mg$^{2+}$ and detergent molecule 3,6,9,12,15,18-Hexaoxaicosane-1,20-Diol.

*b* Data taken from crystal structure of *Dechloromonas aromatica* chlorite dismutase (PDB 3Q08) bound to heme at pH 6.5.
7.3.5 Molecular Weight and Oligomerization State

The predicted molecular weight of HemQ (SaCld) from *S. aureus* based on amino acid sequence is 29.39 kDa and the molecular weight of the purified protein according to MALDI-TOF MS analysis is 29.30 kDa. To determine the native molecular weight and the oligomeric state of the enzyme, analytical gel filtration was carried out on the apo- and holo-proteins. Chromatograms of individual column runs of the protein standards and both the apo- and holo-proteins are overlayed in Figure 7.6. The inset shows the linear relationship between the log of the molecular weight and the elution time of each globular protein standard. Apo-HemQ elutes near the column’s void volume and gives an estimated molecular weight of 1200 kDa, which suggests a very high order multimer, a non-globular protein structure, or a highly disordered but soluble protein. By contrast, holo-HemQ has an apparent molecular weight of approximately 187 kDa, suggesting an oligomeric state of ~6. Analytical gel filtration carried out in a similar fashion for DaCld suggested a homotetramer in solution; however, crystallography showed the functional protein to be a pentamer.\(^7,20\) Other Cld-family proteins have shown a similar, relatively small level of deviation between gel filtration estimates and crystallographic measurements of oligomerization states.\(^21-23\) Aside from a subset of Clds having a short sequence (<200 amino acids)\(^24\), all Clds examined to date form stable pentamers in the solid state. The Cld from *Azospirae oryzae*, for example, appears to be a hexamer in solution/gel filtration studies but a pentamer according to crystallography and mass spectrometry.\(^22\) Three structures of non-perchlorate respiring bacterial Clds have been solved without heme bound and all are pentamers in the solid state. Nevertheless, the addition of heme to apo-HemQ appears to induce a change in the organization of the protein’s oligomeric state or a structural change to a more ordered state at the tertiary but not secondary level, as suggested by CD spectra for the holo- and apo-protein forms.
Figure 7.6 Gel Filtration Chromatography and Molecular Weight Determination of Apo- and Holo-HemQ (SaCld). A. Overlay of size exclusion chromatograms for apo-HemQ, holo-HemQ, and molecular weight standards run separately on S200-sephacryl GE Biosciences resin in 0.05 M potassium phosphate with 0.15 M NaCl at pH 7.0 and 4 °C at 0.5 mL/minute. The absorbance, expressed as mAU, was measured at 280 nm to detect total protein. The molecular weight standard peaks (- - - -) are from left to right thyroglobulin (670 kDa), α-globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (13.5 kDa). Approximately 10 mg/mL of Apo-HemQ (− − − −) eluted near the void volume and resulted in an estimated molecular weight of 1200 kDa and the holo-HemQ (        ) sample (~ 10 mg/mL with 1 equivalent heme added) eluted to give a molecular weight of 187 kDa. Inset contains a linear display of the size-exclusion chromatogram with the ratio of elution to void volume plotted as a function of the log of the molecular weight of protein standards (○) and fit to a line. Apo-HemQ (■) and holo-HemQ (●) are shown on where they lie on the curve.

7.3.6 Heme and Porphyrin binding to apo-HemQ (SaCld)

7.3.6.1 UV-Visible Titrations

The affinity of apo-HemQ (SaCld) for heme (dissolved hemin or Fe(III)-protoporphyrin IX/heme b) was determined by UV-Vis spectroscopic titration. To distinguish between free heme and the bound species, parallel titrations were performed using a cuvette containing no protein and the sample containing ~ 2 μM apo-HemQ. The difference of the two spectra was plotted as a function of the concentration of heme added, shown in Figure 7.7. The equation fit well to an equilibrium binding isotherm, giving a $K_d = 0.7 ± 0.2$ μM. This value is relatively high for a specific protein-cofactor interaction, which would more typically reside in the nanomolar range.
7.3.6.2 Fluorescence Quenching

To confirm the UV/vis results and to compare the affinities of apo-HemQ (SaCl) for heme and metal-free porphyrin, fluorescence quenching was used as a second observable for describing protein/ligand interactions. Apo-HemQ fluoresces from ∼300-350 nm when excited at 280 nm. This fluorescence emission is quenched with the addition of heme and was thus used as a method to determine binding affinities for both heme and protoporphyrin IX (Figure 7.8). From fluorescence quenching the heme/protein $K_d$ was determined to be $1.68 \pm 0.16 \mu M$, which is similar to that found using UV/Vis. The affinity of apo-HemQ (SaCl) for protoporphyrin IX is slightly lower at $2.2 \pm 0.1 \mu M$. Heme affinities for various proteins are organized in Table 7.3. HemQ (SaCl) from *M. tuberculosis* was shown to bind heme relatively poorly with a predicted $K_d$ of 30-40 µM. The CDE structural family members YfeX, EfeB, and a well-studied representative DyP have been shown to bind heme and PPIX with relatively high affinity, with $K_d$'s in the nanomolar range. Such values are expected for an enzyme/cofactor pair. These proteins have...
been predicted to have a variety of functions including oxidation of Fe(II) to Fe(III), removal of iron from exogenous heme or deferrochelatase activity, and general peroxidase activity against undefined substrates. Proteins involved in heme acquisition, trafficking, metabolism, or homeostasis, including IsdC and IsdG, tend to bind heme in the micromolar range.\textsuperscript{28,29} The former protein's function is to acquire heme extracellularly and transport it into the cell; the latter functions as a heme oxygenase. Similarly, the heme transport/chaperone protein PhuS (\textit{Pseudomonas aeruginosa}) binds heme with a $K_d$ of 0.18 µM.\textsuperscript{30} The relatively low affinity is thought to be important for its role in delivering heme to heme oxygenase for degradation. In short, proteins such as these that use heme in a non-cofactor role tend to bind heme with much lower (µM) affinity than their enzymatic counterparts. By virtue of its heme affinity, \textit{S. aureus} HemQ (SaCld) appears to fall into the non-enzymatic group. The similar affinity for iron-free protoporphyrin IX (PPIX) suggests that the protein is not selective for heme over porphyrin, and could in principle bind a heme precursor \textit{in vivo}.

7.3.7 Enzyme Activities and Transient Kinetics

Holo-HemQ was examined for chlorite dismutase, peroxidase, and catalase reactivity under steady-state conditions. No chlorite decomposition was detected up to 20 mM chlorite at 25 °C. The results of the peroxidase and catalase activity measurements are summarized and compared to other heme and CDE family proteins in Tables 7.4 and 7.5. At pH 6.8, the enzyme exhibits relatively weak peroxidase activity with the reductant 2,2'-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid or ABTS ($K_{M(H_2O_2)} = 3.4 \pm 0.4$ mM; $k_{cat} = 1.6 \pm 0.1$ s\(^{-1}\)heme\(^{-1}\)). Using a saturating amount of hydrogen peroxide (15 mM) and varying the concentration of ABTS, a $K_{M(ABTS)}$ of $30 \pm 8$ µM and a $k_{cat}$ of $1.8 \pm 0.2$ s\(^{-1}\) were measured. At higher concentrations of ABTS (>0.2 mM), steady diminution of the measured initial rate with increasing [ABTS] is observed, suggesting substrate inhibition. The measured $k_{cat}/K_{M(H_2O_2)}$ of $4.8 \times 10^2$ M\(^{-1}\)s\(^{-1}\) is roughly 4 orders of magnitude lower than in a canonical peroxidase ($4.2 \times 10^6$ M\(^{-1}\)s\(^{-1}\) for HRPC with H\(_2\)O\(_2\)/ABTS at pH 7.0).\textsuperscript{31} Using peracetic acid (PAA) as the oxidant a much lower $K_m$ of $247 \pm 106$ µM (PAA), higher
Figure 7.8 Equilibrium Binding of apo-HemQ (SaCl) for Heme and Protoporphyrin IX Measured by Fluorescence Quenching. A. Binding isotherm for the quenching of apo-HemQ (~5 μM) in 0.1 M potassium phosphate buffer pH 6.8 by heme. The percent of quenching observed is plotted as a function of heme added and fit to a quadratic binding equation taking into account the concentration of protein (see Materials and Methods). A $K_d = 1.68 \pm 0.16 \mu M$ was determined for heme. Inset shows quenching of apo-HemQ fluorescence by addition of heme (excitation $\lambda = 284$ nm; emission $\lambda = 300 – 400$ nm; 5 μM in 0.1 M potassium phosphate pH 6.8, 25°C). Heme was added from 1.4-111 μM and spectra were collected after several minutes of incubation to allow equilibrium to be reached. B. Binding isotherm for protoporphyrin IX (PPIX). The binding of PPIX was monitored in the same fashion as heme using a stock solution of PPIX (1 mM) dissolved in DMSO and diluted into 0.1 M potassium phosphate pH 6.8. A $K_d = 2.21 \pm 0.10 \mu M$ was determined.
<table>
<thead>
<tr>
<th>Protein &amp; Organism</th>
<th>Ligand (Method Used)</th>
<th>$K_d$ (nM)</th>
<th>pH</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HemQ (Cld) S. aureus</strong></td>
<td>Heme (UV/Vis)</td>
<td>720 (0.21)</td>
<td>6.8</td>
<td>This Work</td>
</tr>
<tr>
<td></td>
<td>Heme (Fluorescence)</td>
<td>1680 (160)</td>
<td>6.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Protoporphyrin IX (Fluorescence)</td>
<td>2210 (100)</td>
<td>6.8</td>
<td></td>
</tr>
<tr>
<td><strong>HemQ (Cld) M. tuberculosis</strong></td>
<td>Heme (UV/Vis)</td>
<td>$3 - 4 \times 10^4$</td>
<td>8.0</td>
<td>4</td>
</tr>
<tr>
<td><strong>YfeX E. coli</strong></td>
<td>Heme</td>
<td>3.9 (1.6)</td>
<td>8.0</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Protoporphyrin IX</td>
<td>4.8 (2.8)</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td><strong>EfeB E. coli</strong></td>
<td>Heme to Apo-EfeB (ITC)</td>
<td>29.6 (4.3)</td>
<td>8.0</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>Heme to PPIX-EfeB (ITC)</td>
<td>214 (50)</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td><strong>BtDyp B. theiotitaomicron</strong></td>
<td>Heme (ITC)</td>
<td>158</td>
<td>7.6</td>
<td>27</td>
</tr>
<tr>
<td><strong>TyrA S. oneidensis</strong></td>
<td>Heme (ITC)</td>
<td>50</td>
<td>7.6</td>
<td>27</td>
</tr>
<tr>
<td><strong>IsdC S. aureus</strong></td>
<td>Heme (UV/Vis)</td>
<td>340 (120)</td>
<td>6.0</td>
<td>28</td>
</tr>
<tr>
<td><strong>IsdG S. aureus</strong></td>
<td>Heme (UV/Vis)</td>
<td>5,000 (1,500)</td>
<td>7.5</td>
<td>29</td>
</tr>
<tr>
<td><strong>PhuS P. aeruginosa</strong></td>
<td>Heme (Fluorescence)</td>
<td>180 (10)</td>
<td>7.8</td>
<td>30</td>
</tr>
<tr>
<td><strong>Myoglobin Sperm whale</strong></td>
<td>Heme (UV, CD, Fluorescence)</td>
<td>$0.88 \times 10^{-4}$</td>
<td>7.0</td>
<td>68</td>
</tr>
</tbody>
</table>

$k_{cat}$ of $52 \pm 9$ s$^{-1}$heme$^{-1}$ were observed, with considerable loss of activity at higher [PAA], likely due to heme destruction. The resulting specificity constant for the acidic oxidant PAA is considerably higher than for $H_2O_2$: $k_{cat}/K_M(PAA) = 2.1 \times 10^5$ M$^{-1}$s$^{-1}$. This enhanced reactivity with PAA, also observed with $DaCl$, may be a result of the low $pK_a$ oxidant not requiring base-catalyzed removal of a proton for complex formation with the ferric heme. HemQ was also monitored as a catalase via the production of $O_2$ from $H_2O_2$ under near anaerobic conditions. Rates of oxygen production follow Michaelis-Menten-like behavior as a function of $H_2O_2$ concentration, yielding a $K_M(H_2O_2) = 2.6 \pm 0.3$ mM, $k_{cat} = 5.2 \pm 0.2$ s$^{-1}$, and $k_{cat}/K_M(H_2O_2) = 3.4 \times 10^3$ (pH 6.8, 25 °C). The low $K_M$ for the catalase reaction is similar to that measured for the peroxidase reaction and again consistent with the lack of an active site base. Biologically speaking, catalases as detoxification enzymes are typically characterized by exceptionally high values for $k_{cat}/K_M$ ($\geq 10^6$ M$^{-1}$ s$^{-1}$). The reactivity observed here for both peroxide-dependent reactions is beneath the threshold commonly measured for biological catalyses with $H_2O_2$. 
HemQ from *M. tuberculosis* and *B. subtilis* exhibited catalase activity (pH 8), with slightly higher turnover numbers than the *S. aureus* protein at pH 6.8 as expected at higher pH (4). Catalase activity was not detected for *DaCld* at any pH. The lack of chlorite decomposition and weak peroxidase and catalase activities of HemQ, in addition to its weak association with heme, suggest a possible non-enzymatic role for the protein *in vivo*.

TABLE 7.4

PEROXIDASE ACTIVITY OF HEMQ (SA CLD) AND RELEVANT HEME PROTEINS

<table>
<thead>
<tr>
<th>Enzyme/ Organism</th>
<th>Oxidant/ Reductant</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$K_m$(Oxidant) (mM)</th>
<th>$k_{cat}/K_m$, oxidant (M$^{-1}$s$^{-1}$)</th>
<th>pH</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>HemQ (Cld) <em>S. aureus</em></td>
<td>$\text{H}_2\text{O}_2$/ ABTS$^a$</td>
<td>97.2 ± 3.3</td>
<td>3.4 ± 0.4</td>
<td>4.8 ± 0.5 x 10$^2$</td>
<td>6.8</td>
<td>This work</td>
</tr>
<tr>
<td>HemQ (Cld) <em>S. aureus</em></td>
<td>$\text{PAA}^b$/ ABTS</td>
<td>3.1 ± 0.6 x 10$^3$</td>
<td>0.3 ± 0.1</td>
<td>2.1 ± 0.9 x 10$^5$</td>
<td>6.8</td>
<td></td>
</tr>
<tr>
<td>HemQ <em>B. subtilis</em></td>
<td>$\text{H}_2\text{O}_2$/ Pyrogallol</td>
<td>380$^c$</td>
<td>NA$^d$</td>
<td>NA</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td>HemQ <em>M. tuberculosis</em></td>
<td>$\text{H}_2\text{O}_2$/ Pyrogallol</td>
<td>350</td>
<td>NA</td>
<td>NA</td>
<td>8.0</td>
<td>4</td>
</tr>
<tr>
<td>HemQ <em>M. tuberculosis</em></td>
<td>$\text{H}_2\text{O}_2$/ Guaiacol</td>
<td>10</td>
<td>NA</td>
<td>NA</td>
<td>8.0</td>
<td></td>
</tr>
<tr>
<td>Cld <em>D. aromatica</em></td>
<td>$\text{H}_2\text{O}_2$/ Guaiacol</td>
<td>12</td>
<td>4.9</td>
<td>4.1 ± 1.5 x 10$^1$</td>
<td>6.0</td>
<td>Ch 7</td>
</tr>
<tr>
<td>Cld <em>D. aromatica</em></td>
<td>$\text{PAA}$/ Guaiacol</td>
<td>168</td>
<td>0.3</td>
<td>9.3 ± 1.8 x 10$^3$</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>DypA <em>R. jostii RHA1</em></td>
<td>$\text{H}_2\text{O}_2$/ ABTS</td>
<td>408</td>
<td>4.1 ± 0.2</td>
<td>16.8 ± 0.5 x 10$^3$</td>
<td>4.5</td>
<td>47</td>
</tr>
<tr>
<td>DypB <em>R. jostii RHA1</em></td>
<td>$\text{H}_2\text{O}_2$/ ABTS</td>
<td>846</td>
<td>7 ± 0.3 x 10$^2$</td>
<td>210 ± 8 x 10$^3$</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>DyP <em>T. cucumeris Dec 1</em></td>
<td>$\text{RB5}^e$/ ABTS</td>
<td>1.5 x 10$^4$</td>
<td>0.03</td>
<td>1.0 x 10$^7$</td>
<td>3.2</td>
<td>40</td>
</tr>
<tr>
<td>HRPC <em>H2O2/ ABTS</em></td>
<td>3.4 x 10$^3$</td>
<td>12 ± 1.3 x 10$^1$</td>
<td>4.2 x 10$^6$</td>
<td>7.0</td>
<td>31</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ABTS = 2,2'-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid]; $^b$PAA = peracetic acid; $^c$Reported as specific activity (min$^{-1}$); $^d$NA = Not available; $^e$RB5 = reactive blue 5
### TABLE 7.5

**CATALASE ACTIVITY OF HEMQ (SA CLD) AND RELEVANT HEME ENZYMES**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Organism</th>
<th>$k_{\text{cat}}$ ($\text{min}^{-1}$)</th>
<th>$K_{m(H2O2)}$ (mM)</th>
<th>$k_{\text{cat}}/K_m$ ($\text{M}^{-1}\text{s}^{-1}$)</th>
<th>pH</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>HemQ (Cld)</td>
<td>S. aureus</td>
<td>5.4 $\pm$ 0.2 x $10^3$</td>
<td>26.2 $\pm$ 2.1</td>
<td>3.4 $\pm$ 0.3 x $10^3$</td>
<td>6.8</td>
<td>This work</td>
</tr>
<tr>
<td>HemQ B. subtilis</td>
<td>1.4 x 10^4</td>
<td>NA\textsuperscript{a}</td>
<td>NA</td>
<td>NA</td>
<td>8.0</td>
<td>4</td>
</tr>
<tr>
<td>HemQ M. tuberculosis</td>
<td>1.7 x 10^4</td>
<td>NA\textsuperscript{a}</td>
<td>NA</td>
<td>NA</td>
<td>8.0</td>
<td>4</td>
</tr>
<tr>
<td>ClD D. aromatica</td>
<td>ND\textsuperscript{b}</td>
<td>ND\textsuperscript{b}</td>
<td>ND\textsuperscript{b}</td>
<td>ND\textsuperscript{b}</td>
<td>6.8</td>
<td>18</td>
</tr>
<tr>
<td>KatG M. tuberculosis</td>
<td>6.1 $\pm$ 0.9 x 10^5</td>
<td>5.18 $\pm$ 0.8</td>
<td>2.0 $\pm$ 12 x 10^5</td>
<td>7.0</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>Catalase Bovine Liver</td>
<td>1.3 x 10^7</td>
<td>93</td>
<td>2.3 x 10^8</td>
<td>7.0</td>
<td>32</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}NA = not available; \textsuperscript{b}ND = not detected

---

#### 7.3.8 Transient Reactions with Oxidants

Figures 7.9 A and B illustrate the spectral changes exhibited by ferric holo-HemQ (SaCld) following rapid mixing with PAA (10 eq) and $\text{H}_2\text{O}_2$ (100 eq) respectively at pH 6.8. No characteristic spectra for Fe-oxygen intermediates are detected, either due to low or no formation of such species or their rapid decay. Concentrations of oxidant ranging from 1-100 equivalents were assayed (data not shown) in order to detect potential reaction intermediates, but none were observed under any conditions. This is in stark contrast to prior measurements with the DaCld R183Q mutant, which is expected to have a similar distal environment to the SaHemQ. This mutant forms an apparent ferryl (Fe(IV)=O) coupled to a porphyrin radical cation (Compound I) at all values of pH tested. Even at very low (< 5) equivalents oxidant bleaching of the heme chromophore is observed, albeit as a very slow process ($t_{1/2} = \text{several hours}$). The reaction with < 5eq PAA results in a decrease in the Soret at 403 nm and the visible band at 510 nm with increased absorbance at ~700 nm, where the appearance of the new visible band suggests production of verdoheme. Following reaction with <5eq $\text{H}_2\text{O}_2$, the Soret shifts from 403 nm to 407 nm prior to bleaching. This suggests the possible formation of Compound III, a common intermediate in the heme degradation pathway. A subsequent increase in absorbance at about 700 nm is again suggestive of slow verdoheme formation. The same product is likewise
observed after H₂O₂-mediated heme lysis in catalases, peroxidases, heme oxygenase, and DaCl'd.

Figure 7.9 UV-Visible Spectrum of the Transient Reaction of holo-HemQ (SaCl'd) with Oxidants

A. Peracetic Acid. Ferric holo-HemQ (~8 μM final) was mixed with an approximately 10-fold excess of peracetic acid (80 μM final) in 0.1 M potassium phosphate pH 6.8 at 25 °C. Spectra shown were recorded between 0.75 ms (——) and 200 s (- - - -). A slow bleaching of the Soret band is observed with no major changes in the visible region (inset). B. H₂O₂. Ferric holo-HemQ (~6 μM final) was mixed with a several fold excess of hydrogen peroxide (1 mM final) in 0.1 M potassium phosphate pH 6.8 at 25 °C. Spectra shown were recorded between 0.75 ms (——) and 1500 s (- - - -). A slight red shift of the Soret band is observed, to ~405 nm, followed by a slow bleaching of the chromophore. No clear changes in the visible region occur (inset).

7.3.9 Heme Chromophore Titration with Oxidants

Because transient kinetics identified no reaction intermediates and showed heme destruction even after addition of less than 5eq of oxidant, we sought to titrate the chromophore with H₂O₂ and chlorite and thereby assess its redox stability. Prior titrations of this kind have shown heme proteins to have a range of stabilities in the presence of these oxidants absence of reductants. Measuring either residual activity after incubation with increasing oxidizing eq or optically titrating DaCl'd gave similar estimates of total turnovers prior to complete loss of heme cofactor. This showed ~17,000 turnovers with chlorite and 500 turnovers with H₂O₂. HRP is inactivated after ~ 600 turnovers in the presence of excess H₂O₂ and without reductant.
7.3.10 Ligand Binding

The equilibrium affinities of cyanide, imidazole and fluoride for holo-HemQ (SaCld) were measured at pH 6.8 via UV/vis spectroscopy (Table 7.6). The HemQ-CN complex Soret intensifies and shifts to 420 nm with a shoulder at 370 nm and a visible peak at 550 nm. An equilibrium-binding constant \( K_d = 8.9 \pm 0.9 \) μM was determined by fitting eq 9.11 to the change in absorbance (425 nm) versus [KCN] (Figure 7.10A). Holo-HemQ has a relatively high affinity for \( \text{CN}^- \) compared to the R183Q mutant of DaCld (Table 7.6) (18), even though structural and sequence comparisons suggest HemQ contains a glutamine residue at the distal pocket position corresponding to R183. This residue, R183, has been shown to interact with bound CO and to stabilize bound anions.\(^{14}\) The results here suggest that the strength of cyanide complexation is influenced by more than just the identity of the hydrophilic distal pocket residue. Similarly, the HemQ-imidazole complex Soret shifts to 414 nm with alpha and beta bands at 570 and 530 nm and loss of the charge-transfer at 630 nm. The \( K_d \) for imidazole at pH 6.8 is 1.0 ± 0.1 mM, Figure 7.10B. In DaCld WT and R183 mutants including R183Q, by contrast, the affinity for imidazole is always in the micromolar range, again suggesting a very different distal environment in HemQ. Finally, no spectral changes were observed with addition of fluoride up to 60 mM, suggesting the protein has a low affinity for negatively charged species, much like the DaCldR183Q or DaCldR183A and distinct from WT DaCld.

### TABLE 7.6

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Protein (Organism)</th>
<th>( K_d ) (μM)</th>
<th>pH</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanide</td>
<td>HemQ (Cld) (S. aureus)</td>
<td>8.94 ± 0.90</td>
<td>6.8</td>
<td>This work</td>
</tr>
<tr>
<td></td>
<td>Cld (D. aromatica)</td>
<td>0.01 ± 0.01</td>
<td>7.0</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Cld R183Q (D. aromatica)</td>
<td>85 ± 6</td>
<td>7.0</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Cld R183K (D. aromatica)</td>
<td>300 ± 20</td>
<td>7.0</td>
<td></td>
</tr>
<tr>
<td>Imidazole</td>
<td>HemQ (Cld) (S. aureus)</td>
<td>1.010 ± 110</td>
<td>6.8</td>
<td>This work</td>
</tr>
<tr>
<td></td>
<td>Cld (D. aromatica)</td>
<td>7.6 ± 0.2</td>
<td>7.0</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Cld R183Q (D. aromatica)</td>
<td>27 ± 1</td>
<td>7.0</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Cld R183K (D. aromatica)</td>
<td>18 ± 1</td>
<td>7.0</td>
<td></td>
</tr>
</tbody>
</table>
Figure 7.10 Equilibrium Ligand Binding to holo-HemQ (SaCld) by UV-Visible Spectroscopy. A. Cyanide. Approximately 8 μM of holo-HemQ was titrated from 0-500 μM potassium cyanide in 0.1 M potassium phosphate buffer pH 6.8 at 25 °C. Each concentration was mixed with protein individually and was allowed to incubate several hours to permit full equilibration before recording the spectra. The difference between the free and bound species was taken at 423 nm and plotted as a function of cyanide. The binding isotherm was fit using a quadratic equation taking the protein concentration into consideration (see Materials and Methods) and resulted in a $K_d = 8.9 \pm 0.9$ μM. B. Imidazole. Approximately 8 μM of holo-HemQ was titrated from 0-10 mM imidazole in 0.1 M potassium phosphate buffer pH 6.8 at 25 °C. Each concentration was mixed with protein individually and was allowed to incubate several hours to permit full equilibration before recording the spectra. The difference between the free and bound species was taken at 418 nm and plotted as a function of imidazole. The binding isotherm was fit using equation 7.1, taking the protein concentration into consideration (see Materials and Methods) and resulted in a $K_d = 1.0 \pm 0.1$ mM.

7.3.11 Characterization of hemQ (cld) Genetic Knockouts

To further understand the function of HemQ (SaCld), a knockout of the gene was created by gene replacement in *Staphylococcus aureus* (strain Newman). ΔhemQ *S. aureus* colonies grown on tryptic soy agar (TSA) are significantly smaller in size (< 1mm) and slightly discolored in comparison to WT colonies, which are white and larger in size (2-3 mm). This apparent small colony variant (SCV) phenotype is observed for other genetic knockouts including the well-studied ΔhemB strain. The hemB gene encodes the tetrapyrrole biosynthesis enzyme δ-aminolevulinic acid dehydratase (also called porphobilinogen synthase), which catalyzes a required step in the common pathways leading to all tetrapyrroles including the various hemes and chlorophyll. SCVs have defects in their respiratory electron transport pathways, often due to menadione or heme auxotrophy. Hence, the roles of aerobic and anaerobic respiratory pathways
were probed, and the effect of heme supplementation tested in each case.\textsuperscript{38-40} Since \( \Delta hemB \) strains have previously been characterized as SCVs and since \( hemB \) has a well-understood role in heme and chlorophyll biosynthesis, this strain was used for comparison to the \( hemQ \) knockout in addition to the WT strain.

### 7.3.12 Culture growth under aerobic conditions

Liquid cultures of small colony variants including both \( \Delta hemB \) and \( \Delta hemQ \) grow very slowly relative to WT (tryptic soy broth, 37°C, and 180 RPM). 5mL cultures of both mutant strains, for example, required \(~48\) hours to reach full saturation while similar WT cultures saturated overnight. When inoculated from a freshly saturated culture, a 50 mL culture of WT reached saturation (\( OD_{600} = 3.0 \)) after about 6h. The \( \Delta hemB \) and \( \Delta hemQ \) cultures, by contrast, grow slowly, experiencing an initial lag and saturating at a lower \( OD_{600} = 1.0 \) after 12 hours (Figure 7.11A). Addition of heme to both the \( \Delta hemB \) and \( \Delta hemQ \) cultures (5 μM) completely restored WT-like growth kinetics (Figure 7.11B).

### 7.3.13 Culture growth under fermentative conditions

The SCV phenotype has been attributed to defects in respiratory electron transport chains. We therefore examined the \( \Delta hemQ \) growth phenotype under fermentative and nitrate-respiring anaerobic conditions. Under anaerobic fermentative conditions with glucose as a fermentable carbon source and no added respiratory electron acceptors, WT cultures of \( S.\ aureus \) grew more slowly than under aerobic conditions, reaching saturation at about 8h and attaining somewhat lower final culture densities (\( OD_{600} = 1.0-1.5 \)) (Figure 7.12A). Under the same conditions, the \( \Delta hemB \) and \( \Delta hemQ \) cultures grew comparably to one another and slowly relative to WT. These cultures began to grow only after a lag of more than 8h and failed to reach saturation within 12h. Addition of heme to the mutant cultures, Figure 7.12B, restored both to WT-like growth kinetics and final culture densities.
Figure 7.11 Aerobic Growth Curves for WT, ΔhemB, and ΔhemQ Strains of *S. aureus* Newman in the Absence and Presence of Exogenous Heme. A. Absence of exogenous heme. The three strains of *S. aureus* Newman cells: wildtype (○), ΔhemB (△), and ΔhemQ (□) cells were grown and monitored at 37°C, 180 rpm, for 12 hours in 150 mL Erlenmeyer flasks. Cultures of ~50 mL tryptic soy broth (TSB) were inoculated (1:100) with saturated overnights and subsequent growth was monitored by taking OD$_{600}$ via spectrophotometer every two hours, each point is an average of duplicate or triplicate measurements. B. Presence of exogenous heme. The three strains of *S. aureus* Newman cells: wildtype (○), ΔhemB (△), and ΔhemQ (□) cells were grown and monitored at 37°C, 180 rpm, for 12 hours in 150 mL Erlenmeyer flasks. Cultures of ~50 mL tryptic soy broth (TSB) supplemented with ~5 μM hemin (from 1 mM stock in 0.1 M NaOH) were inoculated (1:100) with saturated overnights and subsequent growth was monitored by taking OD$_{600}$ via spectrophotometer every two hours, each point is an average of duplicate or triplicate measurements.
Figure 7.12 Anaerobic Fermentative Growth Curves for WT, ΔhemB, and ΔhemQ Strains of S. aureus Newman in the Absence and Presence of Exogenous Heme. A. Absence of exogenous heme. The three strains of S. aureus Newman cells wildtype (○), ΔhemB (△), and ΔhemQ (□) cells were grown and monitored at 37 °C, 180 rpm, for 12 hours in air-tight 50 mL falcon tubes. Cultures of ~50 mL tryptic soy broth (TSB) were inoculated (1:100) with saturated overnights and subsequent growth was monitored by taking OD$_{600}$ via spectrophotometer every two hours via syringe, each point is an average of duplicate or triplicate measurements. B. Presence of exogenous heme. The three strains of S. aureus Newman cells wildtype (○), ΔhemB (△), and ΔhemQ (□) cells were grown and monitored at 37 °C, 180 rpm, for 12 hours in air-tight 50 mL falcon tubes. Cultures of ~50 mL tryptic soy broth (TSB) supplemented with ~5 μM hemin (from sterile filtered 1 mM stock in 0.1 M NaOH) were inoculated (1:100) with saturated overnights and subsequent growth was monitored by taking OD$_{600}$ via spectrophotometer every two hours via syringe, each point is an average of duplicate or triplicate measurements.
7.3.14 Culture growth under anaerobic/nitrate-respiring conditions

Under anaerobic nitrate-respiring conditions with glucose as a carbon source and no other added respiratory electron acceptors, WT cultures of *S. aureus* again grew more slowly than under aerobic conditions, reaching saturation at about 10-12h and attaining somewhat lower final culture densities (OD$_{600}$ = 1.0-1.5) (Figure 7.13A). Under the same conditions, the ΔhemB and ΔhemQ cultures grew slowly relative to WT. While neither reached full saturation within 12 hours, it appears that the ΔhemQ cultures grew more rapidly after the 8h lag period reaching OD$_{600}$ ~ 0.5 after 12 hours. In the presence of exogenous heme both the ΔhemB and ΔhemQ begin to reach saturation after a 4 hour lag period, and both seem to outperform the wild-type strain that saturates after 4 hours at OD$_{600}$ = 1.0 as shown in Figure 7.13B.

7.3.15 Porphyrin Metabolite Profiles

WT, ΔhemQ, and ΔhemB cultures were quantitatively analyzed for heme and its precursors via exact-mass LCMS. Standard curves generated and used for metabolite determination are shown in Figure 7.14. The results show substantially different profiles for each strain grown under aerobic and anaerobic conditions (Figure 7.15). Under aerobic conditions, the most abundant porphyrin species in WT cells was iron protoporphyrin IX (heme b), with approximately 1.9 ± 0.4 nanomoles per gram of cell pellet. The ΔhemB cells contained significantly less heme b (0.73 ± 0.1 nanomoles/g cell pellet), though surprisingly, were not entirely heme-free in spite of the fact that the gene for an early and presumably non-redundant step in the heme biosynthetic pathway had been knocked out. The source of heme in this strain is unclear. The ΔhemQ cells contained less heme b on average than the WT (1.2 ± 0.4 nanomoles per gram), although the quantities measured for the two strains are the same within error. By contrast, cells grown under anaerobic fermentative conditions had significantly less heme b. Only WT *S. aureus* had detectable levels (0.27 ± 0.07 nanomoles per gram).

Among the heme precursors, only coproporphyrin was observed to accumulate under any condition tested. Under aerobic conditions, WT *S. aureus* produced a small amount of
Figure 7.13 Anaerobic Nitrate-Respiring Growth Curves for WT, Δ hemB, and Δ hemQ Strains of S. aureus Newman in the Absence and Presence of Exogenous Heme. A. Absence of exogenous heme. The three strains of S. aureus Newman cells wildtype (○), Δ hemB (△), and Δ hemQ (□) cells were grown and monitored at 37 °C, 180 rpm, for 12 hours in air-tight 50 mL falcon tubes. Cultures of ~50 mL tryptic soy broth (TSB) supplemented with 20 mM sodium nitrate were inoculated (1:100) with saturated overnights and subsequent growth was monitored by taking OD<sub>600</sub> via spectrophotometer every two hours via syringe, each point is an average of duplicate or triplicate measurements. B. Presence of exogenous heme. The three strains of S. aureus Newman cells wildtype (○), Δ hemB (△), and Δ hemQ (□) cells were grown and monitored at 37 °C, 180 rpm, for 12 hours in air-tight 50 mL falcon tubes. Cultures of ~50 mL tryptic soy broth (TSB) supplemented with 20 mM sodium nitrate and ~5 μM hemin (from sterile filtered 1 mM stock in 0.1 M NaOH) were inoculated (1:100) with saturated overnights and subsequent growth was monitored by taking OD<sub>600</sub> via spectrophotometer every two hours via syringe, each point is an average of duplicate or triplicate measurements.
coproporphyrin (0.45 ± 0.06 nanomoles per gram) and ΔhemB had none above the limits of detection. ΔhemQ cell pellets by contrast accumulated large amounts of coproporphyrin: 2.5 ± 0.5 nanomoles per gram, a greater than five-fold increase relative to WT. A similar trend though with smaller quantities of coproporphyrin is observed in anaerobic/fermentatively grown cultures. ΔhemQ and WT strains produced 0.5 ± 0.08 and 0.09 ± 0.01 nanomoles per gram of coproporphyrin respectively, again an approximate five-fold increase for the mutant.

The ΔhemQ cells make more heme compared to ΔhemB and show significant build-up of coproporphyrin. This phenotype of coproporphyrin build-up has been observed in other knockouts heme biosynthesis genes. Removal of hemY (protoporphyrinogen oxidase) from B. subtilis yields accumulation of coproporphyrin. It was proposed that the ΔhemY cells accumulated protoporphyrinogen, their supposed substrate, it ultimately inhibited or disregulated coproporphyrinogen oxidase, making it unable to perform catalysis. Additionally, hemH (Ferrochelatase) from B. subtilis yields accumulation of coproporphyrin. One explanation for this centered on the last three enzymes; coproporphyrinogen oxidase, protoporphyrinogen oxidase and ferrochelatase forming a functional complex where substrates and products could be channeled from active sites without being exposed to solvent. The absence of one enzyme could disrupt the entire process leaving coproporphyrin to accumulate. Porphyrin metabolites have been shown to be cytotoxic and complex formation, stable or transient, has been suggested and even observed as means of controlling their side reactivity.
Figure 7.14 LCMS Standard Curves of Uroporphyrin and Coproporphyrin Each curve was generated from a serial dilution of a stock solution of each porphyrin in acetonitrile. Freshly prepared solutions stored in amber vials (to reduce light absorption) were run immediately by the LCMS method described in the text. The peak area of three measurements was averaged and plotted as a function of porphyrin concentration. Linear fits to the data were used to determine concentrations of unknown samples.
Figure 7.15 LCMS Standard Curves of Protoporphyrin IX and Hemin. Each curve was generated from a serial dilution of a stock solution of each porphyrin in acetonitrile. Freshly prepared solutions stored in amber vials (to reduce light absorbption) were run immediately by the LCMS method described in the text. The peak area of three measurements was averaged and plotted as a function of porphyrin concentration. Linear fits to the data were used to determine concentrations of unknown samples.
Figure 7.16 LCMS Standard Curve of Internal Standard. The curve was generated from a serial dilution of a stock solution of each porphyrin in acetonitrile. Freshly prepared solutions stored in amber vials (to reduce light absorption) were run immediately by the LCMS method described in the text. The peak area of three measurements was averaged and plotted as a function of porphyrin concentration. Linear fits to the data were used to determine concentrations of unknown samples.

Figure 7.17 Porphyrin Abundance for Wild-Type, ΔhemQ, and ΔhemB S. aureus Newman Cells. Cell cultures (50 mL) were grown under standard conditions in tryptic soy broth (TSB) at 37 °C, 180 RPM, for approximately 16 hours before harvesting. Aerobic samples were grown in 150 mL Erlenmeyer flasks and anaerobic samples in filled 50 mL falcon tubes. Harvested cells were lysed and porphyrins extracted and analyzed by LCMS as described in the Materials and Methods sections. The bar graph represents the estimated levels of A. heme and B. coproporphyrin in nanomoles of metabolite per gram of wet cell pellet for wild type (white), ΔhemQ (dark grey) and ΔhemB (light grey) strains. Error bars are the result of standard deviations taken from analysis of three separate cell cultures.
7.4 Discussion

Heme proteins are central to many cellular processes. Many have consequently been extensively characterized. Genome sequencing and allied systems-biology techniques have, however, demonstrated the existence of families and superfamilies of microbial heme proteins that have yet to find firm biological functions. We undertook a combined genetic and biochemical study of a Cld or C-family protein from *S. aureus* in order to better define the probable biological roles of the family in this and possibly a broader cross section of organisms bearing the encoding gene.

Detailed investigations of the protein’s chemical and catalytic properties showed it to be strikingly unreactive toward a range of common hemoprotein substrates in either the steady or transient state. Though the protein family derives its name from the ability of its first characterized members to catalytically detoxify chlorite (\(\text{ClO}_2^- \rightarrow \text{O}_2 + \text{Cl}^-\)),\(^{7,21}\) we observed no chlorite-directed reactivity for the *Sa* Cld. Moreover, the protein is only modestly reactive with peroxide, with values of \(k_{\text{cat}}\) and \(k_{\text{cat}}/K_{\text{M(H}_2\text{O}_2)}\) for the peroxide-mediated oxidation of ABTS approximately 2- and 5-orders of magnitude below those measured for the structurally related DyP peroxidase at its pH optimum.\(^{41}\) Such suppressed steady state kinetic parameters have been previously measured for the Cld-catalyzed peroxidase reaction using proteins from several other species. Similarly, the catalase reaction has \(k_{\text{cat}}\) and \(k_{\text{cat}}/K_{\text{M(H}_2\text{O}_2)}\) values that are 4- and 3-orders of magnitude beneath those measured for bovine catalase.\(^{32}\) These parameters suggest that peroxide is not an avid substrate for *Sa* Cld. Unlike DyP enzymes and similar to *Da* Cld, it appears to lack an active site base in the distal pocket. Sequence comparisons predict that *Sa* Cld has a glutamine residue as the sole polar distal pocket residue, while DyPs have an aspartate as an active site base and *Da* Cld has an arginine.\(^1\) In keeping with such a relatively hydrophobic distal pocket, the *Sa* Cld protein has exceedingly low equilibrium affinity for fluoride and exhibits no “alkaline transition” to form a stable Fe(III)-OH species (from the ferric water complex) at basic pHs. In these ways, *Sa* Cld resembles the *Da* Cld R183Q mutant, in which the distal pocket is populated with a similar set of hydrophobic residues and the weakly polar glutamine.\(^{18}\) However, if the non-biological but much more strongly acidic oxidant peracetic acid \([\text{CH}_3(\text{CO})\text{CH}_2\text{COOH} \rightleftharpoons \text{CH}_3(\text{CO})\text{CH}_2\text{COO}^- + \text{H}^+]\),
pKₐ = 8.2) is used, the peroxidase reactions for SaCld and the WT/mutant DaCld are significantly more robust, both in terms of turnover and k_{cat}/K_M, again suggesting that the lack of an active site base limits the peroxide reactivity of each protein. At the same time, the lack of any observed Fe-oxygen intermediates using either oxidant is unique among these proteins to SaCld. The highly hydrophobic distal environment of this protein appears to be incapable of stabilizing the electron-deficient oxygen atom of the strongly polar ferryl.

Perhaps even more remarkably, SaCld appears to be only conditionally stable. While circular dichroism suggests its monomeric structure is intact whether or not heme is bound, analytical gel filtration indicates that the protein's tertiary structure changes dramatically in the apo form. Moreover, heme (or equivalently, protoporphyrin IX) binding was found to be relatively weak (micromolar K_D's) compared to what one would expect for a redox cofactor, and closer to the range observed for proteins that either transport or use heme as a substrate. At the same time, heme association with the protein was limited to a very small window of pH: between 6-8, compared with 4.5-10 for DaCld.

Taken in conjunction with the protein's observed minimal reactivity and predicted lack of key catalytic active site residues, these results strongly suggest a non-catalytic role for SaCld. The conditional stability of the protein – here shown to be dependent on both pH and heme content, with the latter additionally sensitive to [H₂O₂] – is reminiscent of the α-Proteobacterial protein Irr, and further points toward a sensor-regulator role. The Irr protein accumulates under iron limitation and represses heme biosynthesis via physically associating with ferrochelatase, the terminal enzyme in the biosynthetic pathway.⁴² When replete, iron in its heme form binds Irr and stimulates Irr's auto-degradation, allowing heme biosynthesis to proceed. When iron is insufficient, Irr binds protoporphyrin IX, dissociates from ferrochelatase, and then has a further role as a DNA-binding transcriptional regulator.⁴³,⁴⁴ Hence, Irr serves to regulate heme biosynthesis at the protein level, preventing the production of redox-active and toxic PPIX when iron is not present, and enforcing the coupling of PPIX to Fe(II) when it is. If SaCld were to serve at least a partially analogous regulatory role at the protein level, the data here suggest that it could potentially serve to sense heme-iron availability, redox status (via H₂O₂), pH, or any/all of
these, as the protein’s aggregation state responds sensitively to each of these conditions. The aggregated protein would presumably be earmarked for proteolytic degradation by the cell’s normal protein degradation machinery.

A role of this kind is consistent with prior work showing the absolute necessity of a heme-bound Cld protein for the final two steps of heme biosynthesis in two representative Gram-positive bacteria, see Figure 7.18 below. Dailey et al. showed that double knock outs in E. coli of the hemG/hemH genes, encoding protoporphyrinogen oxidase and ferrochelatase, are not complemented by functionally equivalent hemY/hemH gene pairs from two different Gram-positive bacteria (B. subtilis and M. tuberculosis). However, including the co-operonic cld gene with hemY/hemH allowed for full complementation. Similar results were observed in vitro, where all 3 proteins were observed to be necessary for successful processing of protoporphyrinogen, the substrate of HemG/Y, and Fe(II) to the final heme product. The primary difference between HemY and HemG is the O₂-dependence and independence of each, respectively. HemY from B. subtilis is similar to the eukaryotic enzyme, a soluble monomer of 51 kDa that requires an FAD-cofactor uses three molecules of O₂ to accept the six electrons from protoporphyrinogen and produce H₂O₂ and protoporphyrin. The eukaryotic enzyme exists as a dimer and is localized to the outer surface of the inner mitochondrial membrane, where the final steps of eukaryotic heme biosynthesis takes places. Both eukaryotic and prokaryotic enzymes are considered membrane associated. A gene for hemY is found in almost all gram-postives, including S. aureus, and is lacking in most gram-negatives. Protoporphyrinogen oxidase activity was recently discovered in gram-negatives from the gene hemG. HemG from E. coli is a smaller protein (22 kDa) compared to hemY that forms a homotetramer in solution and uses an FMN cofactor. The gram-negative enzyme was recently shown to couple the six electrons from protoporphyrinogen to electron transporters like ubiquinone that under aerobic conditions, transfers the electrons to O₂, under anaerobic conditions this enzyme transfers electrons to menaquinone or fumarate and nitrate reductases. This allows for heme biosynthesis under either respiratory condition and couples the anabolic reaction to ATP synthesis via electron transport. Membrane association in eukaryotes is understood to effect proper coupling of the two toxic entities, PPIX and Fe(II), and
could serve an analogous role in Gram-negative bacteria. It is not clear how Gram positive organisms enforce PPIX/Fe(II) coupling. The Cld gene product could play some role in this, as it could as well serve to regulate heme biosynthesis in response to cellular redox status (H₂O₂), pH, or Fe availability. Interestingly, immunoprecipitations carried out here and in the prior study failed to detect interactions between SaCld and any other protein, including HemG or HemH, though it is possible that weak or transient complexation, low protein copy number, the growth conditions used, or other factors could be responsible for the observed result.

The necessity of SaCld for heme biosynthesis is dramatically illustrated by phenotypic characterization of the cld knock out. The first indication that a defect involving heme might be present came from examination of Δcld colonies on solid media. These are <1 mm, as opposed to ~3 mm for WT S. aureus, suggesting that the knock outs are small colony variants (SCVs). The SCV phenotype has been observed in clinical S. aureus isolates for many decades, and has more recently been associated with a variety of defects in respiratory pathways. Some of the better characterized SCVs, in fact, include the ΔhemB knock out and other auxotrophs for either heme or menadione. The latter molecule is a primary electron carrier in the electron transport chain. Heme auxotrophy is analogously believed to exert its influence via the loss of cytochromes and terminal respiratory reductases that are coupled to ATP synthesis. We therefore investigated the Δcld growth phenotype in the liquid phase under aerobic, glucose-fermentation, and nitrate-respiring conditions, observing significant growth lags and attenuated final culture densities for the mutants in every case. Supplementation of the culture media with heme likewise restored growth to WT levels under each condition, indicating that the knock out exerts its influence on growth whether energy generation is via respiratory or fermentative means. Identical results were obtained for ΔhemB, where HemB catalyzes an early (the second) step in heme
Figure 7.18 Prokaryotic Heme Biosynthesis. The accepted reaction pathway for heme b biosynthesis from glutamyl-tRNA showing the intermediates formed in green lettering, the proper enzyme names in grey, and the genes associated with that function found in both gram-negative and gram-positive bacteria shown in blue.
biosynthesis. The restoration of growth via heme b supplementation suggests that the heme
defect in \( \Delta \text{cld} \) is global and that the influence of \( \text{cld} \) is not confined either to one particular heme b
derivative (e.g., heme a or o) or to respiratory pathways. Examination of the porphyrin profiles for
\( \Delta \text{cld} \), \( \Delta \text{hemB} \), and WT cultures grown under aerobic and fermentative conditions further indicated
substantial accrual of coproporphyrin (the spontaneously oxidized version of coproporphyrinogen)
specifically by \( \Delta \text{cld} \). This observation is consistent with a defect near the terminus of the heme
biosynthesis pathway, deletion mutants of protoporphyrinogen oxidase (\textit{hemY}) and
ferrochelatase (\textit{hemH}) in \textit{B. subtilis} were both shown to accumulate coproporphyrin.\textsuperscript{44,51} This
was taken as direct evidence of enzyme complex formation where the final three enzymes of the
biosynthetic pathway shuttled substrates and products between one another to avoid toxicity
associated with each. The removal of one entity would thus result in loss of any reactions in the
final three steps. Structural modeling studies have given any evidence that protein-protein
interactions are possible and a complex has been observed between ferrochelatase (\textit{hemH}) and
protoporphyrinogen IX (\textit{hemY}) from \textit{B. subtilis} using co-immunoprecipitation and immunogold-
labeling with electron microscopy.\textsuperscript{52} No complex formation has been observed with
coproprophyrinogen oxidase enzymes, though the toxicity of the substrate and product make it a
likely candidate.

In the commonly accepted pathway of heme biosynthesis the product of
uroporphyrinogen III decarboxylase, coproporphyrinogen III, is converted to protoporphyrinogen
IX as shown in Figure 7.18 above.\textsuperscript{53} The reactions releases 4 electrons and 2 molecules of
carbon dioxide and can be catalyzed by one of two different enzymes, an \( \text{O}_2 \)-dependent reaction
(Coproprophyrinogen III Oxidase, CPO) or and \( \text{O}_2 \)-independent reaction (Coproprophyrinogen
Dehydrogenase, CPDH). The \( \text{O}_2 \)-dependent enzyme is encoded by the \textit{hemF} gene in bacteria
and is only present in subclasses of \textit{Proteobacteria} and cyanobacteria.\textsuperscript{47} A \textit{hemF} gene has been
identified in several gram-negative species; including \textit{E. coli}, \textit{P. aerugenosa}, and \textit{V. cholerae}, but
has not been identified in gram-positives like \textit{S. aureus} and \textit{B. subtilis}. The enzyme from \textit{E. coli}
has been characterized and has no clear cofactor requirement, however it was found that metal
chelation treatment diminished activity that could only be restored with addition of manganese.\textsuperscript{54}
Four histidine residues and a highly conserved tryptophan were identified as catalytically important, and the histidines were posited to play a role in Mn coordination. The O$_2$-independent enzyme encoded by hemN is found in most prokaryotes, even those that also have a hemF gene. HemN has been identified in gram-positive species like S. aureus and B. subtilis. The HemN enzyme from E. coli has been characterized and has a requirement for S-adenosyl-L-methionine (SAM) and an additional cytoplasmic component to accept electrons; originally proposed to be NADP$^+$.\textsuperscript{55,56} E. coli hemN contains an O$_2$-sensitive iron-sulfur cluster and has three conserved cysteine residues critical for activity. Both hemN and hemF from Pseudomonas aeruginosa were found to be under transcriptional control by anaerobic and denitrification regulators, Anr and Dnr respectively and surprisingly both proteins were induced under anaerobic conditions.\textsuperscript{57} Mutation of both genes does not abolish growth suggesting another enzyme of redundant function. With the recent identification of the O$_2$-independent protoporphyrinogen IX oxidase, HemG from E. coli, coupling electron transfer from protoporphyrinogen to the respiratory chain via electron acceptors like ubiquinone, cytochromes, fumarate and nitrate reductases, it is possible that a similar coupling could take place with coproporphyrinogen.\textsuperscript{49} An alternate electron acceptor could bind to HemN, or another unidentified coproporphyrinogen dehydrogenase, and couple the 4 electrons to the respiratory chain for energy production.

It has also been observed in vitro that coproporphyrinogen III can be catalytically converted to its oxidized counterpart coproporphyrin III, releasing 6 electrons and without decarboxylation. This reaction is catalyzed by some bacterial protoporphyrinogen oxidases (PPO), the enzyme that typically catalyzes the O$_2$-dependent oxidation of protoporphyrinogen, using three equivalents of O$_2$ to accept the six electrons from protoporphyrinogen IX forming H$_2$O$_2$ as side product in the penultimate step of heme biosynthesis.\textsuperscript{58} PPO’s are encoded by the gene hemY in bacteria and is found in most gram-positive species including S. aureus and B. subtilis.\textsuperscript{37} HemY from B. subtilis was shown to perform the same O$_2$-dependent reaction on coproporphyrinogen III as it does canonically on protoporphyrinogen III.\textsuperscript{45,59} It is a peripheral membrane protein and requires an FAD cofactor for catalysis. The reaction occurred with a $k_{\text{cat}}/K_m$ that is 12-fold higher for coproporphyrinogen compared to protoporphyrinogen, bringing
into question the true substrate of the enzyme. Other PPO’s from eukaryotic organisms and some bacterial organisms, like *M. Xanthus*, do not have coproporphyrinogen oxidase activity and only have modest sequence homology (23%) with the *B. subtilis* enzyme. Coproporphyrinogen oxidation catalyzed by *B. subtilis* hemY was thought to be undesirable and it was proposed to be somehow masked *in vivo*, perhaps by an enzyme complex. A deletion mutant of hemY in *B. subtilis* did, however, accumulate coproporphyrin instead of the assumed protoporphyrinogen and it was also proposed that coproporphyrinogen oxidation by hemY may be desirable *in vivo*. Another unidentified enzyme was thought to be responsible for the decarboxylative conversion of oxidized coproporphyrin III to protoporphyrin IX, though no such activity has been identified. As mentioned above, HemG from *E. coli* has been shown to transfer electrons from protoporphyrinogen to oxygen via ubiquinone, cytochrom *bo*$_{3}$ and cytochrom *bd* oxidase or to fumarate and nitrate via menaquinione and fumarate or nitrate reductase. This was one of the first examples of anabolic heme biosynthesis being coupled to catabolic ATP generation from electron transport chains, and may be more prevalent in other systems. It seems possible that an analogous reaction for coproporphyrinogen III oxidation by hemY could occur with hemG, and to our knowledge that has not been tested. The question of whether this is desirable would again have to be asked, however one could proposed that cells starved for energy could couple the six electrons from coproporphyrinogen to electron transport for a much needed energy burst. The resultant coproporphyrin is potentially toxic; however a mechanism for its detoxification could be present as suggested elsewhere. Alternatively, another enzyme could exist that performs the transfer of electrons form coproporphyrinogen III to the electron transport chain producing coproporphyrin. Again the fate of coproporphyrin would have to be assessed.

Another pathway for the oxidation of coproporphyrinogen to coproporphyrin has been shown to occur, where peroxidases used reduced porphyrins, such as uroporphyrinogen III, coproporphyrinogen III, and protoporphyrinogen III, as electron sources for hydrogen peroxide reduction. This activity was observed when inhibitors of PPO activity, mostly herbicides, were used, causing reduced porphyrins to accumulate and become available to peroxidases. It was also shown *in vitro* that horseradish peroxidase rapidly oxidizes uroporphyrinogen III to
uroporphyrin III, 60 nanomoles uroporphyrin formed per minute per mg of protein, and likely uses H$_2$O$_2$. Peroxidase-dependent oxidation of coproporphyrinogen III and protoporphyrinogen IX was also observed by YfeX, a proposed DyP-type peroxidase from *E. coli*. This work showed the enzyme behaved as a typical DyP-type peroxidase and reacted with a variety of reductants like pyrogallol and cibacron blue. This supported the argument that YfeX is not a heme deferrochelatase and accumulation of porphyrin in cells overexpressing YfeX is caused by its peroxidase activity on endogenous porphyrinogens. It has not been determined if the oxidation of reduced porphyrins by peroxidases is a legitimate function in nature and may just be a result of the promiscuity of many peroxidases for reductants.

Associations between *SaCld* and cellular redox status are also clear from microarray and proteomic studies. Transcription of the *cld* from *Bacillus* species, *ywfI*, is induced in response to a number of conditions, including anaerobic nitrate and nitrite respiration as well as fermentation with glucose as the carbon source in the presence and absence of pyruvate. Similar up-regulation of YwfI protein production was observed in a proteomics study under all anaerobic conditions tested: fermentative, nitrate or nitrite-respiring. The *ywfI* gene appears to be controlled by the redox regulator *fnr* which is in turn controlled by the respiration-regulating *resDE*, as an *fnr* mutant exhibits reduced *ywfI* induction under fermentative condition. The *ywfI* is adjacent to and possibly co-regulated with a *pta* (phosphotransacetylase) gene responsible for the reversible phosphorylation of acetyl-CoA during fermentative acetate formation, suggesting an additional association of the gene with anaerobiosis.

A recent proteomic investigation of the peroxide stress response in *B. licheniformis* also indicated moderate upregulation in the production of YwfI. Cells stressed by the addition of 50 μM H$_2$O$_2$ undergo several changes at the DNA, protein, and metabolite level to accommodate and detoxify the reduced oxygen species. Under these conditions, cells induce the glyoxylate cycle, which involves bypassing part of the tricarboxylic acid cycle and use of acetate for energy production. Several of the heme biosynthesis genes were also detected at higher levels, primarily those involved in the initial steps leading to uroporphyrinogen. A massive induction (141.6 fold transcriptome change) of an unidentified homolog of ferrochelatase, BLi04115, was also noted.
Both of these increases are likely necessary to produce enough heme to accommodate the parallel induction of catalase and peroxidase enzymes like KatA.

These results suggest questions that will guide future work. The affinity and reactivity of SaCld with coproporphyrin or coproporphyrinogen needs to be examined. It is clear that redundant functions for the steps of heme biosynthesis is a common strategy to enable production under various conditions, S. aureus contains at least one known gene, hemN, necessary for coproporphyrinogen oxidation and SaCld could be another candidate, especially considering no O$_2$-dependent coproporphyrinogen oxidase has been found. Dailey et. al. did not see heme formation using HemQ, HemY and HemH in the presence of coproporphyrinogen and Fe(II), suggesting this is not true or another cofactor is required. $\Delta$cld cells make a substantial amount of heme, especially compared to the hemB knockout where a redundant function is not expected, suggesting biosynthesis is happening but not as efficiently as possible. It is not clear whether poor growth in $\Delta$cld was caused by insufficient heme or coproporphyrin toxicity, and testing growth conditions under limited light may help to determine the mechanism of deficiency. It has been suggested by others that iron starved cells also accumulate coproporphyrin suggesting an iron-dependent regulatory step of heme biosynthesis at this critical point. Protein-based regulation of chlorophyll biosynthesis is common in plants, and may be shared by some lower eukaryotes. Additionally, iron deficient cells may need a mechanism with which to remove excess coproporphyrin requiring a set of degradative enzymes to detoxify harmful products, both a legitimate hypotheses for the function of SaCld. It is also important to consider that regulation of cld gene in B. subtilis is induced under anaerobic fermentative and nitrate-respiring conditions and that heme biosynthesis likely undergoes multiple changes with these conditions. The absence of an O$_2$-independent protoporphyrinogen oxidase in many gram-positives is one potentially missing function. The identification of increased heme production by HemY and HemH in presence of HemQ observed by Dailey is very intriguing, in particular the enhanced activity of HemY with HemQ present. This suggests HemQ may be providing or is the electron acceptor necessary to complete the reaction, or is in some facilitating electron transport via O$_2$. Activity assays with the two enzymes together would be beneficial, especially monitoring O$_2$. 
consumption to determine the mechanism of activation. Supplementation with additional cofactors may also provide further evidence. It is expected that further biochemical characterization and microbiological studies, in addition to pursuit of enzyme complexes will help to answer these questions surrounding the cld genes. The function of annotate Cld’s in non-perchlorate respiring bacteria will give further insight into how dismutase activity came into existence and reveal possible moonlighting roles for the enzyme.

7.5 Summary and Conclusions

Chlorite dismutase from gram-positive *S. aureus* does not behave like Cld’s characterized from proteobacteria. It has no detectable dismutase activity, limited peroxidase activity, and below typical, but observable catalase activity. No Fe-oxygen intermediates are observed under transient reaction conditions. The purified protein contains little bound heme, but binds the cofactor with a $K_d = 720$ nm (UV/visible titration). Upon cofactor binding the protein’s quaternary structural changes and appears to be a hexamer in solution. The holo-enzyme is stable from pH 6-8 and no alkaline transition is observed. It binds the ligand cyanide at 8.9 μM and imidazole at 1 mM, suggesting different electronic heme pockets compared to bona fide chlorite dismutases. The deletion mutant of the cld gene in *S. aureus* forms a small colony variant, grows poorly in liquid media under aerobic and anaerobic conditions and the phenotype is rescued by the addition of excess heme. Cells lacking the cld gene show disrupted heme biosynthesis and accumulate coproporphyrin. Together these data show that SaCld is distinct from the *Dechloromonas aromatica* enzyme in many ways including their heme pocket architecture – SaCld also appears to be distinct from DaCldR183Q, though their distal pockets are very similar at the sequence level. These results suggest a role for SaCld in catalysis or regulation of the final steps of heme biosynthesis which may require the kind of challenging redox chemistry that DaCld can perform. Further studies will reveal if these gene products have some shared function and will reveal how amino acid changes manifested into dismutase activity.
7.6 Acknowledgements

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7.7 References


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CHAPTER 8

CONCLUSIONS AND SUMMARY OF STUDIES ON OXYGEN CONSUMING AND PRODUCING BIOMOLECULES AND THEIR SIGNIFICANCE TO REDOX BIOCHEMISTRY

Abstract – This chapter provides a concise overview of the key results presented in this thesis. It highlights the most critical observations that permitted classification and characterization of two redox enzyme families. A brief summary of the biological context of each family is given with a reiteration of the goals of the research and each study is kept in the context of redox biochemistry and how enzymes adapt to environmental pressures like the appearance of oxygen in the atmosphere.

8.1 Introduction

The work presented in this thesis covers a variety of different topics in microbial biochemistry and each section has unique aspects. A continuing theme emerges exemplifying how microbes and, in particular, the enzymes they use respond to environmental change. The key environmental change illustrated here is the increased level of oxygen in the atmosphere that occurred billions of years ago. Reactions with O₂ require sophisticated mechanisms that reduce the generation of radical oxygen species (ROS) and then novel reactions that can detoxify those species that do accumulate. These redox reactions require efficient catalytic mechanisms that take eons of evolution to perfect. This work probes the structures and functions of two redox enzyme families that have adapted to environmental pressures including increased O₂. Environmental pressure is biologically apparent as species adapt new ways of living; those adaptations or changes are less clear at the biochemical where enzyme reactions provide the foundation for survival. Not only has oxygen played a pivotal role, but also other environmental
changes like a need for acquiring iron or adapting to utilizing novel compounds like oxochlorates. The following summarizes the key findings on how microbes have manifested change at the biochemical level and how efficient redox reactions are achieved. Understanding these biochemical processes of oxygen activation, chlorite dismutation, gene function, and evolution help us understand how the world around us works and how to live in harmony with Nature.

8.2 Ornithine Monooxygenase

The first series of studies describes the fungal enzyme L-ornithine monooxygenase (OMO) and characterizes the enzyme’s catalytic mechanism for L-ornithine amine hydroxylation using molecular oxygen. Hydroxylation reactions epitomize how Nature utilizes molecular O\(_2\) not for respiration but for creating molecular biodiversity as well as detoxifying xenobiotic compounds. The reaction studied here is the first committed step in the biosynthesis of fusaricinone, a siderophore used by the pathogenic fungus Aspergillus fumigatus. This siderophore chelates iron with extremely high affinity both extracellularly for acquisition and intracellularly for iron storage and trafficking. The reaction catalyzed by OMO is essential for its biosynthesis and genetic knockouts of the OMO gene sidA have made the organisms avirulent. These findings suggest the enzyme could be a good target for antifungal compounds as this reaction and siderophore use is not common in eukaryotes.

OMO is a flavin-dependent monooxygenase that is found in many species of bacteria and fungi that produce siderophores. It is closely sequence-related to the yucca enzymes found in plants that are also believed to hydroxylate amines, but for hormone biosynthesis. There are numerous flavin-dependent enzymes that utilized molecular oxygen, as the cofactor is well suited for redox biochemistry. Two groups of characterized FAD-dependent monooxygenases have emerged each using a different mechanism to perform catalysis. These enzymes must achieve two catalytic goals: productively form a reactive oxygen intermediate without formation of hydrogen peroxide and hydroxylate the appropriate substrate. The means by which these goals are achieved are distinct in the two mechanistic groups. One group, classified as “bold”, reacts with NADPH and O\(_2\) in the absence of substrate and stabilizes the reactive intermediate until
hydroxylateable substrate is present. The other, “cautious”, only forms the reactive intermediate after binding to desired substrate. Prior to this work, the position of siderophore monooxygenases (SMOs) was unclear and, as we have shown, use a modified version of the “bold” strategy that allows for a high level of specificity.

The second chapter of the thesis reports on the biochemical properties of the heterologously expressed and purified enzyme as well as the steady-state kinetic mechanism. The approximately 40 kDa protein appears to exist as a dimer in solution and contains only about 40-50% bound flavin adenine dinucleotide (FAD). Two methods can be used to measure the steady-state reaction: the UV-visible trace of NADPH consumption and the measure of $O_2$ consumption on a Clark-type oxygen electrode. Both techniques were used to study the substrate relationships between NADPH, $O_2$, and L-ornithine. These data reveal that reversible relationships between NADPH and L-ornithine and between $O_2$ and L-ornithine occurred with saturation in the third substrate. A reversible connection was observed between NADPH and $O_2$, but only under sub-saturating L-ornithine concentrations, and when the third substrate was saturating and irreversible relationship was observed. This data suggested a mechanism where NADPH binds first followed by L-ornithine and $O_2$. To support this work product inhibition using NADP$^+$ and hydroxy-L-ornithine was applied. The patterns observed here supported the work in the forward reaction and the competitive inhibition from NADP$^+$ against NADPH demonstrated that is the last product to leave and likely remains bound throughout catalysis.

The subsequent chapter is a continuation of the mechanistic determination, but an application of transient-state and spectroscopic studies. The FAD intermediates used for the hydroxylation reaction have distinct UV-visible spectra that can be observed easily on a stopped-flow timescale for simple kinetic analysis. The transient-state work is in agreement with steady-state and support the proposed mechanism. The key results show that the rate of FAD reduction is independent of L-ornithine and the C4a-(hydro)peroxy flavin intermediate can form in the absence of the hydroxylateable substrate. In addition, the C4a-(hydro)peroxy flavin intermediate is only observed with reduction by NADPH, and not chemical reductants like dithionite, and is very stable in the presence of NADP$^+$. Both of these features are characteristic of “bold” catalytic
strategies where a reactive intermediate is formed in the absence of hydroxylateable substrate and NADP⁺ serves to protect the intermediate from solvent. However, in the presence of L-ornithine it was found that formation of C4a-(hydro)peroxy flavin is enhanced, but only by a modest amount that does not suggest tight regulation. Hydroxylation was shown to occur regardless of whether the intermediate formed in the absence or presence of L-ornithine. These data led us to propose a mechanism where NADPH binds and reduced the FAD, followed by either L-ornithine addition or O₂ activation, the data suggest L-ornithine is first depending on its availability; this is followed by hydroxylation and hydroxy-L-ornithine release, and last NADP⁺. This mechanism came as somewhat of a surprise as this enzyme and its family members are known to be highly specific for L-ornithine and this mechanism is generally used for non-specific enzymes. Further work was necessary to provide a better idea of how specificity is achieved.

In the first Appendix, A, a series of L-ornithine related compounds were used to study their effect on the steady and transient-state reactions of studying the L-ornithine monooxygenase. These results show a substantial rate enhancement for O₂ activation in the presence of the amino acid L-arginine. In addition, the presence of L-arginine increases the coupling ratio (the ratio NADPH consumed to hydroxylated product produced) with L-ornithine from 87% to 100%. These two key points, and the fact that L-lysine, one methylene unit longer than L-ornithine, does not have this effect but rather uncouples the reaction and destabilized the C4a-(hydro)peroxy flavin to product H₂O₂, support a role for L-arginine as a small molecule enzyme regulator. L-arginine is the metabolic precursor to L-ornithine and a mechanism of feed-forward rate enhancement may be in place in order for cells to rapidly produce siderophores to meet biological needs.

8.3 Chlorite Dismutase

The versatility of catalytic reactions performed by heme cofactors warrants it the title of Nature’s Swiss-army knife. Much like flavin, heme has played an essential role in an organism’s ability to respire and react with oxygen. Different types of heme-dependent enzymes appear to be flexible toward novel redox reactions. The relationship between heme and its protein
environment dictates a specific function and prokaryotes contain a variety of heme-dependent enzymes that take advantage of the cofactor's catalytic versatility. Many organisms often exist in toxic, dangerous, and erratic environments where they need metabolic adaption to survive. An example of this is found in a species of a-proteobacteria named Dechloromonas aromatica. This organism was discovered to have the ability to respire oxochlorates like perchlorate. These are primarily man-made molecules generated for a variety of uses from bleaching paper to rocket fuel. Electrons from perchlorate are transferred as the molecule is reduced to chlorate and then subsequently to the end product chlorite, while protons are transferred creating an electrochemical gradient. The heme-dependent enzyme chlorite dismutase (Cld) is responsible for detoxification of chlorite (ClO$_2^-$) to harmless chloride anion (Cl$^-$) and oxygen (O$_2$). Not considering photosystem II, Cld is the only enzyme system found in nature able to form an O-O bond. This reaction is the subject of numerous studies and an effort to determine the structural properties that dictate its unknown mechanism are presented in three of the following chapters. Studies on mutations of the critical distal pocket arginine residue are reported, in addition to an effort to understand the detailed chemical mechanism of the reaction with chlorite through alternate oxidants like hydrogen peroxide and peracetic acid. Perhaps one of the more interesting facets of chlorite dismutase is its appearance in many species with non-perchlorate respiring needs or machinery. Structure and sequence analysis suggest that these enzymes found in other bacterial families have non-dismutase function and an effort to determine that function of one of those gene products is presented in chapter 9.

In Appendix B background studies of chlorite dismutase Arg183 mutants is supplied as immediate reference and context for following chapters. It was known from previous structural and kinetic work that the distal pocket arginine (Arg183) played a critical role in dismutation. The pH-dependent change in enzyme activity and distal pocket polarity is the proposed result of a conformational change in this residue or possible deprotonation of the guanidinium group with a \( pK_a \) of 6.5. To test the hypothesis, three point mutants were made and studied for their reactivity and heme pocket characterization using spectroscopy, ligand binding, and reduction potentials. Arginine 183 mutated to lysine, glutamine and alanine were all generated and examined. All
three mutants retained chlorite dismutase activity, but with greatly diminished values of \( k_{\text{cat}}/K_m \). The mutants show no pH-dependent behavior in dismutase reactivity suggesting that Arg183 is directly or indirectly causing the previously observed pH-dependent shift. The anion binding affinity for each mutant is also greatly diminished suggesting a role for Arg183 in anion stabilization during the reaction. It is confirmed here that the pKa 6.5 observed is caused by the availability of a positive charge in the distal pocket. A conformation model is favored, however deprotonation of the guanidinium group is not ruled out.

The following chapter, 5, reports results of the reaction of WT DaCld with oxidants hydrogen peroxide (H\(_2\)O\(_2\)) and peracetic acid (PAA). This study was pursued for two reasons: the active site architecture of DaCld is very similar to canonical peroxidases that have well-characterized Fe(IV)-oxo intermediates that dictate its reactivity and can be studied by UV/visible techniques and secondly the reaction with chlorite (ClO\(_2^\cdot\)) cannot be measured adequately using UV/visible spectroscopy and stopped-flow techniques due to the vigorous (O\(_2\) bubbles) reaction and rapid rates. The active sites of both DaCld and most peroxidases have a proximal histidine with an H-bonding partner and distal arginine, however DaCld does not contain a distal base like histidine or aspartic acid found in many peroxidases and the repercussions of this may reveal interesting insight into the evolution of DaCld being closely related to DyP-type peroxidases. The study of the reaction with H\(_2\)O\(_2\) shows limited reactivity and requires many equivalents of oxidant to form what are likely heme degradation intermediates, as expected since DaCld contains no active site base capable of deprotonating H\(_2\)O\(_2\) (pK\(_a\) = 11). However, the reaction with PAA (pK\(_a\)=8.0) is much more revealing. At or below pH 6 the enzyme forms a clear Fe(IV)=O with a porphyrin based radical, also called Cpd I, that decays slowly to Cpd ES as the radical migrates toward an active Trp residue. At pH 8 and above the reaction proceeds more rapidly with PAA, however the first intermediate observed is Cpd ES suggesting the radical has already migrated. These results suggest the O-O bond of peracetic acid is cleaved by a heterolytic mechanism to form Cpd I opposed to a homolytic mechanism that would give Cpd II directly. This supports a model where the Cl-O bond in chlorite is broken heterolytically to form Cpd I and hypochlorite that can then rebound to react with Cpd I and form O\(_2\) and Cl\(^-\). These data also provide more
evidence for pH dependent behavior in the enzyme active site as Arg 183 loses its positive presence at high pH and radical migration occurs much more rapidly.

In the next chapter, DaCld and its R183Q mutant are studied for their ability to bind hydrogen cyanide. This ligand has acted as a surrogate for hydrogen peroxide binding in heme enzymes for many years and here provides further insight into the role of Arg 183 in the dismutation reaction of Cld. The ligation of cyanide by heme enzymes is accompanied by a large shift in the UV/Visible spectrum of the free enzyme that can be monitored spectroscopically, permitting the measurement of association and dissociation rates. A pH-dependent study of this kind revealed critical aspects of peroxidase and myoglobin mechanisms and here provides more evidence for an arginine conformational model. Rates of association increase for wild-type and R183Q as a function of pH, but are nearly 3 orders of magnitude greater for wild-type. This occurs with an apparent $pK_a$ of ~8.9 for both enzymes, corresponding to deprotonation of HCN in solution that occurs at pH 9.1 and preferential interaction with deprotonated cyanide. This agrees with previously measured equilibrium binding results. Interestingly, the rates of dissociation of the DaCldWT-CN complex increase with pH and the R183Q-CN complex dissociation rates remain constant with pH. Increasing pH appears to cause Arg183 to lose its distal charge either by conformational flexibility or deprotonation at pH 6.5. The association rates observed with DaCldWT rank among the fastest for any heme-dependent enzymes validating its preference for anionic ligands.

Chapter 7 of the thesis shifts from mechanistic studies of enzyme substrate complexes and chemical reactions toward a proteomic investigation set out to determine annotated chlorite dismutase’s function in *Staphylococcus aureus*. This organism has no known ability to respire perchlorate and sequence alignments reveal the absence of the critical arginine in the distal pocket both suggesting a non-dismutase role. The goal of this work is not only to correct the wrongly annotated gene but also to understand the relationship between this enzyme and the bona fide dismutase studied in prior chapters. A functional assignment will provide insight into how the dismutase enzyme evolved so rapidly, oxochlorates only being widespread in the past fifty years, as well present a potential antibiotic target as many pathogenic species have this gene
and eukaryotes do not. Heterologous expression and purification of Cld from *S. aureus* (*SaCld* or *HemQ*) was performed. The purified protein has minimal heme bound and relatively low affinity for the cofactor, compared to *DaCld* that purifies with nearly one heme equivalent bound per monomer. The approximately 29 kDa protein appears to undergo structural changes upon binding of heme as the oligomerization goes from what appears to be a soluble protein aggregate to a hexamer. The heme-bound protein has a similar UV/Visible spectrum as *DaCld*, however it has no detectable chlorite dismutase activity and limited peroxidase and catalase activities. Peroxidase activity has been observed for *DaCld*, yet no catalase activity has been detected showing the differences between these two enzymes. In addition the apo-protein appears to bind protoporphyrin IX (heme minus Fe) with similar affinity compared to heme. Microbiological studies show that the genetic knockout of the cld gene in *S. aureus* forms a small-colony variant on solid media, suggesting the inability to synthesize respiratory cofactors like heme or menadione. The position of the cld gene in the genome of some species of Actinobacteria reveal it may play a role in heme biosynthesis and prior work from another group showed that the enzyme was necessary to complete the final two steps of the biosynthetic pathway, though it plays no clear enzymatic role. Growth of ∆cld cells in liquid culture is poor, but can be complemented with addition of heme to the media. Immunoprecipitation experiments were performed using the FLAG-tagged protein as the bait, but no protein-binding partners were discovered. In addition *S. aureus* cells deficient in cld were examined for porphyrin metabolites using a liquid-chromatography mass spectrometry technique developed in our lab. This method allows for the quantification of heme biosynthesis intermediates and can give valuable information when heme metabolism is expected to be disrupted. The results from this show that coproporphyrin accumulates in the ∆cld cells and heme levels are about half of that observed for wild-type. Overall the results do not point to a specific single function for the *SaCld*, however several potential roles have been ruled out narrowing the possibilities. Potential roles are proposed for future work including catalytic or regulatory function in heme biosynthesis.

To better understand the how enzymes work it is important to consider them in an evolutionary context. All of the enzymes studied here have to some degree been effected by
environmental pressures that forced an organism to adapt. Those changes that occur at the biochemical level allow organisms to survive and perform the reactions and processes they desperately need. Comprehension of basic reactions and mechanism at the biochemical level permits us to understand how changes are adopted and how evolution manifests itself. As microbes continue to evolve in a changing environment better knowledge of their evolutionary strategies will be essential. The growing number of antibiotic resistance pathogenic organisms is creating a public health crisis and the multitude of pests that disrupt agriculture that could potentially become pesticide resistant may possibly cause a serious global food shortage. Research that seeks to comprehend biochemical reactions will facilitate resolution to serious social issues regarding both antibiotic and pesticide resistance, it will also drive production of new strategies and targets for future antimicrobials.
APPENDIX A

REGULATED O₂ ACTIVATION IN FLAVIN-DEPENDENT MONOOXYGENASES

Abstract – It was shown in the two previous chapters that L-ornithine monooxygenase from *A. fumigatus* (OMO) is mechanistically similar to its well-studied distant homologues from mammalian liver. The latter are highly promiscuous in their choice of substrates, while OMO is unusually specific. This presents a puzzle: how do OMO and other FMOs of the biosynthetic classes achieve such specificity in spite of their bold mechanism? The results in this chapter show substantial enhancement in the rate of O₂ activation in OMO in the presence of L-arginine, which acts as a small molecule regulator. Such protein-level regulation could help explain how this and related biosynthetic FMOs manage to couple O₂ activation and substrate hydroxylation to each other and to the appropriate cellular conditions. Given the essentiality of Fe to *A. fumigatus* and the avirulence of the OMO gene knockout, inhibitors of OMO are likely to be drug targets against this medically intractable pathogen.

A.1 Introduction

As previously described flavin-containing monooxygenases (FMOs) catalyze the oxygenation of diverse small organic molecules using O₂, NADPH, and the flavin adenine dinucleotide (FAD) cofactor.¹,² Genome sequencing has shown this family of enzymes to be widespread in bacteria, fungi, plants, and animals, though the mammalian FMOs are the only ones to have received significant prior attention.³⁻⁹ Like the cytochrome P450s, the mammalian liver FMOs are involved in the degradation of xenobiotics. FMOs from other sources, by contrast, appear to be primarily involved in the biosynthesis of diverse and important natural products. These include the siderophores upon which many pathogenic microorganisms depend for the
uptake and storage of nutritionally essential iron, and without which they can lose their virulence.

These FMOs have consequently been proposed as promising drug targets, particularly against the medically intractable fungal pathogen *Aspergillus fumigatus*, which depends on a single FMO (SidA, OMO) for all siderophore production and for virulence. FMOs likewise play key roles in the biosynthesis of the primary plant hormone, auxin. The spatial and temporal regulation of auxin biosynthesis is important for controlling plant growth, though how this is achieved at the transcript and protein levels is yet unclear.

Like all monooxygenases, the biosynthetic FMOs must avoid uncoupling O\textsubscript{2} activation from substrate hydroxylation that would result in the waste of reducing equivalents and release of toxic H\textsubscript{2}O\textsubscript{2}. At the same time, FMOs must direct the oxidizing power of activated O\textsubscript{2} toward the correct substrate. It was previously shown that OMO activates O\textsubscript{2} to generate an FAD-C4a-hydroperoxide intermediate (FAD-OOH) that acts as the hydroxylating agent (Figure A.1). As with the well characterized mammalian liver enzymes, this species is strikingly stable in the presence of bound NADP\textsuperscript{+} (t\textsubscript{1/2} = 33 min, 25 °C, pH 8). Similar results were obtained for the OMO from the bacterium *Pseudomonas aeruginosa*, PvdA. Such a "bold" catalytic strategy is consistent with the biodegradative role of the liver FMOs that hydroxylate literally hundreds of structurally variable nucleophiles including amines, thiols, and halogenated compounds. The FMOs are constitutively expressed; the FAD-OOH is formed and simply persists until intercepted by a substrate. Continuous delivery of substrates to the liver minimizes the steady release of H\textsubscript{2}O\textsubscript{2} from FAD-OOH that otherwise would occur.

In spite of sharing a bold mechanism, OMO exhibits high substrate specificity. As will be shown, hydroxylation of the L-Orn side chain amine by OMO is almost completely coupled to NADPH oxidation. D-Orn and 1,4-diaminobutane, isosteric with or smaller than L-Orn, have coupling ratios near 60%. L-Lys, though one methylene unit longer than L-Orn, is not hydroxylated, nor are any of a series of structurally related compounds used in this study. Similar stringency in substrate preference appears to be common in FMOs involved in siderophore biosynthesis. It is therefore a goal to understand how OMO achieves such specificity in spite of its bold mechanism. No crystal structure for a substrate-stringent FMO yet exists (see refs
Reactions catalyzed by OMO

24 and 25). The indole monooxygenase from the bacterium *Methylophaga* sp. strain SK1 has recently been structurally characterized, but its substrate promiscuity was described as comparable to that of the human liver FMO.\textsuperscript{24,25} Substrate preferences for the structurally characterized *Schizosaccharomyces pombe* FMO have not been described. Hence, it is not yet possible to explain substrate specificity or allosteric regulation (described herein) in simple structural terms. However, given the long-lived FAD-OOH observed in OMO, it potentially shares important features with the *Methylophaga* enzyme: an NADP\(^+\) -binding site in an interdomain cleft that protects the FAD-OOH from solvent, and an Asn residue that stabilizes the hydroperoxide via H-bonding.

Defining substrate interactions with OMO would appear to be critical for understanding specificity. It was previously shown that, unlike the liver FMOs, in which the FAD-OOH/substrate reaction is strictly second order, OMO forms a quasi-stable complex with its substrate prior to hydroxylation.\textsuperscript{17,26} This was indicated by competitive inhibition between L-Orn and the N\(^\epsilon\)-hydroxy-L-ornithine (L-Orn-N\(^\epsilon\)-OH) product in the steady state. Additionally, the plot of the rate constant for the reaction between FAD-OOH and L-Orn vs [L-Orn] \((k_{\text{FAD}}\text{, Figure A.3})\) saturates with an apparent \(K_d\) of 1.1 mM (pH 8), indicating binding. It was further demonstrated that an interaction between L–Orn and a second, reduced enzyme form (E-FADH\(^+\), Figure A.3) occurs. Specifically, the rate constant for FAD-OOH formation from the reduced enzyme and O\(_2\) \((k_{\text{FAD-OOH}})\) increases by about an order of magnitude in the presence of saturating L-Orn (apparent \(K_d = 310\) \(\mu M\)).
The observed L-Orn-dependent enhancement in $k_{\text{FAD-OOH}}$ led us to look for more potent regulators that could potentially stimulate formation of the reactive species in the presence of the appropriate substrate, or under biologically relevant conditions. These issues were addressed by comparing the effects of L-Orn and a series of related compounds on (1) the rate constant $k_{\text{FAD-OOH}}$ and (2) the rate constant describing the conversion of FAD-OOH to the oxidized FAD ($k_{\text{FAD}}$). The latter occurs along with hydroxylation of the substrate if present, or with the loss of $\text{H}_2\text{O}_2$. (Substrate hydroxylation involves the intermediacy of a short-lived FAD-OH species that strongly resembles FAD-OOH and which, for the present analysis, is not considered.)

A.2 Experimental Procedures

A.2.1 Standard procedures, chemicals, and equipment

All reagents were obtained from commercial sources and used without further purification unless otherwise stated. Substrate/substrate analogs in this study: L-Ornithine, L-Lysine, $N^6$-tert-butoxycarbonyl-(BOC)-L-lysine, L-Citrulline, $N^5$-Hydroxy-L-Ornithine, L-Arginine, 5-Aminopentanoate, L-Glutamine, L-Histidine, D-Ornithine, 1,4-Diaminobutane, L-2,4-diaminobutyric acid, n-octylamine. Spectrophotometric and steady-state kinetic measurements were made at 25 °C using a Varian Cary 50 spectrophotometer equipped with a Peltier-style thermostat as described in chapter 2. Transient kinetics were measured using a Hi-Tech Scientific DX-2 stopped flow spectrometer with diode array or photomultiplier tube detection, as described in chapter 3. All reactions were carried out in 100 mM Tris-$\text{SO}_4$ buffer at pH 8, unless otherwise noted. Protein concentrations were routinely measured by the Bradford assay. Ultrapure Milli-Q water was used in the preparation of all reagents. All data plots shown in the paper were produced using Kaleidagraph. Data fits by non-linear regression were produced by the same software for the steady-state data, and by Kinetic Studio for the stopped-flow data.
A.2.2 Overexpression and purification of OMO

See Chapter 2.2 Experimental Procedures for a full description of this method.

A.2.3 Steady-state kinetic studies

Steady-state kinetic studies were performed similar to those described in prior sections. Briefly, all reactions were initiated with enzyme in the absence of added FAD. Rates were referenced to the concentration of flavin-containing enzyme subunit, where the FAD titer was determined as described above. Reactions were monitored continuously via the oxidation of NADPH and all reactions were carried out at 25 °C. NADPH oxidation was monitored via UV/vis spectrophotometry (ε\text{NADPH, }340\text{nm} = 6220 \text{ cm}^{-1}\text{M}^{-1}). The buffer was added to a cuvette and the spectrophotometer was zeroed at 340 nm. NADPH was added and mixed by pipetting (final volume, 200 μL). The reaction was then initiated with enzyme. The slow NADPH oxidase activity of OMO was monitored for 10 seconds, and was then followed by the addition of a small volume of amino acid stock (see above for list of compounds). Rates were determined from linear regression fits to the initial linear portion of the curves. Initial rate measurements were performed in triplicate or greater. Average values are plotted with +/- one standard deviation as the error bar. Where noted, kinetic parameters were determined from plots of initial rate (v\text{i}) versus substrate concentration fit to the Michaelis-Menten equation, v\text{i}/[E] = k\text{cat}[S]/(K_m + [S]), where [E] = concentration of FAD-bound subunit. Unweighted curves were fit by nonlinear regression to 3 the full collection of data points, with three replicates at each [S]. Errors reported in values for k\text{cat} or K_m are sums of the least-square differences between the computed and actual curves. K_D values were determined using a non-linear least-squares fit of rate constants plotted as a function of ligand concentration ([L]) to the Langmuir isotherm analogous to the Michaelis Menton equation: y = k_{maximum}[L]/(K_D + [L]) + b, where b is the y-intercept of the given curve. The value for b was fixed at the appropriate measured, 0-added-compound values.
A.2.4 Hydroxylamine Detection

Analyses for various hydroxylamine products were carried out by oxidizing them (in KI/H$_2$SO$_4$) to their corresponding deaminated forms plus nitrite. The nitrite was then detected using a modified form of the Griess assay (see Figure 4.2 below), in which the nitrite is quantitatively reacted with sulfanilic acid to generate the corresponding diazonium salt. This reactive species then couples with α-naphthylamine to generate a strongly absorbing azo dye, which can be readily detected spectrophotometrically. Quantification of the nitrite was achieved with the use of a standard curve. Reactions were assayed for hydroxylamine as follows: In a 1.5 mL micro-centrifuge tube, 90 μL of the reaction to be analyzed was mixed with 10 μL of 0.3 M sulfuric acid. 100 μL 1% (w/v) sulfanilic acid in 30% (v/v) acetic acid was added followed by 40 μL 1.3% (w/v) potassium iodide solution in glacial acetic acid. The sample was vortexed to mix evenly and allowed to incubate for 5-7 minutes at room temperature. I$_2$ forming in the solution was cleared with 40 μL 0.1 M sodium thiosulfate solution followed by the addition of 40 μL 0.6% (w/v) α-naphthylamine solution in 30% (v/v) acetic acid. The solution was allowed to incubate at room temperature for 15 min. The absorbance of each solution was measured at 529 nm. A standard curve using hydroxylamine (from 0-160 μM NH$_2$OH) was constructed and used to determine the concentration of hydroxylated product.

![Figure A.2 Assay for determining hydroxylamine-derived nitrite.](image-url)

A.2.5 Rapid reaction studies

Transient kinetic reactions of reduction of the enzyme by NADPH, oxidation by O$_2$, and conversion of the FAD-OOH intermediate to the fully oxidized FAD-containing enzyme were performed similar to those described in prior sections with a few modifications. They were done...
using stopped-flow spectrophotometric techniques at 25 °C in single-mixing mode, in the presence/absence of substrate analogs. For all experiments, the enzyme was placed inside a gas-tight tonometer and made anaerobic via repeated cycles of evacuation and purging with purified and hydrated nitrogen or argon gas. Solutions before mixing consisted of 20-40 μM FAD-containing enzyme subunit. These were mixed with an equal volume of reactant solution (containing substrate or a substrate analog) prepared in the same buffer from a second syringe. For anaerobic experiments, the solution in the second syringe was equilibrated with Ar or N₂ via bubbling. A diode array detector was used for most experiments, with wavelengths monitored over the complete (300-700 nm) visible range.

**A.2.6 Reactions of reduced OMO with O₂**

All measurements were made using diode array detection. The single-mixing mode was used to monitor reduced enzyme after mixing with buffer that contained defined concentrations of either O₂ and substrate analog alone or O₂ and L-Orn in combination. ⁴

**A.2.7 Reactions with NADPH**

The anaerobic reduction of OMO (10 μM enzyme plus 100 μM NADPH, final concentrations) was monitored at 450 nm via diode array detection. Additional measurements were made with 200 μM NADPH plus either 200 μM NADP⁺ or 5 mM (saturating) L-Orn, in order to determine their effect on the reduction rate. For subsequent experiments, reduced enzyme solutions were prepared anaerobically in a tonometer by titrimetric addition of 1 eq of NADPH via a titration syringe.
A.3 Results and Analysis

A.3.1 Effects of L-Orn and Related Compounds on Rate of Formation of the C4a-hydroperoxide (FAD-OOH) Intermediate

Formation of FAD-OOH from the NADPH-reduced enzyme was monitored via stopped flow UV/vis spectroscopy at 370 nm (Figure A.3A), following mixing of the reduced enzyme under Ar with an air saturated solution of buffer containing 5 mM of one of the following: 1,4-diaminobutane (DAB), 5-aminopentanoic acid, L-2,4-diaminobutyric acid, L-citrulline (L-Citr), L-lysine, N\(^\text{ε}\)-tert butoxycarbonyl-(BOC)-L-lysine, L-glutamine, L-histidine, n-octylamine, L-arginine, D-ornithine, L-Orn-N\(^\text{ε}\)-OH, or L-ornithine. Of these, only L-Orn, L-Lys, L-Citr, L-Arg, and DAB had an appreciable effect on the observed rate constant for FAD-OOH formation (Tables A.1 and Appendix D Table A.2).

For L-Orn, L-Lys, L-Citr, and L-Arg (Figure A.6), the concentration dependence of \(k_{\text{obs}}\) was determined, and from this, \(k_{\text{FAD-OOH}}\) was extrapolated (Figure A.3B, Table A.1). The rate constant increases ca. 14-fold in the presence of saturating L-Orn, consistent with prior work in chapter 3.\(^{17}\) In the presence of either L-Lys or L-Citr, the rate constants increased about half as much. L-Arg, by contrast, has a much more dramatic effect, causing an increase in \(k_{\text{FAD-OOH}}\) of greater than 2 orders of magnitude. This demonstrates that L-Arg is a fairly strong effector of \(O_2\) activation.
Figure A.3 (A) Reaction of reduced OMO with O₂ to form FAD-OOH in the presence of saturating (5 mM) l-Arg, as monitored by stopped flow UV/vis spectroscopy over time (measurement times listed). The initial spectrum is shown in red, and the final in blue. Spectra measured in between are shown in gray. See ref 17 for analogous data for l-Orn and Figure A.4 (l-Lys). (B) Concentration dependence of $k_{obs}$ for this conversion (fit to a single exponential at 370 nm; see Figure A.5) in the presence of l-Orn, l-Lys, l-Arg, and l-Citr. These have measured apparent $K_d$ values of 310 ± 40 μM, 130 ± 30 μM, 620 ± 70 μM, and 4.4 ± 0.9 mM respectively. Associated values for $k_{FAD-OOH}$ extrapolated from these data at saturating concentrations for each compound are listed in Table A.1.
Figure A.4 FAD-OOH formation in the presence of L-Lys. The reaction of reduced OMO (red spectrum) with $O_2$ to form FAD-OOH (blue spectrum) in the presence of 5 mM L-Lys was monitored by stopped flow UV/vis spectroscopy. Spectra monitored at the intermediate times listed on the plot are shown in grey. See Figure A.3A above for analogous data measured in the presence of L-Arg. Note the much longer time scale required to achieve maximal production of the FAD-OOH intermediate in the presence of L-Lys.
Figure A.5 Representative kinetic traces at 370 nm (symbols) showing formation of FAD-OOH in the presence of A) buffer, B) L-Orn and L-Lys, and C) L-Arg and D) L-Citr with data fit (lines) to a single exponential to obtain the rate constant $k_{\text{obs}}$, and $k_{\text{FAD-OOH}}$ is taken as the maximal value extrapolated as each compound reaches saturation. The data from A-D are shown on a logarithmic time scale in plot E) Each reaction contains 15-20 μM enzyme reduced with 1 eq NADPH, which is subsequently mixed with air-saturated buffer containing 5 mM of the given compound. The different time scales for FAD-OOH formation in the presence of the given compounds are illustrated by these traces.
TABLE A.1
RATE CONSTANTS, APPARENT RATE CONSTANTS, AND COUPLING RATIOS MEASURED IN THE PRESENCE OF THE GIVEN COMPOUNDS

<table>
<thead>
<tr>
<th>compound</th>
<th>$k_{\text{red}}$ (s$^{-1}$)</th>
<th>$k_{\text{fadooh}}$ (s$^{-1}$)</th>
<th>$k_{\text{fad}}$ (s$^{-1}$)</th>
<th>compd-OH:NAPH$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.21 (0.03)</td>
<td>1.28 (0.13)</td>
<td>0.041 (0.00)</td>
<td>0</td>
</tr>
<tr>
<td>L-Orn</td>
<td>1.46 (0.16)</td>
<td>19.8 (0.5)</td>
<td>2.58 (0.05)</td>
<td>0.87 (0.06)</td>
</tr>
<tr>
<td>D-Orn</td>
<td>ND$^d$</td>
<td>1.78 (0.01)</td>
<td>0.142 (0.004)</td>
<td>0.63 (0.03)</td>
</tr>
<tr>
<td>DAB</td>
<td>ND</td>
<td>4.74 (0.16)</td>
<td>0.151 (0.001)</td>
<td>0.59 (0.02)</td>
</tr>
<tr>
<td>L-Lys</td>
<td>2.26 (0.05)</td>
<td>9.0 (0.3)</td>
<td>1.98 (0.04)</td>
<td>0.03 (0.01)</td>
</tr>
<tr>
<td>L-Arg</td>
<td>20.3 (0.8)</td>
<td>144 (4.5)</td>
<td>0.038 (0.00)</td>
<td>0.06 (0.03)</td>
</tr>
<tr>
<td>L-Citr</td>
<td>13.3 (0.7)</td>
<td>16.9 (1.0)</td>
<td>0.157 (0.002)</td>
<td>0.10 (0.001)</td>
</tr>
<tr>
<td>L-Orn-N$^\epsilon$OH</td>
<td>ND</td>
<td>1.13 (0.02)</td>
<td>0.04 (0.00)</td>
<td>ND</td>
</tr>
<tr>
<td>L-Orn + L-Orn-N$^\epsilon$OH</td>
<td>ND</td>
<td>13.5 (0.4)</td>
<td>0.34 (0.01)</td>
<td>ND</td>
</tr>
<tr>
<td>L-Orn + L-Lys</td>
<td>1.74 (0.12)</td>
<td>15.6 (0.3)</td>
<td>0.157 (0.002)</td>
<td>0.89 (0.06)</td>
</tr>
<tr>
<td>L-Orn + L-Arg</td>
<td>13.4 (0.66)</td>
<td>48.5 (1.1)</td>
<td>1.75 (0.07)</td>
<td>1.03 (0.07)</td>
</tr>
</tbody>
</table>

$^a$Concentration dependencies were measured from rate constants when 5 mM of the added compound was observed to have a >5-fold effect on the step of interest relative to no-compound added controls (see Figures A.3B and A.7B). Extrapolated rate constants obtained from such data are underlined. Otherwise, apparent rate constants at 5 mM each of the given compounds are reported. The average of three measurements is given with the standard deviation as the error in parentheses. All measurements were made at 25 °C in 100 mM Tris-SO$_4$ buffer, pH 8.

$^b$Several additional compounds not shown here were examined for their effects on the reaction kinetics. See text.

$^c$Coupling ratios are defined as the amount of hydroxylated product formed measured by the method described in 4.2.4 divided by the amount of NADPH consumed measured by UV/Vis. Ratios were measured in the presence of limiting amounts of NADPH.

$^d$ND = not determined.
TABLE A.2
FITTED RATE CONSTANTS FOR FAD-OOH FORMATION ($k_{\text{FAD-OOH}}$) AND CONVERSION TO OXIDIZED FAD ($k_{\text{FAD}}$) AND HYDROXYLAMINE PRODUCT DETECTION FOR VARIOUS COMPOUNDS IN THE ABSENCE OR PRESENCE OF 5 MM L-ORN. 

<table>
<thead>
<tr>
<th>Compound</th>
<th>$k_{\text{FAD-OOH}}$</th>
<th>$k_{\text{FAD-OOH}}$</th>
<th>$k_{\text{FAD}}$</th>
<th>$k_{\text{FAD}}$</th>
<th>Compound-</th>
<th>OH:NADPH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(s$^{-1}$)</td>
<td>(s$^{-1}$)</td>
<td>(s$^{-1}$)</td>
<td>(s$^{-1}$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>1.28 ± 0.13</td>
<td>ND</td>
<td>0.041 ± 0.0</td>
<td>0.038 ± 0.0001</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0.5 mM L-Orn</td>
<td>12.3 ± 0.90</td>
<td>ND</td>
<td>0.72 ± 0.1</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>5 mM L-Orn</td>
<td>18.8 ± 1.50</td>
<td>ND</td>
<td>1.98 ± 0.01</td>
<td>ND</td>
<td>0.87 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>0.5 mM L-Lys</td>
<td>7.5 ± 0.60</td>
<td>20.2 ± 1.0</td>
<td>0.87 ± 0.04</td>
<td>0.46 ± 0.04</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>5 mM L-Lys</td>
<td>9.0 ± 0.10</td>
<td>15.6 ± 0.3</td>
<td>1.61 ± 0.03</td>
<td>0.16 ± 0.00</td>
<td>0.03 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>0.5 mM L-Arg</td>
<td>52.0 ± 0.5</td>
<td>23.1 ± 0.9</td>
<td>0.03 ± 0.00</td>
<td>2.28 ± 0.02</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>5 mM L-Arg</td>
<td>118.9 ± 4.1</td>
<td>48.5 ± 1.1</td>
<td>0.038 ± 0.0</td>
<td>1.75 ± 0.07</td>
<td>0.06 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>0.5 mM L-Citr</td>
<td>2.44 ± 0.05</td>
<td>ND</td>
<td>0.04 ± 0.00</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>4 mM L-Citr</td>
<td>7.4 ± 0.2</td>
<td>ND</td>
<td>0.048 ± 0.0</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>0.5 mM 1,4-Diaminobutane</td>
<td>1.83 ± 0.03</td>
<td>15.2 ± 0.3</td>
<td>0.051 ± 0.0</td>
<td>2.14 ± 0.02</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>5 mM 1,4-Diaminobutane</td>
<td>4.74 ± 0.27</td>
<td>14.8 ± 0.5</td>
<td>0.151 ± 0.00</td>
<td>2.21 ± 0.01</td>
<td>0.59 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>0.5 mM 5-Aminopentanoate</td>
<td>1.52 ± 0.06</td>
<td>17.4 ± 0.2</td>
<td>0.042 ± 0.0</td>
<td>1.97 ± 0.07</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>5 mM 5-Aminopentanoate</td>
<td>1.75 ± 0.09</td>
<td>18.3 ± 0.3</td>
<td>0.057 ± 0.0</td>
<td>1.97 ± 0.01</td>
<td>0.1 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>0.5 mM D-Orn</td>
<td>1.51 ± 0.06</td>
<td>20 ± 1</td>
<td>0.057 ± 0.0</td>
<td>2.17 ± 0.05</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>5 mM D-Orn</td>
<td>1.78 ± 0.01</td>
<td>19.1 ± 0.7</td>
<td>0.142 ± 0.0</td>
<td>2.16 ± 0.05</td>
<td>0.63 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>0.5 mM L-2,4-Diaminobutyrate</td>
<td>1.48 ± 0.07</td>
<td>19.8 ± 0.8</td>
<td>0.035 ± 0.0</td>
<td>2.32 ± 0.07</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>5 mM L-2,4-Diaminobutyrate</td>
<td>1.19 ± 0.1</td>
<td>18.4 ± 0.2</td>
<td>0.021 ± 0.0</td>
<td>2.08 ± 0.03</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>0.5 mM N$^6$-Boc-L-Lys</td>
<td>1.85 ± 0.03</td>
<td>18.5 ± 0.6</td>
<td>0.06 ± 0.0</td>
<td>2.44 ± 0.18</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>5 mM N$^6$-Boc-L-Lys</td>
<td>3.94 ± 0.17</td>
<td>18.8 ± 0.1</td>
<td>0.23 ± 0.00</td>
<td>0.84 ± 0.04</td>
<td>0.02 ± 0.005</td>
<td></td>
</tr>
<tr>
<td>0.5 mM L-Glutamine</td>
<td>1.54 ± 0.04</td>
<td>18.5 ± 0.2</td>
<td>0.04 ± 0.0</td>
<td>2.40 ± 0.13</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>5 mM L-Glutamine</td>
<td>1.54 ± 0.07</td>
<td>17.0 ± 0.2</td>
<td>0.052 ± 0.0</td>
<td>2.76 ± 0.15</td>
<td>0.00 ± 0.003</td>
<td></td>
</tr>
<tr>
<td>0.5 mM L-Histidine</td>
<td>1.53 ± 0.01</td>
<td>17.6 ± 0.1</td>
<td>0.047 ± 0.0</td>
<td>2.29 ± 0.06</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>5 mM L-Histidine</td>
<td>1.27 ± 0.10</td>
<td>17.2 ± 0.4</td>
<td>0.081 ± 0.0</td>
<td>2.16 ± 0.08</td>
<td>0.00 ± 0.0004</td>
<td></td>
</tr>
<tr>
<td>0.5 mM n-Octylamine</td>
<td>1.61 ± 0.07</td>
<td>18.2 ± 0.4</td>
<td>0.040 ± 0.0</td>
<td>2.66 ± 0.19</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>5 mM n-Octylamine</td>
<td>1.49 ± 0.05</td>
<td>19.8 ± 0.3</td>
<td>0.049 ± 0.0</td>
<td>2.77 ± 0.13</td>
<td>0.01 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>0.32 mM L-Orn-OH</td>
<td>1.68 ± 0.00</td>
<td>17.0 ± 0.3</td>
<td>0.723 ± 0.0</td>
<td>1.56 ± 0.04</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>3.2 mM L-Orn-OH</td>
<td>1.13 ± 0.02</td>
<td>13.5 ± 0.4</td>
<td>1.98 ± 0.01</td>
<td>0.34 ± 0.01</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

*aReactions were carried out using 15 μM enzyme subunit at 25 °C in 100 mM Tris-SO$_4$ buffer, pH 8.
*b Measured at 370 nm. *c Measured at 450 nm. *d ND = not determined.
A.3.2 Effects of L-Orn and Related Compounds on Rate of Conversion of C4a-hydroperoxide (FAD-OOH) to Oxidized FAD

We subsequently examined the effects of the same full series of compounds on FAD-OOH conversion to FAD ($k_{FAD}$, Figure A.1; spectra shown in Figure A.7A). Except in the presence of L-Orn, D-Orn, and 1,4-diaminobutane, $H_2O_2$ is overwhelmingly the major product (Tables A.1 and A.3). L-Lys stimulates the FAD-OOH/FAD conversion at ~ 60% of the rate constant measured in the presence of L-Orn, and with an identical apparent $K_d$ value (Figure A.7B). L-Arg, by contrast, has no effect on this step. This strongly suggests that L-Arg discriminates between the reduced and FAD-OOH enzyme forms, selectively stimulating activation of $O_2$ by the reduced species. L-Arg thus possesses the characteristics of a nonsubstrate allosteric regulator, although it is important to emphasize that in the absence of structural data the actual physical binding sites for L-Arg and L-Orn on the reduced and FAD-OOH enzyme forms, respectively, are unknown and could overlap. It is possible that the positively charged side chain of L-Arg promotes the rate-limiting one-electron reduction of $O_2$ to superoxide that precedes recombination of the resulting two radicals to form FAD-OOH as discussed in chapter 1 ($FADH^- + O_2 \rightleftharpoons \{FADH+O_2^+\} \rightarrow \text{FAD-OOH}$). L-Orn and L-Lys, also positively charged but smaller than L-Arg, have much smaller effects on $k_{FAD-OOH}$. The roughly isosteric L-Citr and L-Orn-N$^\epsilon$-OH by contrast have uncharged side chains at neutral pH. L-Orn-N$^\epsilon$-OH has no measurable effect on FAD-OOH formation, and L-Citr has a small effect with a very high apparent $K_d$ (4.4 mM, Figure A.3B).
A.4 Discussion

In the presence of excess (5 mM) L-Orn and L-Arg together, $k_{\text{FAD-OOH}}$ is smaller than in L-Arg alone. This is expected as the two compete for the regulatory site (on E-FADH$^-$) and L-Orn is a much less efficient allosteric effector than L-Arg. The value of $k_{\text{FAD}}$ is only slightly diminished, and the reaction remains completely coupled (Table A.1). This suggests that L-Arg does not compete strongly with L-Orn for the E-FAD-OOH binding site.

By contrast, L-Lys (Figure A.5) interacts strongly with the E-FAD-OOH enzyme form. L-Lys is not hydroxylated (Table A.1) but stimulates the release of H$_2$O$_2$ from FAD-OOH with a rate constant ($k_{\text{FAD}}$, uncoupled) that approaches that measured for the FAD-OOH/FAD conversion that occurs with hydroxylation of L-Orn (Figure A.7B). It is possible that the E-FAD-OOH substrate-binding pocket is structurally well defined and that it admits L-Lys but will not accommodate the larger L-Arg.

The mechanism for O$_2$ control described here differs from that used by the FAD-dependent aromatic hydroxylases, such as para-hydroxybenzoic acid hydroxylase (PHBH).$^{2,27,28}$ PHBH is strongly regulated at the level of FAD reduction, which is $10^4$-fold faster when the correct, para-hydroxybenzoate substrate is bound. Substrate “proofreading” is followed by motions of the protein and FAD that alternately expose and protect the cofactor from solvent.$^{29}$ Interestingly, excess L-Arg and its neutral isostere L-Citr have a moderate (~18- and 10-fold respectively) influence on the rate constant for reduction of FAD ($k_{\text{red}}$), indicating that they bind the oxidized/FAD form of the enzyme, while L-Lys and L-Orn have no effect (Figure A.9). Since $k_{\text{red}}$ partially limits the rate of turnover of L-Orn, L-Arg and L-Citr are expected to influence the magnitude of $k_{\text{cat}}$, which they indeed do (Table A.3). However, $k_{\text{FAD}}$ is also partially rate limiting but unaffected by L-Arg or L-Citr. The composite effects of these compounds on $k_{\text{cat}}$ are consequently relatively small.
Figure A.7 (A) Conversion of the OMO FAD-OOH to oxidized FAD and H₂O₂ in the presence of saturating (5 mM) l-Arg as monitored by stopped flow. (B) Concentration dependence of $k_{obs}$ for the conversion in (A) (fit to a single exponential at 450 nm; see Figure A.8) in the presence of l-Orn or l-Lys. These have measured apparent $K_d$ values of 1.1 ± 0.09 and 1.2 ± 0.1 mM respectively. Note that l-Orn-NeOH is the major product in the presence of l-Orn, while the enzyme converts O₂ to H₂O₂ in l-Arg and l-Lys. Values for $k_{\text{FAD}}$ extrapolated from these data at saturating concentrations for each compound are listed in Table A.1.
Figure A.8 Representative kinetic traces at 450 nm (symbols) showing conversion of FAD-OOH to oxidized FAD in the presence of A) buffer and L-Arg, B) L-Orn, and C) L-Lys with data in each case fit (lines) to a single exponential to obtain the rate constant $k_{obs}$. The data from A-C are shown on a logarithmic time scale in plot D). In each case, a FAD-OOH–NADP$^+$ complex reacts with substrate to form L-Orn-OH and oxidized FAD, or with non-substrate to form $\text{H}_2\text{O}_2$ and oxidized FAD. Each reaction contains 15-20 μM enzyme reduced with 1 eq NADPH, which is subsequently mixed with air-saturated buffer containing 5 mM of the given compound.
TABLE A.3

STEADY STATE KINETIC PARAMETERS

<table>
<thead>
<tr>
<th>varied substrate</th>
<th>second amino acid</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (varied substrate) (mM)$^b$</th>
<th>$k_{cat} / K_m$ (varied substrate) (mM$^{-1}$s$^{-1}$)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Orn</td>
<td>-</td>
<td>0.46 ± 0.01</td>
<td>0.35 ± 0.06</td>
<td>1.29 ± 0.08</td>
</tr>
<tr>
<td>L-Lys</td>
<td>-</td>
<td>0.39 ± 0.04</td>
<td>0.47 ± 0.02</td>
<td>0.82 ± 0.17</td>
</tr>
<tr>
<td>L-Orn 1 mM L-Lys$^c$</td>
<td>0.46 ± 0.02</td>
<td>3.9 ± 0.6</td>
<td>0.12 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>L-Orn 1 mM L-Arg$^c$</td>
<td>0.93 ± 0.03</td>
<td>1.05 ± 0.13</td>
<td>0.89 ± 0.12</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Initial rates were monitored spectroscopically via disappearance of the UV/visible absorbance associated with NADPH. The average of three measurements is reported, with the standard deviation given as the error. All measurements were made at 25 °C in 100 mM Tris-SO$_4$ buffer, pH 8. $^b$L-Orn was the variable substrate in each case except for the second line of the table, where L-Lys was used alone. The measured initial rates reflect NADPH oxidase activity uncoupled from hydroxylation (H$_2$O$_2$ as product). $^c$Competitive (1-2 time $K_m$ (amino acid) rather than saturation (≥5 mM) amounts of the second amino acid were used in order to determine the nature of its interaction with the L-Orn binding forms of the enzyme.

The regulating mechanism described here also differs from that observed in cytochrome P450s, in which substrate binding displaces a water molecule, causing a spin state change in the ferric iron. This allows for a $10^5$-fold more rapid reduction to Fe(II). Once reduced, the iron can readily activate O$_2$. In OMO, the primary locus of regulation is not FAD reduction but O$_2$ activation by the reduced FADH$_2$; the gating molecule is not substrate but an allosteric effector.

The identification of L-Arg as an allosteric regulator of L-Orn hydroxylation has interesting biological implications (Figure A.10). L-Orn is the initial precursor to both the secreted fusariline and intracellular ferricrocin siderophores in A. fumigatus. Under conditions of iron starvation, siderophore production in this organism is prodigious, commandeering up to 10% of the total cellular biomass. The cell consequently requires efficient ways of generating L-Orn and steering it toward siderophore biosynthesis in response to the Fe status. The metabolic commitment required for siderophore production moreover must be balanced against the need to maintain other cellular functions. L-Orn is produced in the mitochondrion, and its pathway for export to the cytosol is upregulated under Fe deprivation. The pathway for converting L-Arg to L-Orn in the cytosol is likewise upregulated in response to Fe stress. Both pathways effectively sacrifice L-Arg to generate L-Orn. Yet, L-Arg itself is vital for protein biosynthesis and cell
Figure A.9 Effects of L-Orn, L-Arg, and L-Citr on rates of NADPH-reduction of OMO. The reaction mixture contained 10 μM enzyme and a final concentration of 15 μM NADPH at 25 °C and A) buffer and L-Orn B) 5 mM L-Arg, C) 4 mM L-Citr. The data at 450 nm (symbols) were fit (lines) to the sum of two exponentials where the first phase accounts for the majority of the amplitude change. The rate constants measured for this phase ($k_{\text{red}}$) were 1.21, 1.46, 5.9 and 13.9 s$^{-1}$ for buffer, L-Orn, L-Citr, or L-Arg, respectively. The remainder of the reaction occurs with rate constants 0.2 s$^{-1}$, 1.2 s$^{-1}$, and 0.8 s$^{-1}$ in the presence of buffer, L-Arg and L-Citr respectively. The observed rate constant for this noticeably slower phase showed no dependence on NADPH, substrate, or substrate analog. These results are consistent those previously described for liver microsomal FMOs, in which the biphasic reaction was explained as being due to the reaction of two enzyme forms. L-Arg has the most substantial effect on $k_{\text{red}}$. (D) Concentration dependence of $k_{\text{red}}$ in the presence of L-Arg and L-Citr, with apparent $K_d$ values of 0.7 ± 0.1 and 4.9 ± 0.9 mM, respectively.
signaling and must be available in sufficient amounts to sustain the cell. Activation of OMO by L-Arg appears to connect the sensed Fe status and siderophore production to cellular homeostasis: siderophore biosynthesis is stimulated only if the cytosolic L-Arg pool is sufficient for all of the cell’s needs. In turn, the cellular L-Arg concentration acts as a metabolic marker of Fe need; recent work by Shrettl et al. has shown that the cellular concentration of L-Arg dramatically increases (>10-fold) under conditions of iron starvation. \(^{16}\)

![Diagram](image)

**Figure A.10** Pathways for L-orn production and conversion into siderophores (mito = mitochondria; cyto = cytoplasm).

The regulatory mechanism identified here could also be critical for understanding regulation of other biosynthetic pathways. In particular, protein-level regulation could play a role in how plants fine-tune the time and place of auxin production, via the plant FMOs (YUC proteins). \(^{31}\) Future work will address these important pathways and the structural basis for the unique allosteric mechanism identified here.

**A.5 Summary**

OMO hydroxylates L-ornithine with about 87% efficiency and does not hydroxylate L-lysine, one methylene unit shorter. This highlights the high specificity of the enzyme. The presence of L-arginine appears to increase the efficiency of the enzyme toward hydroxylation of L-ornithine to 100%, without hydroxylation of L-arginine. The presence of L-lysine has no effect on the enzyme’s ability to effectively hydroxylate L-ornithine. In regard to kinetics, the rate of hydroperoxyflavin formation is modestly enhanced by L-ornithine (~14-fold as shown in Chapter 3
and here). Surprisingly, L-arginine enhances this rate almost 2-fold and L-lysine has no effect. Alternatively, L-lys accelerates peroxyflavin decay at a similar rate to L-ornithine, although in an unproductive manner. L-arginine has no effect on this step of the reaction. From this data it seems that specificity is achieved at the hydroperoxyflavin formation step and the metabolic precursor of L-ornithine, L-arginine, participates in this regulation.

### A.6 Acknowledgements

Professor Dave Ballou (Department of Biochemistry, University of Michigan) and Garrett Moraski are thanked for providing critical feedback on this manuscript. The Mobashery and Miller laboratories are acknowledged for providing reagents. R.E.F. was supported by a National Institutes of Health Chemistry-Biochemistry-Biology (CBBI) Interface Program and NIH Training Grant T32GM075762NIH-CBBI. J.A.M. was supported by NIH R01GM090260.


### A.7 References


APPENDIX B
UNDERSTANDING HOW THE DISTAL ENVIRONMENT DIRECTS REACTIVITY IN CHLORITE DISMUTASE: SPECTROSCOPY AND REACTIVITY OF ARG183 MUTANTS

Abstract – *Dechloromonas aromatica* chlorite dismutase (DaCld) catalyzes the highly efficient dismutation of chlorite (ClO$_2^-$) into molecular oxygen (O$_2$) and chloride ion (Cl$^-$). Prior studies of wild-type DaCld have indicated two pH sensitive moieties; one is the heme, and Arg183 in the distal heme pocket has been hypothesized to be the second. Using site-directed mutagenesis, this active site residue has been examined to understand the roles of positive charge and hydrogen bonding. Three mutants, Arg183 to Lys (R183K), Arg183 to Gln (R183Q), and Arg183 to Ala (R183A) were investigated to determine their respective abilities toward decomposition of chlorite, spin state and coordination states of their ferric and ferrous forms, their cyanide and imidazole binding affinities, and their reduction potentials. UV–Visible and resonance Raman spectroscopy showed that DaCld(R183A) contains five-coordinate high-spin (5cHS) heme, the DaCld(R183Q) heme is a mixture of five-coordinate and six-coordinate high spin (5c/6cHS) heme, and DaCld(R183K) contains six-coordinate low-spin (6cLS) heme. In contrast to wild-type (WT) DaCld, which exhibits pKa values of 6.5 and 8.7, all three ferric mutants exhibited pH-independent spectroscopic signatures and kinetic behaviors. Steady state kinetic parameters of the chlorite decomposition reaction catalyzed by the mutants suggest that in WT DaCld the pK$_a$ of 6.5 corresponds to a change in the availability of positive charge from the guanidinium group of Arg183 to the heme site. This could be due to either direct acid-base chemistry at the Arg183 side chain or a flexible Arg183 side chain that can access various orientations. Current evidence is most consistent with a conformational adjustment of Arg183. A properly oriented Arg183 is critical for the stabilization of anions in the distal pocket and for efficient catalysis.
Characterization of the Arg183 mutants presented in this chapter provides a foundation for work in the following chapters using the same mutant proteins.

B.1 Introduction

Heme proteins carry out a variety of chemical functions, often with striking specificity. These include the sensing and trafficking of small molecules, catalysis of one- and two-electron oxidations (peroxidases, peroxygenases), monoxygenation (cytochrome P450s), chlorination (chloroperoxidase), two- and four-electron reductions (N- and S-oxide reductases; cytochrome c oxidase), electron transfers (cytochromes), and heme trafficking (hemophores). The catalytic versatility of the heme is directed toward a particular function by the protein environment. A diverse body of literature has focused on reducing the complexity of the protein structure down to the fewest possible elements responsible for controlling active site chemistry. The proximal heme ligand is an important variable, ranging from a strong and polarizable thiolate, to imidazole/imidazolate, or phenolate (Cys, His, or Tyr, respectively). The distal side of the heme is often characterized by either an open coordination site or a labile iron ligand, with residues in the surrounding distal pocket controlling ligand access, the electrostatic environment, H-bonding and, as a consequence of all these factors, the heme-mediated chemistry.

Chlorite dismutase (Cld) is a relatively recently discovered heme enzyme that is associated with respiratory perchlorate reduction in several Proteobacteria. Enzymes from perchlorate respirers detoxify chlorite (ClO$_2^-$), the end product of perchlorate (ClO$_4^-$) respiration, by converting it to Cl$^-$ and O$_2$. This remarkable reaction is only the second well-described means of generating the O–O bond in biology. It is likewise difficult to reproduce efficiently using synthetic porphyrins. The enzyme from the perchlorate respiring organism Dechloromonas aromatica (DaCld) is fast, highly efficient (maximum $k_{cat} = 2.0 \pm 0.6 \times 10^5$ s$^{-1}$, $k_{cat}/K_M = 3.2 \pm 0.4 \times 10^7$ M$^{-1}$s$^{-1}$, pH 5.2, 4 °C), and highly specific, producing one molecule of O$_2$ from each ClO$_2^-$ even in the presence of large amounts of potential peroxidase (1-electron donating) substrates. Because the vast majority of environmental perchlorate is man-made, it has only recently become a significant contaminant. When or how the chlorite decomposition reaction evolved is an open
question. Interestingly, Cld homologs are found in a very broad array of bacterial and archaeal phyla, suggesting that the protein family is both ancient and necessary for reactions other than chlorite detoxification. Based on sequence and structural analyses, their active site structures and particularly the residues in the pocket above the open coordination position on the heme are likely diverse.\textsuperscript{7} We therefore aim to understand, in the context of well-described structure-activity models for heme proteins, how the distal environment of \textit{DaCld} and other proteins from this family directs their reactivity.

\textit{DaCld} has been crystallographically and spectroscopically characterized and shown to have an unusual active site structure, illustrated in Figure B.1.\textsuperscript{8} It has a proximal His that is hydrogen bonded to the carboxylate side chain of Glu220. A similar histidine-aspartate hydrogen bonding pair is common in heme peroxidases \textsuperscript{9}, where it has been postulated to be important for the heterolytic cleavage of the coordinated peroxide O–O bond that initiates the peroxidase catalytic cycle. In spite of the His–Glu interaction, which might be expected to lend anionic character to the ligating histidine, the resonance Raman (rR) spectrum of ferrous Cld indicates a weak Fe–His bond relative to the peroxidases. This suggests that the His–Glu interaction in \textit{DaCld} is in turn relatively weak, and that the ligating His is neutral in character.\textsuperscript{6} With the exception of an arginine residue (Arg183), the distal pocket contains only hydrophobic side chains that form a sterically constrained pocket over the porphyrin plane. The guanidinium group of Arg183 hydrogen bonds to a heme-bound nitrito ligand in the \textit{DaCld} crystal structure at pH 9 \textsuperscript{6}, and to the heme-bound thiocyanate in the Cld from \textit{Azospira oryzae}.\textsuperscript{10} At pH 6.5, the side chain in \textit{DaCld} bridges the heme-bound nitrito ligand and a molecule of the buffer system (2-(N-morpholino)ethanesulfonic acid). However, even though there is no buffer anion present in the pH 9.0 structure, the relative position of the heme and Arg183 side chain do not change significantly between the \textit{DaCld} crystal structures at pH 6.5 and 9.0.\textsuperscript{9} By contrast, in the otherwise very similar structures of the imidazole and cyanide-bound Cld from \textit{Nitrospira defluvii}, the guanidinium side chain is turned away from the distal pocket and facing the protein surface. When this residue is mutated to lysine, its amine side chain forms a hydrogen bond to an Fe-bound water.\textsuperscript{11}
The various conformations assumed by the side chain in these structures suggest that it is mobile. Its hydrogen bonding patterns likewise suggest that it is potentially important for forming and stabilizing anion-bound forms of the protein, functions known to be served by the distal Arg in peroxidases.\textsuperscript{12} Evidence suggests that it also plays a role in polarizing the heme-bound hydroperoxide ligand in these proteins, thereby promoting heterolytic cleavage of the O–O bond. It subsequently serves to stabilize the ferryl [Fe\textsuperscript{IV}=O] porphyrin cation radical species (Compound I) via hydrogen bond donation to the ferryl oxygen atom.\textsuperscript{13-16} Analogous functions for the DaCld distal arginine are possible.

To further understand the role of Arg183 in the O\textsubscript{2}−-generating ClO\textsubscript{2}− decomposition reaction, this residue has been substituted by a positively charged lysine, a neutral amide glutamine, and small, hydrophobic alanine. The effects of these substitutions on solution structure, axial heme ligation, reactivity, and the reduction potential of these mutants were probed by UV-visible and resonance Raman (rR) spectroscopy, steady-state kinetics of chlorite decomposition, and Fe\textsuperscript{3+}/Fe\textsuperscript{2+} potential determination. The results are consistent with a conformationally mobile Arg183 side chain having roles in stabilizing the enzyme-substrate complex and promoting O–Cl bond cleavage.

\textbf{Figure B.1} Active site of \textit{DaCld}–nitrite complex (PDB entry 3Q08)
B.2 Experimental Methods

B.2.1 Generation of DaCld(R183Q), DaCld(R183K), and DaCld(R183A)

Site directed mutagenesis of DaCld was carried using a Quick Change PCR mutagenesis kit (Stratagene). The following primers were used to construct the three mutants, where the altered codon is underlined: 5’-GTGAACGTCAAGCAGAAGTTGTACC-3’ (R183Q), 5’-CCTATCTTGTAACGTCAAGAAGTTGTACC-3’ (R183K) and 5’-CCTATCTTGTAACGTCAAGGTTGTACC-3’ (R183A). The reaction products were digested with Dpn1 to isolate the non-methylated (mutated) DNA and transformed into competent E. coli cells (NovaBlue) by heat shock. Transformants were plated on kanamycin supplemented LB agar. Sequencing was used to confirm the desired nucleotide mutations. The plasmids were transformed into E. coli TunerDE3 competent cells (EMD Biosciences) by heat shock.

B.2.2 Protein purification.

The mutant proteins were expressed in E. coli TunerDE3 cells and purified according to the WT procedure previously described. For DaCld(R183A), hemin was solubilized in DMSO and added during the sonication process (~60 μM).

B.2.3 Steady state kinetics of chlorite decomposition

O₂ production from chlorite decomposition was monitored continuously using a Clark electrode. The electrode was equilibrated to the set temperature for at least 1 h and calibrated to the expected O₂ concentration in air saturated, double distilled Milli-Q water, adjusted for temperature and daily atmospheric pressure. The temperature was maintained at 4 °C by a circulating water bath (Thermo-Fisher Scientific) with a custom built chamber jacketing the reaction vessel. Reactions were carried out in 1.5 mL volumes of 100 mM citrate-phosphate buffer at pH 6.8 with 0.080-500 mM of sodium chlorite added from a stock made in the same
buffer. Initial rates were determined by linear least-squares regression fits to the initial 5–10% of the progress of reaction curves (LoggerPro, VrTech).

**B.2.4 Catalase activity**

For catalase activity measurements, [O$_2$] was continuously monitored via Clark electrode. Equilibration and calibration were performed as described above, except with calibration carried out on an N$_2$ saturated solution containing ditihionite. For each measurement, buffer alone was incubated for 5 minutes under N$_2$ gas so that the initial [O$_2$] was between 0 and 50 mM. The probe was then inserted into the solution and equilibrated. H$_2$O$_2$ was added to a final concentration of 50 mM via gas-tight Hamilton syringe and a baseline was measured. Enzyme (1-2 mM heme) was added via gas-tight syringe and evolved O$_2$ measured. Initial rates were determined by linear least-squares regression fits to the initial 5-10% of the progress of reaction curves (Kaleidagraph).

**B.2.5 Peroxidase activity**

Initial rates of tetraguaiacol formation from the standard peroxidase substrate guaiacol were monitored on a Varian Cary 50 spectrometer at 25°C at 460 nm ($\text{I}_{470} = 26 \text{ mM}^{-1} \cdot \text{cm}^{-1}$). Oxidation of 2,2’-azino-bis(3-ethyl-benzthiazoline-6-sulphonic acid (ABTS) to the ABTS radical was monitored at 414 nm using $\text{e}_{414} = 36 \text{ mM}^{-1} \cdot \text{cm}^{-1}$.$^{18}$ Reactions were carried out in a quartz cuvette containing a saturating concentration of guaiacol (8.35 mM, 140 µL) or ABTS (79-84 mM) and 10 µL of the protein in 20 mM phosphate buffer (2.07 µM in heme) at the desired pH (final volume 160 µL). The reaction was performed in a 100 mM phosphate buffer at pH 6.8 and 25°C. The reaction was initiated by the addition of 10 µL of a H$_2$O$_2$ stock prepared in the same buffer. Initial rates were determined by linear least-squares regression fits to the initial 5-10% of the progress of reaction curves (Kaleidagraph). The H$_2$O$_2$ concentration was varied from 0.04-116 mM.
B.2.6 UV/visible pH titrations

UV-visible spectra were measured on a Varian Cary 50 spectrometer. Protein samples were titrated at 25 °C over three pH ranges. The following buffers were used: 50 mM citrate (pH 3 to 6.3), 100 mM phosphate (pH 6.7 to 10.93), and 100 mM disodium phosphate (pH 8.77 to 13). Each titration was carried out in an 8 mL reaction with the protein at either ~1.8 µM heme or ~3.4 µM heme, where heme concentration was determined via the pyridine hemochrome assay. The solutions were constantly stirred and the pH continuously monitored using a pH meter with a glass electrode (Corning pH 430). Small volumes of HCl (1 M) or NaOH (1 M) were added to the solution and the pH measured, after which 200 µL aliquots were withdrawn and analyzed by UV/vis. The spectra were adjusted for dilution.

B.2.7 Resonance Raman spectroscopy

Samples for rR experiments were prepared at 20 to 90 µM DaCl in 100 mM citrate at pH 5.8, 100 mM sodium phosphate at pH 6.8 and 7.5, 100 mM Tris/HCl at pH 8.5, and 100 mM Ches at pH 10.0. Resonance Raman spectra were obtained with 406.7, 413.1, or 441.6 nm excitation from Kr⁺ and HeCd lasers, respectively, using the 135° backscattering geometry. The spectrometer was calibrated against Raman frequencies of toluene, DMF, acetone, d⁶-dimethylsulfoxide, and methylene bromide. Data were collected at ambient temperature from samples in spinning 5-mm NMR tubes. UV-visible spectra were recorded before and after rR experiments to probe whether the samples had been irreversibly altered in the laser beam. Laser power at ferric and ferrous samples ranged from 6 to 18 mW with 406.7 nm excitation and from 2 to 5 mW for ferrous-CO samples with 413.1 nm excitation; no spectral artifacts due to photoinduced chemistry were observed with these irradiation powers.
B.2.8 CO complexes of WT and mutant DaClds

Ferrous Cld was generated anaerobically at 20 °C by treatment of ferric Cld with an excess of buffered stock sodium dithionite solution. The corresponding CO complexes were prepared by flushing the ferrous proteins with $^{12}$CO or $^{13}$CO. Protein concentrations were typically 20-40 μM Cld in 100 mM MES pH 5.6, 100 mM citrate at pH 5.8, 100 mM sodium phosphate at pHs 6.2, 6.8 and 7.5, 100 mM Tris/HCl at pHs 8.0, 8.6, 9.1, and 100 mM CHES at pH 10.0. The Cld−CO samples in D$_2$O were prepared in a manner analogous to those in H$_2$O except that the solution conditions were 100 mM Mes pD 5.6 with 88% D$_2$O/12% H$_2$O.

B.2.9 Equilibrium binding of ligands

For each spectrophotometric titration, an ~8.0 μM enzyme sample was prepared in a 200 μL volume of the appropriate buffer in a quartz cuvette and its spectrum recorded. Solutions of the ligands (imidazole, azide, fluoride and cyanide) were prepared in the same buffers at 0.002 – 200 mM and added in 5 μL aliquots. Spectra were repeatedly measured after each addition until the reaction mixtures had reached equilibrium. When the titration appeared complete, a ligand stock of 10-fold higher concentration was added in 5 μL aliquots in order to be sure that a clear endpoint had been reached. Difference spectra were generated from spectra which had been corrected for sample dilution. The wavelength of maximum absorbance change was used to construct a plot of ΔAbs versus [L]$_T$ (total concentration of added ligand) and fit by least squares regression (Kaleidagraph) to an equilibrium isotherm of the form $\Delta$Abs=$\Delta$Abs$^\infty$[L]$_T$/(KD+[L]$_T$). The pH profiles were fitted using two or one pK$_a$ equations, as previously described.$^6$ Here, c is a pH-independent measure of the parameter y (here, the $K_D$ for a given ligand), and $K_{a[1]}$ and $K_{a[2]}$ describe its dependence on protonation state.
B.2.10 Redox potentials

The reduction potentials of the WT and mutant DaClds were measured via the UV/visible titrimetric method of Massey (UV-2550 Shimadzu UV-Vis spectrophotometer, University of Michigan, Palfey Laboratory) (20). Briefly: 2 μM of WT-DaCl was introduced into the body of an anaerobic cuvette with ~20 μM of the reduction dye potassium indigo tetrasulfonate (E = -46 mV (21)), 200 μM xanthine and 1 μM of benzyl viologen in 0.1 M potassium phosphate buffer at pH 7 and 25 °C. Benzyl viologen was used to ensure rapid equilibrium. An appropriate amount of xanthine oxidase was placed in the side arm of the cuvette and several cycles of evacuation and flushing with argon gas were used to eliminate any oxygen. The spectrum of the solution of the enzyme and the dye was recorded. The reduction began as soon as the xanthine oxidase was mixed with the enzyme. Spectra were recorded every 2 minutes and the reaction was stopped when the dye and the enzyme were fully reduced (minimum of 3 hours). The extent of reduction of the dyes was monitored at 416 nm (an isosbestic point for the enzyme spectra as the enzyme was reduced). The extent of reduction of the enzyme was monitored at 432 nm. This is not an isosbestic point for the dye, so the contributions from oxidized and reduced dye were subtracted. Reduction of the Clid mutants (R183A, R183K and R183Q) was inhibited by the indicator dyes. Therefore, reduction potentials were determined by reducing the enzyme with the xanthine/xanthine oxidase system and then adding the indicator dyes. The indicator dyes reacted immediately with the enzymes and the potentials were calculated based on the extent of

\[
\log(y) = \log \left( \frac{c}{1 + \frac{[H^+]}{K_a^{(1)}}} \right)
\]

(Equation B.1)

\[
\log(y) = \log \left( \frac{c}{1 + \frac{[H^+]^{2}}{K_a^{(2)}}} \right)
\]

(Equation B.2)

\[
\log(y) = \log \left( \frac{c}{1 + \frac{[H^+]^{1}}{K_a^{(1)}}} \right)
\]

(Equation B.3)
reactions. The same dye as WT-DaCld was used for the mutant R183Q and the redox dyes pyocyanin (E = -34 mV) and 5-hydroxy-1,4-napthoquinone (E = -3 mV) were used for the mutants R183K and R183A, respectively.

B.3 Results and Analysis

B.3.1 Reactivity of DaCld R183 Mutants with Chlorite Ion

The steady state kinetic parameters for chlorite decomposition catalyzed by WT DaCld, DaCld(R183Q), DaCld(R183K), and DaCld(R183A) were determined at pH 6.8 and 4 °C (Table B.1).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}/K_m$ ($\text{M}^{-1}\text{s}^{-1}$)$^a$</th>
<th>$k_{cat}$ ($\text{s}^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT Cld</td>
<td>0.3</td>
<td>$2.0 \times 10^7$</td>
<td>$6 \times 10^3$</td>
</tr>
<tr>
<td>R183K</td>
<td>42 ± 12</td>
<td>$2.5 \times 10^4$</td>
<td>$(1.0 \pm 0.1) \times 10^3$</td>
</tr>
<tr>
<td>R183Q</td>
<td>50 ± 9.0</td>
<td>$6.9 \times 10^3$</td>
<td>$(3.5 \pm 0.2) \times 10^2$</td>
</tr>
<tr>
<td>R183A</td>
<td>15 ± 4.3</td>
<td>$6.2 \times 10^3$</td>
<td>$91 \pm 6$</td>
</tr>
<tr>
<td>HRP-C$^c$</td>
<td>$(11.5 \pm 1.1) \times 10^{-3}$</td>
<td>$4.6 \times 10^6$</td>
<td>$52.5 \pm 3.9$</td>
</tr>
<tr>
<td>WT Cld</td>
<td>1.60 ± 0.2</td>
<td>$(1.5 \pm 0.2) \times 10^3$</td>
<td>$2.5 \pm 0.2$</td>
</tr>
<tr>
<td>R183K</td>
<td>30.5 ± 5.1</td>
<td>$(2.3 \pm 0.4) \times 10^2$</td>
<td>$7.0 \pm 0.4$</td>
</tr>
<tr>
<td>R183Q</td>
<td>12.6 ± 3.0</td>
<td>$5.6 \pm 1.4$</td>
<td>$0.070 \pm 0.005$</td>
</tr>
<tr>
<td>R183A</td>
<td>9.70 ± 1.7</td>
<td>$4.3 \pm 0.8$</td>
<td>$0.042 \pm 0.002$</td>
</tr>
</tbody>
</table>

$^a$For the reactions in the top section, chlorite is the sole substrate. For reactions in the bottom section, the concentration of H$_2$O$_2$ was varied at a constant and saturating concentration of ABTS. As described in Experimental Procedures, initial rates were determined by monitoring the production of ABTS radical.

The WT reaction has a $k_{cat}/K_m$ of $2 \times 10^7$ M$^{-1}$s$^{-1}$, which is 103-fold higher than that of DaCld(R183K) and 104-fold higher than that of either DaCld(R183Q) or DaCld(R183A). Because the Arg to Gln and Lys mutations have little effect on $k_{cat}$, the changes in $k_{cat}/K_m$ are primarily attributable to the sensitivity of $K_m$ to Arg183 mutations. This suggests that a positive charge
and/or a polar, H-bond-donating residue (Q or K) is important for efficient formation of the Michaelis complex with chlorite. Both $k_{cat}$ and $K_M$ are significantly altered in DaCld(R183A). Plots of log($k_{cat}$) and log($k_{cat}/K_m$) versus pH for DaCld- (R183Q) (Figure B.2) show only small variations with pH. This is in contrast to the WT enzyme, for which bimodal pH profiles were observed for both log($k_{cat}$) and log($k_{cat}/K_m$), indicating an optimally active low pH (pH 6.5) and less active higher-pH forms of the enzyme.

![Figure B.2](image)

**Figure B.2** pH-rate profiles of the R183Q mutant with chlorite. Plots of log($k_{cat}$) (○) and log $k_{cat}/K_m$ (●) as a function of pH using chlorite at 4 °C in citrate/phosphate buffer.

### B.3.2 Catalase and Peroxidase Activity of DaCld R183 Mutants

No catalase activity from either the wild-type or the R183Q mutant (pH 6.8, 0.1 M phosphate, and 25 °C) was observed. In the presence of one-electron-reducing substrates, the WT enzyme exhibits modest peroxidase (Table B.1) activity. Both $k_{cat}$ and $k_{cat}/K_{M,\text{peroxide}}$ for WT DaCld are roughly 4 orders of magnitude smaller than the same parameters measured for the chlorite decomposition reaction. Comparison of the mutants to the WT enzyme shows that $k_{cat}/K_{M,\text{peroxide}}$ is strongly diminished in the mutants while $k_{cat}$ either decreases by 1 order of magnitude for DaCld(R183Q) or slightly increases for DaCld(R183K). Similar to the chlorite reaction, having a polar H-bonding and/or charged residue at position 183 appears to be important for maintaining a high $k_{cat}/K_{M,\text{oxidant}}$. 

297
pH Dependence of the Axial Coordination and Spin-state with a pKa of 8.7. UV-visible and rR characterization of WT DaCl showed that the enzyme undergoes a transition from its five-coordinate high-spin (5cHS) acidic form to its low-spin, ferric hydroxide form with a pKa of 8.7. UV-visible pH titrations for the three Arg183 mutant proteins were conducted over acidic (pH 3–6.8), neutral (pH 7–10), and basic (pH 8–12) ranges (Figures B.3–B.5). In contrast to WT DaCl, the mutants do not exhibit a pH transition below pH 10. Above pH 10, the dramatic loss of extinction in the Soret, α, and β bands is consistent with the loss of heme from the protein. Heme loss likewise occurs at low pH. On the basis of the heme spectrum, the mutant enzyme folds are stable from pH 5 to 9.9 (R183A) (Figure B.4), from pH 5.6 to 10.8 (R183K) (Figure B.5), and from pH 5.1 to 10.1 (R183Q) (Figure B.3).

![Figure B.3 UV visible pH titration for the R183Q mutant](image)

**Figure B.3** UV visible pH titration for the R183Q mutant (A) pH 6.8, 6.5, 6.1, 5.8, 5.5, 5, 3.8 (B) pH 6.8, 7.3, 7.7, 8.2, 8.7, 9.4, 10, 10.5, 10.9 and (C) pH 8.4, 9.3, 9.9, 10.3, 10.6, 10.9, 11.6, 12.1.
Figure B.4 UV/visible pH titrations for mutant R183A at (A) pH 6.8, 6.5, 6.1, 5.8, 5.5, 5, 3.8 (B) pH 6.8, 7.2, 7.5, 7.9, 8.3, 8.9, 9.5, 9.9, 10.2, 10.4, 10.6 and (C) pH 8.4, 9.3, 9.9, 10.3, 10.6, 10.9, 11.6, 12.1.
B.3.3 *DaCld*(R183A)

Like the acidic of the WT enzyme, the heme in *DaCld*(R183A) is 5cHS. At pH 6.8, the UV–visible spectrum of *DaCld*(R183A) (Figure B.6) is similar to that of WT [λmax at 393 (Soret), 509 (Q), and 648 nm (CT)]. The resonance Raman (rR) spectrum of the ferric protein at pH 6.8 is shown in Figure B.7 with its pH dependence shown in Figure B.8. Over the pH range examined, those spectra exhibit ν4, ν3, and ν2 bands at 1372, 1496, and 1566 cm⁻¹, respectively. These frequencies are indicative of a 5cHS heme. The alanine side chain in *DaCld*(R183A) cannot serve as a hydrogen bond donor to stabilize an anionic distal heme ligand. This likely accounts, at least in part, for the fact that this mutant does not form a hydroxide complex at alkaline pH values where the protein is stable. This conclusion is supported by the 5cHS rR signature of *DaCld*(R183A) at pH 10.0.
B.3.4 DaCld(R183Q)

The heme in DaCld(R183Q) exists as an equilibrium mixture of 5cHS and six-coordinate high-spin (6cHS) heme. Its UV–visible spectrum has a red-shifted Soret band maximum (403 nm) relative to that of WT, a shoulder at 380 nm, a broad α and β envelope at 509 nm, and a charge transfer (CT) maximum at 633 nm (Figure B.6 and Table B.2). Charge transfer bands are observed between 600 and 650 nm for HS but not LS ferric heme complexes, and their wavelengths are indicative of coordination number. Spectra of 5cHS species typically have a CT band at ≥640 nm, while the band is closer to 630 nm for 6cHS hemes. Additionally, a shoulder on the Soret band at 380 nm usually indicates the presence of a 5cHS species.22 The DaCld(R183Q) UV–visible spectrum with its shoulder at 380 nm and a CT band around 630 nm therefore suggests a mixture of 5cHS and 6cHS species. The A_{Soret}/A_{280} absorptivity ratio (Table B.2) reflects the relative abundance of 6cHS versus 5cHS species. The low ASoret/A380 ratio for WT and DaCld(R183A) is indicative of a primarily 5cHS species, while the higher ratio for DaCld(R183Q) suggests a mixture of 5c- and 6cHS heme. The rR spectrum of DaCld(R183Q) (Figure B.7A) exhibits core size marker bands, v4 and v3, at frequencies that confirm the presence of HS ferric heme. The band in the v3 region is broad, consistent with the presence of both 5c- and 6cHS hemes (v3 at 1493 and 1485 cm^{-1}, respectively). The rR spectra for this mutant, like the UV–visible spectra and kinetic parameters, are also insensitive to pH values between 6.8 and 10.0 (Figure B.8). The lack of a pH-dependent shift in the rR signature from a 5cHS or 6cHS species to a 6cLS species, which would be expected if a ferric hydroxide had formed, suggests that the sixth heme ligand of the 6cHS species is not hydroxide; the more likely possibility is that the proton-accepting Gln183 stabilizes a heme-bound water molecule that does not undergo proton loss.

B.3.5 DaCld(R183K)

Ferric DaCld(R183K) has a six-coordinate low-spin (6cLS) heme over the entire pH range examined. The UV–visible spectrum of DaCld(R183K) (Figure B.6 and Table B.2) has a sharp, red-shifted Soret peak (410 nm) relative to that of the WT Cld spectrum. The Soret wavelength
together with the α and β bands at 530 and 560 nm, respectively, and the lack of a CT band in the visible region of the spectrum are characteristic of 6cLS heme complexes with bis-nitrogen axial ligation. The rR spectrum of DaCld(R183K) at pH 6.8 shown in Figure B.8 has its core size marker bands, ν4, ν3, and ν10, at 1373, 1507, and 1641 cm$^{-1}$, respectively. These frequencies confirm a ferric, 6cLS heme. The high-frequency, Soret-excited rR spectra of ferric DaCld(R183K) are pH-independent between pH 6.8 and 10.0 (Figure B.8). The lack of pH dependence in both the UV–visible and rR data argues against hydroxide being the sixth heme ligand. Instead, the data [the UV–visible features and detailed rR (see below)] support a His/Lys axial ligand pair in which the neutral form of Lys183 or HS but not LS ferric heme complexes, and their wavelengths are indicative of coordination number. Spectra of 5cHS species typically have a CT band at ≥640 nm, while the band is closer to 630 nm for 6cHS hemes. Additionally, a shoulder on the Soret band at 380 nm usually indicates the presence of a 5cHS species. The DaCld(R183Q) UV–visible 1899 coordinates to the heme iron. The p$K_a$ of Lys183 (free lysine pKa = 10.53) must therefore be lower than 6.8 in the vicinity of the nearby Lewis acidic ferric iron center.

Figure B.6. UV–visible spectra for WT and DaCld mutants at pH 6.8 in 100 mM phosphate buffer. The spectrum of WT DaCld is represented with a thick black solid line with a Soret band at 393 nm. The R183A mutant (−−−) has a spectrum similar to that of WT with a Soret band at 391 nm. The R183Q (---) and R183K (thick gray line) mutants have Soret bands at 403 and 410 nm, respectively.
TABLE B.2

UV-VISIBLE ABSORBANCE $\lambda_{\text{max}}$ VALUES (NANOMETERS) FOR WT AND MUTANT DaCLDS AT pH 6.8 AND 10.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>pH</th>
<th>Soret</th>
<th>CT 2</th>
<th>$\beta$</th>
<th>$\alpha$</th>
<th>CT 1</th>
<th>spin state</th>
<th>$\nu$ (cm$^{-1}$)</th>
<th>$A_{\text{Soret}}$/$A_{280}$</th>
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<tr>
<td>WT Cld</td>
<td>6.8</td>
<td>392</td>
<td>509</td>
<td>~536</td>
<td>~644</td>
<td>5cHS</td>
<td></td>
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<tr>
<td>R183K</td>
<td></td>
<td>410</td>
<td>-</td>
<td>~530</td>
<td>~560</td>
<td>6cLS</td>
<td></td>
<td>1507</td>
<td>2.6</td>
</tr>
<tr>
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<td></td>
<td>403</td>
<td>509</td>
<td>~530</td>
<td>~635</td>
<td>6c/5cHS</td>
<td></td>
<td>1485/1493</td>
<td>1.5</td>
</tr>
<tr>
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<td></td>
<td>391</td>
<td>509</td>
<td>~530</td>
<td>~644</td>
<td>5cHS</td>
<td></td>
<td>1496</td>
<td>1.1</td>
</tr>
<tr>
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<td>408</td>
<td>-</td>
<td>533</td>
<td>576</td>
<td>~608</td>
<td>6cHS/LS</td>
<td>1479/1506</td>
<td>2.05</td>
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<tr>
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<td></td>
<td>410</td>
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<tr>
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<td>391</td>
<td>-</td>
<td>~530</td>
<td>~625</td>
<td>5cHS</td>
<td></td>
<td>1496</td>
<td>1.1</td>
</tr>
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</table>

Figure B.7 Soret-excited rR spectra of Cld mutants at pH 6.8. Samples were prepared in 100 mM sodium phosphate (pH 6.8): (A) high-frequency window and (B) low-frequency window. Spectra of WT Cld at pH 6.8 and 10.0 (100 mM CHES) are included for comparison to those of the mutants.
[Figure B.8. Soret-excited, high frequency window rR spectra of Cld mutants as a function of pH. Samples were prepared in 100 mM sodium phosphate, pH 6.8, 100 mM Tris/HCl pH 7.8, 100 mM Tris/HCl pH 8.8, 100 mM Ches pH 10.0 and 100 mM MES pH 5.5. Spectra were collected with 406.7 nm excitation and 14 mW at the sample. A) Cld(R183K) spectra compared to the spectrum of WT Cld at pH 10, B) Cld(R183A) spectra, and C) Cld(R183Q) spectra compared to WT Cld at pH 6.8. *indicates plasma emission lines.]

B.3.6 Effects of Mutations on Heme Conformation

The low-frequency region of the mutant rR spectra is shown in Figure B.8B. Assignments for the HS DaCld(R183Q) and DaCld- (R183A) vibrations are made by analogy to HRP23 and Mb24 and indicated in Figure B.8B. The δ(CβCaCb) bending modes for the vinyl groups [DaCld(R183Q), 411 and 421 cm⁻¹; DaCld(R183A), 423 cm⁻¹] are very similar to those observed for WT DaCld. The δ(CβCcCd) bending modes for the propionyl groups [DaCld(R183Q), 365 and 379 cm⁻¹; DaCld- (R183A), 369 and 384 cm⁻¹] are clearly resolved in the DaCld(R183Q) and DaCld(R183A) spectra but not in the WT spectrum. The latter has a broad
shoulder at 380 cm$^{-1}$ on the propionate bending band (367 cm$^{-1}$). These data suggest that the environment of at least one of the propionate groups in $DaCld$(R183Q) and $DaCld$(R183A) is perturbed relative to the WT heme. The LS $DaCld$(R183K) spectrum has multiple bands in the vinyl bending range [$\delta$(C$\beta$CaCb), 412–430 cm$^{-1}$] and a $\delta$(C$\beta$CcCd) band at 367 cm$^{-1}$. Compared to those of alkaline WT $DaCld$, which is also 6cLS, the frequencies of $\nu$8 and the $\delta$(C$\beta$CcCd) bands of $DaCld$(R183K) are shifted down by 2 and 4 cm$^{-1}$, respectively. Arg183 therefore clearly influences the conformation of at least one of the peripheral heme propionate groups in solution. Although the crystal structure does not reveal any direct interaction, the distance between the guanidinium group of Arg183 and the carboxylate group of the 7-propionate could be bridged by a hydrogen- bonded water molecule. In the mutants, this interaction would range from less favorable to impossible, releasing the 7-propionate group to adopt a different conformation.

### B.3.7 Axial Heme Coordination and Spin State of Ferrous $DaCld$ Mutants

The visible spectra of ferrous $DaCld$(R183Q) (Soret band at 434 nm and $\alpha$ and $\beta$ bands at 558 and 585 nm, respectively) and ferrous $DaCld$(R183A) (Soret band at 433 nm and $\alpha$ and $\beta$ bands at 559 and 585 nm, respectively) are consistent with 5cHS ferrous heme. This is confirmed by the coordination and spin state marker bands, $\nu$3 and $\nu$4, for $DaCld$(R183Q) and $DaCld$(R183A) near 1473 and 1357 cm$^{-1}$, respectively, in the high-frequency rR spectra (Figures S5 and S6 of the Supporting Information). This frequency signature is typical of 5cHS ferrous heme and is very similar to that observed for ferrous WT $DaCld$ (1474 and 1359 cm$^{-1}$).$^6$ The UV–visible spectra of ferrous $DaCld$(R183K) indicate that it has an alkaline transition in the pH range of 6.8–10.0 (Figure B.11). The acidic form has its Soret band at 432 nm and $\alpha$ and $\beta$ bands at 556 and 588 nm, respectively. This is very similar to the spectrum observed for ferrous WT $DaCld$ (433, 556, and 587 nm) and for ferrous soybean seed coat peroxidase (434, 555, and 582 nm),$^{23}$ and slightly blue-shifted relative to the spectra of ferrous HRP-C, CCP, BP,$^{25}$ and CIP.$^{26}$ Thus, the spectrum of acidic ferrous $DaCld$(R183K) is consistent with a 5cHS heme. When the pH is increased, the Soret band of $DaCld$(R183K) shifts to 423 nm and the $\alpha$ and $\beta$ bands move to 528 and 558 nm, respectively. This suggests that the 5c heme is binding a sixth
ligand under alkaline conditions. The acid–base transition of the heme is also observed in the pH dependence in the 406.7 nm excited rR of ferrous DaCld(R183K) (Figure B.7). The acidic ferrous form has a $\nu_3$ (1474 cm$^{-1}$) and a $\nu_4$ (1358 cm$^{-1}$) consistent with a 5cHS heme and similar to those observed for ferrous WT DaCld. Between pH 7.8 and 8.8, a second $\nu_3$ band starts to appear at 1493 cm$^{-1}$, consistent with the formation of 6cLS ferrous DaCld(R183K) under alkaline conditions. This behavior supports the conclusion that at low pH the amine side chain of Lys183 is protonated and unable to ligate the heme; in its alkaline form, Lys183 is deprotonated and able to bind to the heme iron, generating a 6cLS heme. This behavior is in contrast to that of ferric DaCld(R183K), where the transition between 5c and 6c hemes is not observed, presumably because protonation of the coordinated amine would have to occur at a pH below the range of enzyme stability.

Figure B.9. High frequency rR spectra of ferrous Cld(R183Q) at pH 7.8 and 10.0. Spectra were acquired with 406.7 nm excitation and 15 mW at the sample. Inset: The low frequency window of the rR spectrum of ferrous Cld(R183Q) at pH 7.8 and 10.0 obtained with 441.6 nm excitation.
Figure B.10. Resonance Raman characterization of ferrous Cld(R183A) in 100 mM sodium phosphate, pH 6.8. (A) The high frequency window rR spectrum acquired with 413.1 nm excitation and 18 mW at the sample. (B) The low-frequency window of the rR spectrum of ferrous Cld(R183A) obtained with 441.6 and 413.1 nm excitation.

Figure B.11 UV-visible spectra of ferrous Cld(R183K) as a function of pH. Samples were prepared in 100 mM sodium phosphate, pH 6.8 or 100 mM CHES pH 10.0 and reduced with sodium dithionite.
B.3.8 Proximal Heme Environment as a Function of Arg183 Mutation

Resonance enhancement of the νFe−His mode increases considerably when the excitation is closer to the Soret band maximum for 5cHS ferrous hemes. In particular, 441.6 nm excitation elicits well-enhanced, low-frequency bands that can be attributed to νFe−His modes. The 441.6 nm-excited low-frequency spectrum of ferrous DaCld(R183A) is compared to its 413.1 nm-excited rR spectrum in Figure B.10. By virtue of its intensity and frequency, the 220 cm⁻¹ band is tentatively assigned to the νFe−His mode of 5cHS ferrous DaCld(R183A). The νFe−His mode for ferrous DaCld(R183Q) is also tentatively assigned to a pH insensitive band at 220 cm⁻¹ (Figure B.9). The pH dependence of the 441.6 nm excited low-frequency spectra of ferrous DaCld(R183K) is shown in the inset of Figure B.12. The 222 cm⁻¹ band is assigned to the νFe−His mode of 5cHS ferrous DaCld(R183K). This vibration is observed at the same frequency in WT DaCld under acidic conditions. The intensity of the νFe−His band decreases as the pH is increased; this is consistent with some of the 5cHS heme being converted to 6cLS heme as seen in the high-frequency rR spectra (Figure B.12). The visible spectra of ferrous DaCld(R183K) at pH 6.8 and 10 are also consistent with this conversion (Figure B.1). The 220–222 cm⁻¹ νFe−His frequency observed for WT and the Arg183 mutant enzymes is typical of a proximal His ligand in which the imidazole (ImH) side chain is H-bonded to a weak H-bond acceptor, such as an amide carbonyl group. Examples include α-Hb and Mb.²⁷ If the proximal His ligand had imidazolate character, as in the peroxidases, the νFe−His frequency would be expected to be ≥230 cm⁻¹.²⁸ The Fe− His stretching band in HRP and CcP decreases 4 to 5 cm⁻¹ in frequency when the distal arginine is replaced with a nonpolar amino acid [WT HRP, 243 cm⁻¹; HRP(R38L), 238 cm⁻¹; WT CcP, 233 and 246 cm⁻¹; CcP(R48L), 242 cm⁻¹]. The proximal side of the heme in DaCld, by contrast, is relatively uninfluenced by mutations in its distal pocket. When the arginine is replaced with lysine in HRP, a 3 cm⁻¹ decrease in the νFe−His frequency is observed but no corresponding change is observed in the spectrum of CcP(R48K).¹² The crystal structure of CcP(R48K) shows that Lys48 maintains the hydrogen bonding network present in WT CcP. At low pH, the same appears to be true for ferrous DaCld(R183K). The small effects of the distal mutation on the Fe−His stretching frequency of ferrous DaCld indicate that the differences in the enzymatic
activity of the mutant DaClds can be attributed to structural and electronic changes within the distal pocket.

Figure B.12 pH dependence of the high-frequency rR spectrum of ferrous DaCld(R183K). Samples were prepared in 100 mM sodium phosphate at pH 6.8, 100 mM Tris-HCl at pH 7.8, 100 mM Tris-HCl at pH 8.8, or 100 mM CHES at pH 10.0 and reduced with sodium dithionite. Spectra were acquired with 406.7 nm excitation and 18 mW power at the sample. The inset shows the pH dependence of the low-frequency window of the rR spectrum of ferrous DaCld(R183K) obtained with 441.6 nm excitation.

B.3.9 Distal Heme Environment Probed with Carbonyl Complexes of WT and Mutant DaClds.

Two CO conformers, identified by isotopic substitution, have been reported for WT Cld–CO. The Fe–C and C–O stretching frequencies for these conformers have been reported at pH 5.8, 6.8, and 10.6. In the low-frequency window, the Fe–C stretching bands for these conformers overlap. The WT Cld–CO spectra were subjected to peak fitting so we could assign the frequencies for these isotope sensitive bands and ascertain the relative peak areas for each conformer (Figure B.13). Here we examined the pH-dependence of WT Cld–CO in greater detail.
to determine if the FeCO vibrational signature of the heme carbonyl is sensitive to the pKₐ of 6.5 observed in the DaClD steady state reaction with chlorite. The high- and low- frequency Soret excited rR spectra of WT ClD–CO are independent of pH over the range of 5.8–9.1 (Figure B.14). The two conformers (νFe–C and νC–O for the so-called “open” form at 493 and 1956 cm⁻¹, respectively and for the “closed” form at 518 and 1929 cm⁻¹, respectively) are observed over the entire pH range examined here; their νFe–C and νC–O vibration frequencies and their relative populations (as judged by their relative peak areas) are independent of pH from 5.6 to 9.1 when the samples are prepared from fresh DaClD. If the protein is subjected to multiple freeze–thaw cycles or is stored frozen for several months, the νFe–C and νC–O bands assigned to the open conformer become sensitive to pH, shifting 5 and 15 cm⁻¹, respectively, over the pH range of 5.6–10.0; further, the closed form ν(C–O) band is no longer observed at pH 10, as reported previously. Changes to spectroscopic heme signatures as a result of storage or sample handling have been reporte for Ccp and KatG.²⁹-³²

The inverse correlation between νFe–CO and νC–O frequencies is plotted for a number of heme–CO proteins and model complexes in Figure B.16. The closed conformer of WT DaClD falls high on the νFe–C–νC–O correlation line, which was interpreted as being due to positive charge near or hydrogen bonding to the bound CO. Residues donating H-bonds to the CO on the distal side of the heme enhance π backbonding, weakening the C–O bond and strengthening the Fe–C interaction. This type of strong distal pocket–CO interaction results in points high on the imidazole line (i.e., νFe–C at 520 cm⁻¹ and νC–O at 1935 cm⁻¹).³³ To assess hydrogen bonding to the CO ligand via the distal pocket Arg183, the rR spectra of WT DaClD–CO at pD 5.6 were examined for deuterium effects in both the νFe–C and νC–O regions of the spectra. No deuterium shift was observed for either conformer. Observation of a deuterium isotope effect on νC–O in D₂O would confirm the presence of H-bonding. However, the deuterium isotope shifts for heme–CO complexes are very small, and their absence does not completely rule out the possibility of H- bonding. Therefore, by virtue of the WT closed conformer’s position on the νFe–C–νC–O correlation line, H-bonding could contribute to the interaction between Arg183 and the heme-bound CO. Alternatively, its position on the νFe–C–νC–O correlation line could be due
Figure B.13 Fit of WT DaCld–CO rR data (pH 5.8) determine the Fe–C stretching frequency of the two conformers. Spectra were acquired with 413.1 nm excitation. Original spectrum is in black, fit peaks are in blue, and the simulated spectrum is in red. The simulated difference spectrum was generated by subtraction of the simulated spectra.

Figure B.14 Soret-excited rR spectra of WT Cld–CO as a function of pH.
to electrostatic polarization of the heme HOMO by distal positive charge that resides on the side chain of Arg183, thereby concentrating electron density in the FeCO unit and increasing the structural manifestations of π backbonding. In the open conformer, this distal positive charge is distanced from the CO ligand by the Arg183 side chain reorienting toward the solvent and away from the heme pocket.

The isotope sensitive bands in the rR spectra of the 6c CO reported previously. Changes to spectroscopic heme signatures as a result of storage or sample handling have been reported for CcP and KatG. The inverse correlation between νFe−CO and νC−O frequencies complexes of the Cld mutants have been identified by comparing their spectra obtained with 12CO and 13CO. Their rR spectra are shown in Figure B.15, and their frequencies and 13C isotope shifts are summarized in Figure B.16 and Table F.1 of Appendix E. Spectra of DaCld(R183Q)−CO at pH 6.8 and 10.0 (Figure B.16 and Figure B.17) reveal two isotope sensitive C−O stretching frequencies at 1935 and 1958 cm−1. The DaCld(R183Q)−CO spectra have a broad feature in the low-frequency region where iron−carbon stretching bands of heme−carbonyl complexes are expected. The 12CO−13CO difference spectrum in Figure B.15 exhibits multiple features that can be attributed to isotope sensitivity. The frequencies of these bands and relative populations of the two conformers were determined by peak fitting (Figure B.17). Two νFe−C frequencies were assigned at 511 and 490 cm−1. Like WT, DaCld(R183Q)−CO exhibits open (490 and 1958 cm−1) and closed (511 and 1935 cm−1) conformers; the small variations with pH are attributed to small changes in the populations of these conformers (see Figure B.17). Further, the DaCld(R183Q)−CO conformers fall below their WT counterparts on the νFe−CO−νC−O correlation line. This suggests that the Gln183 is not as polarizing in its interactions with the bound CO, presumably because it lacks the distal positive charge supplied by Arg183 in the WT enzyme. The percentage of the total area of the νFe−C bands assigned to the open and closed conformers also differs between WT (79% open, 21% closed) and R183Q (65% open, 35% closed). Assuming that the relative resonance enhancements of this mode do not change substantially between the WT and R183Q protesin, the closed conformer is more favored in the mutant than in WT DaCld.
Figure B.15. Soret-excited rR spectra of the isotopomers of CO complexes of DaCld Arg183 mutants at pH 6.8. Spectra were acquired with 413.1 nm excitation and 2 mW power at the sample.

Figure B.16 vFe–C–vC–O correlation plot comparing CO complexes of WT and Arg183 Cld mutants with peroxidases and myoglobin. The data used to generate the plot are listed in Table B.3.
Figure B.17 Resonance Raman spectra and fits for the low frequency data for the isotopomers of Cld(R183Q)-CO at (A) pH 6.8 and (B) pH 10.0. The original data is in black; blue indicates the component peaks and red shows the calculated fit.

The Soret-excited rR spectra of DaCld(R183K)-CO (Figure B.15) reveal three isotope sensitive bands at 491, 575, and 1956 cm$^{-1}$. They are assigned to $\nu_{Fe-C}$, $\delta_{FeCO}$, and $\nu_{C-O}$ modes, respectively. These vibrations are not sensitive to pH, as judged by comparison of the DaCld(R183K)-CO spectra at pH 5.5, 6.8, and 10.0. These data indicate that there is a single form of DaCld(R183K)-CO analogous to the open form of WT. The isotopically sensitive bands in the DaCld(R183A)-CO complex are observed at 488, 572, and 1964 cm$^{-1}$ and are assigned to the $\nu_{Fe-C}$, $\delta_{FeCO}$, and $\nu_{C-O}$ vibrations, respectively (Figure B.15). These features report a single form of DaCld- (R183A)-CO. The Fe–CO stretching frequency is the lowest observed for any of the DaCld–CO complexes that have been characterized to date, while the C–O stretching frequency is the highest, consistent with little or no nonbonded distal interaction with the bound CO ligand. Similar to the WT DaCld, the locations of the Arg183 mutants of this mode do not change substantially between the WT and R183Q proteins, the closed conformer is more favored in the mutant than in WT DaCld. The Soret-excited rR spectra of DaCld(R183K)-CO (Figure
B.15) on the νFe−C−νC−O correlation plot are consistent with 6c heme carbonyl complexes in which the heme is coordinated to a proximal histidine. All three Arg183 mutations exhibit a decrease in the observed νFe−C frequency and an increase in the νC−O frequency relative to that of WT. Their positions on the νFe−C−νC−O correlation line relative to WT are consistent with the decrease in dπ−π* backbonding expected with weakening polar interactions between the distal heme pocket and the bound CO ligand.

While the CO complexes of DaCld(R183Q) and WT are able to access two conformers, substitution of Arg183 with lysine or alanine eliminates the closed conformer: only the open form is observed in their rR spectra. For DaCld(R183A), the electrostatic interactions that stabilize the closed WT DaCld−CO conformer (H-bonding and/or charge interaction) are lost. DaCld(R183A)−CO falls very close to Mb(H64A)−CO and Mb(H64L)−CO on the νFe−C−νC−O correlation plot. In these Mb mutants, the distal histidine that normally donates H-bonds to the bound CO has been replaced with a hydrophobic amino acid residue. The diminished π back-bonding in these Mb mutants is reflected in a 17−18 cm$^{-1}$ decrease in their Fe−C stretching frequencies and an increase of 19 cm$^{-1}$ in the C−O stretching frequencies relative to those of WT Mb (Table B.3).$^{34−36}$

The observation of a single DaCld(R183K)−CO conformer with little interaction between the distal pocket and the bound CO was unexpected. Because the charge and protonation state of lysine are similar to that of Arg183, one may predict that two CO conformers analogous to those of the WT enzyme would be formed. Between pH 5.8 and 10, DaCld(R183K) exhibits a single CO complex that falls slightly above the open form of DaCld(R183Q)−CO on the νFe−C−νC−O correlation plot. This corresponds to a weakly interacting distal pocket−CO pair. Lys183 appears flexible in the placement of its side chain. The crystal structure of ferric NdCld(R173K) showed the distal lysine hydrogen bonded to a sulfate ion well above the heme iron and not to the water molecule coordinated to the heme.$^{11}$ The solution characterization of ferric DaCld(R183K) presented above clearly shows that the lysine side chain coordinates to the iron. The lack of a closed form of DaCld−(R183K)−CO suggests that, although the distal pocket structure can reorganize to allow coordination of the Lys183 side chain to the heme iron,
TABLE B.3
COMPARISON OF Fe-CO VIBRATIONAL FREQUENCIES FOR SEVERAL HEME PROTEINS THAT WERE USED TO CONSTRUCT FIGURE B.16 IN THE CHAPTER. THE FREQUENCIES ARE REPORTED FOR pH 6.8 UNLESS OTHERWISE INDICATED. THE FREQUENCIES FOR CLD\textsuperscript{13}CO COMPLEXES ARE IN PARENTHESES.

<table>
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<th>$v_{C-O}$</th>
<th>$\Delta v_{Fe-C-Mut} - \Delta v_{Fe-C-WT}$</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
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<td>1929(1984)</td>
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<tr>
<td>open</td>
<td>494(491)</td>
<td>1954(1908)</td>
<td>-3/+2\textsuperscript{b}</td>
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<tr>
<td>DaCld(R183K) open</td>
<td>491(488)</td>
<td>1956(1914)</td>
<td>-27/+27\textsuperscript{c}</td>
<td>This work</td>
</tr>
<tr>
<td>DaCld(R183Q closed</td>
<td>511(506)</td>
<td>1935(1891)</td>
<td>-7/+6</td>
<td></td>
</tr>
<tr>
<td>open</td>
<td>490(487)</td>
<td>1958(1915)</td>
<td>-3/+4\textsuperscript{a}</td>
<td></td>
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<tr>
<td>DaCld(R183A) open</td>
<td>488(486)</td>
<td>1964(1918)</td>
<td>-6/+10\textsuperscript{b}</td>
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<td></td>
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<tr>
<td>HRP acidic pH</td>
<td>492</td>
<td>1672</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HRP pH 6 form I</td>
<td>539</td>
<td>1906</td>
<td></td>
<td></td>
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<tr>
<td>form II</td>
<td>516</td>
<td>1934</td>
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<td></td>
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<tr>
<td>HRPC alkaline pH</td>
<td>530</td>
<td>1934</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 6</td>
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<td>1941.5</td>
<td>-24/+35.5</td>
<td>(37,38)</td>
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<tr>
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<td>496</td>
<td>1944</td>
<td>-1/+7.5</td>
<td></td>
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<tr>
<td>alkaline pH</td>
<td>525</td>
<td>1924</td>
<td>-14/+18 (form I)</td>
<td></td>
</tr>
<tr>
<td>pH 6-9.5</td>
<td>530</td>
<td>1922</td>
<td>+9/-10 (form II)</td>
<td></td>
</tr>
<tr>
<td>Ccp pH 7 alkaline</td>
<td>503</td>
<td>1948</td>
<td></td>
<td>(29)</td>
</tr>
<tr>
<td>pH</td>
<td>500</td>
<td>1941</td>
<td>-30/+19</td>
<td></td>
</tr>
<tr>
<td>Ccp(R48L) acidic</td>
<td>500</td>
<td>1951</td>
<td>-3/+3</td>
<td>(33)</td>
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<td>1944</td>
<td>-22/+22</td>
<td>(39)</td>
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<tr>
<td>Ccp(R48L) alkaline</td>
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<td>1944</td>
<td></td>
<td></td>
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<tr>
<td>pH 6</td>
<td>512</td>
<td>1944</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SW Mb pH 8.4</td>
<td>507</td>
<td>1947</td>
<td></td>
<td>(40)</td>
</tr>
<tr>
<td>SW Mb pH 7.0</td>
<td>489</td>
<td>1966</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SW Mb pH 2.6</td>
<td>490</td>
<td>1966</td>
<td>-17/+19</td>
<td>(34,35)</td>
</tr>
<tr>
<td>SW Mb(H64A)</td>
<td>449</td>
<td>1966</td>
<td>-18/+19</td>
<td>(34-36)</td>
</tr>
<tr>
<td>SW Mb(H64L)</td>
<td>525</td>
<td>1923</td>
<td></td>
<td>(32)</td>
</tr>
</tbody>
</table>
driving force for H-bond formation with bound CO is not sufficient to promote reorganization of the
distal pocket. Finally, it is interesting to note that the open conformers of DaCld and its Arg183
mutants go down the correlation line in order of decreasing chlorite decomposition activity (WT >
R183K > R183Q > R183A) and decreasing heme pocket polarity. This supports our current
hypothesis that the positive charge in the pocket, rather than H-bonding, provides the most
important structural basis for the enzyme’s chlorite decomposition activity.

Resonance Raman data for the CO complexes of peroxidases and peroxidase mutants
are summarized in Figure B.16 and Table B.3 of Appendix E for comparison with the DaCld–CO
complexes reported here. Heme peroxidases typically have histidine and arginine residues in
their distal heme pockets. The distal interactions in the peroxidases are stronger than those in
DaCld, because their distal His and Arg residues act in concert. For example, comparison of WT
DaCld–CO to the CO complexes of cytochrome c peroxidase (CcP) and horseradish peroxidase
(HRP) shows that the Arg–CO interaction in the more strongly interacting closed DaCld
conformer is still weaker than the distal CO interaction in these peroxidases. At neutral pH, the
CO in the CcP–CO complex is hydrogen bonded to the distal arginine via a water molecule.42
This is a relatively strong interaction with its location on the vFe−C−vC−O correlation line well
above the closed WT DaCld–CO. When the distal histidine in CcP (His52) is replaced with a
nonpolar residue, its heme environment approaches that of closed WT DaCld–CO. Acidic and
basic forms of CcP- (H52L)–CO are observed; the acidic form is slightly higher than the closed WT DaCld–CO form on the correlation line, while the basic form falls between open and closed WT DaCld.\textsuperscript{39} Replacement of the CcP distal arginine generating CcP(R48L) yields a CO complex that is well below closed WT DaCld–CO but still above open WT Cld–CO on the vFe–C–vC–O correlation plot. Two conformers of HRP–CO that fall on the imidazole vFe–C–vC–O correlation line have been reported.\textsuperscript{42} The form highest on the correlation line is attributed to direct interaction of the distal Arg with CO; the second conformer, with its weaker distal interaction, is attributed to interaction between CO and the distal histidine. When the distal histidine is mutated in HRP, its CO complex is still higher on the correlation line than WT DaCld. However, if the distal Arg is removed, the two HRP(R38L)–CO conformers fall between the open and closed conformers of WT DaCld–CO, reflecting the effect of the polar His remaining in the pocket.

\textbf{B.3.10 Affinity of Ferric Mutant Clds for Exogenous Ligands}

The equilibrium affinity of DaCld(R183K), DaCld(R183Q), and DaCld(R183A) for a series of ligands was measured as a function of pH from pH 5 to 10 to assess the effects of the R183 side chain on the stabilities of exogenous ligand complexes with ferric DaCld. The maximal measured values for $K_D$ are reported in Table B.3.

\textbf{TABLE B.4}

\begin{center}
\begin{tabular}{lcccc}
\hline
ligand & WT & R183K & R183Q & R183A \\
\hline
KCN & 4 & 300 ± 20 & 85 ± 6 & 11000 ± 2000 \\
Imidazole & 9.6 ± 0.3 & 18 ± 1 & 27 ± 1 & 21 ± 1 \\
$N_3^-$ & 8.3 ± 0.1 & NB\textsuperscript{a} & NB\textsuperscript{a} & NB\textsuperscript{a} \\
F$^-$ & 15000 ± 1000 & NB\textsuperscript{a} & NB\textsuperscript{a} & NB\textsuperscript{a} \\
\hline
\end{tabular}
\end{center}

\textsuperscript{a}No binding observed optically up to 400 mM added ligand.


**B.3.11 Hydrogen Cyanide**

A representative titration of a mutant protein with hydrogen cyanide is shown for DaCld(R183A) in Figure B.18A. The Soret band shifts from 391 to 420 nm with an isosbestic point at 411 nm. Values of $\Delta A$ were determined from the difference spectra in Figure B.18B and used to generate the plot of $\Delta A$ versus total ligand concentration in Figure B.18C. Hydrogen cyanide ($\text{HCN} \leftrightarrow \text{H}^+ + \text{CN}^-$; $pK_a = 9.1$) binds with high affinity to WT DaCld, with approximately 20- and 75-fold less affinity to DaCld(R183Q) and DaCld(R183K), respectively, and with substantially (close to 3000-fold) diminished affinity for DaCld(R183A) at pH 7. The sigmoidal shape of the DaCld(R183Q) and DaCld(R183A) $pK_a$ versus pH curves for HCN binding (Figure B.19A) is similar to the shape of the curve for binding of HCN to WT Cld, generating similar $pK_a$ values [DaCld(R183Q), $pK_{a1} = 5.8 \pm 0.2$ and $pK_{a2} = 8.6 \pm 0.2$; DaCld(R183A), $pK_{a1} = 6.3 \pm 0.1$ and $pK_{a2} = 8.6 \pm 0.1$; WT, $pK_{a1} = 6.0 \pm 0.7$ and $pK_{a2} = 8.8 \pm 0.76$]. The higher $pK_a$ likely corresponds to the acid dissociation of HCN. The lower turning point was previously assigned to a protein-based deprotonation event. However, it seems more likely that it is not a true $pK_a$ but reflects the pH at which CN$^-$ is fully converted to HCN, particularly as the same turning point is observed for the WT and each mutant. The plateau in the lower-pH region of the plots indicates that neutral HCN enters the distal pocket with appreciable affinity, with binding likely facilitated in the active site by the Lewis acidity of Fe(III), which facilitates deprotonation of HCN. WT DaCld and the Gln and Ala mutants each have a stronger affinity for CN$^-$ than HCN, by 2–2.5 orders of magnitude, in spite of the greatly diminished affinity of the mutants for anionic azide or fluoride (see below). However, the $pK_a$ versus pH curves for DaCld(R183Q) and DaCld(R183A) are shifted $\sim 2.5$ and $\sim 3$ log units lower than that of WT DaCld, respectively. This indicates that the mutants have substantially lower affinity than WT for both CN$^-$ and HCN.

By contrast, the affinity of cyanide for DaCld(R183K) is essentially insensitive to pH (Figure B.19A). Because lysine and hydrogen cyanide have somewhat similar $pK_a$ values (10.5 and 9.3, respectively) and spectroscopic data for DaCld(R183K) suggest that the $pK_a$ for Lys183 in the enzyme is lower than that for free lysine, both of these species could be deprotonated at similar pH values. Over the pH range examined and in the absence of HCN, Lys183 is
deprotonated and forms a stable heme Fe–Lys species. This complex has to be dissociated for the formation of the heme–cyanide complex. At higher pH values, the concentration of cyanide ion in solution increases and the enzyme has a higher affinity for the anion than the hydrogen cyanide. However, the plot of $pK_D$ versus pH may be flat because the energy yield from the increased affinity of the enzyme for cyanide ion is offset by the higher free energy cost of displacing of the heme-bound Lys183 at alkaline pH.

**B.3.12 Imidazole**

The imidazole affinity for DaCld(R183Q) and turning point was previously assigned to a protein-based deprotonation event. However, it seems more likely that it is DaCld(R183A) is substantial and relatively unchanged compared with that for WT DaCld. A curve with one $pK_a$ was found for the plots of $pK_D$ versus pH for imidazole and DaCld(R183Q) and DaCld(R183A) (Figure B.19B). The $pK_{a1}$ of $6.7 \pm 0.1$ and the $pK_{a1}$ of $6.8 \pm 0.1$ appear to correspond to the $pK_a$ values of imidazolium and imidazole ($pK_a = 7$). The amine side chain of DaCld(R183K) forms a strong complex with the ferric heme and must be displaced by any exogenous ligand. Imidazole binds DaCld(R183K) with an affinity similar to that for WT near neutral pH. Above pH 7, the affinity begins to decrease, again suggesting that the energy cost of displacing Lys183 becomes sufficiently large that imidazole cannot effectively compete for the heme iron. Clearly, the heme has a strong affinity for amine and imidazole ligands regardless of the residue at position 183.
Figure B.18 Representative plots illustrating data quality and analysis for \( \text{DaCl}(\text{R183A}) \)-ligand titrations. (A) Titration of KCN at pH 7.8 in a 0.1 M citrate/phosphate buffer. Spectra were measured after addition of cyanide to final concentrations of 0, 24, 48, 163, 272, 377, 478, 574, 770, 959, 92, 1.1, 1.3, 20, 39, and 57 mM. The initial and final spectra are presented as thick black lines, and spectra are corrected for dilution. The inset shows an expanded view of the visible region of the spectrum. (B) Difference plot generated from the data in panel A. (C) Plot of \( \Delta \text{Abs} \) 386 as a function of ligand concentration. The wavelength of maximum absorbance change was used to construct a plot of \( \Delta \text{Abs} \) 386nm versus \([\text{L}]_T\) (total concentration of added ligand, HCN) and fit by least squares regression to an equilibrium isotherm of the form \( \Delta \text{Abs} = \Delta \text{Abs}^\text{max}[\text{L}]_T/(K_\text{D} + [\text{L}]_T) \). The \( K_\text{D} \) in this case was determined to be 553 ± 45 μM.
B.3.13 Anions

The affinity of all of the mutants for the azide and fluoride anions is effectively lost (ligands added at ≤ 400 mM) (Table B.3). Hence, with the exception of CN⁻, Arg183 is essential for stabilizing the binding of the anions examined in this study.

B.3.14 Reduction Potential

The reduction potential for WT DaCld was measured at −23 mV versus the standard hydrogen electrode (SHE), similar to the −21 mV potential of Cld from *Ideonella dechloratans*⁴³.
but very different from that reported for *A. oryzae* (Az) Cld (158 mV)\textsuperscript{10} (Table B.4). A typical data set for the reduction potential measurement of the DaCld Arg183 mutants is shown in Figure B.20 (the plot of the Nernst equation is represented in the inset); the values for the potentials for all three Arg183 mutants are given in Table B.4. Several factors together control the reduction potential of heme proteins, including the electronic characteristics of the axial heme ligand(s), the polarity of the distal environment, and the bonding and non-bonding interactions between the heme and amino acid residues or solvent molecules in the heme vicinity.\textsuperscript{44}

**TABLE B.5**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th><strong>E'</strong> (Fe\textsuperscript{3+}/Fe\textsuperscript{2+}) (V vs. NHE)</th>
<th>Ref</th>
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</thead>
<tbody>
<tr>
<td>WT CcP from <em>S. cerevisiae</em></td>
<td>-0.189</td>
<td>44</td>
</tr>
<tr>
<td>CcP (R48K) from <em>S. cerevisiae</em></td>
<td>-0.186</td>
<td>44</td>
</tr>
<tr>
<td>WT APX from soybean</td>
<td>-0.206</td>
<td>44</td>
</tr>
<tr>
<td>APX (R38K) from soybean</td>
<td>-0.214</td>
<td>44</td>
</tr>
<tr>
<td>HRPC</td>
<td>-0.278</td>
<td>46</td>
</tr>
<tr>
<td>Cld from <em>A. oryzae</em></td>
<td>-0.158</td>
<td>10</td>
</tr>
<tr>
<td>Cld from <em>I. dechloratans</em></td>
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<td>43</td>
</tr>
<tr>
<td>WT Cld from <em>D. aromatica</em></td>
<td>-0.023</td>
<td></td>
</tr>
<tr>
<td>Cld(R183A) <em>D. aromatica</em></td>
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<td></td>
</tr>
<tr>
<td>Cld(R183K) <em>D. aromatica</em></td>
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</tr>
<tr>
<td>Cld(R183Q) <em>D. aromatica</em></td>
<td>-0.034</td>
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</tr>
</tbody>
</table>

\textsuperscript{a}All potentials are referenced to SHE and measured at 25 °C and pH 7. In the case of *DaCl*ds, 100 mM phosphate buffer was used.

In the case of WT DaCld and the Arg183 mutants examined here, their proximal ligation is unperturbed, based on strong similarities among their Fe–His stretching frequencies (vide supra). Thus, differences in their reduction potentials likely reflect mutation-induced changes in the distal pocket. In the simplest analysis, the Fe(III)/Fe(II) potential is expected to be lowered (ferric species stabilized) by the introduction of a negative charge in the distal pocket and increased by a positive charge. At the same time, charged and polar residues can stabilize axial ligands to the iron or distal water molecules, electrostatically stabilizing the ferric state. Hence,
the influence of distal residues on oxidation state is complex. Myoglobin has a distal histidine in an otherwise hydrophobic distal pocket, yielding a potential of 46 mV.\textsuperscript{45} Peroxidases by contrast have a much more polarizing distal environment with a neutral histidine and positively charged arginine. Accordingly, their reduction potentials are considerably lower.\textsuperscript{46} A wide range of reduction potentials have been reported for peroxidases (from \(-0.028\) V for \textit{Mycobacterium tuberculosis} KatG to \(-0.320\) V for cucumber basic peroxidase) in part because of variations in their electronic structures.\textsuperscript{46} As it possesses just a distal arginine and no histidine, \textit{DaCld} has a more hydrophobic pocket with a less electropositive character than typical heme peroxidases. This is in keeping with a measured reduction potential at the less negative end of the peroxidase range. In both \textit{DaCld} and well-studied heme peroxidases, the arginine also serves to stabilize ligands bound to Fe(III) via hydrogen bonding. Hence, although it is positively charged, the arginine can contribute to the overall stability of the ferric state.

The reduction potential for \textit{DaCld}(R183K) was measured at \(-18\) mV (pH 7); this is 5 mV higher than the WT \textit{DaCld} potential. Notably, at this pH, the ferrous and ferric forms of the protein

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure.png}
\caption{Titration of \textit{DaCld} and potassium indigo tetrasulfonate (dye) with xanthine oxidase used in the determination of its redox potential. An anaerobic solution of 2 μM Cld and 2 μM dye in 0.1 M potassium phosphate buffer (pH 7) with 1 μM benzyl viologen and 200 μM xanthine was reduced with an appropriate amount of xanthine oxidase. The spectra shown were recorded at 0, 50, 104, 158, 216, 266, 320, and 376 min. At time zero, the dye and \textit{DaCld} are fully oxidized with maxima at 393 and 592 nm, respectively (---); at 376 min (blue line), they are fully reduced with a maximum at 432 nm for \textit{DaCld}. The inset shows the logarithmic plot of the Nernst equation used to determine the reduction potential. The data were normalized to the concentration of Cld, and the wavelengths of 432 and 416 nm were used to determine the reduction potential via the relationship \(\ln(\frac{E_{\text{ox}}}{E_{\text{red}}}) = 0.73 \times \ln(\frac{\text{Dye}_{\text{ox}}}{\text{Dye}_{\text{red}}}) - 0.88.\)\end{figure}
are both 5cHS, so the change in oxidation state does not bring about a change in coordination. In DaCld- (R183A), which likewise remains 5cHS, the charge of the distal pocket is diminished by replacing the long and positively charged arginine side chain with the short and nonpolar methyl group of alanine. An increase in reduction potential of 19 mV relative to that of WT DaCld was observed, indicating that the ferrous form is relatively more stable in the mutant. The inability of the hydrophobic DaCld(R183A) pocket to stabilize anions, hydrogen-bonded water molecules, or hydroxide at higher pH values would cut off these possible avenues for electrostatically compensating for the higher charge of Fe(III). Finally, in the DaCld(R183Q) mutant, the reduction potential is 11 mV more negative than in the WT protein. DaCld(R183Q) is a mixture of 5cHS and 6cHS (presumably water-bound) heme, and coordination number affects reduction potential. The mixed coordination apparently supports a slightly more peroxidase-like potential, stabilizing the ferric state. The reduction potentials for several peroxidase distal Arg mutants have been reported. For the ascorbate peroxidase mutant, APX(R38K) (ER38K − EWT = −8 mV) and CcP(R48K) (ER48K − Ewt = 3 mV), only small changes in the potential relative to that of WT are observed. On the basis of electrostatic considerations alone, no change in reduction potential is expected when the charged arginine is replaced with a similarly charged lysine. The effect of replacing the distal arginine with nonpolar residues in peroxidases is variable. In CcP(R48L), APX(R38A), and HRP-C(R38L), the mutations shift the reduction potentials to a substantial degree but in opposite directions: for CcP(R48L), ER48L − EWT = 25 mV; for APX(R38A), ER38A − EWT = −33 mV; and for HRP-C(R38L), ER38L − EWT = −35 mV. Loss of charged and polar residues in the distal pocket, its effect on the imidazolate character of the proximal histidine, and the effects of both on the potential all appear to depend on the peroxidase structure. Notably, the DaCld distal pocket does not contain hydrogen-bonding partners for the distal arginine, nor is this residue connected by hydrogen bonds to the proximal pocket.

B.4 Discussion

The Clds from perchlorate-respiring bacteria catalyze a biologically unusual O−O bond-forming reaction via a heme cofactor with great efficiency in a structurally unique active site. We
have sought to understand the role of the residue at the position of Arg183 in dictating the chemical, spectroscopic, and electrostatic properties of DaCld through its substitution with neutral (Gln), positively charged (Lys), and hydrophobic (Ala) residues and the use of CO as a probe. These data are important for understanding both the rapid O₂ forming reaction catalyzed by DaCld and the likely reactivities of other proteins in the large and phylogenetically diverse Cld protein family, most of which are predicted to have a glutamine, serine, or alanine residue at the position corresponding to Arg183 of DaCld.⁷ Surprisingly, all of the mutants, including DaCld(R183A) that lacks any polar distal pocket residue, are catalytically active toward chlorite. The steady state turnover number (kcat) decreases only 6-fold in DaCld(R183K), close to 20-fold in DaCld(R183Q), and 65-fold in DaCld(R183A). The Km and kcat/Km values, by contrast, are much more strongly affected: kcat/Km decreases by 3–4 orders of magnitude in all three mutants. This suggests that Arg183 is particularly important for efficient formation of the Michaelis complex. Consistent with this observation, Arg183 is critical for stabilizing bound anions, including hydroxide. WT DaCld forms a hydroxide complex at moderately alkaline pH (pKₐ=8.7).⁶ The three Arg183 mutants examined here, by contrast, exhibit no analogous alkaline transition. By the same token, with the exception of the π-acceptor CN−, none of the anionic ligands binds with measurable affinity to DaCld(R183Q), DaCld(R183K), or DaCld(R183A), and the affinity of the Fe(III) for CN− in the mutants is substantially diminished. The distal arginine forms hydrogen bonds with bound anions in several Cld crystal structures (vide infra). R183A offers no such hydrogen bonding capabilities, and R183Q is likely an insufficient H-bond donor to stabilize complexes with OH⁻, F⁻, and N₃⁻. On the other hand, the primary amine of R183K, rather than acting as a hydrogen bond donor, itself binds to the ferric heme over the entire pH range for which the protein is stable. The Lewis acidity of the ferric iron and the hydrophobicity of the distal pocket likely assist in deprotonating R183K (pKₐ of free lysine = 10.53). Similar to the R183K side chain, imidazole binds avidly to the ferric heme. It does so regardless of the identity of the residue at position 183, and with a pKₐ in the plot of pKₐ versus pH corresponding to the imidazolium/imidazole deprotonation. This suggests that the WT and mutant distal pockets cannot accommodate exogenous cations, but the endogenous R183K can be deprotonated at the
heme. The WT enzyme has been shown to have several pH-efficient formation of the Michaelis complex. Consistent with this observation, Arg183 is critical for stabilizing bound anions, including hydroxide. WT DaClid forms a hydroxide complex at moderately alkaline pH (pKₐ = 8.7). The three Arg183 mutants examined here, by contrast, exhibit no analogous alkaline transition. By the same token, with the exception of the π-acceptor CN⁻, none of the anionic ligands binds with measurable affinity to DaClid(R183Q), DaClid(R183K), or DaClid(R183A), and the affinity of the Fe(III) for CN⁻ in the mutants is substantially diminished. The distal arginine forms hydrogen bonds with bound anions in several Clid crystal structures (vide infra). R183A offers no such hydrogen bonding capabilities, and R183Q is likely an insufficient H-bond donor to stabilize complexes with OH⁻, F⁻, and N₃⁻. On the other dependent features in the pH range below the alkaline transition (pKₐ = 8.7). These could involve the polar-protic Arg183, either directly or indirectly, particularly as its side chain has a clear role in stabilizing Fe(III)-bound anions. This nonbonded interaction could be either a hydrogen bond or a salt bridge. The pH profiles of the steady state kinetic parameters for catalytic chlorite decomposition (k₉ and k₉/K₉) exhibit a number of turning points, indicating acid–base interconversions. Points at pH 4.8 and 10.3 define the limits of the enzyme’s activity. Outside this range, irreversible inactivation of the enzyme occurs. The UV–visible spectra suggest that inactivation correlates with unfolding of the enzyme and dissociation of the heme from the protein. A pKₐ at pH 6.5 was also observed and was hypothesized to arise from acid dissociation of the Arg183 guanidinium group, largely because it is the only active site residue with a protic side chain. By virtue of its proximity to the ferric iron center and its otherwise hydrophobic neighbors, we proposed that Arg183 could have a pKₐ of 6.5. Similarly low pKₐ values and acid–base roles for protein-bound arginine have been proposed in a number of other enzymes, including fumarate reductase, polysaccharide lyase, inosine 5’-monophosphate dehydrogenase, and photo-system II. Mutation of Arg183 to Gln results in a loss of the transition in the plots of log k₉ and log k₉/K₉ versus pH observed at pH 6.5 for WT DaClid. This suggests that this pKₐ indeed involves Arg183, either directly or indirectly. Support for an indirect effect of pH on Arg183 comes from the rR spectra of the DaClid(R183) mutants (discussed below) and structural data. Recent crystal structures of the homologous Clids

327
from Candidatus *Nitrospira defluvii* (NdCld), *Nitrosbacter winogradskyi* (NwCld), and the DaCld enzyme show two accessible distal arginine conformers. In the structures of WT NdCld−CN and NdCld−ImH, the distal arginine is rotated away from the exogenous ligand such that it is not positioned over the porphyrin plane.\(^\text{11}\) In the structure of NwCld, the distal arginine is similarly rotated away from the porphyrin plane and outside of the distal pocket, forming a hydrogen bond to a nearby asparagine residue. The heme iron binds to a water molecule that forms part of a hydrogen-bonded water chain that appears to gain access to the active site when the arginine is rotated out. By contrast, in the DaCld−nitrito structure (pH 9), the Arg183 side chain is hydrogen bonded to the ligand and directly above the porphyrin plane.\(^\text{8}\) A similar arginine conformer was observed in the structure of the thiocyanate complex of the Cld from *A. oryzae*.\(^\text{10}\) The two types of side chain conformers are illustrated in **Figure B.21**. The repositioning of the distal arginine side chain in the NwCld and NdCld structures clearly displaces positive charge from the heme pocket. On the basis of the steady state kinetics results for the WT and mutant DaClds described above, the displacement of charge from the distal pocket appears to reduce anion affinity and reaction efficiency.

The observed pKa at 6.5 for WT DaCld could be due to an analogous structural shift in the flexible Arg183 side chain. In the closed conformer, the Arg183 side chain is over the porphyrin plane and points into the distal pocket, as in DaCld− ONO\(^-\). In the open conformer, the Arg183 side chain points away from the porphyrin plane and is oriented outside the distal pocket, as seen in NdCld−CN, NdCld−ImH, and NwCld−OH\(_2^\). The mechanism by which pH could bias the populations of the open and closed conformers in DaCld is uncertain. However, a conformer model accounts for the pH profiles of \(k_{\text{cat}}\) and \(k_{\text{cat}}/K_m\) via pH-dependent modulation of positive charge within the nonbonded interaction distance of the bound substrate. It also suggests a dynamic opening and closing of the heme pocket by a residue that can modulate access of the substrate to the heme, stabilize the enzyme− substrate complex, and possibly facilitate release of the product chloride ion.
Figure B.21 Comparison of open (top and middle) and closed (bottom) conformers hypothesized to modulate DaCld activity. The proteins are drawn as cyan cartoons with important residues depicted as sticks and elements designated by color: green for carbon, red for oxygen, blue for nitrogen, and orange for phosphorus. The iron atom is shown as a rust-colored sphere. In each case, hydrogen bonding interactions that are important for maintaining the distal arginine conformation are depicted as dotted lines. The top panel shows the active site of the Cld from *N. winogradskyi* (PDB entry 3QPI) with water as its axial ligand. This water molecule is at the center of a hydrogen bonding network that connects the iron to the distal arginine in its open conformation. The distal arginine is also hydrogen bonded to a glutamine. The middle panel shows the active site of the cyano–Cld complex from *Ca. N. defluvii* (PDB entry 3NN2). The Fe-bound cyanide ligand is connected to the distal arginine via a hydrogen bonding network containing a molecule of solvent (polyethylene glycol), again in the open conformation. The side chain is further hydrogen bonded to two backbone carbonyls. The bottom panel shows the DaCld–nitrito complex (PDB entry 3Q09). The distal arginine assumes its closed conformation. These figures were generated using PyMOL (http://www.pymol.org).
The pH-dependent resonance Raman spectra of the ferric WT and mutant DaClds are consistent with a conformer model. The rR spectra of the WT ferric enzyme clearly showed formation of the heme−OH complex with a pKa of 8.7 but were independent of the pH 6.5 transition reported by the enzyme activity. The insensitivity of the rR features to the lower pKa is consistent with the associated acid–base chemistry occurring at a site remote from the heme iron. The FeCO vibrational signature of the heme carbonyl could be sensitive to the pKa at 6.5 if the transition results in conformational reorganization of the Arg183 side chain. The closed and open Arg183 conformations described above would be expected to impose distinct H-bonding and/or electrostatic environments on the terminal oxygen atom of coordinated CO. Under the influence of a positive charge or a H-bond donor, the closed conformer should fall high on the νFe−C−νC−O backbonding correlation line. By contrast, the open conformer would be expected to fall low and to the right, a position consistent with minimal nonbonded interaction between CO and the distal pocket. The two conformers seen in the rR spectra of WT DaCld−Co and DaCld(R183Q)−CO complexes are consistent with these expectations. Because glutamine does not contain a titratable proton, the observation of both closed and open active site forms for this mutant supports the conformer model rather than a distal acid/base model. The single conformer (open form) observed for the carbonyl complexes of DaCld(R183K) and DaCld(R183A) indicates that Lys and Ala cannot close the heme site in a manner analogous to that of Arg or Gln. In the case of Ala, the reason is most likely the lack of side chain bulk; for Lys, the reason is not apparent. Finally, the pH independence of the WT−CO complexes reported here is curious and suggests that the heme carbonyl is perhaps not an accurate mimic of the enzyme–substrate complex. Indeed, unlike the ferric–chlorite complex, the CO complex contains ferrous iron and a charge-neutral diatomic axial ligand. It is possible that, as a consequence, it cannot drive the pH-dependent reorientation of the Arg183 side chain in the same manner or to the same extent as the ferric–anion complex. Like Arg183 in the WT enzyme, Gln183 can access the open and closed conformers. We hypothesize that the closed form stabilizes coordination of a water molecule to the resting heme, giving rise to the observed mixed 5c/6cHS states. Given the lack of a clear LS heme signature in the spectra, even at high pH, we conclude that the pKa for formation...
of the heme hydroxide in this mutant is >10. This is consistent with the lack of an observed alkaline transition in the UV-vis titration of this mutant. The distribution of open and closed conformers in DaCld(R183Q)−CO is slightly pH-dependent, suggesting that the shorter Gln183 amide side chain is more sensitive than the guanidinium group of Arg183 to pH-driven reorientation.

The amine side chain of DaCld(R183K) does not interact with the distal pocket but instead binds to the heme Fe itself. Both the UV-visible and rR data support coordination of the primary amine side chain of Lys183 over the entire pH range in which the ferric enzyme is soluble. Consistent with this observation, the pH profiles of \( k_{cat} \) and \( k_{cat}/K_m \) are featureless and offset to lower values than their WT counterparts (Figure B.2). The ferrous enzyme contains a 5cHS heme under acidic conditions, but under basic conditions, a 6cLS ferrous complex is formed. The 6cLS ferrous Cld is probably the result of coordination of Lys183 to the Fe(II). The difference in pKa for coordination of the Lys to the heme iron from \(<6.8\) for the ferric enzyme to \(~9\) for the ferrous enzyme can be attributed to the weaker Lewis acidity of Fe(II) relative to Fe(III). Binding of a distal lysine to the heme has precedence in the R48K distal pocket mutant of CcP. UV-visible and rR data indicate that ferric CcP(R48K) is 6cLS with a pKa for conversion to a 5cHS species below pH 5.2.\(^{51}\) The 6cLS species that predominates at neutral and alkaline pH values is attributed to coordination of the Lys side chain. Ferrous CcP(R48K) also undergoes a conversion from 5cHS to 6cLS ferrous heme between pH 7.0 and 8.533 in a manner similar to that of ferrous DaCld(R183K) (Figure B.12). Conformational mobility in the distal Arg likewise has precedence in heme peroxidases. In the 1.2 Å crystal structure of resting CcP, the distal arginine (Arg48) has two positions: one in which Arg48 is “out” toward the heme propionates and a second in which Arg48 is positioned “in” toward the heme iron.\(^{52}\) These out and in positions of Arg48 in the CcP crystal structure are proposed to be analogous to the open and closed conformers, respectively, observed here for ferric DaCld. In CcP Compound I, the in position of Arg48 is observed exclusively, allowing it to donate a hydrogen bond to the ferryl oxygen atom.\(^{54}\) The distal arginine is found in a similar in position in Compound I of HRP.\(^{53}\) Observation of the closed conformer of DaCld in solution under acidic conditions where the enzyme is most active is
consistent with the closed conformer or in position of Arg183 stabilizing its substrate complex and possibly directing the reactivities of its intermediates, in both the reactions with chlorite and H$_2$O$_2$. What do these results say about the likely roles of other enzymes in the chlorite dismutase family, given that they are known to have a diversity of residues at position 183, including Gln? The reactivity of the mutants is of note here. Arg183 could conceivably be necessary for polarizing and therefore promoting heterolytic cleavage of the Cl−O bond, or subsequently stabilizing the resulting Compound I−hypochlorite pair. If bond cleavage were instead homolytic, resulting in neutral chlorine monoxide and Compound II, roles for Arg in positioning the substrate and stabilizing Compound II could be proposed. However, all three mutants, including the alanine mutant, evolve O$_2$. In other words, Cl−O bond cleavage and subsequent stabilization of intermediates and leaving groups can occur in DaCld containing a distal Gln, Lys, or Ala and no other polar residues. Many peroxidases are known to cleave the chlorite Cl−O bond when using chlorite as a “shunt” reagent for the generation of reactive Compound I. However, unlike DaCld and the R183 mutants described here, peroxidases do not efficiently promote the recombination of the hypochlorite− Compound I or chlorine monoxide−Compound II pair. The hydrophobic and sterically confined nature of the Cld distal pocket appears to be important for protecting the reactive intermediates and promoting their reaction by rebound. (Notably, no uncoupling of chlorite consumption and O$_2$ generation was observed in any of the mutants studied here.) It is therefore possible that other Cld proteins could have some O$_2$ evolving capabilities even if they lack a distal Arg, though their efficiency is expected to be diminished. It was likewise interesting to observe no catalase and only weak peroxidase reactivity in any of the DaCld mutants. While such a lack of reactivity is consistent with the lack of a distal His or Asp base in the active site, it raises further questions about the likely biological and chemical roles of other Cld family proteins, which are often still described in genome annotations as peroxidases. In addition, the dye decolorizing peroxidases (DyPs) and EfeB proteins, which share a monomer structure with the Clds and collectively make up the CDE (Chlorite dismutase, Dye decolorizing peroxidase, EfeB) superfamily, possess both distal Asp and Arg residues. However, their hemes are flipped 180° relative to the heme in the Cld proteins, putting their distal arginine within close hydrogen bonding.
distance of one of the propionic acid side chains. Hence, the distal Arg in these proteins is conformationally constrained by this hydrogen bond. Moderate to substantial reactivities of DyP and EfeB proteins toward \( \text{H}_2\text{O}_2 \) have been reported.\textsuperscript{7,50} What do these results suggest about design elements that could be important in synthetic catalysts for \( \text{O}_2 \) generation or, focusing specifically on the O–O bond-forming step, water reduction? Site isolation and the proximity of the two reactive O atoms would appear to be critical for promoting O–O bond formation. It is achieved in photosystem II by having both the high-valent metal–oxo species and the attacking nucleophilic or radical oxygen species bound to adjacent manganese and calcium sites on the manganese cluster. In \( \text{DaCld} \), the high-valent metal–oxo species and the very reactive leaving group (which could be a nucleophilic hypochlorite or a chlorine monoxide) are generated concurrently in a confined and chemically inert pocket, promoting their recombination. Notably, efforts aimed at reacting a premade \( \text{DaCld} \) Compound I with exogenously added hypochlorite have to date not resulted in generation of \( \text{O}_2 \).\textsuperscript{1}

**B.5 Summary and Conclusions**

R183 occupies a key position structurally and serves as the unique charged or polar entity in the \( \text{DaCld} \) distal pocket. Mutations at this position do not completely eliminate \( \text{O}_2 \)-evolving reactivity with chlorite. A positively charged residue at position 183 (Arg or Lys) is important for maintaining a high \( k_{\text{cat}} \), though the largest effect for all substitutions (Lys, Gln, and Ala) was a substantially increased value for \( K_m \). Consistent with this observation, R183 appears to be essential for maintaining the affinity of the ferric site for anions, including the hydroxide-bound alkaline form. Resonance Raman data are consistent with the R183 side chain accessing a closed conformation that maximizes positive charge in the vicinity of the heme iron, supporting the most efficient reactivity in the steady state. The ferric heme is somewhat less oxidizing than in a typical heme peroxidase, in either WT \( \text{DaCld} \) or the mutants, and the distal pocket less electrostatically polarizing. Rotation of the R183 side chain away from the porphyrin plane is expected to further reduce the amount of positive charge in the distal pocket. The fact that the WT and R183 mutants all successfully produce \( \text{O}_2 \) suggests an especially important role for the
pocket in promoting the rebound reaction between the high-valent Fe−O intermediate and the leaving group. It also suggests that Cld family proteins could still retain some chlorite decomposition activity, even though most appear to lack a distal Arg. This reactivity could be limited by their ability to stabilize a bound anionic substrate and/or leaving group.

B.6 Acknowledgements

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B.7 References


