EXPLORING THE ROLE OF THE AXIAL LIGAND IN THIOLATE-LIGATED HEME ENZYMES: SPECTROSCOPY OF HIGH-VALENT IRON INTERMEDIATES OF CHLOROPEROXIDASE

A Thesis in Chemistry
by
Kari Lea Lunder Stone

© 2008 Kari Lea Lunder Stone

Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

May 2008
The thesis of Kari Lea Lunder Stone was reviewed and approved* by the following:

Michael T. Green  
Assistant Professor of Chemistry  
Thesis Advisor  
Chair of Committee

J. Martin Bollinger, Jr.  
Associate Professor of Biochemistry and Molecular Biology  
Associate Professor of Chemistry

Carsten Krebs  
Associate Professor of Biochemistry and Molecular Biology  
Associate Professor of Chemistry

John H. Golbeck  
Professor of Biochemistry and Biophysics  
Professor of Chemistry

Ayusman Sen  
Professor of Chemistry  
Head of the Department of Chemistry

*Signatures are on file in the Graduate School
Cytochromes P450 (P450) are thiolate-ligated heme enzymes that play critical roles in many biological processes, including metabolism of xenobiotics. Thiolate-ligated heme enzymes are unique among heme proteins because they catalyze oxygen insertion reactions. Recently, an X-ray absorption spectroscopy study of the ferryl form of chloroperoxidase (CPO-II) suggested a novel role for thiolate-ligation. It revealed that CPO-II was not an authentic ferryl (iron(IV)oxo), but rather a protonated ferryl with a long Fe-O bond length of 1.82 Å (typically ferryls have Fe-O bond lengths near 1.65 Å). This finding has important implications for P450 hydroxylation chemistry where a basic ferryl may provide the driving force to perform hydrogen atom abstraction. The hydroxylation mechanism for P450’s describes two high-valent iron intermediates. These intermediates are highly reactive. Due to almost identical first coordination spheres of chloroperoxidase (CPO) and P450’s, CPO has been utilized as a model to investigate high-valent iron intermediates of P450 reaction chemistry.

To gain insight into the reactive intermediate of P450 enzymatic catalysis, X-ray absorption measurements of chloroperoxidase compound I were performed. CPO-I is an authentic ferryl species (a doubly bonded Fe-O bond with a length of 1.65 Å) and strong axial-ligand interactions result in a long Fe-S bond length of 2.48 Å. This is the first unambiguous structural characterization of a thiolate-ligated compound I species and provides a first glimpse into the reactive intermediate of P450 reaction chemistry.

We have also examined the protonation state of CPO-II with the use of multiple spectroscopies: Mössbauer spectroscopy coupled with density functional calculations, X-
ray absorption spectroscopy, ENDOR spectroscopy and resonance Raman spectroscopy.

A Badger’s rule analysis of the EXAFS-determined Fe-O bond length of CPO-II (1.82 Å) affords an Fe-O stretching frequency of $v_{\text{Badger}} = 563 \text{ cm}^{-1}$. A deuterium and $^{18}$O sensitive stretching frequency, $v_{\text{Fe-OH}} = 565 \text{ cm}^{-1}$, was located by resonance Raman spectroscopy in remarkable agreement to what is predicted from Badger’s rule.

All evidence, to date, suggests that the basic ferryls are a general feature of thiolate-ligated heme enzymes. This provides a reasonable explanation of why Nature utilizes the electron donating thiolate ligand to perform some very demanding oxidizing reactions.
# TABLE OF CONTENTS

LIST OF FIGURES.............................................................................................................. viii

ACKNOWLEDGEMENTS...................................................................................................... xv

Chapter 1  Introduction.................................................................................................... 1
  1.1 P450’s: an Introduction ................................................................................ 1
  1.1.1 Catalytic Cycle of P450 Enzymes ...................................................... 4
  1.2 Chloroperoxidase: an Introduction .................................................................. 6
    1.2.1 Structure of Chloroperoxidase......................................................... 7
    1.2.2 Reactions of Chloroperoxidase....................................................... 11
  1.3 Chloroperoxidase as a Model for P450 Reaction Chemistry ......................... 12
  1.4 Oxygen Rebound Mechanism ..................................................................... 14
  1.5 Role of the Thiolate Ligand ...................................................................... 16
    1.5.1 Compound II of Chloroperoxidase is Basic ...................................... 17
    1.5.2 Importance of Ferryl pKa.................................................................. 20
  1.6 Parameterization of Badger’s Rule for Fe-O Bonds..................................... 22
  1.7 Utilization of Mössbauer Spectroscopy Coupled with Density Functional
      Calculations to Determine Ferryl Protonation States ................................... 23
  1.8 Topics to be Addressed in this Thesis ..................................................... 24
  1.9 References ................................................................................................. 25

Chapter 2   Structure of Thiolate-Ligated Compound I ............................................. 36
  2.1 Abstract ......................................................................................................... 36
  2.2 Introduction .................................................................................................. 37
  2.3 Results and Discussion ................................................................................ 39
    2.3.1 Structure of Thiolate-Ligated Compound I......................................... 41
    2.3.2 Effect of Photoreduction .................................................................. 46
    2.3.3 Discussion ......................................................................................... 49
  2.4 Conclusion ................................................................................................... 52
  2.5 References ................................................................................................... 53

Chapter 3  Chloroperoxidase Compound II is Basic: Implications for P450
      Hydroxylation Chemistry .................................................................................. 58
  3.1 Abstract ........................................................................................................ 59
  3.2 Introduction .................................................................................................. 60
  3.3 Results and Discussion ................................................................................. 65
    3.3.1 Calculations of Mössbauer Parameters ............................................ 65
    3.3.2 Mössbauer Spectroscopy of Chloroperoxidase Compound II ............ 69
    3.3.3 Cryogenic Reduction of CPO-I ....................................................... 74
    3.3.4 Preparation of CPO-II under Different Conditions ......................... 86
3.4 Chemical Modification of Histidine Residues in CPO

3.4.1 Electron Nuclear Double Resonance of Cryoreduced CPO-II

3.4.2 Resonance Raman Spectroscopy of CPO-II

3.4.3 Preparation and Characterization of a Single Fe(IV) Species in CPO-II

3.4.4 Identifying the Minor Ferryl Species in a CPO-II

3.4.5 Generation of a Single Fe(IV)-Hydroxide in CPO-II: $m$-CPBA or Acetone?

3.4.6 The Role of the Acetone in Producing a Single Ferryl Species in CPO-II

3.5 Conclusion

3.6 References

Chapter 4  Summary and Conclusions

4.1 Thiolate-ligated Compound I

4.2 Observation of Two Ferryl Species in Chloroperoxidase Compound II

4.3 Resonance Raman Spectroscopy Provides Direct Evidence of an Iron(IV)-Hydroxide in Chloroperoxidase Compound II

4.4 Identification of the Minor Ferryl Species in Chloroperoxidase Compound II

4.5 Conclusion

4.6 References

Chapter 5  Materials and Methods

5.1 Preparation and Purification of Chloroperoxidase and Isotopically-Labeled Chloroperoxidase

5.2 Preparation of CPO-I for X-Ray Absorption Spectroscopy

5.3 X-ray Absorption Data Collection and Analysis of CPO-I

5.4 Photoreduction Analysis of CPO-I

5.5 Preparation of $^{57}$FeCl$_3$

5.6 Synthesis of $^{33}$SO$_4^{2-}$

5.7 Synthesis of Alkyl Peroxides

5.8 Total Peroxide Assay

5.9 Hydrogen Peroxide Assay

5.10 Synthesis of Perbenzoic Acids

5.11 Synthesis of $^{18}$O Peracetic Acid

5.12 Preparation of CPO-II for Mössbauer Spectroscopy

5.13 Preparation of CPO-I for Cryoreduction

5.14 Cryogenic Reduction of CPO-I and Annealing

5.15 Mössbauer Spectroscopy

5.16 Computational Methods

5.17 Preparation of CPO-II for Resonance Raman Spectroscopy

5.18 Resonance Raman Spectroscopy
5.19 Photo-reduction/Degradation Analysis ....................................................... 155
5.20 Cryoreduction of CPO-II for ENDOR Spectroscopy ................................. 155
5.21 Preparation of CPO-II for Mössbauer and Resonance Raman ................. 156
5.22 Chemical Modification of Chloroperoxidase .......................................... 157
5.23 Chlorination Assay for Chloroperoxidase ............................................. 157
5.24 Preparation of CPO-II for X-ray Absorption Spectroscopy ...................... 158
5.25 Preparation of CPO-I for Cryoreduction, Mössbauer, and Resonance Raman Experiments ................................................................. 158
5.26 References ............................................................................................. 159
LIST OF FIGURES

1.1: Ferric protoporphyrin IX. This structure is found in many heme-containing enzymes, including P450 enzymes and some peroxidases.................................3

1.2: Catalytic cycle of P450 enzymes. Upon substrate binding, water is released from low-spin ferric enzyme (a) to produce five-coordinated, high-spin ferric enzyme (b). (b) is easily reduced to ferrous enzyme (c). Dioxygen binds which is in equilibrium with a ferric superoxide complex (d). This complex is reduced to form a ferric-peroxy complex (e) which is easily protonated to form the ferric-hydroperoxy complex (f). Another protonation event affords heterolytic cleavage of the O-O bond to produce an Fe(IV)O-radical species (g). ....................................................................................................................5

1.3: Proton shuttle of chloroperoxidase. Asp-106, His-105, and Glu-183 delivers protons to the active site.................................................................10

1.4: Oxygen rebound mechanism. Compound I abstracts a hydrogen atom from substrate to form a carbon radical and a protonated ferryl. Radical recombination with the protonated ferryl produces hydroxylated product and regenerates the ferric state.................................................................15

1.5: Oxygen rebound mechanism. Compound I abstracts a hydrogen atom from substrate to produce a protonated compound II (the rebound intermediate). Radical recombination leaves hydroxylated product and regenerates the ferric resting state. Distances shown in black are from density functional calculations and distances in red are EXAFS-derived bond distances.................19

1.6: The relationship of ferryl pKa and redox potential of compound I in performing hydrogen atom abstraction of cyclohexane [D(O-H) ~ 99 kcal/mol]...........................................................................................................21

2.1: A representative EPR spectrum of CPO-I sample used for X-ray absorption data collection. EPR spectra for ferric CPO (A) and CPO-I (B) containing ~9% ferric CPO are shown. Spectrum recorded at x-band (9.368 GHz) at 20 K with 1.027 mW of power and 4.58 G modulation amplitude. All spectra were recorded at the same conditions for comparison.........................................................40

2.2: Fe-K X-ray absorption edges for ferric CPO (black, dotted line) and CPO-I (red line). Inset displays a 10x magnification of the pre-edge region.............43

2.3: EXAFS spectra and Fourier Transforms of (A) raw data and (B) Fourier filtered data of the first two shells. Blacks lines show experimental data and red lines are best fits to the data. Black lines in the Fourier transform show
region used for Fourier filtering. The fits shown here were obtained over the region \( k = 3-15 \, \text{Å}^{-1} \). .................................................................44

2.4: Fe-K X-ray absorption edge of CPO-I upon continuous scans. The first scan is shown in red. .................................................................47

2.5: EXAFS-determined results for ferric CPO, CPO-II and CPO-I. The Fe-O bond lengths show typical coordination chemistry where the Fe-O bond shortens with increasing oxidation state. The Fe-O bond shortens from 2.11 Å to 1.65 Å. The Fe-S bond shortens the opposite behavior lengthening from 2.24 Å in ferric CPO to 2.4 Å in CPO-II to 2.48 Å in CPO-I. The Fe-S bond is the longest heme alkanethiolate bond reported. This illustrates the strong interactions between the donating thiolate ligand and the anionic oxo ligand. ....51

3.1: Oxygen rebound mechanism of Cytochrome P450. Compound I abstracts a hydrogen atom from substrate forming the rebound intermediate. This figure illustrates that (except for the presence of the hydrocarbon radical) the P450 rebound intermediate and a thiolate-ligated compound II are equivalent. Distances in red are from X-ray absorption measurements on CPO-II and distances in black are from density functional calculations. .........................62

3.2: Large active-site model of the protonated form of chloroperoxidase compound II. ........................................................................67

3.3: Mössbauer spectrum of CPO-II pH 6.5, 250 ms quench time. Similar results were obtained with a 28 ms quench. Spectrum A shows raw data; solid line is best fit to two quadrupole doublets, \((\delta = 0.10 \, \text{mm/s}, \Delta E_Q = 2.06 \, \text{mm/s})\) and \((\delta = 0.11 \, \text{mm/s}, \Delta E_Q = 1.59 \, \text{mm/s})\). Spectrum B was obtained from a Fourier transform analysis of the data, which removes the linewidth of the \(^{57}\text{Co}(\text{Rh})\) source and highlights the presence of two distinct species. Parameters were obtained by fitting raw data. .................................................................70

3.4: Mössbauer spectra (hash marks) of CPO-II recorded at 4.2 K in externally applied magnetic fields of 4 T and 7 T oriented parallel to the \( \gamma \)-beam. The solid lines are simulations using the following parameters and assuming the slow relaxation limit. Simulations were performed assuming \( S = 1, D = +23 \, \text{cm}^{-1}, E/D = 0.0, g = (2.1, 2.1, 2.0), \eta = 0, \) and \( A/gN\beta_N = (-20, -20, -7) \, \text{T} \) for both species and using the Mössbauer parameters (\( \delta \) and \( \Delta E_Q \)) listed in Table 3.1. The red (blue) lines above the data are the individual contributions of the major (minor) component .........................................................73

3.5: Cryoreduction and annealing of CPO-I. CPO-I containing glycerol is prepared and exposed to \( \gamma \)-irradiation at 77 K, which reduces CPO-I forming an unprotonated CPO-II. This species is annealed at 193 K to allow for proton delivery. .................................................................75
3.6: Cryoreduction of ferric CPO. A) Mössbauer spectrum of ferric CPO. Solid line is simulated using a spin Hamiltonian formalism in the slow relaxation limit with the following parameters: \( S = 1/2, \mathbf{g} = (1.84, 2.26, 2.63), \delta = 0.30 \text{ mm/s}, \Delta E_Q = 2.9 \text{ mm/s}, \eta = -2, \) and \( A/g_N B_N = (-46.0, +7.9, +28.4) \text{T}. \) B) Mössbauer spectrum of cryoreduced ferric CPO. Deconvolution of the spectrum reveals 51% ferric CPO remains. C) Mössbauer spectrum of ferrous species generated upon cryoreduction of ferric CPO. The raw data is fit to one quadrupole doublet (solid line) with parameters typical for a high-spin ferrous heme: \( \delta = 0.85 \text{ mm/s} \) and \( \Delta E_Q = 2.49 \text{ mm/s}. \)

3.7: Annealing of CPO-I. A) Mössbauer spectrum of CPO-I. Deconvolution of this spectrum reveals 10% ferric CPO (solid line). CPO-I is simulated using a spin Hamiltonian formalism in the slow relaxation limit with parameters nearly identical to those previously published: \( \text{Seff} = 1/2, \mathbf{g} = (2.0, 2.0, 2.0), \delta = 0.15 \text{ mm/s}, \Delta E_Q = 1.02 \text{ mm/s}, \eta = 0, \) and \( A/g_N B_N = (-30.5, -30.5, -6.0) \text{T} \) (red, solid line). B) Mössbauer spectrum of CPO-I annealed at -80°C for 30 minutes. Ferric CPO increases (solid line) from 10% to 74%. C) Difference spectrum (B-A). Features pointing upward indicate species that are decaying and features pointing downward indicate species that are growing in.

3.8: Cryoreduction of CPO-I. A) Mössbauer spectrum of CPO-I before cryoreduction. The spectrum contains 22% ferric CPO (solid line) and 78% CPO-I. B) Mössbauer spectrum of cryoreduced CPO-I. C) Difference spectrum (B-A) highlighting the fraction of sample that is reduced during cryoreduction. Adding back 53% of CPO-I (blue line) to the difference spectrum results in the red line. A sharp quadrupole doublet (a) grows in that is identical to cryoreduced ferric CPO. D) After removing appropriate contributions of ferric CPO, and cryoreduced ferric CPO, and CPO-I; the Mössbauer spectrum of cryoreduced CPO-I remains that is fit to one quadrupole doublet with the following parameters: \( \delta = 0.12 \text{ mm/s} \) and \( \Delta E_Q = 1.41 \text{ mm/s}. \)

3.9: Annealing of cryoreduced CPO-I. A) Mössbauer spectrum of cryoreduced CPO-I annealed at -80°C for 30 minutes. The Mössbauer spectrum of cryoreduced CPO-I before (solid line) and after (hash marks) annealing. The amount of CPO-I decreases (‘b’), the quadrupole doublet of the ferryl generated by cryoreduction (red bracket) decreases in intensity. The intensity of ferric CPO increases (‘c’) and a new quadrupole doublet grows in (blue bracket). B) Difference spectrum (Figure 3.9A - Figure 3.8D) highlights the new ferryl species generated during annealing. C) Removing appropriate amounts of other components (33% ferric CPO, 4% CPO-I, and 18% cryoreduced ferric CPO) results in the Mössbauer spectrum of annealed cryoreduced CPO-I.
3.10: A) The Mössbauer spectrum for the species generated during the annealing process. This spectrum is similar to the spectrum in Figure 3.9C but it was generated from spectra collected over a narrower range of Doppler velocities. The solid line shows a minority species (6% of the total sample) with $\delta = 0.29$ mm/s, $\Delta E_Q = 2.30$ mm/s, which is consistent with a ferrous-oxy complex. B) Mössbauer spectrum of cryoreduced and annealed CPO-I obtained by removing contributions from the minority species represented by the solid line in (A). The quadrupole doublets shown in (B) represent 29% ($\delta = 0.10$ mm/s, $\Delta E_Q = 2.08$ mm/s) and 10% ($\delta = 0.11$ mm/s, $\Delta E_Q = 1.60$ mm/s) of the total sample.

3.11: Mössbauer spectrum of CPO-II, pH 4.5, recorded at 4.2K in a 40 mT magnetic field applied parallel to the $\gamma$ beam. The spectrum shows raw data; solid line is best fit to two quadrupole doublets. The parameters for the protonated ferryl ($\Delta E_Q=2.08$ mm/s, $\delta=0.10$ mm/s, ~66%) and the ferryl ($\Delta E_Q=1.58$ mm/s, $\delta=0.11$ mm/s, ~34%) are similar to parameters measured for CPO-II at pH 6.5.

3.12: Mössbauer spectrum of CPO-II prepared with hydrogen peroxide as the oxidant. The spectrum shows raw data; solid line is best fit to two quadrupole doublets. A) The sample contained 65% ferric CPO and was removed from the raw data. B) The remaining absorption showed two quadrupole doublets: ($\Delta E_Q=2.08$ mm/s, $\delta=0.10$ mm/s, 69%) and ($\Delta E_Q=1.58$ mm/s, $\delta=0.11$ mm/s, 31%). These parameters are similar to parameters measured for CPO-II with peracetic acid as the oxidant.

3.13: Mössbauer spectrum of CPO-II, pH 6.5, prepared with $p$-phenolsulfonic acid as the reductant. Spectra were collected over a range of Doppler velocities of approximately ± 4 mm/s (left panel) or ± 8 mm/s (right panel). Removal of 20% of ferric CPO from the raw data (A) yields the spectra shown in (B). Removal of 35% of CPO-I yields the spectra shown in (C). These spectra have been simulated with two quadrupole doublets with the following parameters: $\delta(1) = 0.10$ mm/s, $\Delta E_Q(1) = 2.07$ mm/s, 32% of total intensity, and $\delta(2) = 0.10$ mm/s, $\Delta E_Q(2) = 1.58$ mm/s, 13% of total intensity. These parameters and the ratio of the relative intensities are similar to those observed for CPO-II with ascorbate as the reductant.

3.15: EPR spectra of ferric CPO (blue) CPO-II (red) and cryoreduced CPO-II (black). CPO-II samples containing 20% glycerol are $\gamma$-irradiated at 77K reducing the EPR-silent Fe(IV)-OH center to an EPR-active Fe(III)-OH center. The Fe(III)-OH species generated by this process is EPR active, and
ENDOR measurements reveal a very strongly coupled (13.4 MHz) exchangeable proton.

3.17: X-band ENDOR spectra of alkaline myoglobin and cryoreduced CPO-II in H2O (red) and D2O (blue) centered at the proton Larmor frequency. * indicates hydroxide resonances in H2O (red) samples.

3.18: Low-frequency resonance Raman data (454.5 nm excitation), oxidation state marker band (\(v_4\)), and EPR spectra of ferric CPO and CPO-II samples (pH 6.5). EPR spectra indicated that CPO-II samples contained < 5% ferric enzyme. The radical signal near \(g = 2\) is attributed to oxidized ascorbate. Arrows highlight movement of the Fe(IV)-OH stretch with isotopic substitution. *, labels the 466 nm line of the argon ion laser. No movement of the oxidation state marker band (\(v_4\)) was observed during data collection. EPR measurements taken after sample irradiation revealed no detectable change in sample composition.

3.19: Top: Overlay of resonance Raman spectra (454.5 nm excitation) of H2O/H2O2 (blue), D2O/H2O2 (black) and H2O/H218O2 (red) preparations of CPO-II with a narrower range of frequencies. Bottom: Difference spectra reveal changes in \(v_{Fe(IV)-OH}\) upon deuterium substitution (black) and 18O substitution (red).

3.20: Mössbauer spectra of CPO-II generated with peracetic acid (A) or meta-chloroperbenzoic acid in 10% acetone (B) and ascorbic acid at pH 6.5. Spectrum A shows the raw data and the solid black line is best fit to two quadrupole doublets (\(\Delta E_Q = 2.05\) mm/s, \(\delta = 0.10\) mm/s, red line) and (\(\Delta E_Q = 1.62\) mm/s, \(\delta = 0.11\) mm/s, blue line), 72% and 28% of the total absorption, respectively. Spectrum B shows raw data and solid line is fit to one quadrupole doublet (\(\Delta E_Q = 2.05\) mm/s, \(\delta = 0.10\) mm/s, red line).

3.21: Low-frequency resonance Raman data (457.9 nm excitation) and Mössbauer spectra of CPO-II prepared with meta-chloroperbenzoic acid in 10% acetone, pH 6.5, in H2O and D2O. Data collection for resonance Raman and Mössbauer was performed on the same sample. Left: overlay of low-frequency resonance Raman of m-CPBA generated CPO-II in 10% acetone reacted in H2O (top, red) and D2O (top, blue) and their difference spectra (bottom, black). Right: Mössbauer spectra of m-CPBA generated CPO-II in 10% acetone prepared in H2O (top) and D2O (bottom).

3.23: Cryogenic reduction of CPO-I. CPO-I in the presence of t-butanol is reduced by \(\gamma\)-irradiation at 77 K. A) Mössbauer spectrum of cryoreduced CPO-I (top), after contributions of CPO-I are removed a quadrupole doublet is highlighted with a blue line (bottom). B) Low-frequency resonance Raman spectra (457.9 nm excitation) of \(^{57}\)Fe-enriched CPO-I (red), \(^{57}\)Fe-
enriched cryoreduced CPO-I (blue) and $^{54}$Fe-enriched CPO-I (black). The difference spectrum of CPO-I and cryoreduced CPO-I is represented by a black, dotted line.

3.24: Overlay of low-frequency resonance Raman spectra (457.9 nm excitation) of CPO-II generated with (A) peracetic acid or (B) m-CPBA/acetone in H$_2$O (red) and D$_2$O (blue). Ascorbate was the reductant in both cases. The difference spectra are shown in black. Arrows indicate high-energy shoulders found in standard preparations of CPO-II.

3.25: Low-frequency resonance Raman spectrum of a standard preparation of CPO-II (457.9 nm excitation). The spectra of CPO-II prepared with peracetic acid is shown in (a) and (b), fit with two Gaussians at 561 cm$^{-1}$ (red line) and 565 cm$^{-1}$ (blue line), and one Gaussian, respectively. The residuals are displayed with the lower black solid line. Black dotted lines represent peaks that are not associated with Fe-OH stretching and were held constant during fitting procedures.

3.27: Mössbauer spectra of CPO-II prepared with m-CPBA in (A) 10% acetone and (B) 10% t-butanol. Their difference spectrum (C) is best fit to one quadrupole doublet with the following Mössbauer parameters: $\Delta E_Q=1.61$ mm/s and $\delta=0.11$ mm/s.

3.28: Mössbauer spectra of CPO-II prepared with peracetic acid (A), m-CPBA in 10% t-butanol (B) and their difference spectrum (C).

3.30: Mössbauer spectrum of CPO-II prepared with H$_2$O$_2$ with a 300x excess of ascorbic acid. The spectrum shows raw data and the solid black line is best fit to two quadrupole doublets ($\Delta E_Q=2.06$ mm/s, $\delta=0.10$ mm/s, red line) and ($\Delta E_Q=1.59$ mm/s, $\delta=0.11$ mm/s, blue line), which is 60% and 40% of the total absorption, respectively.
LIST OF TABLES

1.1: Spectroscopic and structural comparisons of chloroperoxidase and P450’s. The table shows X-ray absorption spectroscopic data where N = coordination number and R = bond distance and UV/visible absorption data for CPO and P450CAM. .................................................................13

2.1: EXAFS fitting results for chloroperoxidase compound I. Raw (A) and Fourier-filtered (B) data were fit over the region k = 3-15 Å⁻¹. Coordination number N, interatomic distance R (Å), mean-square deviation in R (the Debye-Waller factor) σ² (Å²), and the threshold energy shift E₀ (eV). The values in parentheses are estimated standard deviations obtained from the diagonal elements of the covariance matrix. Empirically, EXAFS uncertainties are ±0.02 Å for R and ±20% for N and σ². The fit-error is defined as \((\sum k^6(\chi_{\text{exptl}} - \chi_{\text{calc}})^2/\sum k^6\chi_{\text{exptl}}^2)^{1/2}\). Fits shown in bold typeface represent the best fit obtained for a sample. Alternative fits with different coordination numbers are also shown. Coordination numbers, N, were constrained during fits. ......................................................................................45

2.2: EXAFS fitting results for chloroperoxidase compound I upon consecutive scans. Raw data were fit over the region k = 3-15 Å⁻¹. Interatomic distance R (Å), mean-square deviation in R (the Debye-Waller factor) σ² (Å²), and the threshold energy shift E₀ (eV). The values in parentheses are estimated standard deviations obtained from the diagonal elements of the covariance matrix. Coordination numbers, N, were constrained for all fits to 4 Fe-N, 1 Fe-S and 1 Fe-O. Empirically, EXAFS uncertainties are ±0.02 Å for R and ±20% for N and σ². The fit-error is defined as \((\sum k^6(\chi_{\text{exptl}} - \chi_{\text{calc}})^2/\sum k^6\chi_{\text{exptl}}^2)^{1/2}\). ............................................................................................48

3.1: Calculated and experimental Mössbauer parameters for chloroperoxidase. Porphine models contained a methyl-mercaptate axial ligand. Constrained optimizations used the ferric crystal structure as a starting point. ......................68

3.2: Mössbauer parameters of 6-coordinate ferryl porphyrin complexes. .................71

3.3: EXAFS fitting results for meta-chloroperbenzoic acid/10% acetone generated chloroperoxidase compound II.................................................................111
ACKNOWLEDGEMENTS

I first would like to acknowledge my research advisor, Professor Michael T. Green. He has afforded me countless opportunities that have been invaluable to my research career. Not only has he been a valuable mentor, but he has also been a friend. He has enabled me to grow as a scientist and I truly appreciate the opportunity to work in his lab. I will forever be grateful that I was able to work with such a truly outstanding scientist. Many people have continued to support me along my educational journey. Thanks to the members of my Ph.D. committee, Professor J. Martin Bollinger, Jr., Professor Carsten Krebs, and Professor John Golbeck. I appreciate all of their support and advice throughout my graduate career. It has been a truly invaluable experience to be surrounded by such wonderful scientists. I would also like to thank my high school chemistry teacher, Ruel Eneboe, who opened my eyes to chemistry and encouraged me to continue in the field. After high school, I attended Augustana College where I interacted with many superb scientists. I am especially grateful to Professor Arlen Viste. He continues to support me in my career even today. After college I went on to graduate school at Penn State where I worked in the laboratory of Professor Richard Koerner. Richard was a brilliant scientist and I certainly keep with me all of the skills and knowledge that I learned working with him. He will certainly be missed. I was blessed to have been able to work with him for a short while. I also thank my labmate, Rachel Behan. She has been my best friend throughout graduate school. We have been through a lot together here at Penn State. She and I have been a team and I will cherish the times that we had, both scientific and non-scientific. I also want to thank other people at Penn
State that have been good friends to me: Pooja Aggarwal, Dan Welna, Lee Hoffart, Brian Gilmartin, and Andrew Wollacott. I also acknowledge my family (Richard Lunder, Sharon Lunder, Kim Lunder, and Kristi Paulsen); their loving support has made my educational journey a fruitful one. My mother has been my role model all of my life and her constant encouragement has made it possible for me to achieve my goals. My father has been the one that has always pushed me to be my best and would never let me settle for less. My sisters have offered me support and friendship throughout my life and they continue to be my support system. I also would like to thank the most important person in my life, David Stone, his love and support has been limitless. He has encouraged me to continue on through some difficult times and I will forever be grateful to him.
Chapter 1

Introduction

Thiolate-ligated heme proteins play critical roles in many physiological processes, including metabolism of xenobiotics and neurotransmission.\textsuperscript{1-6} Significance of thiolate-ligated heme proteins stems not only from their obvious biological importance but also from a general interest to harness their synthetic potential because they catalyze the insertion of oxygen into a variety of organic substrates with a high degree of regio- and stereo-selectivity.\textsuperscript{7} Thiolate-ligated heme enzymes are unique among oxidative heme proteins because they catalyze the insertion of an oxygen atom into alkanes. It has been shown in nonenzymatic systems that oxygen transfer reactivity scales with the reduction potentials of the metal-oxo center.\textsuperscript{8,9} Therefore, Nature’s choice of the electron donating thiolate ligand to perform some of the most difficult oxidative chemistry is surprising since thiolate-ligation has been shown to lower redox potentials several hundred millivolts.\textsuperscript{10} The role of the axial ligand in thiolate-ligated heme enzymes in performing oxygen transfer reactions will be explored.

1.1 P450’s: an Introduction

Cytochromes P450 (P450) are ubiquitous in nature and have been isolated in mammalian tissues,\textsuperscript{1,6} plants,\textsuperscript{11} insects\textsuperscript{12} and bacteria,\textsuperscript{13} among others. P450’s are
hydroxylating enzymes that perform vital roles in the synthesis and metabolism of many biologically important compounds and xenobiotics.\textsuperscript{1, 2, 6} For instance, the human enzyme, CYP2B6, has been implicated in the metabolism of artemisinin, which is employed to treat resistant strains of malaria, and pethidine (commonly referred to as Demerol), which is a powerful pain reliever, among other drug targets.\textsuperscript{1} Some P450’s are highly specific in the kinds of substrates they will oxidize, while human CYP3A4 metabolizes almost 50% of the pharmaceuticals currently on the market.\textsuperscript{6}

These enzymes are Nature’s detoxifiers, but they can also exhibit detrimental effects on human health in cases where metabolism products have toxic or carcinogenic effects.\textsuperscript{14} Human CYP2A6 catalyzes the metabolic activation of nitrosamines (found in cigarette smoke), which are potent carcinogens once they have been hydroxylated.\textsuperscript{15}

P450’s contain a ferric protoporphyrin IX moiety, shown in Figure 1.1, with a cysteinate axial ligand that is stabilized by at least two hydrogen bonds donated from backbone amides.\textsuperscript{16} The active sites of P450’s are found to be considerably more hydrophobic in nature than peroxidases and catalases. There are no obvious acid/base catalyst residues close to the heme iron. Instead, P450’s utilize a network of hydrophilic side chains that create a water chain to deliver protons to the active site.\textsuperscript{16}

P450’s perform a diverse set of oxidative reactions: some examples include aliphatic and aromatic hydroxylation,\textsuperscript{17} epoxidation,\textsuperscript{18} N-dealkylation,\textsuperscript{19, 20} O-dealkylation,\textsuperscript{21} and sulfoxidation.\textsuperscript{18}
1.1: Ferric protoporphyrin IX. This structure is found in many heme-containing enzymes, including P450 enzymes and some peroxidases.
1.1.1 Catalytic Cycle of P450 Enzymes

The catalytic cycle of P450 enzymes is shown in Figure 1.2. This mechanism demonstrates how P450 enzymes perform oxidative chemistry derived from dioxygen. Resting state ferric P450 is low-spin with a water molecule taking the 6th coordination position (a). Upon substrate binding, water is released forming high-spin, five-coordinate ferric (b). The high-spin ferric P450 has a more positive redox potential, which is easily reduced to the ferrous state (c). High-spin ferrous is susceptible to ligand binding where carbon monoxide can act as an inhibitor forming a stable ferrous-CO complex, or dioxygen binds forming a ferrous-dioxygen complex, which resembles a ferric-superoxide spectroscopically (d). Injection of a second electron produces the ferric-peroxo complex (e) that is easily protonated to form the ferric-hydroperoxo complex (f). A second protonation event affords heterolytic cleavage of the O-O bond forming the Fe(IV)O-radical species (g). This intermediate is highly reactive and little spectroscopic evidence is available for this species.
1.2: Catalytic cycle of P450 enzymes. Upon substrate binding, water is released from low-spin ferric enzyme (a) to produce five-coordinated, high-spin ferric enzyme (b). (b) is easily reduced to ferrous enzyme (c). Dioxygen binds which is in equilibrium with a ferric superoxide complex (d). This complex is reduced to form a ferric-peroxo complex (e) which is easily protonated to form the ferric-hydroperoxo complex (f). Another protonation event affords heterolytic cleavage of the O-O bond to produce an Fe(IV)O-radical species (g).
1.2 Chloroperoxidase: an Introduction

Clutterbuck and coworkers first discovered chloroperoxidase (CPO) in 1940 in which an enzyme in the marine fungus Caldariomyces fumago was shown to chlorinate β-ketoadipic acid. For two decades no further research was published on CPO. In 1959, Lowell Hager and coworkers began an extensive research career investigating many aspects of CPO and other peroxidases. The isolated chlorinating enzyme was found to be a monomeric protein that contains a ferric heme moiety with a molecular weight of ~ 45 kDa. CPO resembles other heme peroxidases in molecular weight (~35 - 50 kDa) and extent of glycosylation.

A vigorous research effort was undertaken to characterize the active site of this unusual enzyme. The ferrous-CO complex of CPO displays an unusually red-shifted UV/visible spectrum for a heme enzyme with a soret at 446 nm, indicative of thiolate ligation. A collection of electron paramagnetic resonance spectroscopy, Mössbauer spectroscopy, resonance Raman spectroscopy, magnetic circular dichroism, X-ray absorption spectroscopy, and X-ray crystallography have confirmed thiolate ligation to the heme iron.

The main function of CPO is to catalyze the hydrogen peroxide-dependent chlorination of cyclopentanedione during the biosynthesis of the antibiotic caldariomycin. Additionally, CPO catalyzes bromination and iodination of a variety of substrates. In the absence of substrate CPO catalyzes the disproportionation of...
hydrogen peroxide. CPO also functions as a classical peroxidase. The versatile chemistry catalyzed by CPO makes it one of the most diverse enzymes known.

1.2.1 Structure of Chloroperoxidase

CPO’s distal heme cavity resembles the active site of a peroxidase. Most notably a proton shuttle, shown in Figure 1.3, is contained in the distal cavity that includes Asp-106, His-105, and Glu-183. It is Glu-183 that presumably acts as an acid/base catalyst that facilitates compound I formation in CPO. This active site architecture differs from P450 enzymes which contain a markedly more hydrophobic distal cavity.

CPO is unique among peroxidases due to a glutamate residue positioned in close proximity to the heme iron that facilitates O-O bond cleavage in contrast to other peroxidases that utilize a histidine or arginine residue. The choice of a glutamate residue may provide insight into the diverse chemistry CPO performs. When Glu-183 is mutated and replaced with a histine residue, chlorination activity is reduced 85%. CPO displays the highest activity at low pH presumably due to the high redox potential that is required to oxidize a chloride anion. This may explain the requirement for a glutamate residue rather than a histidine residue in the active site of CPO.

The ability of CPO to chlorinate substrate was found to be dependent on an essential histidine residue from chemical modification of histidine residues by reaction with diethylpyrocarbonate. NMR measurements of CPO reveal the presence of an exchangeable proton attributable to a histidine residue 8 Å away from the iron center. An examination of the X-ray crystal structure identifies this amino acid as His-105.
is likely that the “essential” histidine residue is His-105 due to the fact it is contained in
the distal active site of CPO. Ideally, the ability to generate mutants of CPO would offer
insight into its structure/function relationships of this unusual enzyme. However, efforts
to generate mutants of CPO have been largely unsuccessful or have been met with low
yields.44,48,49 Two studies utilized Aspergillus niger as a vehicle to produce recombinant
CPO and described the production of an E183H mutant. The low yield of recombinant
CPO makes it difficult to obtain amounts necessary for spectroscopy.

Due to the high quality X-ray crystal structure of CPO, researchers were able to
identify fourteen glycosylation sites and twenty-one different kinds of sugar molecules.40
25-30% of the molecular weight of CPO comes from glycosylation. The role of
glycosylation is unknown; although, it has been shown that when glycosylation sites are
removed from CPO, the enzyme functions normally.44

Interestingly, the X-ray crystal structure of CPO revealed the presence of a Mn\textsuperscript{+2}
ion bound to a heme propionate.40 The function/role, if any, of the Mn\textsuperscript{+2} ion is unknown.
CPO can be produced with or without manganese depending on growth conditions;
however, once the manganese ion is bound it cannot be removed.50 Also, if CPO folds in
the absence of manganese ion the high affinity manganese-binding site remains vacant
and is not accessible to Mn\textsuperscript{+2}.47 The manganese ions have to be present upon maturation
processing of the protein for binding to CPO to occur. NMR, UV/visible absorption
spectroscopy, and electron paramagnetic resonance have supported these conclusions.

Ferric CPO is five-coordinate and high-spin at room temperature, while a
temperature-dependent spin transition is observed by Mössbauer spectroscopy.34 An
electron paramagnetic resonance (EPR) spectrum of ferric CPO at 1.6 K is essentially all
low spin with g-values of 2.61, 2.26, and 1.83.\textsuperscript{33} Electron nuclear double resonance (ENDOR) measurements of ferric CPO reveal that a water molecule holds the sixth coordination site,\textsuperscript{51} while the X-ray crystal structure at ambient temperature measured a water molecule 3.4 Å away from the heme iron which is too distant to be a ligand.\textsuperscript{40, 51} These studies show that the temperature-dependent spin transition in CPO is due to a water molecule in the distal pocket binding to the ferric center. P450’s also have a water molecule bound to the sixth coordination position making the resting enzyme low-spin.
1.3: Proton shuttle of chloroperoxidase. Asp-106, His-105, and Glu-183 delivers protons to the active site.
1.2.2 Reactions of Chloroperoxidase

CPO performs four different types of reactions: chlorination,\textsuperscript{30, 42, 43, 52, 53} dismutation of hydrogen peroxide,\textsuperscript{43} peroxidase reactions,\textsuperscript{43} and monooxygenase reactions.\textsuperscript{54-57}

CPO catalyzes the chlorination of substrate with the addition of hydrogen peroxide and Cl\textsuperscript{−} ion. Horseradish peroxidase has the ability to chlorinate substrate but only with the use of NaClO\textsubscript{2} rather than Cl\textsuperscript{−} ion.\textsuperscript{58} Chlorination by CPO is proposed to proceed through compound I and an Fe(III)-OCl intermediate.\textsuperscript{53} The steady-state kinetics of chlorination of monochlorodimedone in the presence of hydrogen peroxide and Cl\textsuperscript{−} ion has been investigated extensively.\textsuperscript{52} In CPO-catalyzed chlorination reactions, compound I forms initially followed by an unknown species that resembles compound II by UV/vis spectroscopy (proposed to be the chlorinating intermediate).\textsuperscript{52} External diffusion of HOCl was suggested to be the chlorinating agent in CPO-catalyzed chlorinations, but later investigations showed that chlorination proceeded at the active site of the enzyme presumably through an enzyme-bound chlorination intermediate.\textsuperscript{52, 53, 58}

A particularly demanding task of heme enzymes is hydrocarbon hydroxylation. Recently, it was discovered that CPO could perform these kinds of reactions, although not equal to the oxidative power as P450 enzymes. For instance, CPO catalyzes benzylic hydroxylation (C-H bond strengths \textasciitilde 85-93 kcal/mol),\textsuperscript{54} while some P450’s catalyze the hydroxylation of the relatively inert cyclohexane (~99 kcal/mol).\textsuperscript{59}
1.3 Chloroperoxidase as a Model for P450 Reaction Chemistry

Spectroscopic properties of heme enzymes are modulated by a number of physical properties, the most important feature is the identity of the fifth ligand to the heme iron. CPO and P450 both contain a thiolate ligand; therefore, they are expected to have very similar spectroscopic properties. The spectroscopic similarities between CPO and P450 are clearly shown in Table 1.1. 

Table 1.1 includes spectroscopic values for complexes of P450 and CPO from X-ray absorption and UV/visible absorption spectroscopies. CPO and P450 both display a characteristic 450 nm peak by UV/vis spectroscopy (specifically 445 nm for CPO and 448 nm for P450CAM) for the reduced CO complex. They are also structurally similar. For example, an X-ray absorption study determined the structure for the oxy complexes of CPO and P450CAM. This EXAFS study put the Fe-S bond length at 2.37 Å and the Fe-O bond length for oxyCPO and oxyP450CAM at 1.77 Å and 1.78 Å, respectively. With striking spectroscopic similarities, CPO has become a valuable model system to investigate high-valent intermediates pertinent to P450 enzymatic catalysis.
1.1: Spectroscopic and structural comparisons of chloroperoxidase and P450’s. The table shows X-ray absorption spectroscopic data where N = coordination number and R = bond distance and UV/visible absorption data for CPO and P450CAM.

<table>
<thead>
<tr>
<th></th>
<th>Fe-S</th>
<th>Fe-O(C)(^a)</th>
<th>Fe-N</th>
<th>(\lambda_{\text{max}})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>R</td>
<td>N</td>
<td>R</td>
</tr>
<tr>
<td><strong>Low spin Fe(III)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPO-H(_2)O</td>
<td>1.0</td>
<td>2.24</td>
<td>5.0</td>
<td>2.02</td>
</tr>
<tr>
<td>P450(_{\text{CAM}})-H(_2)O</td>
<td>1.0</td>
<td>2.20</td>
<td>5.0</td>
<td>2.02</td>
</tr>
<tr>
<td><strong>High spin Fe(III)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPO</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P450- substrate bound</td>
<td>1.0</td>
<td>2.28</td>
<td>4.0</td>
<td>2.04</td>
</tr>
<tr>
<td><strong>Low spin Fe(II)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPO</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P450(_{\text{CAM}})-CO</td>
<td>1.0</td>
<td>2.33</td>
<td>1.2</td>
<td>1.72</td>
</tr>
<tr>
<td>CPO- (_2)O (_2)</td>
<td>1.4</td>
<td>2.37</td>
<td>1.3</td>
<td>1.77</td>
</tr>
<tr>
<td>P450(_{\text{CAM}})-O(_2)</td>
<td>1.3</td>
<td>2.37</td>
<td>1.1</td>
<td>1.78</td>
</tr>
<tr>
<td><strong>High spin Fe(II)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPO- ferrous</td>
<td>0.6</td>
<td>2.34</td>
<td>3.0</td>
<td>2.08</td>
</tr>
<tr>
<td><strong>High Valant</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPO-I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P450-I(_{\text{CAM}})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP119-I(^b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Fe bound to carbon in the CO complexes

\(^b\) Compound I was generated by reaction of meta-chloroperoxybenzoic acid with P450. P450-I’s have been observed by rapid scan stopped-flow spectrophotometry, but in low yields.
1.4 Oxygen Rebound Mechanism

The commonly accepted mechanism for hydroxylation of P450 enzymes is called the oxygen rebound mechanism (shown in Figure 1.4).\textsuperscript{69, 70} Compound I abstracts a hydrogen atom from substrate to form a protonated ferryl species and a substrate radical. Radical recombination with the protonated ferryl forms hydroxylated product and regenerates the ferric resting state. A large amount of experimental and theoretical research supports the oxygen rebound mechanism as the operative mechanism in P450 chemistry.\textsuperscript{17, 28, 69-72} For instance, experimentally measured kinetic isotope effects support a hydrogen atom abstraction mechanism for hydroxylation ($k_H/k_D \sim 11-14$).\textsuperscript{73}

Further evidence for the oxygen rebound mechanism came from recent work by Brian Hoffman and coworkers. They employed $\gamma$-irradiation of the reduced oxyP450\textsubscript{CAM} complex at 77 K to initiate steps in the P450 catalytic cycle.\textsuperscript{28, 74} They describe the generation of the end-on peroxo ferriheme that converts to the hydroperoxo complex upon annealing. Further annealing at 200 K produced the product 5-exo-hydroxycamphor. Their results are significant because they demonstrated for the first time the ability to trap heme-peroxo intermediates in the P450 catalytic cycle. Through deuterium substitution, their observations provide evidence that the P450 reaction cycle (at least in P450\textsubscript{CAM}) proceeds through an iron-oxo pathway, rather than through a hydroperoxo insertion pathway. These observations support the commonly accepted oxygen rebound mechanism, which requires the participation of compound I.
1.4: Oxygen rebound mechanism. Compound I abstracts a hydrogen atom from substrate to form a carbon radical and a protonated ferryl. Radical recombination with the protonated ferryl produces hydroxylated product and regenerates the ferric state.
1.5 Role of the Thiolate Ligand

Controversy continues on the role of thiolate ligand in P450 reaction chemistry. Since thiolate-ligated heme proteins are unique in their ability to perform oxygen transfer reactions it has been suggested that the thiolate may be playing a vital role in driving their chemistry.\textsuperscript{22} Dawson et al. suggested that the thiolate ligand may assist in dioxygen activation. They proposed that the electron donating ability of the thiolate might push electron density into the trans position weakening the O-O bond whereby facilitating heterolytic cleavage and forming compound I.\textsuperscript{23}

Experimental evidence for the electron donating ability of the thiolate ligand came from a direct comparison of the measured pKa of 1-propanethiol bound to ferric P450\textsubscript{CAM} and ferric myoglobin.\textsuperscript{75} The measured pKa of the thiol was substantially lower when complexed with ferric myoglobin (< 4.0 vs. 6.7). This study showed quantitatively that a ligand bound to P450\textsubscript{CAM} is more basic than when the same ligand is bound to ferric myoglobin thus demonstrating the electron donating ability of the thiolate. This observation lends support to the hypothesis that the thiolate ligand is directly involved in facilitating O-O bond cleavage.
1.5.1 Compound II of Chloroperoxidase is Basic

Recently an EXAFS report determined that the ferryl form of CPO (CPO-II) is protonated at pH 6.5. This study revealed a longer than expected Fe-O bond length of 1.82 Å (typically a doubly bonded iron(IV)-oxo bond length is near 1.65 Å), but this value was in good agreement with a density functional calculation of a thiolate-ligated Fe(IV)-OH heme unit (1.81 Å). This finding has important implications for P450 hydroxylation chemistry. In the oxygen rebound mechanism, an Fe(IV)-oxo radical species abstracts a hydrogen atom from substrate to form a protonated ferryl species, which subsequently hydroxylates the substrate radical. The ability of metal-oxos to perform hydrogen abstraction has been shown to scale with the strength of the O-H bond that is formed upon hydrogen atom abstraction. For heme enzymes, this energy depends on the redox potential of compound I and the pKa of the ferryl species, compound II. Equation 1.1 highlights the importance of the ferryl pKa to perform hydrogen atom abstraction.

\[
D(O-H) = 23.06 \times E_{\text{cmpd-I}}^{0} + 1.37 \times pK_{a_{\text{cmpd-II}}} + 57\pm2 \text{ (kcal/mol)}
\]

The ability of P450’s to perform hydroxylation chemistry is enhanced by the basic ferryl of compound II. The pKa of CPO-II was estimated to be at least 95% protonated at pH 7 due to an unchanging UV/visible absorption spectrum throughout the enzyme’s pH stability (~ 3 – 7), which puts the pKa at > 8.2. EXAFS-measured bond distances of CPO-II and density functional calculations of high-valent forms of a thiolate-ligated
porphine are shown in Figure 1.5. Distances shown in black are from density functional calculations while distances shown in red are from EXAFS measurements.

Lending support to this theory is that the ferryl stretching frequency of CPO-II had not been identified. Three separate reports utilized resonance Raman spectroscopy to characterize high-valent intermediates of CPO.\textsuperscript{78-80} An \textsuperscript{18}O sensitive band in CPO-II was not located in any of these reports. A possibility remained that the ferryl oxygen was exchanging with bulk water, but even upon reaction of ferric CPO with H\textsubscript{2}\textsuperscript{18}O\textsubscript{2} in H\textsubscript{2}\textsuperscript{18}O, an \textsuperscript{18}O sensitive band was not located between 655 cm\textsuperscript{-1} – 875 cm\textsuperscript{-1}.\textsuperscript{79} The researchers speculated that the Fe-O resonance was not enhanced.
1.5: Oxygen rebound mechanism. Compound I abstracts a hydrogen atom from substrate to produce a protonated compound II (the rebound intermediate). Radical recombination leaves hydroxylated product and regenerates the ferric resting state. Distances shown in black are from density functional calculations and distances in red are EXAFS-derived bond distances.
1.5.2 Importance of Ferryl pKa

Recently, other (non-thiolate) compound II’s have been reported to contain basic ferryls determined from X-ray crystal structures. Catalase compound II, horseradish peroxidase compound II, myoglobin compound II, and cytochrome c peroxidase compound ES have been reported to have Fe-O bond lengths of 1.87 Å, 1.84 Å, 1.92 Å, and 1.87 Å, respectively. These reports are in conflict with EXAFS and resonance Raman experiments that generally yield shorter Fe-O bonds. Can these long bonds in X-ray crystal structures be associated with photoreduction of the high-valent iron center? The source of these long Fe-O bonds is unclear, but it is well-known that powerful synchrotron radiation can produce potent reducing equivalents in the form of solvated electrons. A clear outlier in this debate is CPO-II. The Fe-O bond of CPO-II has been measured by EXAFS spectroscopy to be 1.82 Å, while the Fe-O stretching frequency is curiously missing.

The ability of P450’s to abstract hydrogen relies, in part, on the ferryl pKa. If this hypothesis is correct then basic ferryls must be a general and unique feature of thiolate-ligated heme enzymes. The ability of the ferryl pKa to increase the driving force for hydrogen atom abstraction is displayed in Figure 1.6. It is known that certain P450’s can hydroxylate cyclohexane. It is shown in Figure 1.6 that if the ferryl pKa played no role in hydrogen atom abstraction, the ability of a P450 to access the C-H bonds of cyclohexane would require a 1.82 eV redox potential of compound I. On the other hand, if the ferryl pKa is ~ 8.2 then a potential around 1.33 eV is adequate to perform hydrogen atom abstraction of cyclohexane.
1.6: The relationship of ferryl pKa and redox potential of compound I in performing hydrogen atom abstraction of cyclohexane [D(O-H) ~ 99 kcal/mol]
### 1.6 Parameterization of Badger’s Rule for Fe-O Bonds

The Fe-O bonds of thiolate and non-thiolate ligated heme systems determined from X-ray crystallography, X-ray absorption spectroscopy, and resonance Raman spectroscopy have been examined by Badger’s rule. Badger’s rule is an empirical formula that relates bond distance to vibrational stretching frequencies. This equation is shown in Eq. 1.2 where $r_e$ is the equilibrium internuclear distance and $\nu_e$ is the vibrational frequency while $C_{ij}$ and $d_{ij}$ are constants.

$$r_e = \frac{(C_{ij}/\nu_e^{2/3}) + d_{ij}}{}$$  \hspace{1cm} 1.2

Badger’s rule has been parameterized for mononuclear iron complexes containing oxo and hydroxo ligands in order to address the issue of ferryl protonation states. A theoretical parameterization of Badger’s rule was achieved by examining 30 complexes with differing axial ligands, oxidations states, and differing protonation states of the oxo ligand. Badger’s rule predicted vibrational frequencies with an average error of 9 cm$^{-1}$ for 17 FeXYPorphyrin complexes (X = no ligand, thiolate, phenolate, or imidazole and Y = O or OH) and an average error of 19 cm$^{-1}$ for some previously unexamined heme and non-heme systems. By applying Badger’s rule to Fe-O bonds measured by X-ray crystallography to their respective resonance Raman stretching frequencies, a clear
inconsistency was observed. These long Fe-O bonds resemble ferric or ferrous hydroxides rather than iron(IV)-hydroxides. A Badger’s rule analysis of the EXAFS-derived bond length for CPO-II (1.82 Å) provides a resonance Raman stretching frequency of $\nu_{\text{Badger}} = 563 \text{ cm}^{-1}$, which is consistent with the inability of previous investigators to locate the Fe-O stretching frequency.\textsuperscript{67} This frequency band would clearly not be located in the frequency region where an authentic ferryl stretching frequency is found (650 cm\textsuperscript{-1} - 950 cm\textsuperscript{-1}).

### 1.7 Utilization of Mössbauer Spectroscopy Coupled with Density Functional Calculations to Determine Ferryl Protonation States

Mössbauer spectroscopy is typically employed to determine the oxidation state and composition of iron containing samples, but is not generally applicable for structural characterizations.\textsuperscript{94} Although recently, it was reported that Mössbauer parameters could be predicted accurately using density functional theory.\textsuperscript{95-97} When analyzing ferryl species, two important Mössbauer parameters are isomer shift and quadrupole splitting. The isomer shift describes the amount of electron density at the nucleus and indicates the oxidation state of the iron. Therefore, when describing ferryl species, it is likely that this parameter will not change dramatically upon protonation of the ferryl. The quadrupole splitting parameter, on the other hand, will most likely change depending on the environment of the iron (ligands). Therefore, we suspected that the quadrupole splitting parameter would change most dramatically upon protonation of the ferryl. In general,
quadrupole splitting parameters from density functional calculations can be obtained within 0.3 mm/s, although errors of 0.3 - 0.5 mm/s are not uncommon. Density functional calculations on Fe(IV)-oxo and Fe(IV)-OH for thiolate and non-thiolate ligated heme systems revealed that protonation of the oxo was accompanied by a large change in $\Delta E_Q \approx 1.0$ mm/s.

1.8 Topics to be Addressed in this Thesis

High-valent iron intermediates of chloroperoxidase (CPO) have been utilized as a model to understand P450 reaction chemistry. I have trapped high-valent iron states in CPO and observed them with a wide variety of spectroscopic methods: stopped-flow spectrophotometry, X-ray absorption spectroscopy, Mössbauer spectroscopy, resonance Raman spectroscopy, and electron paramagnetic resonance, among others. Utilizing freeze-quench technology, I generated high-valent iron intermediates on the millisecond time-scale in which enzyme was rapidly mixed with oxidant and the reaction mixture was sprayed into liquid ethane (-180°C). I utilized spectroscopy to observe trapped intermediates in order to extract physical parameters such as structure, oxidation states, and spin states. My recent results have provided insight into the role of thiolate-ligation in P450 proteins by utilizing CPO as a model for P450 catalysis.

Herein I describe the structure and spectroscopy of high-valent intermediates of chloroperoxidase. In Chapter 2, I describe the first structure of thiolate-ligated compound I from X-ray absorption spectroscopy, which provided a glimpse into the elusive
intermediate of P450 reaction chemistry. Problems with photoreduction were encountered during irradiation of the sample by the synchrotron beam. In this case, it is determined that only samples that have been exposed to the beam a maximum of thirty minutes were suitable to be used for data analysis. In Chapter 3, the problem of ferryl protonation was investigated utilizing multiple spectroscopies: Mössbauer spectroscopy coupled with density functional calculations, ENDOR spectroscopy, X-ray absorption spectroscopy and resonance Raman spectroscopy. Surprisingly, the Mössbauer spectrum revealed that CPO-II is in fact a mixture of two ferryl intermediates in an ~70:30 ratio and many techniques were employed to determine the identities of these ferryl species. I also describe the first direct evidence for the existence of an iron(IV)-hydroxide by applying resonance Raman spectroscopy. To date CPO-II is the only compound II where the Fe(IV)-OH stretching frequency has been identified.

1.9 References


Chapter 2

Structure of Thiolate-Ligated Compound I


2.1 Abstract.

Cytochromes P450 (P450) are thiolate-ligated heme proteins that perform critical roles in many biological processes, e.g. metabolism of xenobiotics. Compound I, an Fe(IV)O-radical species, is proposed to be the primary oxidant in the consensus P450 enzymatic mechanism, although unambiguous detection of this intermediate has not been realized. Chloroperoxidase compound I (CPO-I) is the only thiolate-ligated compound I that has been characterized by a number of spectroscopies. To provide insight into the
reactive intermediate of P450 chemistry, we performed X-ray absorption spectroscopy studies on CPO-I. CPO-I is determined to be an authentic ferryl (Fe-O bond length of 1.65 Å) with a long Fe-S bond, 2.48 Å. The long Fe-S bond can be understood by the trans effect with increasing interactions with the anionic oxo ligand. CPO-I was highly susceptible to photoreduction, which resulted in longer Fe-O bonds and short Fe-S bonds accompanied with large Debye-Waller factors.

2.2 Introduction

The generally accepted hydroxylation mechanism of P450 enzymes involves a poorly characterized iron(IV)-oxo radical species, called compound I. It is proposed to be analogous to compound I’s of other oxidative heme enzymes (horseradish peroxidase (HRP), chloroperoxidase (CPO), and catalase). This species is highly reactive and has avoided spectroscopic observation, except for rapid-scan stopped-flow spectrophotometry conducted by Ishimura and Sligar and co-workers. P450-I was generated by reaction of ferric P450 with meta-chloroperbenzoic acid (through the so-called peroxide shunt pathway), but very low yields (~ 1 – 3%) of intermediate were observed. Alternatively, Davydov, Hoffman and co-workers attempted to observe P450-I by cryogenic γ-irradiation of oxyP450CAM followed by step-wise annealing to allow for proton transfer. The peroxy (of the D251N mutant) and the hydroperoxo intermediates were identified. Although P450-I was not detected, hydroxylated product formation was observed.
Through deuterium substitution, it was reasoned that the process must go through an iron-oxo species, indicating participation of compound I leading to hydroxylation.\textsuperscript{13}

Although compound I of thiolate-ligated heme proteins are proposed to be analogous to other compound I’s, e.g. histidine ligated or phenolate ligated, experimental and theoretical evidence suggests that they might possess an entirely different electronic structure.\textsuperscript{9, 14} For example, the EPR spectrum of a thiolate-ligated compound I (CPO-I) is unique. It displays a spin-spin coupling scheme between an $S = 1$ Fe(IV) and an $S = 1/2$ radical species, but with strong antiferromagnetic coupling ($J \sim -35$ cm$^{-1}$),\textsuperscript{9} in comparison to other heme peroxidases ($J \sim +0-10$ cm$^{-1}$)\textsuperscript{8, 10, 15-17} and porphyrin model complexes ($J \sim +25-50$ cm$^{-1}$)\textsuperscript{18, 19} Green demonstrated theoretically that ligand spin density presents an explanation for the observed antiferromagnetic coupling in CPO-I, which deviates from the standard porphyrin-radical cation model.\textsuperscript{14}

CPO, like P450’s, possesses a thiolate ligated to the heme iron. With an almost identical first coordination sphere, CPO is a valuable model system to study P450 reaction chemistry due to their spectroscopic and structural similarities.\textsuperscript{20-23} Structural determination of P450 catalytic intermediates is limited by the ability to trap them in yields that are amenable for spectroscopic analysis. Chloroperoxidase compound I (CPO-I) is the only thiolate-ligated compound I that can accumulate in yields suitable to be investigated by: Mössbauer,\textsuperscript{9} EPR,\textsuperscript{9} resonance Raman,\textsuperscript{24-26} and UV-Vis spectroscopies\textsuperscript{27} with application of rapid freeze-quench and stopped-flow techniques.

To gain insight into the reactive intermediate of P450 chemistry, we performed X-ray absorption measurements on CPO-I. During the course of obtaining X-ray absorption data of CPO-I, photoreduction of the high-valent iron intermediate was a concern. We
analyzed the extent of photoreduction of CPO-I at 10 K over time. The standard method of analyzing extent of photoreduction in X-ray absorption spectroscopy is by monitoring the oxidation-state dependent absorption edge. Although, as we observed in this study, it was also important to measure changing EXAFS parameters with consecutive scans. Here, we present the first structural characterization of a thiolate-ligated ferryl-radical species. This X-ray absorption study of CPO-I provides us with a structural model of the primary oxidant in P450 enzymatic catalysis.

2.3 Results and Discussion.

CPO-I accumulates to greater than 90% and decays exclusively to ferric enzyme, as previously determined by Mössbauer spectroscopy. Therefore, all species are EPR active and concentrations of both ferric CPO and CPO-I can be assessed using EPR spectroscopy. For each individual quench an EPR sample was prepared (a total of 4 EPR samples). Standard ferric samples were used to quantify amount of ferric CPO in freeze-quenched CPO-I samples assuming a 50% packing factor. The quality of all samples used for X-ray absorption experiments was determined in this fashion. A representative EPR spectrum of CPO-I used in X-ray absorption experiments is shown in Figure 2.1. The top (A) EPR spectrum is ferric CPO and the bottom (B) spectrum is a CPO-I sample used in X-ray absorption experiments; the g-values are specified therein. All CPO-I samples were run at the same EPR experimental conditions for comparison and were shown to contain greater than 90% CPO-I.
2.1: A representative EPR spectrum of CPO-I sample used for X-ray absorption data collection. EPR spectra for ferric CPO (A) and CPO-I (B) containing ~ 9% ferric CPO are shown. Spectrum recorded at x-band (9.368 GHz) at 20 K with 1.027 mW of power and 4.58 G modulation amplitude. All spectra were recorded at the same conditions for comparison.
2.3.1 Structure of Thiolate-Ligated Compound I

X-ray absorption near edge spectroscopy (XANES) is an atom-specific technique that is a useful tool to probe changes in oxidation state of an atom of interest, specifically iron in this case. These Fe-K X-ray absorption edges display a feature which is attributed to an 1s $\rightarrow$ 3d transition, which is dipole forbidden but can gain intensity from small amounts of 4p mixing. This feature is displayed in the pre-edge region of the X-ray absorption spectrum. The Fe-K X-ray absorption edge for ferric CPO (black, dotted line) and for CPO-I (red line) is shown in Figure 2.2. The absorption edge of CPO-I is shifted to higher energy, ~1eV, indicating a greater binding energy of the 1s electron for CPO-I as it is expected due to an increased oxidation state of CPO-I. The X-ray absorption spectrum of CPO-I exhibits a large pre-edge 1s $\rightarrow$ 3d feature that is typical of a ferryl species.

To minimize effects of photoreduction, only data from the first scan on an unexposed spot was used for data analysis (a total of 16 data sets were averaged). The fits of the extended X-ray absorption fine structure region of the CPO-I spectrum (EXAFS) and the corresponding Fourier transform of CPO-I are shown in Figure 2.3. The EXAFS spectrum and the Fourier transform of the raw data (black lines) and the best fit to the data (red lines) are shown. The top spectrum (A) is the raw data and the bottom spectrum (B) is the Fourier filtered data. The lines in the top Fourier transform spectrum show the region used for Fourier filtering (first two shells). The EXAFS region of CPO-I
could be fit from $k = 1-16 \text{ Å}^{-1}$ without significantly different results. The best fit obtained for the data was in the region of $k = 3-15 \text{ Å}^{-1}$. Table 2.1 lists the iron-ligand bond distances obtained by fitting the EXAFS region of the CPO-I spectrum. Interatomic bond distances ($R$), Debye-Waller factors ($\sigma^2$) and threshold energy shifts ($E_0$) were allowed to float during each successive fit. Coordination numbers ($N$) were varied systematically, but were constrained during each individual fit. The best fit to the data is shown in bold typeface.
2.2: Fe-K X-ray absorption edges for ferric CPO (black, dotted line) and CPO-I (red line). Inset displays a 10x magnification of the pre-edge region.
2.3: EXAFS spectra and Fourier Transforms of (A) raw data and (B) Fourier filtered data of the first two shells. Blacks lines show experimental data and red lines are best fits to the data. Black lines in the Fourier transform show region used for Fourier filtering. The fits shown here were obtained over the region $k = 3-15 \ \text{Å}^{-1}$. 

2.1: EXAFS fitting results for chloroperoxidase compound I. Raw (A) and Fourier-filtered (B) data were fit over the region $k = 3-15$ Å$^{-1}$. Coordination number $N$, interatomic distance $R$ (Å), mean-square deviation in $R$ (the Debye-Waller factor) $\sigma^2$ (Å$^2$), and the threshold energy shift $E_0$ (eV). The values in parentheses are estimated standard deviations obtained from the diagonal elements of the covariance matrix. Empirically, EXAFS uncertainties are ±0.02 Å for $R$ and ±20% for $N$ and $\sigma^2$. The fit-error is defined as $(\sum k^6(\chi_{\text{exptl}} - \chi_{\text{calc}})^2/\sum k^6\chi_{\text{exptl}}^2)^{1/2}$. Fits shown in bold typeface represent the best fit obtained for a sample. Alternative fits with different coordination numbers are also shown. Coordination numbers, $N$, were constrained during fits.

<table>
<thead>
<tr>
<th></th>
<th>Fe-N</th>
<th></th>
<th>Fe-S</th>
<th></th>
<th>Fe-O</th>
<th></th>
<th>$E_0$</th>
<th>Error</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$N$</td>
<td>$R$</td>
<td>$\sigma^2$</td>
<td>$N$</td>
<td>$R$</td>
<td>$\sigma^2$</td>
<td>$N$</td>
<td>$R$</td>
</tr>
<tr>
<td>Raw A</td>
<td>4</td>
<td>2.000(3)</td>
<td>0.0016(1)</td>
<td>1</td>
<td>2.473(9)</td>
<td>0.0061(9)</td>
<td>1</td>
<td>1.653(6)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.996(3)</td>
<td>0.0016(2)</td>
<td>0</td>
<td>1.650(6)</td>
<td>0.0041(7)</td>
<td>0</td>
<td>1.650(6)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.992(3)</td>
<td>0.0015(2)</td>
<td>1</td>
<td>2.467(9)</td>
<td>0.0054(9)</td>
<td>0</td>
<td>1.650(6)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.990(3)</td>
<td>0.0015(2)</td>
<td>0</td>
<td>1.650(6)</td>
<td>0.0041(7)</td>
<td>0</td>
<td>1.650(6)</td>
</tr>
<tr>
<td>Filtered B</td>
<td>4</td>
<td>2.007(2)</td>
<td>0.0017(1)</td>
<td>1</td>
<td>2.478(5)</td>
<td>0.0069(6)</td>
<td>1</td>
<td>1.654(3)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2.002(3)</td>
<td>0.0017(1)</td>
<td>0</td>
<td>1.651(4)</td>
<td>0.0033(4)</td>
<td>0</td>
<td>1.651(4)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.992(3)</td>
<td>0.0016(2)</td>
<td>1</td>
<td>2.470(10)</td>
<td>0.0060(11)</td>
<td>0</td>
<td>1.651(4)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.991(4)</td>
<td>0.0016(2)</td>
<td>0</td>
<td>1.651(4)</td>
<td>0.0033(4)</td>
<td>0</td>
<td>1.651(4)</td>
</tr>
</tbody>
</table>
2.3.2 Effect of Photoreduction

Photoreduction is a concern when collecting X-ray absorption data, but some complexes are more susceptible to photoreduction than others. Photoreduction of CPO-I was first observed by the X-ray absorption edge shifting to lower energy indicating reduction of the sample accompanied by a decrease in the intensity of the $1s \rightarrow 3d$ pre-edge feature. Photoreduction of the high-valent iron intermediate was evident even after the first scan. The X-ray absorption edges for CPO-I upon consecutive scans are shown in Figure 2.4.

The effects of photoreduction on the EXAFS parameters of CPO-I can clearly be seen in Table 2.2. Individual scans were analyzed separately (as a function of time) and not averaged as a whole data set. In all fits, coordination numbers (N) were constrained to 4 nitrogens, 1 oxygen, and 1 sulfur. As shown in the table, the second scan exhibits larger Debye-Waller factors for the sulfur and the oxygen. By the third scan the length of the Fe-S bond shortens to 2.36 Å and the Fe-O bond lengthens to 1.67 Å. In the fifth scan data set, where CPO-I has been exposed 2.5 hours, the disorder is so large that a reasonable fit cannot be obtained. In the case of the CPO-I data set, even moderate X-ray exposure (~ 1.5 hours or three scans) to CPO-I samples is accompanied by longer Fe-O bonds and shorter Fe-S bonds. Because of the tendency of CPO-I to reduce in the beam, only the first scan on each spot was used for structural determination of CPO-I.
2.4: Fe-K X-ray absorption edge of CPO-I upon continuous scans. The first scan is shown in red.
2.2: EXAFS fitting results for chloroperoxidase compound I upon consecutive scans. Raw data were fit over the region $k = 3-15$ Å$^{-1}$. Interatomic distance $R$ (Å), mean-square deviation in $R$ (the Debye-Waller factor) $\sigma^2$ (Å$^2$), and the threshold energy shift $E_0$ (eV). The values in parentheses are estimated standard deviations obtained from the diagonal elements of the covariance matrix. Coordination numbers, $N$, were constrained for all fits to 4 Fe-N, 1 Fe-S and 1 Fe-O. Empirically, EXAFS uncertainties are ±0.02 Å for $R$ and ±20% for $N$ and $\sigma^2$. The fit-error is defined as $\left(\sum k^6(\chi_{\text{exptl}} - \chi_{\text{calc}})^2/\sum k^6\chi_{\text{exptl}}^2\right)^{1/2}$.

<table>
<thead>
<tr>
<th></th>
<th>Fe-N</th>
<th></th>
<th>Fe-S</th>
<th></th>
<th>Fe-O</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R$</td>
<td>$\sigma^2$</td>
<td>$R$</td>
<td>$\sigma^2$</td>
<td>$R$</td>
<td>$\sigma^2$</td>
<td>$E_0$</td>
<td>Error</td>
</tr>
<tr>
<td>1 scan</td>
<td>2.002(3)</td>
<td>0.002(0)</td>
<td>2.474(9)</td>
<td>0.006(1)</td>
<td>1.654(5)</td>
<td>0.004(1)</td>
<td>-12.7(12)</td>
<td>0.3373</td>
</tr>
<tr>
<td>2 scans</td>
<td>2.010(4)</td>
<td>0.002(0)</td>
<td>2.454(14)</td>
<td>0.010(2)</td>
<td>1.654(9)</td>
<td>0.007(1)</td>
<td>-11.3(12)</td>
<td>0.3630</td>
</tr>
<tr>
<td>3 scans</td>
<td>1.995(3)</td>
<td>0.002(0)</td>
<td>2.362(38)</td>
<td>0.023(8)</td>
<td>1.671(16)</td>
<td>0.012(2)</td>
<td>-15.5(12)</td>
<td>0.3366</td>
</tr>
<tr>
<td>4 scans</td>
<td>1.997(4)</td>
<td>0.002(0)</td>
<td>2.389(57)</td>
<td>0.025(10)</td>
<td>1.679(68)</td>
<td>0.027(10)</td>
<td>-15.9(16)</td>
<td>0.3835</td>
</tr>
<tr>
<td>5 scans</td>
<td>2.008(4)</td>
<td>0.003(0)</td>
<td>2.389(44)</td>
<td>0.015(9)</td>
<td>1.397(132)</td>
<td>0.053(24)</td>
<td>-14.6(14)</td>
<td>0.4049</td>
</tr>
</tbody>
</table>
2.3.3 Discussion

We have provided the first unambiguous structural determination of a thiolate-ligated compound I utilizing X-ray absorption spectroscopy. Previously, a crystallographic study by Schlichting et al. used cryogenic reduction techniques of the ferrous-oxy complex of P450\textsubscript{CAM} to examine the catalytic pathway of P450\textsubscript{CAM}\textsuperscript{30}. They report a structural description of P450-I, although yields of P450-I did not allow for accurate determination of metal-ligand bond lengths. Our X-ray absorption measurements determine that CPO-I has an Fe-O bond length of 1.65 Å. This bond length is typical for a doubly bonded Fe(IV)-oxo species compared to the Fe-O bond lengths of other known ferryls: 1.64 Å for HRP-I and HRP-II and 1.66 Å for FeO(TMP)+.\textsuperscript{19, 31, 32} A Badger’s rule analysis of the CPO-I ferryl stretching frequency (\(\nu_{\text{FeO}} = 790 \text{ cm}^{-1}\)) provides an Fe-O bond length of 1.654 Å, which is excellent agreement with the EXAFS-derived Fe-O bond length.\textsuperscript{24-26, 33} The trans effect is highlighted by the interaction between the oxo ligand and the axial-thiolate ligand which is reflected by the very long Fe-S bond (2.48 Å). This distance is unusually long for an iron-alkanethiolate bond. A search of the Cambridge Structural Database for iron-thiolate bonds relevant to proteins yielded an Fe-S bond length range of 2.21 Å - 2.40 Å and an average distance of 2.27 Å.\textsuperscript{34, 35} The longest iron-alkanethiolate bond reported previously from either EXAFS spectroscopy or small molecule X-ray crystallography is 2.40 Å.\textsuperscript{23}
Figure 2.5 shows the EXAFS-determined bond lengths for ferric CPO, CPO-II, and CPO-I. These complexes have formal iron oxidation states of III, IV, and V, respectively. The trans influence is highlighted across the series as the Fe-O bond shortens from 2.11 Å to 1.65 Å while the iron-thiolate bond lengthens from 2.24 Å to 2.48 Å. This illustrates the strong axial interactions between the donating thiolate ligand and the anionic oxo ligand. A similar trend is observed in terminal oxo and hydroxo ligands of Fe(III) and Mn(III)Tris[(N’-tert-butylureaylato)-N-ethyl]aminato (Borovik’s ligand).\textsuperscript{36, 37} Due to the trans effect, the metal-nitrogen bond trans to the oxygen ligand in these complexes lengthens by ~ 0.1 Å after going from the hydroxo to the oxo complex.

This X-ray absorption study of CPO-I displays dramatic effect of X-ray damage to the sample. Both the shifting X-ray absorption edge and changing EXAFS parameters were analyzed as a function of number of scans to determine extent of photoreduction. Even with a moderate amount of exposure (~1.5 hours or three scans), photoreduction of CPO-I was accompanied with an elongated Fe-O bond and a shortening of the Fe-S bond.
2.5: EXAFS-determined results for ferric CPO, CPO-II and CPO-I. The Fe-O bond lengths show typical coordination chemistry where the Fe-O bond shortens with increasing oxidation state. The Fe-O bond shortens from 2.11 Å to 1.65 Å. The Fe-S bond shows the opposite behavior lengthening from 2.24 Å in ferric CPO to 2.4 Å in CPO-II to 2.48 Å in CPO-I. The Fe-S bond is the longest heme alkanethiolate bond reported. This illustrates the strong interactions between the donating thiolate ligand and the anionic oxo ligand.
Oxidation of the sulfur axial ligand may also contribute to the long Fe-S bond in CPO-I. Theoretically determined bond lengths of P450-I range from 2.44 Å - 2.69 Å. Gas-phase calculations of a thiolate-ligated porphine model provides and iron-thiolate bond length of 2.69 Å with a sulfur spin density of $\sigma_s = 0.82$, while quantum mechanics/molecular mechanics methods provide Fe-S bond lengths of (2.44 Å – 2.63 Å) and sulfur spin densities of ($\sigma_s = 0.24 – 0.39$). The results of QM/MM calculations seem to be in agreement with our experimentally determined bond length, which may suggest partial spin density distribution on the thiolate-ligand. Experiments are underway in our laboratory to determine magnitude of spin density on the thiolate ligand.

### 2.4 Conclusion

The first unambiguous structure of a thiolate-ligated compound I has been described using X-ray absorption spectroscopy. These results reveal the presence of a doubly bonded oxo ligand and a long Fe-S bond in CPO-I. Axial interactions between the thiolate and oxo ligands result in a long 2.48 Å Fe-S bond. The strong trans influence exhibited in CPO-I results in part from the donating nature of the thiolate ligand. The role of the axial ligand is unclear, but the electron donating ability of the axial-thiolate ligand in P450’s has been suggested to play important roles in oxygen activation and hydrogen abstraction chemistry.
The elusive intermediate of P450 chemistry has not been fully spectroscopically characterized. P450’s and CPO have been shown to possess virtually identical first coordination spheres and it is likely that our findings provide the best structural description, to date, of the highly reactive P450-I, while the electronic structure of thiolate-ligated compound I remains unsettled.

2.5 References


Chapter 3

Chloroperoxidase Compound II is Basic: Implications for P450 Hydroxylation Chemistry

Data in this chapter is adapted from:


3.1 Abstract

Cytochrome P450 enzymes are thiolate-ligated heme enzymes that perform oxygen transfer reactions in many biological processes. We have utilized the thiolate-ligated heme enzyme, chloroperoxidase, as a model to investigate P450 chemistry. Recently, a novel role for thiolate ligation in heme enzymes was suggested where the ferryl form of chloroperoxidase (CPO-II) was determined to be protonated at neutral pH. Researchers suggested that the increased pKa increases the driving force for hydrogen atom abstraction in P450 hydroxylation chemistry. We have examined the protonation state of CPO-II by utilizing multiple spectroscopies and experimental techniques. Mössbauer spectroscopy of CPO-II reveals the presence of two Fe(IV) species in an ~70:30 ratio that is pH invariant. Mössbauer spectroscopy coupled with density functional calculations, cryogenic reduction and annealing of CPO-I, and ENDOR spectroscopy all provide evidence for the protonation of the ferryl unit in CPO-II. Direct evidence for the Fe(IV)-hydroxide species in CPO-II was provided by applying resonance Raman spectroscopy. We determined that by utilizing 10% acetone in a preparation of CPO-II one ferryl species is produced and identified the ratio of ferryl intermediates in a standard preparation of CPO-II as two iron(IV)-hydroxides. All evidence, to date, suggests that thiolate-ligated heme enzymes contain basic ferryls.
3.2 Introduction

The consensus mechanism for P450 hydroxylation chemistry involves a hydrogen atom abstraction mechanism called the oxygen rebound mechanism.\(^1,2\) Recently, an X-ray absorption report suggested a novel role for thiolate ligation where the ferryl form of chloroperoxidase (CPO-II) contained a long Fe-O bond (1.82 Å).\(^3\) The Fe-O bond distance measured in CPO-II is longer than what is expected for an authentic ferryl species (1.65 Å), but agrees with a density functional calculation of a protonated ferryl heme species (1.81 Å).\(^4\) This result has important implications for P450 hydroxylation chemistry. Mayer and coworkers have shown that the ability of metal-oxos to perform hydrogen atom abstraction scales with redox potential of the oxidized metal center and the pKa of the reduced form.\(^5,7\) For heme enzymes, compound I abstracts a hydrogen atom from substrate producing a protonated ferryl species (the rebound intermediate). Radical recombination with the protonated ferryl produces the hydroxylated product and regenerates the ferric state. This mechanism is shown in Figure 3.1.

\[
D(O-H) = 23.06 \times E_{\text{compd-I}}^0 + 1.37 \times \text{pKa}_{\text{compd-II}} + 57 \pm 2 \text{ (kcal/mol)} \tag{3.1}
\]

Equation 3.1 highlights the importance of ferryl pka’s to provide the driving force for hydrogen atom abstraction and suggests that Nature may be utilizing basic ferryls in thiolate-ligated heme enzymes to allow for hydrogen atom abstraction and subsequent hydroxylation at biologically relevant reduction potentials of compound I. This theory rests upon the two important assumptions: the oxygen rebound mechanism is operative
P450 hydroxylation chemistry and that basic ferryls are a general and unique feature of thiolate-ligated heme enzymes. The former is continually supported by experimental and theoretical reports.\textsuperscript{8-10} The second assumption is not as clearly established. We have provided evidence that thiolate-ligated heme enzymes are basic.
3.1: Oxygen rebound mechanism of Cytochrome P450. Compound I abstracts a hydrogen atom from substrate forming the rebound intermediate. This figure illustrates that (except for the presence of the hydrocarbon radical) the P450 rebound intermediate and a thiolate-ligated compound II are equivalent. Distances in red are from X-ray absorption measurements on CPO-II and distances in black are from density functional calculations.
Interest in ferryl (Fe(IV)-oxo) protonation states has increased. X-ray crystal structures of other (non-thiolate) compound II species have reported the presence of long Fe-O bonds; researchers have inferred protonation from their findings. X-ray crystal structures of catalase compound II (1.84 Å),

myoglobin compound II (1.92 Å),

horseradish peroxidase compound II (1.84 Å),

cytochrome c peroxidase ES (1.87 Å)

have revealed Fe-O bond lengths. These results are in conflict with resonance Raman and EXAFS reports that generally indicate authentic ferryl species for these systems.

Reports of long Fe-O bonds in heme compound II species come from X-ray absorption spectroscopy and X-ray crystallography experiments that do not allow for the direct measurement of protonation. This assignment is unusual because ferryl species are generally thought to be electrophilic in nature. Reports of synthetic ferryl porphyrins are numerous, but reports of synthetic FeIV-OH species are rare. Bruice, Kadish, and Que independently reported generation of FeIV-OH species, but these systems were not structurally characterized. In short, although implicated in synthetic and enzymatic systems, no direct evidence for the existence of an iron(IV)-hydroxide species has been reported in the literature.

The origin of long Fe-O bonds in the X-ray crystal structures of compound II species is not determined, but it is well known that the synchrotron radiation that is used for data collection can produce potent reducing equivalents in the form of solvated electrons. Density functional calculations of Fe(III)-OH porphyrin complexes predicts Fe-O bonds lengths of ~1.81 Å - 1.85 Å. Could the presence of long Fe-O bonds in these X-ray crystal structures be a product of photoreduction by the synchrotron beam?
Due to inconsistencies in the literature, we have set out to utilize methods to determine ferryl protonation states that do not suffer the consequences of photoreduction. We have utilized Mössbauer and resonance Raman spectroscopies.

Mössbauer spectroscopy is a powerful technique to study iron-containing metalloproteins that quantitatively monitors composition of the sample, but is is not typically utilized to determine structural parameters.\(^{27}\) Recently, it was determined that density functional calculations could accurately predict Mössbauer parameters. Typical errors of quadrupole splitting parameters are \(\sim 0.3\) mm/s,\(^{28}\) although errors of 0.3 – 0.5 mm/s are not uncommon.\(^{29}\) With the guidance of density functional calculations, we examined the protonation state of CPO-II by Mössbauer spectroscopy.

All of the techniques used initially to characterize the protonation state of CPO-II in this chapter were indirect methods where protonation was inferred. Resonance Raman spectroscopy was applied to determine the protonation state of CPO-II where the Fe-OH unit can directly be observed utilizing isotopic substitution. The Fe-O stretching frequency of CPO-II had not previously been observed for CPO-II in the region where an authentic ferryl would be located (700 - 900 cm\(^{-1}\)). Three separate attempts have sought to locate this Raman band.\(^{30-32}\) A Badger’s rule analysis of the EXAFS-derived Fe-O bond length would predict an \(v_{\text{Badger}} = 563\) cm\(^{-1}\).\(^{4}\) Using Badger’s rule as a guide, we examined the protonation state of CPO-II by resonance Raman spectroscopy.
3.3 Results and Discussion

3.3.1 Calculations of Mössbauer Parameters

The Mössbauer parameters of a thiolate-ligated protonated ferryl heme or an unprotonated ferryl heme were not known. Therefore, it was unclear if the Mössbauer parameters would change significantly upon protonation of the ferryl. To start, the accuracy of Mössbauer parameters was determined for our system. We performed calculations on a thiolate-ligated porphine in a variety of spin states and oxidation states and compared the results of our calculations to experimental results.

The results of our calculations are shown in Table 3.1.\textsuperscript{33-35} It is clearly shown that there is good agreement between the calculated isomer shifts of a thiolate-ligated porphine complex and the experimentally determined isomer shifts. The quadrupole splitting parameters do not perform as well, but they do display appropriate trends in sign and magnitude. Calculations were performed on a large active-site model of CPO, shown in Figure 3.2. It is demonstrated in Table 3.1 that the quadrupole splitting parameters for the large active-site model are greatly improved while the isomer shifts remain very similar. This is most likely because the interactions from the proximal helix were included which mimic the active site of CPO more closely than the simple porphine model.

Application of density functional calculations on the large active-site model of the ferryl and the protonated ferryl yielded isomer shifts that are very similar, but the
quadrupole splitting parameters differ by 1.06 mm/s. Taken together, we found that
density functional calculations perform very well for the prediction of Mössbauer
parameters for different oxidation states and spin states of CPO.
3.2: Large active-site model of the protonated form of chloroperoxidase compound II.
### 3.1: Calculated and experimental Mössbauer parameters for chloroperoxidase

Porphine models contained a methyl-mercaptate axial ligand. Constrained optimizations used the ferric crystal structure as a starting point.

<table>
<thead>
<tr>
<th>Distal Ligand</th>
<th>Oxidation State</th>
<th>Spin State</th>
<th>Optimized Porphine</th>
<th>Constrained Optimization</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>$\delta$</td>
<td>$\Delta E_q$</td>
<td>$\delta$</td>
</tr>
<tr>
<td>none</td>
<td>II</td>
<td>2</td>
<td>0.68</td>
<td>-1.85</td>
<td>0.67</td>
</tr>
<tr>
<td>CO</td>
<td>II</td>
<td>0</td>
<td>0.31</td>
<td>0.22</td>
<td>0.30</td>
</tr>
<tr>
<td>water</td>
<td>III</td>
<td>1/2</td>
<td>0.38</td>
<td>-2.66</td>
<td>0.38</td>
</tr>
<tr>
<td>none$^b$</td>
<td>III</td>
<td>5/2</td>
<td>0.43</td>
<td>0.60</td>
<td>0.39</td>
</tr>
<tr>
<td>oxo-H</td>
<td>IV</td>
<td>1</td>
<td>0.08</td>
<td>1.84</td>
<td>0.10</td>
</tr>
<tr>
<td>oxo$^-$</td>
<td>IV</td>
<td>1</td>
<td>0.13</td>
<td>0.63</td>
<td>0.12</td>
</tr>
</tbody>
</table>

a) Constrained optimizations used the ferric crystal structure as a starting point. These calculations differed from the smaller (porphine only) set in that a portion of the proximal helix was included and all atoms except for an inner core surrounding the Fe (~30 atoms) were frozen during geometry optimizations.

b) Experimental parameters for the high-spin ferric form of CPO are not available. Values shown are from measurements on cytochrome P450.
3.3.2 Mössbauer Spectroscopy of Chloroperoxidase Compound II

The Mössbauer spectrum of CPO-II is shown in Figure 3.3. Some samples of CPO-II contained minor amounts of ferric CPO (<15%), this component was removed before data fitting procedures. Two asymmetric lines make up the Mössbauer spectrum that is deconvoluted into two quadrupole doublets with similar isomer shifts. In order to enhance the resolution of the spectrum, the line widths of $^{57}$Co(Rh) source were removed using a Fourier transform technique (Figure 3.3B). The presence of two distinct quadrupole doublets is clearly shown.

Fits to the raw data assuming equal line widths of the two quadrupole doublets yields a major (red line) component (74 ± 4%) with an isomer shift of $\delta = 0.10 \pm 0.03$ mm/s and a quadrupole splitting of $\Delta E_Q = 2.06 \pm 0.03$ mm/s. These parameters are identical to the Mössbauer parameters predicted for a protonated ferryl (Table 3.1). The minor (blue line) species (26 ± 4%) has parameters similar to other known Fe(IV)-oxo heme species with an isomer shift of $\delta = 0.11 \pm 0.03$mm/s and a quadrupole splitting of $\Delta E_Q = 1.59 \pm 0.05$ mm/s (Table 3.2).

Fits using line widths that vary no more than 15% yielded 68 ± 7% of the major ferryl species and 32 ± 7% of the minor ferryl species, while nearly identical Mössbauer parameters are observed in all fits.
3.3: Mössbauer spectrum of CPO-II pH 6.5, 250 ms quench time. Similar results were obtained with a 28 ms quench. Spectrum A shows raw data; solid line is best fit to two quadrupole doublets, ($\delta = 0.10$ mm/s, $\Delta E_Q = 2.06$ mm/s) and ($\delta = 0.11$ mm/s, $\Delta E_Q = 1.59$ mm/s). Spectrum B was obtained from a Fourier transform analysis of the data, which removes the linewidth of the $^{57}$Co(Rh) source and highlights the presence of two distinct species. Parameters were obtained by fitting raw data.
3.2: Mössbauer parameters of 6-coordinate ferryl porphyrin complexes.

<table>
<thead>
<tr>
<th>System</th>
<th>Trans Ligand</th>
<th>Oxidation/Spin State</th>
<th>$\delta$ [mm/s]</th>
<th>$\Delta E_0$ [mm/s]</th>
</tr>
</thead>
<tbody>
<tr>
<td>JRP-I</td>
<td>histidine</td>
<td>IV (S=1)</td>
<td>0.10</td>
<td>1.33</td>
</tr>
<tr>
<td>JRP-II</td>
<td>histidine</td>
<td>IV (S=1)</td>
<td>0.03</td>
<td>1.59</td>
</tr>
<tr>
<td>CCP-I</td>
<td>histidine</td>
<td>IV (S=1)</td>
<td>0.05</td>
<td>1.55</td>
</tr>
<tr>
<td>HRP-I</td>
<td>histidine</td>
<td>IV (S=1)</td>
<td>0.08</td>
<td>1.25</td>
</tr>
<tr>
<td>HRP-II</td>
<td>histidine</td>
<td>IV (S=1)</td>
<td>0.03</td>
<td>1.61</td>
</tr>
<tr>
<td>CPO-I</td>
<td>cysteine</td>
<td>IV (S=1)</td>
<td>0.14</td>
<td>1.02</td>
</tr>
<tr>
<td>Mb-II</td>
<td>histidine</td>
<td>IV (S=1)</td>
<td>0.09</td>
<td>1.43</td>
</tr>
<tr>
<td>FeOT PP</td>
<td>1-mLm</td>
<td>IV (S=1)</td>
<td>0.11</td>
<td>1.26</td>
</tr>
<tr>
<td>FeOT PP</td>
<td>pyridine</td>
<td>IV (S=1)</td>
<td>0.10</td>
<td>1.56</td>
</tr>
<tr>
<td>FeOT MP</td>
<td>chloride</td>
<td>IV (S=1)</td>
<td>0.07</td>
<td>1.35</td>
</tr>
<tr>
<td>FeO(TMP- B$_{4}$)**</td>
<td>methanol</td>
<td>IV (S=1)</td>
<td>0.02</td>
<td>1.28</td>
</tr>
<tr>
<td>FeO(TMP- Cl$_{4}$)**</td>
<td>methanol</td>
<td>IV (S=1)</td>
<td>0.08</td>
<td>1.43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Avg.</td>
<td>0.08</td>
<td>1.39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RMSD</td>
<td>0.04</td>
<td>0.17</td>
</tr>
</tbody>
</table>
In order to identify the spin states of the two states in CPO-II, we recorded the Mössbauer spectra in parallel magnetic fields of 4 T and 7 T, shown in Figure 3.4. The solid lines indicate the components of the major ferryl species (red line) and the minor ferryl species (blue line). Both species were assumed to have similar parameters that are typical for ferryl hemes.\textsuperscript{27, 44} The solid black line is a simulation assuming the slow relaxation limit and using the following parameters: $S = 1$, $D = +23 \text{ cm}^{-1}$, $E/D = 0.0$, $g = (2.1, 2.1, 2.0)$, $\eta = 0$ and $A/g_S\beta_N = (-20, -20, -7) \text{ T}$ for both species using the isomer shifts and the quadrupole splittings previously mentioned. The data is consistent with both species having an $S = 1$ ground state.
3.4: Mössbauer spectra (hash marks) of CPO-II recorded at 4.2 K in externally applied magnetic fields of 4 T and 7 T oriented parallel to the γ-beam. The solid lines are simulations using the following parameters and assuming the slow relaxation limit. Simulations were performed assuming $S = 1$, $D = +23$ cm$^{-1}$, $E/D = 0.0$, $g = (2.1, 2.1, 2.0)$, $\eta = 0$, and $A/g_\parallel \beta_N = (-20, -20, -7)$ T for both species and using the Mössbauer parameters ($\delta$ and $\Delta E_Q$) listed in Table 3.1. The red (blue) lines above the data are the individual contributions of the major (minor) component.
3.3.3 Cryogenic Reduction of CPO-I

We have the ability to quantitatively monitor the iron states of CPO and observe subsequent chemistry upon cryogenic reduction and annealing by Mössbauer spectroscopy. CPO-I is known to be an authentic ferryl and, when it is exposed to γ-irradiation at 77 K, reduction of the high-valent metal center produced an unprotonated CPO-II. Annealing of the cryoreduced CPO-I allows for protonation producing the protonated ferryl. This process is shown in Figure 3.5.
3.5: Cryoreduction and annealing of CPO-I. CPO-I containing glycerol is prepared and exposed to γ-irradiation at 77 K, which reduces CPO-I forming an unprotonated CPO-II. This species is annealed at 193 K to allow for proton delivery.
The results of these cryogenic reduction and annealing experiments are illustrated in Figures 3.6 - 3.9. Figure 3.8 shows the cryoreduction of CPO-I and Figure 3.9 shows the annealing of cryoreduced CPO-I. Two control experiments were performed: 1) annealing of CPO-I (Figure 3.6) and 2) cryogenic reduction of ferric CPO (Figure 3.7). The yield of CPO-I was not 100% and, therefore, the Mössbauer parameters of cryoreduced ferric CPO was measured. The decay of CPO-I needed to be analyzed by Mössbauer spectroscopy because it was had been shown by Pickard and coworkers that CPO-I decays preferentially to ferric.45

Figure 3.6 displays the cryoreduction of ferric CPO. Figure 3.6A show the Mössbauer spectrum of ferric CPO. The solid line is a simulated using the spin Hamiltonian formalism in the slow relaxation limit with the following parameters: $S = 1/2$, $g = (1.84, 2.26, 2.63)$, $\delta = 0.30$ mm/s, $\Delta E_Q = 2.9$ mm/s, $\eta = -2$, and $A/g_s\beta_N = (-46, 7.9, 28.4)$ T. Figure 3.6B shows the cryoreduction of ferric CPO. One quadrupole grows in upon reduction. Deconvolution of the spectrum reveals 51% of ferric CPO remains. Removing contributions from ferric CPO leaves Figure 3.6C. A ferrous species is produced upon cryoreduction with the following parameters: $\delta = 0.85$ mm/s and $\Delta E_Q = 2.49$ mm/s. These parameters are consistent with the Mössbauer parameters typical for a high-spin ferrous heme species.27 Other minor features are visible upon reduction, but these features are not entirely understood.
3.6: Cryoreduction of ferric CPO. A) Mössbauer spectrum of ferric CPO. Solid line is simulated using a spin Hamiltonian formalism in the slow relaxation limit with the following parameters: $S = 1/2$, $g = (1.84, 2.26, 2.63)$, $\delta = 0.30 \text{ mm/s}$, $\Delta E_Q = 2.9 \text{ mm/s}$, $\eta = -2$, and $A/g_N\beta_N = (-46.0, +7.9, +28.4) \text{ T}$. B) Mössbauer spectrum of cryoreduced ferric CPO. Deconvolution of the spectrum reveals 51% ferric CPO remains. C) Mössbauer spectrum of ferrous species generated upon cryoreduction of ferric CPO. The raw data is fit to one quadrupole doublet (solid line) with parameters typical for a high-spin ferrous heme: $\delta = 0.85 \text{ mm/s}$ and $\Delta E_Q = 2.49 \text{ mm/s}$. 
Figure 3.7 shows the decay of CPO-I upon annealing at –80°C. Figure 3.7A shows the Mössbauer spectrum of CPO-I prior to annealing. Deconvolution of this spectrum reveals 10% ferric CPO (black solid line) remains. CPO-I is simulated using a spin Hamiltonian formalism in the slow relaxation limit with parameters that are almost identical to those previously published: \( S_{\text{eff}} = 1/2 \), \( g = (2.0, 2.0, 2.0) \), \( \delta = 0.15 \text{ mm/s} \), \( \Delta E_Q = 1.02 \text{ mm/s} \), \( \eta = 0 \), and \( A/g_N \beta_N = (-30.5, -30.5, -6.0) \text{ T} \) (red solid line). Annealing the CPO-I sample at –80°C for 30 minutes produces the Mössbauer spectrum shown in Figure 3.7B. It shown that ferric CPO (black solid line) increases from 10% to 74%. Figure 3.7C is the difference spectrum (B-A). The features pointing upward indicate species that are decaying and features pointing downward indicate features that are growing in. These results show that CPO-I decays through a two-electron process directly to ferric CPO. There is no evidence that CPO-I decays through even minor amounts of CPO-II.
3.7: Annealing of CPO-I. A) Mössbauer spectrum of CPO-I. Deconvolution of this spectrum reveals 10% ferric CPO (solid line). CPO-I is simulated using a spin Hamiltonian formalism in the slow relaxation limit with parameters nearly identical to those previously published: $S_{\text{eff}} = 1/2$, $g = (2.0, 2.0, 2.0)$, $\delta = 0.15$ mm/s, $\Delta E_Q = 1.02$ mm/s, $\eta = 0$, and $A_g / g N C_N = (-30.5, -30.5, -6.0)$ T (red, solid line). B) Mössbauer spectrum of CPO-I annealed at –80°C for 30 minutes. Ferric CPO increases (solid line) from 10% to 74%. C) Difference spectrum (B-A). Features pointing upward indicate species that are decaying and features pointing downward indicate species that are growing in.
Figure 3.8 shows the cryoreduction of CPO-I at 77 K. Figure 3.8A is the Mössbauer spectrum of CPO-I before cryoreduction experiments. Deconvolution of the spectrum reveals that the spectrum is made up of 22% ferric CPO (black solid line) and 78% CPO-I. Figure 3.8B shows the effect of cryoreduction of CPO-I. A sharp quadrupole doublet grows in upon reduction. Figure 3.8C shows the difference spectrum (B-A) highlighting the fraction of sample that is reduced during cryoreduction. Adding back 53% CPO-I (blue line) to the difference spectrum results in the red line. The Mössbauer spectrum reveals the presence of two quadrupole doublets. One of these species is identical to cryoreduced ferric CPO (13%) denoted with an (a). Removal of appropriate contributions from ferric CPO, cryoreduced CPO and CPO-I results in the Mössbauer spectrum of cryoreduced CPO-I in Figure 3.8D. This quadrupole doublet is fit to the following parameters: $\delta = 0.12 \text{ mm/s}$ and $\Delta E_Q = 1.41 \text{ mm/s}$. These parameters are consistent with Mössbauer parameters of other known ferryl heme species (as seen in Table 3.2). In short, cryoreduction of CPO-I results in an unprotonated CPO-II.
3.8: Cryoreduction of CPO-I. A) Mössbauer spectrum of CPO-I before cryoreduction. The spectrum contains 22% ferric CPO (solid line) and 78% CPO-I. B) Mössbauer spectrum of cryoreduced CPO-I. C) Difference spectrum (B-A) highlighting the fraction of sample that is reduced during cryoreduction. Adding back 53% of CPO-I (blue line) to the difference spectrum results in the red line. A sharp quadrupole doublet (a) grows in that is identical to cryoreduced ferric CPO. D) After removing appropriate contributions of ferric CPO, and cryoreduced ferric CPO, and CPO-I; the Mössbauer spectrum of cryoreduced CPO-I remains that is fit to one quadrupole doublet with the following parameters: $\delta = 0.12$ mm/s and $\Delta E_Q = 1.41$ mm/s.
Figure 3.9 shows the effect upon annealing of cryoreduced CPO-I. The Mössbauer spectrum of cryoreduced CPO-I annealed at –80°C for 30 minutes is shown in Figure 3.9A. The red bracket indicates the quadrupole doublet before annealing and the blue bracket highlights the new quadrupole doublet produced after annealing. The peak denoted by ‘a’ is associated with cryoreduced ferric CPO. Other species are highlighted in the Mössbauer spectrum, such as the amount of CPO-I decreases denoted by ‘b’ and the amount of ferric CPO increases (‘c’). Figure 3.9B is a difference spectrum (Figure 3.9A – Figure 3.8D) that highlights the new species that was generated upon annealing of cryoreduced CPO-I. Removing appropriate amounts of other components represented in the Mössbauer spectrum (33% ferric CPO, 4% CPO-I, and 19% cryoreduced ferric CPO) results in the spectrum shown in Figure 3.9C. Annealing cryoreduced CPO-I allows for protonation of the unprotonated ferryl species, producing a new quadrupole doublet with an enlarged quadrupole splitting ($\Delta E_Q = 2.06$ mm/s). This is identical to the Mössbauer parameters of the major ferryl species measured in freeze-quenched CPO-II. This provides evidence that CPO-II is in fact a protonated ferryl species.
3.9: Annealing of cryoreduced CPO-I. A) Mössbauer spectrum of cryoreduced CPO-I annealed at 
-80°C for 30 minutes. The Mössbauer spectrum of cryoreduced CPO-I before (solid line) and after (hash marks) annealing. The amount of CPO-I decreases ('b'), the quadrupole doublet of the ferryl generated by cryoreduction (red bracket) decreases in intensity. The intensity of ferric CPO increases ('c') and a new quadrupole doublet grows in (blue bracket). B) Difference spectrum (Figure 3.9A - Figure 3.8D) highlights the new ferryl species generated during annealing. C) Removing appropriate amounts of other components (33% ferric CPO, 4% CPO-I, and 18% cryoreduced ferric CPO) results in the Mössbauer spectrum of annealed cryoreduced CPO-I.
The Mössbauer spectrum of the species generated from the annealing of cryoreduced CPO-I from Figure 3.9C with data collected with a smaller range of Doppler velocities is shown in Figure 3.10. Figure 3.10A shows the contribution of a minority species (6% of the total intensity, $\delta = 0.29$ mm/s and $\Delta E_Q = 2.30$ mm/s). This species is consistent with a ferrous-oxy complex, although the mechanism to generate this species by this method is unclear. After the contribution of the minority species is removed, the reference Mössbauer spectrum for annealed cryoreduced CPO-I is shown in Figure 3.10B. Interestingly, the Mössbauer spectrum upon annealing of cryoreduced CPO-I consists of two quadrupole doublets that represent 29% ($\delta = 0.10$ mm/s and $\Delta E_Q = 2.08$ mm/s) and 10% ($\delta = 0.11$ mm/s and $\Delta E_Q = 1.60$ mm/s) of the total intensity. Within the error of the data, these Mössbauer parameters are essentially identical to the Mössbauer parameters of a freeze-quenched sample of CPO-II. It is demonstrated by these cryoreduction and annealing experiments that: 1) CPO-II contains a protonated ferryl and 2) the ratio of ferryl intermediates remains unchanged.
3.10: A) The Mössbauer spectrum for the species generated during the annealing process. This spectrum is similar to the spectrum in Figure 3.9C but it was generated from spectra collected over a narrower range of Doppler velocities. The solid line shows a minority species (6% of the total sample) with $\delta = 0.29 \text{ mm/s}$, $\Delta E_Q = 2.30 \text{ mm/s}$, which is consistent with a ferrous-oxy complex. B) Mössbauer spectrum of cryoreduced and annealed CPO-I obtained by removing contributions from the minority species represented by the solid line in (A). The quadrupole doublets shown in (B) represent 29% ($\delta = 0.10 \text{ mm/s}$, $\Delta E_Q = 2.08 \text{ mm/s}$) and 10% ($\delta = 0.11 \text{ mm/s}$, $\Delta E_Q = 1.60 \text{ mm/s}$) of the total sample.
3.3.4 Preparation of CPO-II under Different Conditions

In an effort to perturb the ratio of ferryl intermediates and to identify and characterize the two ferryl species, we have prepared CPO-II utilizing different reaction conditions. If the minor ferryl species of CPO-II were indeed an Fe(IV)-oxo, then the possibility remained that the reaction conditions (pH 6.5) described previously was near the ferryl pKa and that by lowering the pH we would generate one protonated ferryl species. The Mössbauer spectrum of CPO-II prepared at pH 4.5 is shown in Figure 3.11. This Mössbauer spectrum of CPO-II at pH 4.5 yields a set of two quadrupole doublets, as previously observed at pH 6.5. The major ferryl species, 66% of the total absorption, has the following Mössbauer parameters: \( \delta = 0.10 \text{ mm/s} \) and \( \Delta E_Q = 2.08 \text{ mm/s} \). 34% of the total absorption is the second ferryl species that has the following Mössbauer parameters: \( \delta = 0.11 \text{ mm/s} \) and \( \Delta E_Q = 1.58 \text{ mm/s} \). These results are nearly identical to the Mössbauer parameters that were recorded for CPO-II generated at pH 6.5. This indicates that the ratio of ferryl intermediates are not merely linked by a protonation event.
Mössbauer spectrum of CPO-II, pH 4.5, recorded at 4.2K in a 40 mT magnetic field applied parallel to the γ beam. The spectrum shows raw data; solid line is best fit to two quadrupole doublets. The parameters for the protonated ferryl ($\Delta E_Q=2.08$ mm/s, $\delta=0.10$ mm/s, ~66%) and the ferryl ($\Delta E_Q=1.58$ mm/s, $\delta=0.11$ mm/s, ~34%) are similar to parameters measured for CPO-II at pH 6.5.
The use of alternative oxidants and reductants to generate CPO-II was applied in an effort to perturb the ratio of ferryl intermediates. The previous CPO-II samples that have been produced utilized peracetic acid as the oxidant. It was shown by Dunford and coworkers that peracetic acid is a superior oxidant and that concentrations of CPO-I can build up to 100%, whereas the use of hydrogen peroxide as the oxidant CPO-I can only accumulate to 88%.46 This is due to CPO’s ability to catalyze the disproportionation of hydrogen peroxide. Shown in Figure 3.12 is the Mössbauer spectrum of CPO-II generated with hydrogen peroxide. After contributions of ferric CPO are removed (Figure 3.12A), the Mössbauer spectrum of hydrogen peroxide generated CPO-II is shown in Figure 3.12B. The spectrum shows the raw data and the solid black line that is best fit to two quadrupole doublets: (ΔE_Q = 2.08 mm/s, δ = 0.10 mm/s, 69%) and (ΔE_Q = 1.58 mm/s, δ = 0.11 mm/s, 31%). The left panel is the Mössbauer spectrum obtained with a narrower range of Doppler velocities. These parameters are similar to parameters measured for CPO-II with peracetic acid as the oxidant.
Mössbauer spectrum of CPO-II prepared with hydrogen peroxide as the oxidant. The spectrum shows raw data; solid line is best fit to two quadrupole doublets. A) The sample contained 65% ferric CPO and was removed from the raw data. B) The remaining absorption showed two quadrupole doublets: $(\Delta E_Q=2.08 \text{ mm/s}, \delta=0.10 \text{ mm/s}, 69\%)$ and $(\Delta E_Q=1.58 \text{ mm/s}, \delta=0.11 \text{ mm/s}, 31\%)$. These parameters are similar to parameters measured for CPO-II with peracetic acid as the oxidant.
We also utilized as p-phenolsulfonic acid as the reductant to replace ascorbic acid from previous preparations of CPO-II. In the case of ascorbic acid, the rate of CPO-I reduction is much faster than the rate of CPO-II reduction allowing CPO-II to accumulate to ≥ 95%. In the case of p-phenolsulfonic acid, the rate of CPO-I reduction is only slightly faster than the rate of CPO-II reduction. To prepare CPO-II using this reductant, the sample was prepared as quickly as possible. Because of the rates of reduction, all three components of CPO are represented in the Mössbauer spectrum. Shown in Figure 3.13 is the Mössbauer spectrum of CPO-II, pH 6.5, prepared with p-phenolsulfonic acid as the reductant. Spectra were collected over a range of Doppler velocities of approximately ± 4 mm/s (left panel) or ± 8 mm/s (right panel). Deconvolution of the spectrum reveals 20% ferric CPO and 35% CPO-I. Shown in Figure 3.13A, the removal of 20% of ferric CPO from the raw data (solid line) yields the spectrum shown in Figure 3.13B. Removal of 35% of CPO-I (black line) yields the spectrum shown in Figure 3.13C. The Mössbauer spectrum in Figure 3.13C is simulated with two quadrupole doublets with the following parameters: (δ = 0.10 mm/s, ΔE_Q = 2.07 mm/s, 32% of total intensity) and (δ = 0.10 mm/s, ΔE_Q = 1.58 mm/s, 13% of total intensity). These parameters and the ratio of their relative intensities (~ 70:30) are similar to those observed for CPO-II with ascorbate as the reductant.

Importantly, the ratio of two ferryl intermediates in CPO-II remains unchanged (~ 70:30) upon lowering the pH, different oxidants and reductants, and even by cryogenic reduction and annealing experiments.
Mössbauer spectrum of CPO-II, pH 6.5, prepared with \(p\)-phenolsulfonic acid as the reductant. Spectra were collected over a range of Doppler velocities of approximately ± 4 mm/s (left panel) or ± 8 mm/s (right panel). Removal of 20% of ferric CPO from the raw data (A) yields the spectra shown in (B). Removal of 35% of CPO-I yields the spectra shown in (C). These spectra have been simulated with two quadrupole doublets with the following parameters: \(\delta(1) = 0.10 \text{ mm/s}, \Delta E_Q(1) = 2.07 \text{ mm/s}, 32\% \text{ of total intensity}, \) and \(\delta(2) = 0.10 \text{ mm/s}, \Delta E_Q(2) = 1.58 \text{ mm/s}, 13\% \text{ of total intensity}. \) These parameters and the ratio of the relative intensities are similar to those observed for CPO-II with ascorbate as the reductant.
3.4 Chemical Modification of Histidine Residues in CPO

Chemical modification of CPO was performed to determine if the histidine residue in the distal cavity plays a role in the production of two distinct ferryl intermediates. An examination of the X-ray crystal structure of CPO provides insight into this phenomenon from a structural prospective. The distal cavity of CPO contains a triad of amino acids that shuttles protons into the active site. Figure 1.3 displays the X-ray crystal structure of CPO and highlights the proton shuttle containing Asp-106, His-105, and Glu-183.

The ability to chlorinate substrate was determined to be dependent on an essential histidine residue from chemical modification of histidine residues in CPO by diethylpyrocarbonate. NMR measurements of CPO reveal the presence of an exchangeable proton 8Å away from the iron center that is attributable to a histidine residue and the X-ray crystal structure identifies this amino acid as His-105. In general, efforts to generate mutants of CPO have largely been unsuccessful or have met with low yields. Therefore, chemical modification was utilized to modify histidine residues in CPO to understand the role that the essential histidine residue plays in the generation of two ferryl intermediates in CPO-II.
Histidine residues of CPO are modified and chlorination activity was followed to maximum modification, which was monitored at 242 nm. Histidine-modified CPO-II can be generated by the reaction of modified-CPO and peracetic acid and ascorbic acid. The Mössbauer spectrum of histidine-modified CPO-II is shown in Figure 3.14. The raw Mössbauer data can be fit to two quadrupole doublets (black line) with the following Mössbauer parameters: \( \Delta E_Q = 2.06 \text{ mm/s}, \delta = 0.10 \text{ mm/s}, 84\% \text{ of the total absorption} \) and \( \Delta E_Q = 1.61 \text{ mm/s}, \delta = 0.13 \text{ mm/s}, 16\% \text{ of the total absorption} \). The red (major) and blue (minor) lines highlight two quadrupole doublets in the Mössbauer spectrum. The Mössbauer parameters are nearly identical to the unmodified protein, but the amount of the minor ferryl species is reduced by ~ 50%.

Modification of histidines residues in CPO is accompanied by a 70% loss of chlorination activity, which is attributed to one “essential” histidine residue. It is reasonable to assign the essential histidine residue as His-105. The Mössbauer spectrum of histidine-modified CPO-II provides evidence that the proton shuttle is playing a role in modulating the ratio of two Fe(IV) intermediates.
3.14: Mössbauer spectrum of histidine-modified CPO-II. The spectrum shows raw data and the solid black line is best fit to two quadrupole doublets ($\Delta E_Q=2.06$ mm/s, $\delta=0.10$ mm/s, red line) and ($\Delta E_Q=1.61$ mm/s, $\delta=0.13$ mm/s, blue line), which is 84% and 16% of the total absorption, respectively. The amount of minority ferryl species is reduced by ~50%.
Our results from Mössbauer spectroscopy indicate that CPO-II is a protonated ferryl species. All evidence accumulated so far is consistent with this assignment. 1) EXAFS measurements put the Fe-O bond length at 1.82 Å, in agreement with a density functional calculation of a thiolate-ligated Fe(IV)-OH porphyrin species (1.81 Å). 2) Density functional calculations of a protonated ferryl species are in excellent agreement with the major ferryl species observed in the Mössbauer spectrum of CPO-II. 3) Cryogenic reduction and annealing experiments enables production of an unprotonated CPO-II and allows for protonation at elevated temperatures. The quadrupole splitting parameter of cryoreduced CPO-I increased from $\Delta E_Q = 1.41$ mm/s to $\Delta E_Q = 2.06$ mm/s upon annealing, which is identical to the major ferryl species in CPO-II.

It was troubling to assign the minor ferryl species as an Fe(IV)-oxo due to it’s pH invariance, which suggests that the ratio of ferryl intermediates is not governed by a protonation event. The invariant ratio of ferryl intermediates agrees with the UV/visible absorption spectrum of CPO-II where the spectrum is unchanged in the pH regime where CPO-II can be prepared (pH 2.5 – 7). All of the Mössbauer results and calculations presented here suggest that CPO-II is at least ~ 70% protonated. Density functional calculations predict a large change in quadrupole splitting parameters upon protonation of the ferryl and this is consistent with what is measured for CPO-II (2.06 mm/s). The Mössbauer parameters of the ferryl species of three P450’s are measured to be larger than what is typical for an authentic ferryl species ($\Delta E_Q = 2.06 – 2.17$ mm/s), suggesting that these ferryl species are also basic ferryls.55
3.4.1 Electron Nuclear Double Resonance of Cryoreduced CPO-II

In order to further characterize CPO-II spectroscopically, we utilized cryogenic reduction techniques to initiate a one-electron reduction of the Fe(IV) center without allowing for further chemistry. CPO-II is EPR-silent and, upon reduction by \( \gamma \)-irradiation at 77 K, a new EPR-active species forms. The EPR spectra measured in this study are shown in Figure 3.15 and the scheme for this process is shown in Figure 3.16. As shown from the EPR spectrum of CPO-II (red), CPO-II is an EPR-silent species where only an ascorbate radical is displayed in its EPR spectrum. Upon exposure to \( \gamma \)-irradiation, a new EPR-active species grows in (black) that is associated with reduction of CPO-II. For comparison, the EPR spectrum of ferric CPO (blue) is displayed. The g-values of cryoreduced CPO-II (2.41, 2.17, 1.93/1.91) are different than those of ferric CPO (2.62, 2.26, 1.83). The new species generated from cryoreduction of CPO-II is a low-spin Fe(III) heme.

Continuous wave x-band ENDOR spectroscopy was applied to examine cryoreduced CPO-II. This spectrum, centered at the proton Larmor frequency, is shown in Figure 3.17. ENDOR spectroscopy of cryoreduced CPO-II (right) prepared in H\(_2\)O (red) and D\(_2\)O (blue) reveals the presence of an exchangeable proton. For comparison, the ENDOR spectrum of alkaline myoglobin is measured (left) in H\(_2\)O (red) and D\(_2\)O (blue). The ENDOR spectrum of cryoreduced CPO-II contains a strongly coupled exchangeable proton resonance at 13.4 MHz. The exchangeable protons in cryoreduced CPO-II and myoglobin hydroxide are assigned as hydroxide resonances. These results provide further evidence for OH\(^-\) ligation in CPO-II.
CPO-II samples containing 20% glycerol are γ-irradiated at 77K reducing the EPR-silent Fe(IV)-OH center to an EPR-active Fe(III)-OH

3.15: EPR spectra of ferric CPO (blue) CPO-II (red) and cryoreduced CPO-II (black). CPO-II samples containing 20% glycerol are γ-irradiated at 77K reducing the EPR-silent Fe(IV)-OH center to an EPR-active Fe(III)-OH
3.16: Cryoreduction of CPO-II. A sample of CPO-II containing 20% glycerol is γ-irradiated at 77 K resulting in reduction of the EPR silent Fe(IV)-OH center. The Fe(III)-OH species generated by this process is EPR active, and ENDOR measurements reveal a very strongly coupled (13.4 MHz) exchangeable proton.
3.17: X-band ENDOR spectra of alkaline myoglobin and cryoreduced CPO-II in H$_2$O (red) and D$_2$O (blue) centered at the proton Larmor frequency. * indicates hydroxide resonances in H$_2$O (red) samples.
3.4.2 Resonance Raman Spectroscopy of CPO-II

All of the spectroscopic methods utilized to characterize CPO-II have provided evidence that it is indeed a basic ferryl species, but all of the techniques used so far do not allow for the direct observation of protonation. We applied resonance Raman to provide direct evidence for OH⁻ ligation.

The low-frequency resonance Raman (454.5 nm excitation) spectra for ferric CPO and H₂O/H₂O₂, D₂O/H₂O₂, and H₂O/H₂¹⁸O₂ preparations of CPO-II are shown in Figure 3.18. Arrows highlight a Raman band that moves upon isotopic substitution. Also shown in Figure 3.18 are the oxidation-state marker bands (ν₄)⁵⁶ and EPR spectra for ferric CPO and CPO-II samples. The oxidation-state marker band for ferric CPO appears at 1375 cm⁻¹ in comparison to samples of CPO-II with an oxidation-state marker band at 1377 cm⁻¹. This is a small, but reproducible, upshift for ν₄ and was used as a tool to monitor quality of resonance Raman samples. CPO-II is EPR silent. The EPR spectra (right) of CPO-II samples used for resonance Raman experiments are also shown in the figure where ferric CPO is essentially gone in all of the CPO-II samples; a radical signal remains at g = 2 attributable to an ascorbate radical.⁴⁵ EPR measurements reveal that all samples used for analysis were ≥ 95% CPO-II.

Figure 3.19 highlights the low-frequency resonance Raman data with a narrower frequency range. In order to minimize effect of photodamage on the samples, all data collected here were compiled from samples exposed to the laser beam < 3 hours. A new
band unique to CPO-II is present in the low frequency resonance Raman spectrum of CPO-II at 565 cm\(^{-1}\) (blue) with a H\(_2\)O/H\(_2\)O\(_2\) preparation. Also shown in Figure 3.19 are D\(_2\)O/H\(_2\)O\(_2\) (black) and H\(_2\)O/H\(_2\)\(^{18}\)O\(_2\) (red) preparations of CPO-II. The new Raman band is shifted to 552 cm\(^{-1}\) when CPO-II is prepared in D\(_2\)O. When CPO-II is prepared in H\(_2\)\(^{18}\)O\(_2\), the Raman band is shifted to 543 cm\(^{-1}\). The difference spectra (lower) highlight the Raman band downshifts upon isotopic substitution of 13 cm\(^{-1}\) with OD\(^-\) and 22 cm\(^{-1}\) for \(^{18}\)OH\(^-\) ligations. These isotopic shifts are consistent with what is predicted for an Fe-OH harmonic oscillator (~ 12 cm\(^{-1}\) and 23 cm\(^{-1}\), respectively).
Low-frequency resonance Raman data (454.5 nm excitation), oxidation state marker band ($v_d$), and EPR spectra of ferric CPO and CPO-II samples (pH 6.5). EPR spectra indicated that CPO-II samples contained < 5% ferric enzyme. The radical signal near $g = 2$ is attributed to oxidized ascorbate. Arrows highlight movement of the Fe(IV)-OH stretch with isotopic substitution. *, labels the 466 nm line of the argon ion laser. No movement of the oxidation state marker band ($v_d$) was observed during data collection. EPR measurements taken after sample irradiation revealed no detectable change in sample composition.
3.19: Top: Overlay of resonance Raman spectra (454.5 nm excitation) of H₂O/H₂O₂ (blue), D₂O/H₂O₂ (black) and H₂O/H₂¹⁸O₂ (red) preparations of CPO-II with a narrower range of frequencies. Bottom: Difference spectra reveal changes in νFe(IV)-OH upon deuterium substitution (black) and ¹⁸O substitution (red).
These resonance Raman results for CPO-II are consistent with OH$^-$ ligation. Another example of OH$^-$ ligation determined from resonance Raman is myoglobin-hydroxide. A resonance Raman report of alkaline myoglobin located an Fe$^{III}$-OH stretching frequency at 550 cm$^{-1}$ that is red-shifted to 538 cm$^{-1}$ upon deuterium substitution.

A 13 cm$^{-1}$ downshift upon deuterium substitution in CPO-II is unprecedented for ferryl hemes. Resonance Raman reports of deuterium substitution in some ferryl heme systems measure Fe-O stretching frequencies higher in energy (≈ 2 – 6 cm$^{-1}$) than when the sample is prepared in H$_2$O. The higher $\nu_{Fe-O}$ frequencies are due to weaker hydrogen bonding in D$_2$O of the Fe(IV)O unit with a histidine residue in the distal cavity.

Due to the presence of a histidine residue in close proximity to the heme iron, many ferryl heme species exhibit different low- and high-pH Fe-O stretching frequencies. Lower pH’s tend to downshift $\nu_{Fe-O}$ frequencies (≈10 cm$^{-1}$). This small change in $\nu_{Fe-O}$ frequencies would be associated with a 0.006 Å increase in Fe-O bond length according to Badger’s rule and would not be consistent with an assignment of an Fe$^{IV}$-OH.$^4$

It was shown by Mössbauer spectroscopy that CPO-II is actually a mixture of two Fe(IV) intermediates in an ~ 70:30 ratio. The major species was assigned as an Fe(IV)-OH from a combination of multiple spectroscopies. The assignment of the minor ferryl species was unsettled. Importantly, in this resonance Raman study no evidence of an Fe(IV)-oxo species was located. This is consistent with three other reports that were unable to locate a ferryl stretching frequency for CPO-II.$^{34-36}$
3.4.3 Preparation and Characterization of a Single Fe(IV) Species in CPO-II

We determined a method to prepare one ferryl species in CPO-II. Reaction of ferric CPO and ascorbic acid with *meta*-chloroperbenzoic acid (*m*-CPBA) in 10% acetone produces a single ferryl species. Acetone is added to the reaction mixture in order to solubilize *m*-CPBA. The 4.2K/54-mT Mössbauer spectrum for the *m*-CPBA intermediate is shown in Figure 3.20B. The raw data was fit to one quadrupole doublet ($\Delta E_Q = 2.05$ mm/s, $\delta = 0.10$ mm/s), which is highlighted with a solid red line. The isomer shift of the *m*-CPBA intermediate indicates that it is an Fe(IV) species. The single ferryl species of the *m*-CPBA intermediate has parameters similar to the major ferryl species of a standard preparation (peracetic acid generated) of CPO-II, shown in Figure 3.20A. The two preparations of CPO-II yield an ~70:30 ratio of Fe(IV) intermediates (standard preparation) and a single ferryl species (*m*-CPBA intermediate).
3.20: Mössbauer spectra of CPO-II generated with peracetic acid (A) or *meta*-chloroperbenzoic acid in 10% acetone (B) and ascorbic acid at pH 6.5. Spectrum A shows the raw data and the solid black line is best fit to two quadrupole doublets ($\Delta E_Q = 2.05$ mm/s, $\delta = 0.10$ mm/s, red line) and ($\Delta E_Q = 1.62$ mm/s, $\delta = 0.11$ mm/s, blue line), 72% and 28% of the total absorption, respectively. Spectrum B shows raw data and solid line is fit to one quadrupole doublet ($\Delta E_Q = 2.05$ mm/s, $\delta = 0.10$ mm/s, red line).
Mössbauer spectroscopy and resonance Raman spectroscopy were applied to determine the identity of the \( m \)-CPBA intermediate. Mössbauer and resonance Raman data collection were performed on the same sample. Figure 3.21 shows the low-frequency resonance Raman data (left) and the Mössbauer spectra (right) of the \( m \)-CPBA intermediate. Right: both the \( m \)-CPBA intermediates generated in \( \text{H}_2\text{O} \) and \( \text{D}_2\text{O} \) have identical Mössbauer parameters (\( \Delta E_0 = 2.06 \text{ mm/s} \) and \( \delta = 0.10 \text{ mm/s} \)). The isomer shift of the \( m \)-CPBA intermediate in \( \text{H}_2\text{O} \) and \( \text{D}_2\text{O} \) indicates an Fe(IV) oxidation state. Left: overlay of low-frequency resonance Raman of \( m \)-CPBA generated CPO-II in 10\% acetone reacted in \( \text{H}_2\text{O} \) (top, red) and \( \text{D}_2\text{O} \) (top, blue) and their difference spectra (bottom, black). Highlighted in the low-frequency resonance Raman spectrum of the \( m \)-CPBA intermediate in \( \text{H}_2\text{O} \) is a peak at 561 cm\(^{-1}\) that is red-shifted to 549 cm\(^{-1}\) when CPO-II is generated in \( \text{D}_2\text{O} \). The observed shift in \( \text{D}_2\text{O} \) is identical to a theoretically predicted Fe-OH harmonic oscillator (12 cm\(^{-1}\)). This result confirms the identity of the single ferryl species in the \( m \)-CPBA intermediate as an iron(IV)-hydroxide species.
3.21: Low-frequency resonance Raman data (457.9 nm excitation) and Mössbauer spectra of CPO-II prepared with meta-chloroperbenzoic acid in 10% acetone, pH 6.5, in H₂O and D₂O. Data collection for resonance Raman and Mössbauer was performed on the same sample. Left: overlay of low-frequency resonance Raman of m-CPBA generated CPO-II in 10% acetone reacted in H₂O (top, red) and D₂O (top, blue) and their difference spectra (bottom, black). Right: Mössbauer spectra of m-CPBA generated CPO-II in 10% acetone prepared in H₂O (top) and D₂O (bottom).
The structural parameters of the Fe(IV)-hydroxide species in CPO-II were determined with a combination of X-ray absorption and Mössbauer spectroscopies. Mössbauer measurements were performed on samples used for X-ray absorption data collection. The best fit of CPO-II prepared with \textit{m}-CPBA in 10\% acetone in the extended X-ray absorption fine structure region (EXAFS) is shown in Figure 3.22 along with its corresponding Fourier transform. Black lines show raw data and red lines show best fits. The fits shown were obtained over the region of $k = 3$-15 Å$^{-1}$. Shown in Table 3.3 are the metal-ligand bond distances for the \textit{m}-CPBA intermediate. Coordination numbers (N) were varied systematically, but were constrained during each individual fit. Fits shown in bold typeface represent the best fit obtained for the sample. In agreement with previous EXAFS measurements on CPO-II, the \textit{m}-CPBA intermediate exhibits a long Fe-O bond length (1.81 Å).\textsuperscript{3} This is in excellent agreement with a density functional calculation of a thiolate-ligated protonated ferryl heme species (Fe(IV)-OH, 1.81 Å),\textsuperscript{4} while an authentic ferryl species has Fe-O bond lengths near ~1.65 Å.\textsuperscript{24,66}
3.22: EXAFS spectrum and Fourier transform of the raw data of CPO-II prepared with \textit{m}-CPBA in 10% acetone. Black lines show raw data and red lines show best fits. The fits shown were obtained over the region of $k = 3-15$ Å$^{-1}$. 
### 3.3: EXAFS fitting results for *meta*-chloroperbenzoic acid/10% acetone generated chloroperoxidase compound II.

<table>
<thead>
<tr>
<th>Fe-N</th>
<th>Fe-S</th>
<th>Fe-O</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>R</td>
<td>σ²</td>
</tr>
<tr>
<td>4</td>
<td>2.002(3)</td>
<td>0.001(0)</td>
</tr>
<tr>
<td>4</td>
<td>1.993(4)</td>
<td>0.001(0)</td>
</tr>
<tr>
<td>4</td>
<td>2.010(3)</td>
<td>0.001(0)</td>
</tr>
<tr>
<td>4</td>
<td>2.005(4)</td>
<td>0.001(0)</td>
</tr>
</tbody>
</table>

Raw data were fit over the region \( k = 3-15 \text{ Å}^{-1} \). Coordination number \( N \), interatomic distance \( R \) (Å), mean-square deviation in \( R \) (the Debye-Waller factor), \( \sigma^2 \) (Å²), and the threshold energy shift \( E_0 \) (eV). The values in parenthesis are estimated standard deviations obtained from the diagonal elements of the covariance matrix. Empirically, EXAFS uncertainties are ±0.02 for \( R \) and ±20% for \( N \) and \( \sigma^2 \). The fit error is defined as: \[ \left[ \sum k^6(\chi_{\text{exptl}}-\chi_{\text{calc}})^2 \sum k^6\chi_{\text{exptl}}^2 \right]^{1/2} \]. Best fit is shown in boldface. Alternative fits with different coordination numbers are also shown. Coordination numbers, \( N \), were constrained during fits.
3.4.4 Identifying the Minor Ferryl Species in a CPO-II

Cryogenic reduction of CPO-I has previously been shown to result in an unprotonated CPO-II. The combination of Mössbauer and resonance Raman spectroscopies of cryoreduced CPO-I was used as a tool to determine the identity of the minor Fe(IV) species in standard preparations of CPO-II.

CPO-I is produced by the reaction of ferric CPO with peracetic acid. CPO-I in the presence of 10% t-butanol (v/v) is exposed to γ-irradiation; this process is shown in the top of Figure 3.23. The cryoreduction reaction is quantitatively monitored by Mössbauer spectroscopy. CPO-I accumulates to ≥90% and, upon exposure of CPO-I to 4 MRad of γ-irradiation, a sharp quadrupole doublet grows in that is consistent with an Fe(IV) species. The resulting Mössbauer spectrum of cryogenically reduced CPO-I is shown in Figure 3.23A. After appropriate amounts of other components are removed, a sharp quadrupole doublet (blue line) remains that is fit to the following Mössbauer parameters: $\Delta E_Q = 1.43 \text{ mm/s}$, $\delta = 0.12 \text{ mm/s}$. These parameters are similar to what has previously been reported. Mössbauer spectroscopy reveals that 51% of CPO-I is reduced by one electron to generate an unprotonated CPO-II, while the remaining ferric enzyme is reduced to a high-spin ferrous species.

The cryogenic reduction of CPO-I was followed by the combination of Mössbauer and resonance Raman spectroscopies on the same sample. Shown in Figure 3.23B are the low-frequency resonance Raman spectra of $^{57}$Fe-enriched CPO-I (red),
$^{57}$Fe-enriched cryoreduced CPO-I (blue) and $^{54}$Fe-enriched CPO-I (black). CPO-I was prepared with both $^{57}$Fe-enriched CPO and $^{54}$Fe-enriched CPO in order to identify the Fe-O stretching frequency. The $\nu_{\text{Fe-O}}$ frequency of CPO-I is determined to be 804 cm$^{-1}$ at 77 K, which is in good agreement with three other resonance Raman reports.$^{30-32}$ Upon cryogenic reduction, a new Raman peak grows in at 796 cm$^{-1}$ while the 804 cm$^{-1}$ peak diminishes. This result is further highlighted by their difference spectrum (shown as a black, dotted line). The new peak at 796 cm$^{-1}$ was assigned as the cryoreduced CPO-I (or unprotonated CPO-II) Fe-O stretching frequency. This result indicates that the minor ferryl species in CPO-II is most likely not an Fe(IV)O species, since enhancement of this Raman band is likely not an issue.
3.23: Cryogenic reduction of CPO-I. CPO-I in the presence of \( t \)-butanol is reduced by \( \gamma \)-irradiation at 77 K. A) Mössbauer spectrum of cryoreduced CPO-I (top), after contributions of CPO-I are removed a quadrupole doublet is highlighted with a blue line (bottom). B) Low-frequency resonance Raman spectra (457.9 nm excitation) of \( ^{57}Fe \)-enriched CPO-I (red), \( ^{57}Fe \)-enriched cryoreduced CPO-I (blue) and \( ^{54}Fe \)-enriched CPO-I (black). The difference spectrum of CPO-I and cryoreduced CPO-I is represented by a black, dotted line.
Since it was known how to prepare CPO-II as a single ferryl species, a comparison of a standard preparation of CPO-II and an \textit{m}-CPBA/acetone preparation of CPO-II revealed differences in their low frequency resonance Raman spectra. Displayed in Figure 3.24 are the low-frequency resonance Raman spectra of peracetic acid generated CPO-II (A) and \textit{m}-CPBA/10\% acetone generated CPO-II (B). H\textsubscript{2}O preparations (red) and D\textsubscript{2}O preparations (blue) of CPO-II and their difference spectra (black) are also shown in Figure 3.24. The low-frequency resonance Raman spectra of two different preparations of CPO-II appear very similar. Close inspection of the peracetic acid generated CPO-II (A) reveals that the hydroxide peak contains a high-energy shoulder. For example, peracetic acid generated CPO-II cannot be fit with a single Gaussian, but rather requires two Gaussians for an appropriate fit. On the other hand, the \textit{m}-CPBA/10\% acetone preparation of CPO-II requires only one Gaussian for the hydroxide peak. Figure 3.25 shows the low-frequency resonance Raman spectrum of a standard preparation of CPO-II. The resonance Raman spectra of CPO-II prepared with peracetic acid is shown in (a) and (b), fit with two Gaussians at 561 cm\(^{-1}\) (red line) and 565 cm\(^{-1}\) (blue line), and one Gaussian, respectively. The residuals are displayed underneath the spectra with a black solid line. A standard preparation of CPO-II cannot be fit with a single Gaussian as displayed clearly from the residual in (b).
3.24: Overlay of low-frequency resonance Raman spectra (457.9 nm excitation) of CPO-II generated with (A) peracetic acid or (B) m-CPBA/acetone in H$_2$O (red) and D$_2$O (blue). Ascorbate was the reductant in both cases. The difference spectra are shown in black. Arrows indicate high-energy shoulders found in standard preparations of CPO-II.
3.25: Low-frequency resonance Raman spectrum of a standard preparation of CPO-II (457.9 nm excitation). The spectra of CPO-II prepared with peracetic acid is shown in (a) and (b), fit with two Gaussians at 561 cm\(^{-1}\) (red line) and 565 cm\(^{-1}\) (blue line), and one Gaussian, respectively. The residuals are displayed with the lower black solid line. Black dotted lines represent peaks that are not associated with Fe-OH stretching and were held constant during fitting procedures.
We also performed resonance Raman and Mössbauer spectroscopies on the same sample. Figure 3.26 displays the low-frequency resonance Raman spectra (left) of the \textit{m}-CPBA intermediate (A) and a standard preparation of CPO-II (B) and their corresponding Mössbauer spectra of both preparations of CPO-II (right). One ferryl species is observed in the Mössbauer spectrum of the \textit{m}-CPBA intermediate, highlighted with a red line, while one peak is observed in the low-frequency resonance Raman spectrum at 561 cm\(^{-1}\). The residual of the Gaussian fit is shown underneath the spectra and is represented as a black line. A standard preparation of CPO-II exhibits two ferryl intermediates in the Mössbauer spectrum, highlighted with a red line (major species) and a blue line (minor species) to highlight two quadrupole doublets. In this case, two peaks contribute to the hydroxide peak in the resonance Raman spectrum at 561 cm\(^{-1}\) and 565 cm\(^{-1}\) highlighted by the red and blue lines, respectively. The black, dotted line indicates peaks associated with CPO and not due to the \(v_{\text{Fe-OH}}\) frequency; these peaks are held constant during Gaussian fitting procedures.

These spectroscopic results provide strong evidence that the minority ferryl species in CPO-II is also an iron(IV)-hydroxide. We, therefore, assigned the ratio of Fe(IV) intermediates in CPO-II as two Fe\(^{IV}\)-OH species.
3.26: Low-frequency resonance Raman (left) (457.9 nm excitation) and Mössbauer spectroscopy (right) of *meta*-chloroperbenozic acid in 10% acetone preparation of CPO-II (A) and peracetic acid generated CPO-II (B) of the same sample. A) *m*-CPBA/acetone CPO-II shown with one Gaussian (red) and one ferryl species (red). B) Peracetic acid generated CPO-II shown with two Gaussians fit for the hydroxide peak (red) and (blue) with the same color scheme to highlight the two ferryl species in the Mössbauer spectrum where the minority species is highlighted in blue and the majority species is highlighted in red.
3.4.5 Generation of a Single Fe(IV)-Hydroxide in CPO-II: \textit{m}-CPBA or Acetone?

The ability to prepare one ferryl intermediate in CPO-II is a consequence of either the solvent or the use an alternative oxidant. The latter seems unlikely since we have previously used different oxidants, but the same mixture of ferryl intermediates resulted. In order to investigate this question, CPO-II was prepared with \textit{m}-CPBA in 10\% \textit{t}-butanol rather than 10\% acetone.

The Mössbauer spectra of CPO-II prepared with \textit{m}-CPBA in 10\% acetone (A) and 10\% \textit{t}-butanol (B) and their subtraction (C) are shown Figure 3.27. It is clearly shown that when the reaction is performed in \textit{t}-butanol rather than acetone a very different result is observed. Two ferryl species in CPO-II are observed in an \textasciitilde 70:30 ratio when prepared in 10\% \textit{t}-butanol. The spectrum that results from their subtraction is fit to one quadrupole doublet ($\Delta E_q = 1.61$ mm/s, $\delta = 0.11$ mm/s). These Mössbauer parameters are consistent with the parameters of the minority species of a standard preparation of CPO-II. Figure 3.28 shows that the Mössbauer spectrum of peracetic acid generated CPO-II (A) is essentially identical to the Mössbauer spectrum of CPO-II generated with \textit{m}-CPBA in 10\% \textit{t}-butanol (B), which is highlighted by their difference spectrum (C). In order to confirm that acetone is producing one ferryl species in CPO-II, the reaction is performed with peracetic acid in the presence of acetone and without acetone. The Mössbauer spectra of peracetic acid generated CPO-II, shown in Figure 3.29, without acetone (A) and in the presence of 10\% acetone (B). Figure 3.29B shows the raw data of CPO-II in
the presence of 10% acetone that was fit to one quadrupole doublet (red line) with the following Mössbauer parameters: $\Delta E_Q = 2.06 \text{ mm/s}$ and $\delta = 0.10 \text{ mm/s}$. These results confirm that addition of acetone to the reaction mixture leads to the production of one ferryl species in CPO-II.
3.27: Mössbauer spectra of CPO-II prepared with \( m \)-CPBA in (A) 10% acetone and (B) 10% \( t \)-butanol. Their difference spectrum (C) is best fit to one quadrupole doublet with the following Mössbauer parameters: \( \Delta E_Q = 1.61 \text{ mm/s} \) and \( \delta = 0.11 \text{ mm/s} \).
3.28: Mössbauer spectra of CPO-II prepared with peracetic acid (A), \textit{m}-CPBA in 10% \textit{t}-butanol (B) and their difference spectrum (C).
Mössbauer spectra comparison of peracetic acid generated CPO-II in 0% (A) and 10% acetone (B). The peracetic acid CPO-II in 10% acetone has Mössbauer parameters of $\Delta E_Q = 2.06$ mm/s and $\delta = 0.10$ mm/s.
3.4.6 The Role of the Acetone in Producing a Single Ferryl Species in CPO-II

The role of the acetone is unknown, but it is not unique in modulating the ratio of ferryl intermediates. For example, generating CPO-II with excess ascorbate pushes the ratio in the other direction. When ferric CPO is reacted with hydrogen peroxide and 300x excess of ascorbic acid (0.3M) the ratio of ferryl intermediates is shifted to ~ 60:40. The Mössbauer spectrum of CPO-II prepared with H2O2 with a 300x excess of ascorbic acid is shown in Figure 3.30. The spectrum shows raw data and the solid black line is best fit to two quadrupole doublets (ΔE_Q=2.06 mm/s, δ=0.10 mm/s, red line) and (ΔE_Q=1.59 mm/s, δ=0.11 mm/s, blue line), which is 60% and 40% of the total absorption, respectively. A reasonable explanation for this phenomenon is that small molecules (acetone or ascorbate) can bind near the distal cavity of CPO and modulate the ratio of Fe^{IV}-OH conformers. These small molecules can shift the ratio of Fe(IV) intermediates in either direction.

Interestingly, the Mössbauer spectra of compound II of P450’s reveal only one intermediate.55 The Mössbauer parameters of all of the known P450- IIIs are similar to the Mössbauer parameters of the major species in CPO-II. The major structural difference of P450’s is that they contain a more hydrophobic distal cavity. Evidence for the role of the proton shuttle in CPO in modulating ratio of Fe(IV) intermediates comes from modification of histidine residues. P450’s do not contain ionizable residues in the active site that would facilitate formation of two Fe(IV) intermediates.57
3.30: Mössbauer spectrum of CPO-II prepared with H₂O₂ with a 300x excess of ascorbic acid. The spectrum shows raw data and the solid black line is best fit to two quadrupole doublets (ΔE_Q=2.06 mm/s, δ=0.10 mm/s, red line) and (ΔE_Q= 1.59 mm/s, δ = 0.11 mm/s, blue line), which is 60% and 40% of the total absorption, respectively.
3.5 Conclusion

A number of inconsistencies are cleared up in this chapter by the assignment of the minor component of CPO-II as an iron(IV)-hydroxide. The ratio of two ferryl intermediates in CPO-II is shown to be pH invariant, which is no longer troubling since we have shown that this ratio is not related by a protonation event. Also, the absence of an $\nu_{Fe-O}$ frequency in CPO-II is in agreement with our results since the minor ferryl species is described as an Fe$^{IV}$-OH. Importantly, this chapter clarifies the status of ferryl protonation in thiolate-ligated heme proteins. Accumulating evidence suggests that ferryls of thiolate-ligated heme enzymes are basic.

The role of the thiolate in these heme enzymes is a subject of debate. The electron donating thiolate has been implicated in promoting oxygen activation and hydrogen atom abstraction. For example, the thiolate ligand increases the basicity of the ferryl species, which increases the driving force to perform hydrogen atom abstraction. This provides a reasonable explanation of why Nature utilizes the electron donating thiolate ligand to perform some very demanding oxidizing reactions.
3.6 References


Chapter 4
Summary and Conclusions

Cytochromes P450 (P450) are hydroxylating enzymes that play critical roles in many biological processes, including production of hormones and metabolism of xenobiotics. P450’s are thiolate-ligated heme proteins that are able to insert an oxygen atom, derived from either dioxygen or peroxide, into substrate. Thiolate-ligated heme proteins (including P450’s, Chloroperoxidase, and Nitric Oxide Synthase) are the only heme enzymes known to perform oxygen transfer chemistry.

Recently, an X-ray absorption study has implicated a novel role for the axial ligand in thiolate-ligated heme enzymes. This study of the ferryl form of chloroperoxidase (CPO-II) revealed that it was not an authentic ferryl (iron(IV)oxo), but rather a protonated ferryl with a longer than expected Fe-O bond length of 1.82 Å (typically ferryls have Fe-O bond lengths near 1.65 Å). This is surprising because Fe(IV)oxo species are generally considered to be electrophilic. The electron donating thiolate makes the Fe(IV)oxo species sufficiently more basic so that it is protonated at neutral pH.

This finding has important implications for P450 hydroxylation chemistry. The generally accepted P450 hydroxylation mechanism, referred to as the oxygen rebound
mechanism, involves an iron(IV)oxo-radical species, called compound I.\textsuperscript{5,6} Compound I abstracts a hydrogen atom from substrate to generate a protonated ferryl (the “rebound intermediate”), radical recombination with the protonated ferryl produces hydroxylated substrate. Mayer and coworkers have shown that the ability of metal-oxos to abstract hydrogen scales with the redox potential of the oxidized metal-oxo and the pKa of the reduced form.\textsuperscript{7,8} This means that for heme enzymes the ability to abstract hydrogen scales with both the redox potential of compound I and the pKa of compound II, suggesting that the presence of basic ferryls in thiolate-ligated heme proteins allows these enzymes to access higher energy C-H bonds.

Chloroperoxidase (CPO) is a thiolate-ligated heme enzyme that shares many spectroscopic signatures with P450’s due to their almost identical first coordination sphere.\textsuperscript{9-11} Intermediates of P450 enzymes are very reactive while intermediates of CPO are much more stable. Due to their common thiolate ligand, it is reasonable to assume that intermediates of CPO should resemble their P450 counterparts. Therefore, I have utilized CPO as a model to understand P450 chemistry. I have trapped high-valent iron states in CPO and observed them with a wide variety of spectroscopic methods: stopped-flow spectrophotometry, X-ray absorption spectroscopy, Mössbauer spectroscopy, resonance Raman spectroscopy, and electron paramagnetic resonance, among others. Utilizing freeze-quench technology, I generated high-valent iron intermediates on the millisecond time-scale in which enzyme is rapidly mixed with oxidant and the reaction mixture is sprayed into liquid ethane (-180°C). I utilized spectroscopy to observe trapped intermediates in order to extract physical parameters such as structure, oxidation states,
and spin states. My recent results have provided insight into the role of thiolate-ligation in P450 proteins by utilizing CPO as a model for P450 catalysis.

4.1 Thiolate-ligated Compound I

To gain insight into the reactive intermediate of P450 enzymatic catalysis (compound I), X-ray absorption measurements of chloroperoxidase compound I was performed.\textsuperscript{12} Compound I of chloroperoxidase (CPO-I), unlike P450’s, is able to accumulate to concentrations that are amenable for numerous spectroscopies: UV/Vis\textsuperscript{13}, resonance Raman\textsuperscript{14-16}, electron paramagnetic resonance\textsuperscript{17}, and Mössbauer\textsuperscript{17}. Similar to P450’s, this intermediate is very reactive. Under certain conditions, CPO-I is generated in >90% yield with the application of rapid freeze-quench technology. X-ray absorption results indicate that CPO-I is an authentic ferryl species (a doubly bonded Fe-O bond with a length of 1.65 Å) and strong axial-ligand interactions result in a long Fe-S bond length of 2.48 Å. These results provided a first glimpse into the elusive intermediate of P450 chemistry.

4.2 Observation of Two Feryl Species in Chloroperoxidase Compound II

Mössbauer spectroscopy was utilized in order to examine the protonation state of CPO-II.\textsuperscript{18} Mössbauer spectroscopy is typically applied to determine composition of iron
containing samples, but is has not typically been applied for structural determination. It was recently determined that density functional theory predicts Mössbauer parameters very accurately (0.2-0.5 mm/s for quadrupole splitting parameters). A density functional calculation on an active site model of CPO predicts a 1.06 mm/s difference for the protonated ferryl and the ferryl. With the combination of density functional calculations and Mössbauer spectroscopy, it was shown that it is possible to differentiate between a protonated ferryl and a ferryl due to the large difference in quadrupole splittings. Surprisingly, Mössbauer measurements revealed that CPO-II is actually two distinct ferryl species locked in an ~70:30 ratio. With the combination of EXAFS spectroscopy, density functional calculations, and cryoreduction and annealing experiments the major species was identified as a protonated ferryl.

4.3 Resonance Raman Spectroscopy Provides Direct Evidence of an Iron(IV)-Hydroxide in Chloroperoxidase Compound II

The observation of a protonated ferryl has important implications for P450 chemistry, where the driving force for hydrogen atom abstraction scales with the both redox potential of compound I and pKa of compound II, suggesting that the presence of basic ferryls allows these enzymes to access higher energy C-H bonds. This theory rests upon the important assumption that basic ferryls are unique to thiolate-ligated heme enzymes. Many other research groups have proposed the presence of protonated ferryls in other heme enzymes (non-thiolate), which has been inferred from long Fe-O bonds in
None of these X-ray structural techniques have directly observed the protonation of a ferryl species.

In an effort to clarify the issue of ferryl protonation states the applicability of Badger’s rule to mononuclear Fe-O was determined. Badger’s rule is an empirical formula that relates bond distance and vibrational frequency. Recently, a theoretical parameterization of Badger’s rule examining over 30 complexes made a direct correlation between resonance Raman stretching frequency and Fe-O bond length. It was found that Fe-O bond length derived from the X-ray crystal structures deviated substantially from the bond length predicted from its resonance Raman stretching frequency. A Badger’s rule analysis of the EXAFS determined bond length of CPO-II (1.82 Å) affords an $\nu_{\text{Badger}} = 563$ cm$^{-1}$. A deuterium and $^{18}$O sensitive stretching frequency was located, $\nu_{\text{Fe-OH}} = 565$ cm$^{-1}$, by resonance Raman spectroscopy in remarkable agreement to what is predicted from Badger’s rule.

4.4 Identification of the Minor Ferryl Species in Chloroperoxidase Compound II

It was recently determined that one ferryl species of CPO-II could be produced with the use of $m$-CPBA in 10% acetone. This result offered an opportunity to fully characterize the minor ferryl species in CPO-II by utilizing resonance Raman and Mössbauer spectroscopies. Two experiments provided evidence for the identity of the minority species in CPO-II: resonance Raman experiments coupled with Mössbauer spectroscopy of cryoreduced CPO-I and low-frequency resonance Raman spectra.
comparison of the \( m \)-CPBA/acetone generated CPO-II and a standard preparation (~70:30 ratio) of CPO-II coupled with Mössbauer spectroscopy. Resonance Raman of cryoreduced CPO-I (unprotonated CPO-II) revealed a new Fe-O stretching frequency of 796 cm\(^{-1}\) upon exposure to \( \gamma \)-irradiation. Comparison of two preparations of CPO-II led to the observation that two ferryl species observed by Mössbauer spectroscopy are associated with two peaks that contribute to the hydroxide stretching frequencies by resonance Raman spectroscopy. These peaks were both red-shifted 12 cm\(^{-1}\) in D\(_2\)O. The first experiment suggests that if the minority species were an Fe(IV)-oxo species then its Fe-O stretching frequency would be observed by resonance Raman spectroscopy because CR-CPO-I, which is essentially an unprotonated CPO-II, is observed upon reduction of CPO-I. Therefore, enhancement of the \( \nu_{\text{Fe=O}} \) frequency is not likely an issue. The second experiment suggests that the peracetic acid generated CPO-II contains two iron(IV)-hydroxides (both peaks red-shift upon deuterium substitution) at 561 cm\(^{-1}\) and 565 cm\(^{-1}\), while the \( m \)-CPBA intermediate contains a single peak at 561 cm\(^{-1}\). The evidence strongly indicates that a standard preparation of CPO-II is in fact two iron(IV)-hydroxide species.

4.5 Conclusion

All evidence, to date, indicates that thiolate-ligated compound II’s are basic.\(^{27-30}\) This may be Nature’s method of providing the driving force to perform hydrogen atom abstraction. In order for this hypothesis to be correct basic ferryls must be a general and unique feature of thiolate-ligated heme proteins. Accumulating evidence on CPO and
P450’s points to the former assumption as being correct; although, conflicting reports have found that other ferryl heme enzymes (non-thiolate) are protonated at neutral pH. No direct evidence of an Fe$^{IV}$-OH unit has been observed in a non-thiolate ligated heme enzyme. CPO-II is the only system where protonation of the ferryl unit has been directly observed leading to the conclusion that thiolate-ligated heme enzymes might be the only heme enzymes to contain basic ferryls.

4.6 References


Chapter 5
Materials and Methods

5.1 Preparation and Purification of Chloroperoxidase and Isotopically-Labeled Chloroperoxidase

Chloroperoxidase (CPO) was obtained from the filamentous fungus Caldariomyces fumago (ATCC 16373). C. fumago was grown in fructose-salts media at 25°C, according to established methods. For \textsuperscript{57}Fe cultures, \textsuperscript{57}FeCl\textsubscript{3} was added as the sole iron source and the media was treated with Chelex (Sigma Aldrich) prior to iron and magnesium sulfate addition. For \textsuperscript{33}S cultures, an equivalent of 5 mg/L of \textsuperscript{33}S was added to the media as the sole sulfur source in the form of \textsuperscript{33}SO\textsubscript{4}\textsuperscript{2-}. After 12 days, the media was collected, filtered through cheesecloth and subjected to two rounds of acetone precipitation. Cold acetone (-20°C) was added to the media slowly with constant stirring to 40%(v/v). Gel precipitation occurred at -20°C after an hour and was removed. Cold acetone (-20°C) was added again for protein precipitation to 60% (v/v). Precipitation of CPO occurred overnight at -20°C. Protein was centrifuged at 6000g for 5 minutes and solution decanted. CPO was dissolved in 20 mM KPhos (pH 5.9), filtered, washed loaded onto a 100 mL SourceQ (GE Healthcare) column and eluted with a linear gradient of 0 - 0.2 M NaCl. Fractions with an Rz (OD\textsubscript{400nm}/OD\textsubscript{280nm}) value of 1.4 were collected.
5.2 Preparation of CPO-I for X-Ray Absorption Spectroscopy

All samples were prepared using a four-syringe ram freeze-quench apparatus (Update Instruments, Madison, WI). 4 mM CPO in 0.1 M KPhos, pH 6.5, was mixed in a 2:1 ratio with 75 mM peracetic acid in the same buffer through an appropriate length aging line in a 4°C water bath and quenched into a 50 mL vial filled with liquid ethane (-180°C) after 10 ms. Ethane was decanted and protein dried under vacuum at -140°C. The protein powder was packed into a delrin sample holder suitable for X-ray absorption studies. Approximately 5 EXAFS samples were prepared with every individual quench (a total of 4 quenches), while EPR samples were also prepared to determine percentage of ferric CPO. EPR spectroscopy was performed on a Bruker Elexsys E-560 at 20K and 1mW of power. Samples were analyzed for ferric CPO content and compared to ferric CPO standards to quantify CPO-I. All EXAFS samples contained >90% CPO-I assuming a 50% packing factor.

5.3 X-ray Absorption Data Collection and Analysis of CPO-I

X-ray absorption measurements were performed at the Stanford Synchrotron Radiation Laboratory on beam line 10-2 held at ~10K with an Oxford Instruments liquid helium flow cryostat. The data was collected using a Si (220) phi=90 double crystal monochromator detuned 50% at 8300 eV for harmonic rejection. Data sets were collected with a Canberra 30-element Ge detector. All XAS measurements were collected in fluorescence mode. The program XAS COLLECT was used for data
acquisition. XAS data collected were analyzed with the curve-fitting program EXAFSPAK with use of *ab initio* phases and amplitudes generated from the program FEFF version 8.X39. Data were collected to $k = 16\text{Å}^{-1}$, where $k$ is the photoelectron wave vector. Each data set was calibrated against Fe foil, set at 7111.2998 eV, to obtain a reliable absorption edge. A Gaussian function was used to remove background X-ray scatter from the pre-edge region before the rising edge of the raw data. Both raw and Fourier-filtered data were fit over various regions of $k$ from 1-16Å$^{-1}$. Fits included first and second shell atoms and one multiple scattering component.

Photo-reduction of the sample was an issue, as judged from a shifting X-ray absorption edge to lower energy. Multiple samples were analyzed. Movement of the sample in the beam allowed analysis of a fresh spot in order to minimize the effects of photoreduction. The beam was 2 mm x 1 mm, so multiple spots could be examined on a single sample. Only the first scan at each spot was taken and averaged together for structural determination. Exposure time was ~30 minutes for each spot.

### 5.4 Photoreduction Analysis of CPO-I

Data from the first scan on an unexposed spot was analyzed for effects of photoreduction with respect to X-ray exposure with consecutive scans. Each individual set of scans was averaged and fit using EXAFSPAK as described above. CPO-I contained 16 sample spots. All averaged raw data and Fourier-filtered data were fit between $k = 3-15 \text{Å}^{-1}$.
5.5 Preparation of $^{57}$FeCl$_3$

$^{57}$Fe (Pennwood Chemicals) metal was added to a vial that contained 1 mL of concentrated HCl and allowed to react at 80°C until $^{57}$Fe was visibly consumed. A typical culture contained 35 μM $^{57}$FeCl$_3$.

5.6 Synthesis of $^{33}$SO$_4^{2-}$

Slices of sodium metal were washed in hexanes and added to 10 mL of hexanes in a round bottom flask. Elemental $^{33}$S was added to the flask. Reaction was finished when sulfur was visibly consumed. NaS was extracted three times with 6M NaOH. 2 mL of 30% H$_2$O$_2$ was added dropwise until the yellow solution turned white. H$_2$O$_2$ was degraded by heating the solution at 60°C in the basic solution until bubble formation halted. Concentration of peroxide was determined. No residual peroxide was present in the $^{33}$SO$_4^{2-}$ solution. Phosphoric acid was added to the $^{33}$SO$_4^{2-}$ solution until pH 5 is reached.
5.7 Synthesis of Alkyl Peroxides

*Methyl peroxide:* The synthesis of methyl peroxide is similar to what has been described previously. Briefly, 5 mL of 30% \( \text{H}_2\text{O}_2 \) and 14 mL water and 5.5 mL of dimethyl sulfate were added together and allowed to chill on an ice bath with constant stirring. 11.6 mL of 40% KOH solution was added drop-wise to the solution keeping the temperature below 10°C. After addition the solution was heated gently (30-35°C) until bubbles ceased to appear. The reaction was left to stir for 24 hours at room temperature to let the residual peroxide degrade. *n-butyl peroxide:* 6.68g of KOH was dissolved in 25 mL of methanol, chilled on ice and mixed with sodium carbonate. The carbonate was allowed to settle and the solution decanted. 19 mL of 30% \( \text{H}_2\text{O}_2 \) was added to a flask and solution was to chilled to below -10°C in an isopentane bath kept at -20°C. The KOH/methanol solution was added drop-wise to the peroxide solution while the solution stayed below -10°C. 25 g of \( n \)-butyl sulfate was added drop-wise while maintaining temperature below -10°C. Solution left to stir on ice for 20 hours. 155 mL of ice water was added to the reaction mixture and unreacted \( n \)-butyl sulfate was extracted with ethyl ether. Solution was neutralized with 50% \( \text{H}_2\text{SO}_4 \) at 0°C and 106 g of \( \text{NH}_4\text{SO}_4 \) was added. \( n \)-butyl peroxide was extracted three times with ethyl ether and evaporated. 16 mL of water was added and the mixture was vacuum distilled. Products were analyzed for total peroxide and hydrogen peroxide content.
5.8 Total Peroxide Assay\textsuperscript{3,7}

5 mL of peroxide solution in 20 mL of 2 M H\textsubscript{2}SO\textsubscript{4} was added to a 1 g KI/10 mL water solution and left in the dark for 90 minutes or until reaction was complete. The solution was titrated with sodium thiosulfate to determine the total peroxide concentration.

5.9 Hydrogen Peroxide Assay\textsuperscript{3,8}

3 mL of a peroxide solution and 0.5 mL of oxytitanium sulfate were added together and the solution was diluted to 50 mL with water. A calibration curve with known concentrations of hydrogen peroxide solutions was constructed in order to determine hydrogen peroxide concentration of the unknown solutions.

5.10 Synthesis of Perbenzoic Acids\textsuperscript{9,10}

Synthesis of perbenzoic acids is similar to what has been described previously. Briefly, 0.5 g MgSO\textsubscript{4} was added to 60 mL of water, 6 g NaOH was added and dissolved, 75 mL of methanol was added to the solution and solution was chilled on ice. 15 mL of 30\% H\textsubscript{2}O\textsubscript{2} was added to the solution with constant stirring. 0.05 mol of benzoyl chloride derivative was added drop-wise while temperature was maintained below 8\degree C. The solution stirred for 10 minutes and 150 mL of 20\% H\textsubscript{2}SO\textsubscript{4} was added. Product was
extracted with three rounds of 50 mL cold dichloromethane, the benzoic acid was extracted with 0.2 M KPhos pH 6.0. The dichloromethane solution was dried over MgSO₄ and filtered. Solution was kept on ice while solvent was evaporated. Crude product was recrystallized from 1:3 ether/petroleum ether. NMR spectra was taken of all perbenzoic acids: perbenzoic acid, phenyl acetic acid, and o-chloroperbenzoic acid.

5.11 Synthesis of ¹⁸O Peracetic Acid

Synthesis of peracetic acid has been described previously and has been modified here to incorporate ¹⁸O labeled peracetic acid. 500 μL of a 2% H₂¹⁸O₂ solution was added to a sodium carbonate solution (0.26 mmol) in water maintained at 0°C. The mixture was maintained 0-5°C upon addition of acetic anhydride (0.26 mmol) and allowed to stir for 45 minutes. Ether was added to the stirring solution followed by addition of sulfuric acid (0.39 mmol). The aqueous layer was saturated with ammonium sulfate and was extracted five times with ether. The ether was allowed to evaporate and the peracetic acid solution was analyzed for total peroxide and hydrogen peroxide content. The peracetic acid was stored at 4°C in 45% acetic acid.

5.12 Preparation of CPO-II for Mössbauer Spectroscopy

4 mM ⁵⁷Fe-enriched chloroperoxidase and 37.5 mM reductant (ascorbic acid or p-phenolsulfonic acid) were reacted with 75 mM oxidant (peracetic acid or hydrogen
peroxide) in appropriate buffer. A 2:1 reaction of protein/reductant solution and oxidant was quenched in cold isopentane (-145°C) after 28 ms or 250 ms. CPO-II prepared with $p$-phenolsulfonic acid as reductant was quenched in 5 ms. All reactions proceeded at room temperature.

5.13 Preparation of CPO-I for Cryoreduction

4 mM $^{57}$Fe-enriched chloroperoxidase was reacted with 80 mM peracetic acid in 100 mM KPhos, pH 6.5, containing 15% (v/v) glycerol. A 2:1 reaction of protein and peracetic acid was quenched into cold isopentane (-145°C) after 10 ms. CPO-I was packed into a delrin cup suitable for Mössbauer measurements.

5.14 Cryogenic Reduction of CPO-I and Annealing

Freeze-quenched 10 ms samples of CPO-I containing glycerol were irradiated at the $\gamma$-irradiation facility of the Breazeale nuclear reactor at Pennsylvania State University using a $^{60}$Co source (35 krad/h), a total dose of 3.6 MRad was applied. Samples were maintained at 77K during irradiation by immersion in liquid $N_2$. The cryoreduced samples were annealed by immersion in a dry ice and isopentane bath (-80°C). All samples were analyzed by Mössbauer spectroscopy before and after cryoreduction and annealing experiments.
5.15 Mössbauer Spectroscopy

Mössbauer spectra were recorded on a spectrometer from WEB research (Edina, MN) operating in the constant acceleration mode in a transmission geometry. Spectra were recorded with the temperature of the sample maintained at 4.2 K. For low-field spectra, the sample was kept inside an SVT-400 dewar from Janis (Wilmington, MA), and a magnetic field of 40 mT was applied parallel to the $\gamma$-beam. For high-field spectra, the sample was kept inside a 12SVT dewar (Janis), which houses a superconducting magnet that allows for application of variable magnetic fields between 0 and 8 T parallel to the $\gamma$-beam. The quoted isomer shifts are relative to the centroid of the spectrum of a metallic foil of $\alpha$-Fe at room temperature. Data analysis was performed using the program WMOSS from WEB research.

5.16 Computational Methods

All calculations were performed with Gaussian 03 using the B3LYP functional. Geometry optimizations were performed with the 6-311G basis set. Quadrupole splittings, the asymmetry parameter, $\eta$, and $^{57}$Fe hyperfine couplings were determined at the optimized geometries using the 6-311G basis set. Isomer shifts were determined at the optimized geometries using Neese’s core properties (CP) basis set. For this basis set, an integration grid containing 199 radial shells with 590 angular points per shell was
used. The electron density at the Fe nucleus was determined using the Atoms In Molecules (AIM) option in Gaussian 03.

Calculations were performed on two types of model systems. The first was a porphine complex that on average contained 44 atoms. The second was a larger active-site model that was taken from a crystal structure.\textsuperscript{13} For CPO, these larger calculations (88 atoms for CPO-II) differed from the smaller set in that a portion of the proximal helix was included and all atoms except for an inner core surrounding Fe ($\approx 30$ atoms) were frozen during geometry optimizations. The inclusion of the proximal helix allows for the consideration of important hydrogen-bonding between the helix and the axial-thiolate, while the geometry constraints allow us to examine structures that more closely resemble those found in the enzyme. Initial geometries were obtained from a crystal structure of ferric CPO. The proximal helix was modeled with five residues (Cys29-Asn33). All residues except cysteine, proline, and glycine were converted into alanine, and the peptide chain was capped with hydrogens. During geometry optimizations, the positions of all atoms except Fe, the distal ligand, the porphyrin-nitrogens, alpha-carbons, meso-carbons, meso-hydrogens, and a portion of the proximal ligand (SCH$_2$CH) were constrained to their position in the crystal structure.

5.17 Preparation of CPO-II for Resonance Raman Spectroscopy

CPO-II samples were prepared for resonance Raman spectroscopy by reacting a solution of 450 mM ascorbate and 1.5 mM $^{57}$Fe-enriched ferric CPO with 30 mM H$_2$O$_2$
(or $\text{H}_2^{18}\text{O}_2$) in a 2:1 mixture. The reaction was quenched in liquid ethane (-180°C) 7 ms after mixing. Reagents were in 100 mM KPhos buffer, pH 6.5. Ethane was decanted and evaporated under vacuum in an isopentane bath (-140°C). Samples were packed into EPR tubes in liquid N$_2$. Deuterated CPO-II was prepared as described above, but CPO was exchanged five times with D$_2$O buffer and all reagents were in D$_2$O buffer. EPR measurements at 77 K revealed that the CPO-II samples contained < 5% ferric enzyme.

### 5.18 Resonance Raman Spectroscopy

Resonance Raman spectra were acquired with a TriVista 555 triple monochromator (Acton Research, 900/900/2400 gr/mm) equipped with a CCD camera (Princeton Instruments, 1340 x 100 pixels). The 454.5 nm and 457.9 nm lines of an argon-ion laser (Coherent I-308) was used for excitation. Power was less than 25 mW at the sample. 900/900/2400 gr/mm gratings provide an instrumental resolution of 1.5 cm$^{-1}$ at 454.5 nm (0.51 cm$^{-1}$ per CCD pixel). Samples were held in an EPR finger-dewar (77 K) in an ~135° back-scattering arrangement. The 2326.5 cm$^{-1}$ vibration of liquid N$_2$ was used for calibration. All Raman spectra contained a smoothly varying background, which was removed, but the data were not smoothed or manipulated in any other way. Locations of the difference spectra extrema were obtained by performing a 21-point Savitzky-Golay smoothing. Application of different background subtractions and different smoothing procedures resulted in minimal changes (< 2 cm$^{-1}$) in Fe(IV)-OH stretching frequencies.
5.19 Photo-reduction/Degradation Analysis

A photo-reduction/degradation study of CPO-II was performed in which the same sample-spot was irradiated continuously for more than 4 hours. No significant change in the sample’s spectrum was observed. The CPO-II oxidation-state marker band at 1377 cm\(^{-1}\) did not change position over the course of 4 hours, and no peaks associated with ferric CPO grew in. EPR measurements (77 K) taken after laser irradiation showed no detectable change in sample composition.

5.20 Cryoreduction of CPO-II for ENDOR Spectroscopy

Samples for cryoreduction and ENDOR spectroscopy were prepared by reacting a solution of 37.5 mM ascorbate and 4 mM ferric CPO with 75 mM peracetic acid in a 2:1 mixture. Reactions were quenched into liquid ethane 28 ms after mixing and packed into EPR tubes. Reagents were in 100 mM KPhos buffer, pH 6.5, and 20% (v/v) glycerol. Samples were examined by EPR and resonance Raman spectroscopies prior to cryoreduction. The EPR measurements revealed that the samples contained less than 5% ferric enzyme, while resonance Raman measurements were indicative of CPO-II (\(v_4 = 1377\) cm\(^{-1}\)). Samples were \(\gamma\)-irradiated (\(^{60}\)Co; total dose of 4.5 Mrad) at the \(\gamma\)-irradiation facility of the Breazeale nuclear reactor at Pennsylvania State University. During
irradiation, samples were maintained at 77 K by immersion in liquid N\textsubscript{2}. Alkaline myoglobin was prepared by dissolving horse heart myoglobin (Sigma) in 50 mM borate buffer, pH/pD 10.4/10.9. Continuous wave X-band ENDOR measurements were performed on a Bruker Elexsys E-560 equipped with an Oxford Instruments liquid helium cryostat and a 150-Watt radio frequency (rf) amplifier. Typical experimental conditions were as follows: sample temperature, 10 K; microwave frequency, 9.43 GHz; microwave power, 40 mW; rf frequency modulation, 20 kHz; rf modulation depth of the rf field, 100 kHz; rf attenuation, 0 dB.

### 5.21 Preparation of CPO-II for Mössbauer and Resonance Raman

4 mM \textsuperscript{57}Fe-enriched chloroperoxidase and 60 mM ascorbic acid were contained in the same syringe and reacted with 75 mM oxidant (peracetic acid or \textit{meta}-chloroperbenzoic acid/[10% acetone or 10% t-butanol (v/v)]). All solutions were in 0.1 M KPhos pH 6.5. A 2:1 reaction of protein/ascorbic acid solution and oxidant was quenched in liquid ethane (-180°C) 28, 85, or 142 ms after mixing. All deuterated CPO-II samples were prepared as described above except that ferric CPO was exchanged five times in deuterated buffer and all reagents were prepared in deuterated buffer. The reaction mixture was packed into a Mössbauer sample holder and an EPR tube for analysis. EPR measurements at 77 K revealed that all samples used for analysis had \( \leq 5\% \) ferric enzyme.
5.22 Chemical Modification of Chloroperoxidase

The procedure for chemical modification of CPO was adapted from Blanke et al.\textsuperscript{14} 100 mL of 35 μM \textsuperscript{57}Fe-enriched CPO in 0.1 M KPhos pH 6.5 was allowed to stir for 30 minutes with 90 mM diethylpyrocarbonate (DEPC) at room temperature. Modification of histidine residues was followed by an absorbance increase at 242 nm. Chlorination activity of CPO was assayed by rate of chlorination of monochlorodimedone. The reaction of DEPC and CPO resulted in modified CPO with a residual activity that was 32% that of the unmodified protein. Excess DEPC was removed by application to a desalting column (Sephadex G-25, GE Healthcare) followed by continuous washing with 0.1 M KPhos pH 6.5 buffer. 2.8 mM DEPC-modified \textsuperscript{57}FeCPO and 50 mM ascorbic acid was reacted with 80 mM peracetic acid in a 2:1 ratio and quenched into liquid ethane after 28ms (0.1 M KPhos pH 6.5). Protein powder was packed into a Mössbauer sample holder for analysis. The sample was determined to contain \leq 5% ferric CPO by EPR measurements at 77 K.

5.23 Chlorination Assay for Chloroperoxidase\textsuperscript{14}

The chlorination of monochlorodimedone by chloroperoxidase has been described previously by Hager et al.\textsuperscript{15} A UV/Vis spectrum of a 3 mL solution of 20 mM KPhos, pH 2.75, with 20 mM KCl and 20 mM H\textsubscript{2}O\textsubscript{2} and 2mM monochlorodimedone was recorded as a zero time point. 10 μL of a chloroperoxidase solution was added to the
solution and a spectrum was recorded every 20 seconds for one minute. The concentration of CPO was determined by the decrease in absorbance at 278 nm.

5.24 Preparation of CPO-II for X-ray Absorption Spectroscopy

4 mM $^{57}$Fe-enriched ferric CPO with 50 mM ascorbic acid was reacted with 100 mM meta-chloroperbenzoic acid in a 2:1 mixture that was quenched in liquid ethane 85 ms after mixing. Both reagents were in 0.1 M KPhos buffer (pH 6.5) containing 10% (v/v) acetone. Dried protein powder was packed into a Mössbauer sample holder that was modified to allow for XAS experiments. EPR measurements revealed that all samples contained ≤5% ferric CPO.

5.25 Preparation of CPO-I for Cryoreduction, Mössbauer, and Resonance Raman Experiments

4 mM $^{57}$Fe enriched chloroperoxidase was reacted with 80 mM peracetic acid in 0.1 M Kphos, pH 6.5, containing 10% (v/v) t-butanol. A 2:1 reaction of protein and peracetic acid was quenched into cold ethane (-180°C) 8 ms after mixing. Protein powder was dried and packed into EPR and Mössbauer sample holders. EPR measurements at 77 K revealed that samples of CPO-I contained ≥90% CPO-I. Freeze-quenched samples of CPO-I containing t-butanol were irradiated at the γ-irradiation facility of the Breazeale nuclear reactor at Pennsylvania State University using a $^{60}$Co
source; a total dose of 4 MRad was applied. Samples were maintained at 77 K during irradiation by immersion in liquid N₂. Mössbauer and resonance Raman measurements were performed before and after irradiation.

5.26 References


VITA

Kari Lea Lunder Stone

Education:

2001-2008: Department of Chemistry, The Pennsylvania State University, University Park, PA 16802.
Advisor: Professor Michael T. Green
Thesis Title: Exploring the Role of the Axial Ligand in Thiolate-Ligated Heme Enzymes: Spectroscopy of High-Valent Iron Intermediates of Chloroperoxidase

1997-2001: Department of Chemistry, Augustana College, Sioux Falls, SD, 57197
Advisor: Professor Arlen Viste

Publications:


Awards:

2007 Schering-Plough Science and Innovation Award for Excellence in Analytical Chemistry, Schering-Plough Research Institute.

2006 Dalanian Fellowship Award, Department of Chemistry, Pennsylvania State University.


2001 Graduate Fellowship, Department of Chemistry, Pennsylvania State University.