SPECTROSCOPIC CHARACTERIZATION OF HIGH-VALENT INTERMEDIATES IN CYTOCHROME P450S AND OTHER HEME ENZYMES

A Thesis in Chemistry by
Rachel Koren Behan

© 2008 Rachel Koren Behan

Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy May 2008
The thesis of Rachel Koren Behan was reviewed and approved* by the following:

Michael T. Green  
Assistant Professor  
Thesis Advisor  
Chair of Committee

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

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

Patrick C. Cirino  
Assistant Professor of Chemical Engineering

Ayusmen Sen  
Professor of Chemistry  
Head of the Department of Chemistry

*Signatures are on file in the Graduate School
ABSTRACT

Our group is particularly interested in the oxygen activation chemistry performed by heme peroxidases and cytochrome P450s. We set out to study compound II in cytochrome P450 and peroxidases because the identity of this intermediate is still the subject of debate. Spectroscopic studies on compound II in peroxidases have indicated that these intermediates are Fe(IV)oxo species. X-ray crystal structures, however, have indicated long Fe-O bonds for the ferryl intermediates in horseradish peroxidase, cytochrome c peroxidase, catalase, and myoglobin suggesting that these intermediates are protonated. To verify the X-ray crystal structure results, we performed both Mössbauer and resonance Raman spectroscopies on the same sample of ferryl myoglobin. Our experiments clearly supported the existence of only the Fe(IV)oxo species from pH 3.5 to 8.5. This result implies that the pKa of ferryl myoglobin is less than 3.5 and suggests that the long Fe-O bonds from the crystal structures are a consequence of photoreduction and does not represent the true nature of the intermediate.

Although ferryl myoglobin was not protonated over a wide pH range, we have shown that compound II in cytochrome P450s is protonated at physiological pH. The ferryl intermediates in P450\textsubscript{BM3}, P450\textsubscript{cam}, and CYP158 were studied by Mössbauer spectroscopy in conjunction with DFT calculations. As was observed in chloroperoxidase compound II, a large quadrupole splitting (and an Fe(IV)-like isomer shift) was obtained for the three intermediates. The experimental Mössbauer parameters also agreed well with the theoretically determined parameters for protonated ferryl
models. High-field Mössbauer measurements on P450\textsubscript{BM3} compound II also confirmed this assignment because this intermediate had an $S = 1$ spin state.

Based on previous experiments concerning the reaction of heme enzymes with peroxynitrite, it was evident that peroxynitrite could be used as an oxidant to generate ferryl intermediates. In our quest to study the electronic structure of P450 compound II, we utilized peroxynitrite as an oxidant to generate P450\textsubscript{BM3} compound II. On characterization of this intermediate using UV/visible, resonance Raman, and Mössbauer spectroscopies, as well as DFT calculations, we determined that the PN intermediate in P450\textsubscript{BM3} was not a ferryl intermediate, but rather an $S = 0$, ferric nitrosyl species. We studied the reaction of peroxynitrite with five other thiolate-ligated heme proteins and found similar results; however, when the reaction was performed with horseradish peroxidase and cytochrome c peroxidase (histidine-ligated heme proteins) a ferryl intermediate was found.
TABLE OF CONTENTS

LIST OF FIGURES ........................................................................................................... vii
LIST OF TABLES .............................................................................................................. xiv
ACKNOWLEDGEMENTS ................................................................................................. xv

Chapter 1 Introduction ................................................................................................. 1
  1.1 The Importance of Heme Proteins ................................................................. 1
  1.2 Peroxidases ....................................................................................................... 3
    1.2.1 Introduction .............................................................................................. 3
    1.2.2 Mechanism .............................................................................................. 4
    1.2.3 Chloroperoxidase .................................................................................... 6
  1.3 Cytochrome P450s ........................................................................................... 7
    1.3.1 Introduction .............................................................................................. 7
    1.3.2 Mechanism .............................................................................................. 9
    1.3.3 Peroxide Shunt Pathway ........................................................................ 11
    1.3.4 Electronic Structure of the Intermediates in the P450 reaction cycle
         from Theoretical and Experimental Techniques ...................................... 12
  1.4 The Role of the Axial Ligand in P450s .......................................................... 35
    1.4.1 Ferryl Protonation ................................................................................... 35
    1.4.2 Protonation state of Chloroperoxidase Compound II: Mössbauer
         and EXAFS spectroscopy ........................................................................... 39
    1.4.3 Badger’s Rule .......................................................................................... 41
  1.5 The Importance of the Ferryl pKa ................................................................. 45
  1.6 Peroxynitrite .................................................................................................... 49
    1.6.1 Reactions of PN with Metalloproteins ................................................... 51
    1.6.2 P450s and PN ........................................................................................ 54
  1.7 Summary of Chapters ...................................................................................... 59
  1.8 References ........................................................................................................ 60

Chapter 2 Resonance Raman and Mössbauer Spectroscopy of Myoglobin
Compound II: Setting an Upper Limit on the Ferryl pKa ............................................. 77
  2.1 Abstract ........................................................................................................... 77
  2.2 Introduction ..................................................................................................... 77
  2.3 Computational Procedures ............................................................................ 85
  2.4 Results and Discussion ................................................................................... 86
  2.5 Conclusion ....................................................................................................... 100
  2.6 References ...................................................................................................... 102

Chapter 3 Evidence for Basic Ferryls in Cytochrome P450s .................................. 108
3.1 Abstract .................................................................................................................. 108
3.2 Introduction ............................................................................................................ 109
3.3 Computational Methods ....................................................................................... 114
3.4 Results and Discussion ......................................................................................... 116
3.5 Conclusion .............................................................................................................. 127
3.6 References ............................................................................................................. 127

Chapter 4 The Reaction of P450BM3 with Peroxynitrite .............................................. 132

4.1 Abstract ................................................................................................................. 132
4.2 Introduction ............................................................................................................ 133
4.3 Computational Procedures .................................................................................... 136
4.4 Results and Discussion ......................................................................................... 137
4.5 Conclusion .............................................................................................................. 150
4.6 References ............................................................................................................. 151

Chapter 5 The Unusual Reaction Between Heme Proteins and Peroxynitrite is
Driven by Heme Axial Ligation .................................................................................. 155

5.1 Abstract ................................................................................................................. 155
5.2 Introduction ............................................................................................................ 155
5.3 Results and Discussion ......................................................................................... 158
5.4 Conclusion .............................................................................................................. 177
5.5 References ............................................................................................................. 181

Chapter 6 Experimental Methods ................................................................................ 186

6.1 Reagents ................................................................................................................. 186
6.2 Protein Expression and Purification ...................................................................... 186
6.3 Preparation of Myoglobin and HRP for Mössbauer Spectroscopy ..................... 191
6.4 Peroxynitrite ......................................................................................................... 195
6.5 Generation of Transient Intermediates Studied ..................................................... 196
6.6 Spectroscopic and Computational Techniques ..................................................... 198
6.7 References ............................................................................................................. 203

Chapter 7 Summary and Conclusions ......................................................................... 205

Appendix Spectroscopic Characterization of the Intermediate Generated in
CYP154 with Peracetic Acid ......................................................................................... 211

A.1 Results and Discussion ......................................................................................... 211
A.2 References ............................................................................................................. 218
LIST OF FIGURES

Figure 1.1: Iron-bound protoporphyrin IX.................................................................2

Figure 1.2: Generic peroxidase reaction cycle. Peroxide oxidizes the ferric resting state to afford compound I, compound I oxidizes substrate yielding compound II, which then returns to resting state ferric with loss of water. .......4

Figure 1.3: Mechanism for compound I formation in peroxidases. The “push-pull” mechanism is believed to contribute to the facile cleavage of the peroxide O-O bond. .................................................................5

Figure 1.4: Proposed Cytochrome P450 reaction cycle. The individual steps will be discussed in detail below. .................................................................10

Figure 1.5: Thiolate-ligated heme porphine models that have been used to calculate the electronic structure of P450 complexes and intermediates. ...........13

Figure 1.6: Example of a QM region used for P450cam, which was chosen for QM/MM calculations. 32 .................................................................................16

Figure 1.7: Two pathways for protonation of the reduced oxyferrous complex. Top pathway represents the double protonation of the distal oxygen in which compound I is formed with no intermediate. The bottom pathway first involves protonation of the proximal oxygen. Protonation of the distal proton results in the release of H$_2$O$_2$. 61 .................................................................23

Figure 1.8: Shaik and coworkers examined the “push effect” of the thiolate ligand on the formation of compound I from the ferric peroxy intermediate. Top, calculation using a thiol axial ligand, bottom is with no axial ligand. The thiol axial ligand lowers the energy of compound I formation by about 80 kcal/mol. 42 .................................................................................26

Figure 1.9: Electronic structure of P450-I from DFT calculations. Natural orbitals of the S=1/2 thiolate. Occupancies are: a) 1.10, b) 1.0, and c) 0.90. The natural orbitals show significant radical character on the thiolate sulfur atom in the gas phase. 70 .................................................................................28

Figure 1.10: Fe-O and Fe-S bond lengths for ferric CPO and its high-valent intermediates.........................................................................................29

Figure 1.11: Consensus rebound mechanism for P450 hydroxylations.................33
Figure 1.12: Two-state reactivity model for a generic FeO⁺ oxidant illustrating the possibility of the use of both the low-spin and high-spin transition states in the hydrogen atom abstraction mechanism. 83 ......................................................... 34

Figure 1.13: Predicted Mössbauer spectra (c) for the ferryl (A, ΔE_Q = 1.00 and δ = 0.08 mm/s) and protonated ferryl (B, ΔE_Q = 2.75 and δ = 0.10 mm/s) histidine-ligated heme models. 107 ................................................................. 39

Figure 1.14: Fe-O bond distance versus 1/ω²/³. The circles represent the calculated bond lengths and frequencies (scaled). The best-fit line through the theoretical data is Badger’s rule. The yellow diamonds represent the EXAFS and resonance Raman data and the black diamonds are from resonance Raman and X-ray crystallography. Figure adapted from reference 93. ................................................................. 42

Figure 1.15: Resonance Raman spectrum of CPO-II prepared with m-CPBA, right, and the corresponding Mössbauer spectrum, left. The Fe-O stretching frequency for this intermediate is at 561 cm⁻¹. Upon D₂O substitution, the Fe-O stretch shifts to 549 cm⁻¹ ................................................................. 44

Figure 1.16: H-atom abstraction reactions performed by CrO₂Cl₂ and MnO₄⁻. Left. Correlations of ΔH⁺ and ΔG⁺ with C-H bond strength for oxidations by CrO₂Cl₂. Right. Scheme used to calculate O-H bond strength in HMnO₄. Both figures were taken from reference 124. ................................................................. 47

Figure 1.17: Strength of the O-H bond as it relates to P450 chemistry. The rebound mechanism is summarized at the top and below it is the equation used ................................................................. 48

Figure 1.18: Reactions of metal porphyrins with PN. Left. Mn porphyrins can react with PN via two pathways. Heterolytic cleavage of the PN O-O bond results in the ability to perform DNA strand scission reactions, whereas homolytic cleavage results in the generation of oxidants capable of the nitration of added phenols. Right. Proposed mechanism for the initial reaction of stoichiometric amounts of PN with Fe⁢III-TMPyP. Reactions of excess PN with Fe™TMPyP appeared to generate the ferryl radical intermediate. These figures were taken from references 155 and 157. ........................................... 52

Figure 1.19: Time course of the reactions of P450_{BM3} and CPO with a large excess of PN. Left. Top. P450_{BM3} spectra over time: 1) 5ms, 2) 320ms, 3) 640ms, 4) 960ms, 5) 1.6s, 6) 3.84s, 7) 15.84s, 8) 27.84s. Bottom spectrum is the kinetic traces for 302nm (PN, circles), 418nm (ferric P450_{BM3}, squares), and 435nm (ferryl P450_{BM3}, triangles). Right. Top. CPO reaction with PN: 1) 5ms, 2) 16ms, 3) 32ms, 4) 128ms, 5) 1.41s, 6) 3.65s, 7) 6.89s, 8) 27.01s.
Bottom spectrum shows the kinetic traces of the reactions. The PN absorbance (302nm, circles), ferric CPO Soret (397nm, squares) and ferryl CPO Soret (434nm, triangles) were followed over the 10s reaction. 

Figure 1.20: Reaction intermediates for the reaction of PN with CPO (left) and CYP119 (right). The inset shows the kinetic traces for the decay of the ferryl intermediate ($\lambda_{\text{max}}$ for the intermediate) at 40ms (CPO) and 40s (CYP119).

Figure 2.1: Resonance Raman spectra over the high frequency region in Mb-II from pH 4.5 to pH 8.5 (431nm excitation). The asterisks represent an oxyferrous contamination at 1351 cm$^{-1}$. 

Figure 2.3: Resonance Raman spectra of Mb-II at pH 5.0 (left) and pH 8.5 (right) using the 501.7nm laser line. The pH 8.5 spectrum was included to show the differences in the rR spectra from pH 8.5 to 5.0. In each spectrum A) is the $^{18}$O spectrum, B) is the $^{16}$O spectrum, and C) is the $^{16}$O-$^{18}$O difference spectrum.

Figure 2.4: Resonance Raman spectrum of Mb-II at pH 4.5 using a 431 nm excitation wavelength. In this spectrum A) is the $^{16}$O spectrum, B) is the $^{18}$O spectrum, and C) is the $^{18}$O-$^{16}$O difference spectrum.

Figure 2.5: Trans effect from DFT calculations exerted by the axial imidazolate ligand in Mb-II models.

Figure 2.6: Proposed mechanism to account for the pH-dependent shift in Fe-O stretching frequency (~10 cm$^{-1}$) in HRP-II and CAT-II, which may also be relevant to Mb-II.

Figure 2.7: Resonance Raman spectrum of Mb$^{III}$-OH (431 nm excitation). Top spectrum is of Mb-OH and bottom is Mb-OD.

Figure 2.8: Models used for DFT calculations on myoglobin complexes. The model on the left is a generic porphine with a imidazole ligated to the heme iron and on the right is the structure taken from the myoglobin crystal structure.

Figure 2.9: Mössbauer spectra (54mT, 4.2K) of Mb-II at pH 8.5, left, and pH 5.2, right. In both spectra the black hash marks represent the experimental data and the blue line is the fit for the experimental data. Included in the pH 5.2 spectrum is a theoretical spectrum for a protonated ferryl in Mb in red.

Figure 2.10: Mb-II generated for Mössbauer (top) and $^{16}$O resonance Raman (bottom) spectroscopies. These samples were generated to confirm the individual experimental results that were reported at these pHs. In the
Mössbauer spectra, the black hash marks represent the raw data and the red line is the fit for the data. The extra species in the pH 5.2 (black solid line) is an oxyferrous Mb contamination.\textsuperscript{51} Figure 2.11: Mössbauer spectrum of Mb-II at pH 4.5. Raw data is represented by the black hash marks, the blue line is the fit of the experimental data, and the red line is the theoretical spectrum from DFT calculations on a protonated ferryl in Mb. Figure 2.12: Mössbauer spectrum of Mb-II at pH 3.5. Raw data is shown in the black hash marks, the blue line is the experimental data from pH 5.2, and the red line is the theoretical spectrum from DFT calculations on a protonated ferryl in Mb.

Figure 3.1: Rebound mechanism for P450 hydroxylations.

Figure 3.2: DFT model for a protonated ferryl intermediate in CYP158A1.

Figure 3.3: Mössbauer spectrum of P450\textsubscript{BM3} PA-generated intermediate (4.2K/54mT). Top spectrum, in purple, is the raw data for P450\textsubscript{BM3}-PA and in black is ferric P450\textsubscript{BM3} that was subtracted from the raw data (44%). The bottom spectrum is of the raw data after subtraction of the ferric P450\textsubscript{BM3} component. The black line is the fit for two quadrupole doublets with $\Delta E_Q = 2.16 \text{ mm/s}$ and $\delta = 0.13 \text{ mm/s}$ (48%) and $\Delta E_Q = 2.41 \text{ mm/s}$ and $\delta = 0.33 \text{ mm/s}$ (8%).

Figure 3.4: High-field Mössbauer spectrum of P450\textsubscript{BM3}-PA at 7T and 4.2K. The top spectrum is the raw data and the bottom spectrum is the data after subtraction of 44% the 7T spectrum of ferric P450\textsubscript{BM3} (top spectrum, solid black line). The fit for the PA intermediate is shown as the solid black line overlaid on the bottom spectrum.

Figure 3.5: Mössbauer spectrum of P450cam-PA intermediate (4.2K/54mT). Top spectrum, in green, is the raw data for P450cam-PA and in black is ferric P450cam that was subtracted from the raw data (71%). The bottom spectrum shows the raw data following subtraction of the ferric P450cam component. The black line is the fit for a single quadrupole doublet $\Delta E_Q = 2.06 \text{ mm/s}$ and $\delta = 0.13 \text{ mm/s}$ (29%).

Figure 3.6: Mössbauer spectrum of CYP158A1-PA intermediate (4.2K/54mT). The raw data for CYP158-PA is shown with the hash marks and the black solid line is the spectrum of ferric enzyme (21%). The bottom spectrum is the raw data after subtraction of the ferric component. The blue line is the fit.
for two quadrupole doublets with the following parameters: $\Delta E_Q = 2.13$ mm/s and $\delta = 0.09$ mm/s (65%) and $\Delta E_Q = 2.42$ mm/s and $\delta = 0.39$ mm/s (14%) 

Figure 3.7: Fe K-edge absorption energies for $P450_{BM3}$-PA and ferric $P450_{BM3}$. Note the shift in the edge energy for the PA intermediate relative to the ferric edge. 

Figure 4.1: Example of $P450_{BM3}$ computational model. This figure represents the nitro- complex. 

Figure 4.2: Stopped-flow spectrophotometry experiments on the $P450_{BM3}$ reaction. The blue spectrum corresponds to ferric $P450_{BM3}$ and the red spectrum is the fully-formed $P450_{BM3}$-PN intermediate (300ms). The time points of the spectra are 10, 20, 40, and 70ms. These spectra correspond to the decreasing absorbance at 302 nm. The inset shows single-wavelength data at 302, 417, and 435 nm. 

Figure 4.3: A) EPR spectra of the $P450_{BM3}$-PN intermediate (red) and ferric $P450_{BM3}$ (black) at the same concentration (T = 20 K, 10 $\mu$W power). B) Mössbauer spectrum of $P450_{BM3}$-PN (4K, 54mT). The hash marks represent the raw data and the solid black line is the fit for the quadrupole doublet. The parameters for this intermediate were $\Delta E_Q = 1.15$ mm/s and $\delta = 0.15$ mm/s. Both spectra indicate that the $P450_{BM3}$-PN intermediate was generated in ~80% yield. 

Figure 4.4: Mössbauer spectra of $P450_{BM3}$-PN (red) and $P450_{BM3}$-II (blue, generated with peracetic acid). The $\Delta E_Q$ for the PN intermediate is 1.15 mm/s, whereas for $P450_{BM3}$-II the $\Delta E_Q = 2.16$ mm/s. 

Figure 4.5: $P450_{BM3}$ $\{FeNO\}_6$ structure from DFT calculations. Calculated Fe-N (1.65 Å), N-O (1.18 Å), and Fe-S (2.38 Å) distances and the Fe-N-O (170.3°) bond angle are shown. 

Figure 4.6: Variable-field Mössbauer spectra of the $P450_{BM3}$-PN intermediate. Spectra were recorded in a 54mT, 6T, and 8T external magnetic field applied parallel to the $\gamma$-beam. The solid blue lines represent a spin Hamiltonian simulation with an S = 0 ground state. An S = 1 simulation was included with the 8T data, in red, for illustrative purposes. 

Figure 4.7: Comparison of the $P450_{BM3}$-PN intermediate and ferric nitrosyl complex. Left. UV/vis spectrum of $P450_{BM3}$-NO (blue) and $P450_{BM3}$-PN (red). The increased absorbance around 300nm is due to excess PN in solution. Right. Comparison of the Mössbauer spectra for A) $P450_{BM3}$-PN
and B) the \(\{\text{Fe-NO}\}\) complex. Raw data is shown as black hash marks and the green solid line is the fit for the raw data. ..................................................... 145

Figure 4.10: Resonance Raman spectra of the \(\text{P450}_{\text{BM3}}\)-PN intermediate using a 413nm excitation wavelength. Left. Low frequency data indicating the Fe-N stretch for the PN intermediate. Two peaks shifted with \(^{15}\text{N}\) substitution, 535 and 550 cm\(^{-1}\). Right. High frequency data in the N-O stretch region. Upon \(^{15}\text{N}\)-substitution, the N-O stretch shifted from 1850 to 1815 cm\(^{-1}\). .............................. 147

Figure 4.11: Reactants, reaction conditions, and observable iron-containing products for the reaction of \(\text{P450}_{\text{BM3}}\) with the following: (Top) Peracetic acid: the first spectroscopically characterizable intermediate during the reaction of \(\text{P450}_{\text{BM3}}\) with PA is compound II, an Fe\(^{IV}\)OH species. (Middle) Peroxynitrite: the reaction of PN with \(\text{P450}_{\text{BM3}}\) results in the formation of a nitrosyl complex (path B). No ferryl intermediates are observed (path A). (Bottom) Nitric oxide: the ferric \(\text{P450}_{\text{BM3}}\)-NO complex and the \(\text{P450}_{\text{BM3}}\)-PN intermediate have identical spectroscopic features............................................. 149

Figure 5.1: Kinetic data for the CPO-PN reaction with (left) PN not treated with MnO\(_2\) and (right) after treatment with MnO\(_2\). The yellow trace is the PN decay at 302nm, the blue trace is ferric CPO monitored at 395nm, the red trace is CPO-PN monitored at 436 nm, and CPO-I monitored at 690nm............. 161

Figure 5.2: CY119-PN intermediate versus the \(\{\text{FeNO}\}\) complex. Left. UV/vis for CYP119-PN (red) and CY119-NO (black). Note: the increased absorbance at 350nm in the CYP119-NO UV/vis spectrum is due to nitrite formed from the reaction of NO with O\(_2\) present in the buffer. Right. Mössbauer spectrum for CYP119-PN. Top is the raw data for CYP119-PN with ferric CYP119 overlaid (black solid line). Bottom is the spectrum of CYP119-PN after subtracting 75% ferric CYP119. The red line is the fit for the quadrupole doublet (\(\Delta E_Q = 1.13\) mm/s and \(\delta = 0.15\) mm/s).................................. 163

Figure 5.3: Mössbauer spectra of PN intermediate (top), spectrum following subtraction of ferric component (middle), and \(\{\text{FeNO}\}\) complex (bottom). A) CYP119, B) P450cam, C) CPO, D) CYP154......................................................... 166

Figure 5.4: UV/vis spectra for HRP and CCP PN, Fe(IV)oxo, and nitrosyl complexes. Left. Time slice of the HRP reaction. Dotted line is the spectrum after 15ms and the solid line is after 2.5s. Middle. HRP-PN at 2.5s (red), HRP-II (blue), and HRP-NO......................................................... 168

Figure 5.5: 4.2K/54mT Mössbauer spectrum of the \(\{\text{FeNO}\}\) complex in HRP. The black hash marks represent the experimental data and the solid line represents the fit for the quadrupole doublet (\(\Delta E_Q = 1.53\) mm/s and \(\delta = 0.06\) mm/s).................................................................. 171
Figure 5.6: Resonance Raman spectra of the PN intermediate, ferryl, and ferric nitrosyl species in HRP and CCP. Top. HRP low frequency and high frequency data: A) Compound II, B) PN intermediate, C) \(\{\text{FeNO}\}_6\) complex, and D) Ferric HRP. Bottom. CCP low frequency and high frequency spectra: A) Compound ES, B) PN intermediate, C) \(\{\text{FeNO}\}_6\) complex, and D) ferric CCP. The ellipses highlight the major differences between these spectra. 172

Figure 5.7: EPR spectra of CCP-PN and CCP-ES at 77K and 8mW power. 176

Scheme 5.8: Summary of PN reactions with heme enzymes. 180

Figure 6.1: Resonance Raman table set up. 199

Figure 6.2: Sample configuration used for low temperature resonance Raman measurements. 200

Figure A.1: EPR spectrum of the CYP154-PA intermediate (20K and 2mW power). The asterisks represent the peaks due to ferric contamination. 213

Figure A.2: Top: EPR spectrum of annealed CYP154-PA sample. The annealing conditions are indicated next to the spectra. Bottom: Radical signal during the annealing of CYP154-PA. Maximum intensity occurred on annealing the sample for 45min at –80°C. 214

Figure A.3: Mössbauer spectra (4.2K/54mT) of CYP154-PA after subtraction of ~40% ferric CYP154. The top spectrum is in a parallel magnetic field and the bottom is in a parallel magnetic field. 215

Figure A.4: 4.2K/54mT Mössbauer spectra of annealed CYP154-PA. Top is the original sample, middle is the spectrum of the species following annealing at –80°C for 30min, and the bottom is the spectrum following annealing at –72°C for 15min. 217

Figure A.5: 4.2K/54mT Mössbauer spectra of CYP154-PA (top) and CR-CYP154-PA (middle). The bottom is the species generated upon cryoreduction. Approximate parameters for this quadrupole doublet are \(\Delta E_q \sim 2.2\) mm/s and \(\delta \sim 0.6\) mm/s. 218
LIST OF TABLES

Table 1.1: Experimental parameters for histidine-ligated compound II species. ........37
Table 2.1: Myoglobin Mössbauer parameters in mm/s. ..............................................95
Table 3.1: Spin densites and bond lengths for the P450 intermediates studied..........116
Table 3.2: P450 compound II Mössbauer parameters (mm/s)..................................117
Table 4.1: Mössbauer parameters for P450BM3 species in mm/s .........................141
Table 5.1: P450BM3-PN reaction under various conditions.................................159
Table 5.2: PN versus {FeNO}^6 in thiolate-ligated proteins...............................162
ACKNOWLEDGEMENTS

I would first like to thank my parents and my brother, Ryan, for their support not only while I was at Penn State, but throughout my whole life. My parents are my biggest fans. My dad played a particularly important role while I was at Penn State by keeping me up to date with Patriots and Red Sox scores from season to season (boo Pittsburgh sports). My mom had the uncanny ability to understand the trials and tribulations of a grad student without ever going to grad school. I am especially grateful to her for her advice and support over the past 5+ years. I should also thank my high school chemistry teacher Kate Myers. She got me interested in chemistry right off the bat. She had so much of an influence on me that I went to Providence College (her alma mater) and majored in Chemistry. While at Providence I also had quite a support group. All of my Chemistry professors were instrumental in my decision to apply to grad school, especially my research advisor Dr. Sheila Adamus.

I would next like to thank all of my friends and coworkers at Penn State. I certainly enjoyed the ride with my friends Pooja Aggarwal, Dr. Dave Stone, Suellen Hopfer, Dr. Dave and Cindy Iwig, and Dr. Martin Peters, to name a few. I would also like to thank my 2007 Co-ed intramural championship softball team for helping us take home the “trophy” for my last year on the team, especially Matt Meketa and Jason Camp. I should next thank Dr. Rob Cicchillo for many LONG nights of drinking in which we would try to forget our many failed experiments, or other frustrations. Finally, I would like to extend a special thanks to Kari Stone. Kari and I have been together since day one (at least for me). Without her so many things would not have been possible. She’s been
a great coworker, drinking buddy, and friend. I like to think that I survived grad school in large part because of her support and I owe her big time for it.

I would like to thank my committee for their help over the years: Prof. Mike Green, Prof. Marty Bollinger, Prof. Carsten Krebs, Prof. Juliet Lecomte, and Prof. Pat Cirino. I should especially thank Mike for his support and help over the past 5 years. I have learned so much from him, not only concerning science, but also aspects of academia that eventually influenced my choice in career. I am indebted to him for his willingness to stay late in lab to help me with my experiments and the many, many, many free dinners and pitchers of beer that he provided me with over the years.

Last, but not least, I would like to thank is my (new) husband Andrew. He has provided me with the love and support that I needed along the way. I am especially grateful for the many massages and back cracks that he has provided during the preparation of this thesis. I can’t wait until we get to Cambridge to begin the rest of our lives together.
Chapter 1

Introduction

1.1 The Importance of Heme Proteins

Heme proteins are found in all organisms ranging from bacteria and plants to humans. These proteins perform a variety of difficult and essential reactions. Heme proteins are comprised of a protein scaffold with an iron-coordinated tetrapyrrole macrocycle (protoporphyrin IX) in the active site, Figure 1.1. Axial coordination sites are occupied by proximal and distal ligands the identity of which is dependent on the enzyme and its function; however, at least one of these ligands (the proximal ligand) is an amino acid residue tethering the heme unit to the protein scaffold. The identity of the proximal ligand is conserved across each heme protein family with few exceptions; for instance, peroxidases are generally ligated by a histidine residue, Cytochrome P450s by a cysteine residue, and catalases by a tyrosine residue.

Typical classes of heme proteins include the cytochromes (key components in respiration and photosynthesis), heme-containing oxygenases (biosynthesis and detoxification), and globins (gas transport). These classes can also be broken down into three main functions: 1) oxygen transport, 2) electron transfer, and 3) catalysis of redox
reactions. Because all of these enzymes contain a heme prosthetic group yet perform very different functions, the difference between these enzymes must stem from the interaction between the heme unit and the protein scaffold. Proteins belonging to the cytochrome class of heme proteins are 6-coordinate being axially ligated by two amino acids. An example of this group is Cytochrome c, which is ligated by a cytsteine proximal residue and a histidine distal residue. Proteins like Cytochrome c are typically involved in electron transfer reactions. The globins, myoglobin and hemoglobin, are the primary proteins involved in dioxygen transport. It is known that dioxygen will only bind to a heme when it is in the Fe(II) oxidation state; therefore, it is believed that the different protein environment in myoglobin and hemoglobin serves to raise the reduction potential of the heme so that the Fe(II) oxidation state is the most stable form. Alternatively, heme oxidases like Cytochrome P450s and peroxidases, exhibit an Fe(III) resting state. In P450s, the reaction cycle is initiated with the binding of substrate, which

Figure 1.1: Iron-bound protoporphyrin IX.
raises the reduction potential of the heme so that it is more easily reduced. Once P450 is in the ferrous oxidation state, dioxygen can bind and the difficult oxygen-insertion reaction can proceed. Peroxidases, on the other hand, react with peroxide to generate potent oxidizing intermediates capable of a variety of reactions. The differences between P450s and peroxidases include the identity of the heme axial ligand (cysteine and histidine, respectively) and the protein superstructure surrounding the active site. The striking differences in protein function across this family of enzymes indicate that the interaction of the surrounding protein residues with the heme can modulate protein function.

The important metabolic role of heme proteins along with their unique chemistry and physical properties has attracted much attention to this group of proteins. Researchers encompassing a variety of disciplines including protein engineering, bioinorganic and bioorganic chemistry, biophysics, biochemistry, molecular biology, and genetics have been intrigued by heme proteins. Though these proteins have been studied for over five decades, a wealth of research is still ongoing regarding this diverse set of proteins.

1.2 Peroxidases

1.2.1 Introduction

Peroxidases are heme-containing proteins that perform a variety of functions. Some of the reactions that peroxidases are capable of performing include the oxidation of
aromatic and heteroatom compounds, epoxidation, free radical polymerization of electron-rich aromatics, and the oxidation of hydrogen peroxide to dioxygen.\textsuperscript{2} These proteins typically have histidine or tyrosine axial ligands; however, a peroxidase called chloroperoxidase has a cysteine axial ligand similar to P450s.\textsuperscript{1}

1.2.2 Mechanism

Horseradish peroxidase (HRP) is one of the best studied peroxidases.\textsuperscript{1} The resting state oxidation state for peroxidases is the Fe(III) state. In the peroxidase mechanism, a peroxide molecule initially coordinates to the heme iron. Following coordination the O-O bond is heterolytically cleaved yielding compound I with release of H\textsubscript{2}O, Figure 1.2. Compound I is extremely well characterized and is most accurately described as a Fe(IV)oxo with a porphyrin a\textsubscript{2}u radical.

![Diagram of generic peroxidase reaction cycle](image)

Figure 1.2: Generic peroxidase reaction cycle. Peroxide oxidizes the ferric resting state to afford compound I, compound I oxidizes substrate yielding compound II, which then returns to resting state ferric with loss of water.
The mechanism of O-O bond heterolysis in peroxidases is slightly different than in P450s. Polar residues above and below the heme in HRP are thought to be involved in an acid-base mechanism often referred to as a “push-pull” mechanism, Figure 1.3.\textsuperscript{3, 4} The pKa of H\textsubscript{2}O\textsubscript{2} is \(~12\), which means that peroxide binds in the protonated form. The distal residue in HRP, His42, is responsible for the “pull” effect in which His42 accepts the proximal proton from H\textsubscript{2}O\textsubscript{2} to form a Fe(III)-OOH species. The “push” effect is achieved through a base on the proximal side of the heme, which is hydrogen bonded to the axial histidine (His170) residue. The resulting negative charge on His170 provides the electron push to facilitate O-O bond cleavage and the formation of compound I.

![Figure 1.3: Mechanism for compound I formation in peroxidases. The “push-pull” mechanism is believed to contribute to the facile cleavage of the peroxide O-O bond.](image)

The distal pocket of peroxidases cannot accommodate large substrates; therefore, peroxidases are limited to performing electron transfer reactions at the heme edge. Following the oxidation of substrate by compound I, compound II is formed. Compound
II is an Fe(IV)oxo species where the porphyrin radical “hole” has been filled by the substrate electron. Compound II is then further reduced to ferric peroxidase and water.

1.2.3 Chloroperoxidase

Chloroperoxidase (CPO) is a glycoprotein secreted from the fungus *Caldariomyces fumago*, which catalyzes hydrogen peroxide-dependent halogenation reactions.\(^1\),\(^5\) Chloroperoxidase is a unique peroxidase in that it possesses a cysteinate axial ligand rather than the typical histidine or tyrosine ligands. The biological role of CPO is as a participant in the synthesis of the natural product caldariomycin.\(^3\) In addition to catalyzing chlorination, bromination, and iodination reactions, CPO functions as a catalase and a peroxidase and also catalyzes P450-like oxygen insertion reactions. The crystal structure of CPO was solved in 1995.\(^6\) Interestingly, although CPO can perform reactions characteristic of peroxidases, catalases, and P450s, the overall fold and 3D structure of the enzyme does not bear a resemblance to any of these enzymes. The distal pocket of CPO bears a close resemblance to that of a peroxidase in that it uses polar amino acids (Glu183) for O-O bond cleavage; however, the proximal region of CPO is more hydrophobic than its peroxidase counterparts. Because the distal pocket of CPO is lined with polar residues yet has a cysteine axial ligand, it has been said that CPO has a peroxidase-like distal pocket and a P450-like proximal pocket. The large distal pocket in CPO, unlike other peroxidases, can accommodate larger substrates, which is important to the versatility of this enzyme.
CPO has been used as a model for P450 chemistry because, unlike P450, the high-valent intermediates in the CPO reaction cycle are reasonably stable (CPO-I: \( \approx 30 \text{ms} \) and CPO-II: \( \approx 2\text{s} \) at room temperature).\(^5,7-12\) It is believed that because P450 and CPO possess the same axial thiolate ligand, the high-valent intermediates in CPO should resemble the geometric and electronic structures of P450 intermediates. This means that by studying compound I and II in CPO, one can better understand the factors that enable P450s to perform such difficult chemistry. Throughout this thesis we will draw upon the spectroscopic similarities between CPO and P450s.

1.3 Cytochrome P450s

1.3.1 Introduction

Cytochrome P450s are thiolate-ligated heme enzymes involved in the biosynthesis and metabolism of a wide variety of compounds and are found in almost all organisms.\(^13\) These enzymes are particularly interesting because they can oxidize a wide range of substrates with high regio- and stereoselectivity. P450s are the main players in the metabolism of xenobiotics, as well as the generation of signaling molecules used for the control of organism development and homeostasis.\(^14\) In mammals, P450s play a critical role in the metabolism of drugs and fat-soluble vitamins, the synthesis of steroids, and the conversion of polyunsaturated fatty acids to biologically active molecules.\(^2,14,15\) Plants and insects take advantage of P450s in a similar manner; for instance, the reactions
performed by plant P450s are often directed towards hormone biosynthesis and insecticide resistance.\textsuperscript{16}

As was discussed above, P450s are found in almost all organisms. These enzymes catalyze a wide variety of reactions, the most difficult of which being the insertion of oxygen into unactivated C-H bonds. Although peroxidases like horseradish peroxidase (HRP) and chloroperoxidase (CPO) possess the same heme prosthetic group as P450s, they are not capable of such difficult reactions. The hydroxylation of unactivated alkanes is unique to P450s. The general reaction for substrate hydroxylation by P450s is as follows:

\[
\text{R-H + NAD(P)H + H}^+ + \text{O}_2 \xrightarrow{\text{P450}} \text{R-OH + NAD(P)}^+ + \text{H}_2\text{O} \quad \text{(1.1)}
\]

Though this is the primary reaction performed by P450s, they are capable of many more chemical transformations. These oxidative reactions include epoxidation, oxidative N- and O-dealkylation, N-hydroxylation, sulfoxidation, oxidative coupling, C-N bond cleavage, oxidative deformylation, and dehydrogenation of substrates such as alkenes, amines, and acetylenes.\textsuperscript{2,14}

Shown in equation 1.1, P450s require the carefully orchestrated delivery of four substrates to produce hydroxylated product. The most important of these substrates are protons and electrons. P450s rely on the action of a reducing cofactor (NADPH or NADH) and an electron transport protein to supply reducing equivalents to the oxygenase active site.\textsuperscript{17} In P450s, this is comprised of either two proteins (a reductase and a ferredoxin protein), or a single reductase flavoprotein. Proton delivery is accomplished through an exquisitely lined channel that extends from the surface of the protein through
the hydrophobic core of the P450 to the heme iron. This channel is lined with two polar amino acids and several water molecules.\textsuperscript{18} Disruption of either proton or electron delivery has been shown to have deleterious effects on the enzyme.

1.3.2 Mechanism

The entire catalytic cycle of P450s has not been characterized at this point, Figure 1.4. There is evidence for several of the initial oxygen binding steps in the catalytic mechanism; however, much of the information available to date concerning the short-lived, reactive intermediates in P450s has been implied from work performed on the high-valent intermediates in peroxidases, especially CPO. Each step in the reaction cycle and an analysis of the electronic structure for each intermediate species will be discussed in detail in the next section.
Briefly, resting state P450 is present in the low-spin ferric oxidation state in which a water molecule is bound in the sixth coordination site. Binding of substrate results in the loss of water from the heme iron, which is accompanied by a spin state change to high-spin ferric. This spin state change is crucial to the reaction cycle because the change from low-spin to high-spin is accompanied by an increase in the redox potential of the heme. For instance, in P450BM3 the binding of substrate results in a 140mV increase in redox potential.\textsuperscript{19, 20} The modulation of the redox potential is required so that reduction of the heme iron is much more facile.

Following the reduction of resting state ferric P450, dioxygen binds to ferrous P450 resulting in an oxyferrous species. This species has a resonance structure in which one

Figure 1.4: Proposed Cytochrome P450 reaction cycle.
electron is transferred from the heme iron to the oxy ligand, generating a ferric-superoxide species. This complex is reduced by one electron followed by the addition of a proton, resulting in a ferric-hydroperoxo species. The ferric-hydroperoxo species is then protonated once again, leading to heterolytic cleavage of the O-O bond. Heterolytic cleavage of the peroxo bond results in the formation of compound I (an Fe(IV)oxo-radical species) and release of a water molecule.

1.3.3 Peroxide Shunt Pathway

Before delving into the discussion of the electronic structure of P450 intermediates, a few words should be dedicated to the discussion of the peroxide shunt pathway. This is the method used to generate all of the P450 intermediates that will be discussed in the proceeding chapters. Shown in figure 1.4, the peroxide shunt pathway involves the reaction of low-spin ferric P450 with a peroxide (ex. hydrogen peroxide, peracetic acid, iodosylbenzene). This method bypasses the steps involving the binding of dioxygen to the heme. The first step of the peroxide-shunt pathway is thus protonation of the ferric peroxy species whereby the peroxo O-O bond is cleaved to yield compound I. The rest of the cycle proceeds in the same manner as with dioxygen. In fact, the peroxygenase activity has been observed in P450s. For example, it has been shown that, in general, the same products are generated using either peroxide or dioxygen as the oxidant in human and rat P450 1A2.21,22
1.3.4 Electronic Structure of the Intermediates in the P450 reaction cycle from Theoretical and Experimental Techniques

Although experimental techniques can provide quite a bit of information about a given metalloenzyme complex, the ability to use computational techniques in conjunction with experiment provides a clear advantage when attempting to understand enzyme chemistry. For instance, while experiments can provide information concerning the absorption spectrum of a complex, calculations can reveal the nature of the individual electronic transitions. There are many computational techniques that have been applied to understanding metalloenzyme chemistry and over time these methods have become faster and more accurate.

The goal of computational methods is to make predictions on the behavior of molecules that can ultimately be tested experimentally. *Ab initio* methods, such as Hartree Fock (HF), set out to solve the Schrödinger equation for a multielectron molecule. Hartree-Fock calculations are the simplest *ab initio* calculations in which each electron is assumed to move in a potential that has been determined by a nucleus and the surrounding electrons. This method does not account for electron correlation. More sophisticated post-HF calculations are required to include correlation effects. These post-HF methods provide a very accurate estimate of the electronic structure of a molecule, but they are extremely time consuming and are too computationally expensive for most molecules of interest today.

Semiempirical methods (MNDO or ZDO) were developed in the 1970s and are methods that have been parameterized to reproduce experimentally observable properties, like heat of formation and functional group geometries. The parameterizations act as a
“starting point” for solving the Schrodinger equation, which makes these calculations much faster (but less accurate) than \textit{ab initio} methods. Because semiempirical methods have been parameterized to match experiment, these methods can be used to calculate a considerable number of parameters. We will see the use of INDO methods in conjunction with configuration interaction (CI), for the calculation of electronic transitions for UV/vis spectra in P450s.

The most common method employed by chemists to evaluate the electronic structure of large molecules is density functional theory (DFT). The main objective of DFT is to replace the many-body electronic wavefunction with the electron density as the basic quantity which determines ground state properties. This method is based off of the observation that there is a correlation between the electron density and the ground state energy of the molecule. These calculations can be used successfully to calculate the ground state properties of a molecule.

\textit{Ab initio}, semiempirical, and DFT methods have been used to calculate the properties of the intermediates in the P450 reaction cycle. Some highlights of these calculations are described below.

![Thiolate-ligated heme porphine models](image)

Figure 1.5: Thiolate-ligated heme porphine models that have been used to calculate the electronic structure of P450 complexes and intermediates.
**Ferric P450.** It was determined experimentally that resting state ferric cytochrome P450 was low spin, \(S = 1/2\). The presence of a 6\(^{th}\) water ligand in the resting form of P450s was confirmed in the crystal structure of P450cam. Small perturbations in ESEEM EPR measurements with \(\text{H}_2^{17}\text{O}\) also confirmed the presence of an axial water ligand in resting state ferric P450\(^{24,25}\). The fact that ferric P450 was a low-spin complex was surprising. All heme proteins that were characterized prior to P450s had a high-spin resting state; therefore, the origin of the \(S=1/2\) ground state was unclear. Since resting state heme complexes in horseradish peroxidase, cytochrome c peroxidase, and met-myoglobin had been shown to have sextet or quartet ground states, it was of interest to computationally determine the origin of the low spin ground state in cytochromes P450.

Semiempirical calculations predicted the high-spin state to be lowest in energy for a porphine methyl mercaptide model, Figure 1.5.\(^{26,27}\) The stability of the sextet state was found to be independent of the axial ligand. However, with the advancement of DFT methods, DFT calculations could be employed on the same models to compare these results to the previous semiemperical results. DFT methods include both exchange and correlation effects, both of which are important for an accurate description of the electronic structure of a molecule. Calculations were performed using both local and non-local DFT methods on porphine models of the P450 active site.\(^{28,29}\) Both methods predicted the \(S=1/2\) state to be the ground state.

Green calculated models of ferric P450 using DFT.\(^{29}\) He found that as the basis set was increased for a ferric P450 model, the amount of sulfur spin density also increased. The increased spin density on the sulfur ligand indicates that there is an increase in ligand character in the iron d-orbitals. If this is the case, then one would
expect an increase in the antibonding character of the metal orbitals, thus the \( e_g \) set would be destabilized relative to the \( t_{2g} \). This poses a reasonable argument for why the doublet ground state is more favorable in these calculations. Interestingly, using the same methods the ligand contribution to the d-orbitals in imidazole-ligated hemes was negligible. If one tracks the amount of Fe-S covalency versus spin state (the amount of interaction between \( d(z^2) - p(z)\sigma^* \)), there is a direct correlation between them: The greater the Fe-S covalency, the larger the stabilization of the low-spin state. Therefore, it was believed that as the Fe-S antibonding interaction, or the Fe-S covalency, increases the system is more accurately represented.

It has been suggested that the existence of spin density on the sulfur atom could be important to the reactivity of the enzyme as well. An analysis of the chemical reactivity of a P450 model was assessed using the Fukui function.\(^\text{30}\) For fixed positions of nuclei, the Fukui function describes the reorganization of electron density for a given molecule due to overall chemical oxidation/reduction. In this case, the changes in the electron density could be attributed to the p- and d-orbitals on the S and Fe atoms, respectively. When a protein dielectric was introduced, slight changes in the energetics of the spin states and the unpaired spin densities were observed, with a decrease in the chemical reactivity of the sulfur in this system.

Advancement of quantum mechanics/molecular mechanics (QM/MM) methods provide the potential for a means to estimate the effects of the protein environment on the electronic structure of the active site, Figure 1.6. Inclusion of the protein environment could influence the geometry of the axial ligands, which would provide a better estimate
of the orientation of the active site found in the protein. The effects of the inclusion of the protein environment were determined for resting state P450cam. The protein environment caused a shortening of the Fe-OH$_2$ bond and an elongation of the Fe-S bond. Spin densities were determined for the doublet, quartet and sextet states in P450cam using B3LYP/CHARMM22. The spin density of the doublet state was mainly localized on the iron atom and did not change much upon inclusion of the protein; however, in the quartet and sextet states spin density was localized on the sulfur and nitrogen atoms, respectively.

Figure 1.6: Example of a QM region used for P450cam, which was chosen for QM/MM calculations. Figure taken from Figure 3 in Schöneboom, J.C., Lin, H., Reuter, N., Thiel, W., Cohen, S., Oligaro, F., Shaik, S. The Elusive Oxidant Species of P450 Enzymes: Characterization by Combined Quantum Mechanical/Molecular Mechanical (QM/MM) Calculations. J. Am. Chem. Soc. 2002, 124 (27), 8145.

Substrate-bound (pentacoordinate) ferric P450 and pentacoordinate ferrous P450. The next step required in the P450 mechanism is the binding of substrate to the protein active site. It is this substrate-binding step that triggers the P450 catalytic cycle. Experimentally this phenomenon is associated with a spin state conversion from the doublet to the sextet state and a change in iron coordination from 6-coordinate, water-
bound to 5-coordinate. The spin state conversion can be monitored by changes in EPR and UV/Visible absorption spectra, while the release of water from the iron has been confirmed from several crystal structures of substrate-bound P450s\textsuperscript{33-35}. It is thought that both the spin state change and the expulsion of H\textsubscript{2}O from the active site make the heme better poised to accept reducing equivalents from a secondary reductant (i.e. P450 reductase or putidaredoxin). These changes with substrate binding have consistently been observed in P450s, and thus is believed to be a significant step in the P450 cycle.

The blue-shift in the Soret band in high spin P450 (417nm to 391nm in P450cam) has been used as an indication of substrate binding; however, it was unknown if the blue-shift was a direct result of a spin state change or slight conformational differences between the substrate-bound and substrate-free species. INDO/ROHF/CI calculations were performed to determine the mechanistic link between the experimentally observed blue-shift and spin state change with substrate binding\textsuperscript{36, 37}. These calculations were performed on identical models (Fe(III)-protoporphyrin-IX-aquo methyl mercaptide complex, Figure 1.5) in the high-spin and low-spin states, which were derived from the ferric crystal structure of P450cam. The difference between the electronic excitations for the Soret excitation in the low-spin and high-spin states was that in the low-spin state there was efficient mixing between the metal d-orbitals and the porphyrin 4e\textsubscript{g}(\pi^*) orbitals. This mixing resulted in the lowering of the e\textsubscript{g} orbitals in relation to the occupied t\textsubscript{2g} orbitals, and thus a red-shift in the Soret. These calculations showed that a spin state change from low- to high-spin is indeed enough to cause a blue-shift in the Soret absorbance.
Pentacoordinate P450 model complexes, both ferric and ferrous oxidation states, have been calculated using a variety of methods.\textsuperscript{28, 37, 38} Using porphine-thiol and porphine-methyl mercaptide models, the sextet and quintet states were the lowest energy states for the pentacoordinate ferric and ferrous oxidation states, respectively. This result was independent of the basis set and functional chosen. The Fe-S bond lengths for these two species have been measured crystallographically. This bond is typically around 2.3 Å. Larger basis sets and the use of the methyl mercaptide model were found to better match experimental results.\textsuperscript{42}

Mössbauer parameters were calculated for the ferric and ferrous pentacoordinate species, which allows us to compare the electronic structure that is calculated by DFT to experiment.\textsuperscript{39} The isomer shift was unaffected by the spin state chosen; however, the quadrupole splitting ($\Delta E_Q$) and asymmetry parameters ($\eta$) were largely affected by spin-state. The calculated Mössbauer parameters for the sextet ground state in ferric P450cam best matched the experimental data for this protein, validating the results of the (B3LYP/B2W)/MM calculations.

This spin state conversion from low-spin P450 to high-spin is also responsible for a shift in the redox potential from -300mV to -170mV in P450cam.\textsuperscript{40} Experimental reduction potentials were compared to computational results to validate these methods. The experimental reduction potential of pentacoordinate ferric P450cam with and without putidaredoxin bound are -170 and -173mV, respectively.\textsuperscript{13, 41} Gas phase calculations on an –SH model resulted in a negative reduction potential of −2.23V. This $\Delta E$ is slightly lower with an –SMe axial ligand.\textsuperscript{42} Using B3LYP/MM, the reduction potential gave a
slightly more reasonable Fe\textsuperscript{III} reduction potential of \(-0.89\)V with a neutral protein environment.\textsuperscript{39} This result is an example of the effects of including the protein environment when determining reduction potentials, though there are still problems with estimating the electrostatic interactions between the QM and MM regions.

\textit{Oxyferrous intermediate.} Once the spin-state change has occurred in ferricytochrome P450 and the iron center has been reduced to the Fe\textsuperscript{II} oxidation state by the appropriate reductant, dioxygen is able to bind to the heme iron. There is a vast amount of experimental characterization of the ferrous-dioxygen species in a variety of P450s. EXAFS and crystal structure data has been collected on this complex in P450cam.\textsuperscript{43, 44} Mössbauer, UV/Visible, and resonance Raman spectroscopies have also been used to characterize the P450cam-oxy complex.\textsuperscript{45, 46} Structural studies found that the O\textsubscript{2} molecule is bound in an “end-on” conformation. A comparison of the spectroscopic properties of P450cam-O\textsubscript{2} to those of known model oxy complexes confirmed these results.\textsuperscript{47} In the P450 reaction cycle, once the O\textsubscript{2} molecule is bound to the heme a second reduction step occurs; however, because this step is rapid, there is no experimental evidence for this second reduction step (a P450cam mutant was capable of forming this complex\textsuperscript{48, 49}). This warranted a theoretical investigation of both the ferrous-oxy (known) and reduced ferrous-oxy (unknown) complexes.

Harris \textit{et al.} performed DFT calculations on both the ferrous and reduced ferrous dioxygen species in a P450 model structure (porphine, SCH\textsubscript{3}, and O\textsubscript{2}) using the B3LYP and BPW\textsubscript{91} functionals.\textsuperscript{50, 51} The calculated geometry using both functionals was in agreement with the experimental bond lengths and bond angles from EXAFS and crystallography measurements.\textsuperscript{43, 44} The electronic and geometric structural information
obtained from these calculations provides insight into the mechanism by which P450s perform oxygen-insertion reactions.

Because the reduced oxyferrous complex has not yet been trapped in P450s, previously calibrated methods were used calculate the electronic and geometric structure of this intermediate. As with the oxyferrous species, the lowest energy O-O binding confirmation for the reduced oxy complex was the “end-on” geometry. The major effect of adding an additional electron to the oxyferrous species was the elongation of the Fe-O and Fe-S bonds. This means that the electron was added to an orbital with significant S-Fe-O character. Interestingly, the O-O bond was not weakened by the addition of the second electron. The ground state determined for the ferrous-O$_2$ species was determined to be a singlet with a low-lying triplet state, in agreement with experiment, and the reduced ferrous-O$_2$ had a calculated doublet ground state consistent with EPR experiments on cryoreduced ferrous-O$_2$ species in P450cam.

The electronic spectra were also calculated for these species using the INDO/S/CI method. The models used in these calculations included a porphine as well as a protoporphyrin IX model with methyl, vinyl, or propionate substituents. The excitations calculated for the ferrous-O$_2$ porphine species exhibited a split Soret spectrum. Although the exact energies did not match experiment, the split Soret feature was observed experimentally. Most of the excitations corresponded to orbitals of porphyrin $\pi$ and sulfur character. The mixing of these orbitals (sulfur p and porphyrin $\pi-\pi^*$) is what caused the split Soret spectrum. The reduced Fe$^{\text{II}}$-O$_2$ calculation also exhibits a split Soret because the mixing between the sulfur p and porphyrin $\pi-\pi^*$ orbitals was
maintained. The Soret peak, however, was red-shifted ~30nm in relation to the Fe$^{II}$-O$_2$ calculated spectrum, which is indicative of an increase in electron density on the ligands in this intermediate.

*Ferric Hydroperoxide.* Double protonation of the reduced oxyferrous complex is proposed to be the next step in the formation of compound I in P450s. It is believed that protonation of the terminal oxygen in the reduced oxy species is crucial to weakening the O-O bond, resulting in the facile cleavage of this bond and the formation of compound I. Kinetic isotope solvent effects were measured in P450cam. These experiments indicated that protonation can only occur after the reduction of the ferrous-oxy complex.$^{54, 55}$ These protonation events occur almost instantaneously after the reduction of the ferrous-oxy complex; therefore, there not much is known about this process. The two potential proton donors are a hydronium ion ($H_3O^+$) in close proximity to the iron, or an external proton source in which the proton is transported into the active site via proton translocations along the proton relay pathway.$^{37}$ Interestingly, in the crystal structure of the ferrous-oxy complex in P450cam reported by Schlichting and coworkers, there are two additional water molecules that enter the active site close to the O$_2$ ligand.$^{43}$ It is believed that these water molecules could assist in the protonation of the reduced ferrous species.

In P450cam, Thr-252 and Asp-251 are believed to be directly involved with rapid proton transfer to the ferric-peroxo species.$^{56-58}$ When Thr-252 was replaced by Ala, hydroxylation of camphor was not observed and hydrogen peroxide was released. This suggests that the protonation of the peroxo ligand is somehow affected by this mutation. The threonine residue is conserved among P450s.$^{59}$
Following these experiments, DFT calculations were performed in order to determine the ramifications of protonation of the proximal versus the distal oxygen in the reduced oxy complex.\textsuperscript{60} These calculations revealed that the protonation of the distal oxygen was more favorable by 18.4 kcal/mol. Protonation of the distal oxygen also resulted in the weakening of the O-O bond and strengthening of the Fe-O bond, both of which are necessary for the heterolytic cleavage of the O-O bond. This process is referred to as proton-assisted dioxygen bond cleavage. Also, when the authors attempted to add a second proton to the distal hydrogen, no stable minimum was found indicating that upon the addition of the second proton, the O-O bond is immediately cleaved.
Harris and Loew also performed DFT calculations in which they initially protonated the proximal oxygen in the ferric-peroxy intermediate, figure 1.7.\textsuperscript{50} In this model the Fe-O bond was weakened, leading to the dissociation of hydrogen peroxide. The same effect was observed if the proximal oxygen in the ferric-hydroperoxo intermediate was protonated instead of the second proton going to the distal oxygen. In either case, the protonation of both oxygen atoms resulted in the formation of a stable...
hydrogen peroxide-bound complex that is weakly bound to the iron, in agreement with the experimental data for Thr-252 mutants in P450cam.

*Influence of the Thiolate Ligand on Compound I formation.* Before compound I can be formed, efficient heterolytic cleavage of the O-O bond must occur. As discussed in the previous section, the inefficient cleavage of this bond can lead to the release of hydrogen peroxide from the active site, which can be harmful to the organism. Therefore, it is likely that Nature has engineered this thiolate ligand into cytochromes P450 so that efficient C-H bond activation can proceed and this harmful reaction can be avoided. A ‘push’ effect by the thiolate has been shown to have a significant effect on the driving force for heterolytic O-O bond cleavage. The axial thiolate is a good electron donor, and this donating ability is strong enough to cause efficient cleavage of the O-O hydroperoxo bond.\(^62,\,63\)

It is well accepted that a negatively charged axial ligand is responsible for providing the “push” necessary to promote O-O bond heterolysis in peroxidases, catalases, and P450s.\(^1\) In P450s, however, it has been suggested that the inherently anionic thiolate-ligand, which is a good pi-donor, provides the extra “push” necessary for efficient O-O bond heterolysis because there is no polar residue in the distal active site to provide the corresponding “pull” effect. Experiments on myoglobin mutants in which the axial histidine was replaced with negatively charged amino acids, cysteine and a tyrosine, exemplified the effect of anionic ligands on the redox potential and reactivity of myoglobin mutants with oxidants.\(^64\)

The H93C and H93Y mutants of human myoglobin were studied to determine their reaction products with cumene hydroperoxide and expoxidation rate with styrene.\(^64\)
In comparison to wild-type myoglobin, the H93C mutant promoted O-O bond heterolysis, whereas the tyrosine axial mutant only slightly increased the ratio of heterolytic to homolytic cleavage. These results indicate that the axial ligand does exert some influence on the reactivity of heme proteins. Although this may not be the definitive reason for the necessity of a thiolate ligand in P450s, it provides a convincing argument to support this view.

An investigation into the axial “push” effect was performed on P450cam through site-directed mutagenesis of residues on the proximal side of the heme. The crystal structure of P450cam revealed the existence of three hydrogen bonds from the peptide backbone of the axial helix to the thiolate residue. The number of hydrogen bonds to the cysteine thiolate is believed to control the electron donating ability of the thiolate. The formation of hydrogen bonds to the cysteine sulfur is a general feature in thiolate-ligated heme proteins. For example, CPO forms two NH-S hydrogen bonds and nitric oxide synthase forms three hydrogen bonds to the sulfur and possibly a fourth from the indole nitrogen from Trp409. In fact, one of the major differences between P450s and CPO in the proximal pocket is the number of hydrogen bonds to the axially ligated sulfur, which could be the origin of the differences in the reactivity between P450s and CPO.

The L358P mutation (effectively reducing the H-bonds to the thiolate from three to two) decreased the reduction potential of ferric P450cam from -134 mV to -170 mV. This reflects the enhanced electron donation of the sulfur ligand to the iron as a result of reducing the number of H-bonds. This result indicates that tertiary structure can modulate the reactivity of the protein.
In an attempt to theoretically gauge the ‘push’ effect in P450s, Ogliaro et al. performed DFT calculations on a porphine and –SH axial ligand model system with O$_2^-$, H$_2$O, OOH⁻, and no ligand to determine their effect on O-O bond cleavage and Fe$^{III}$/Fe$^{II}$ reduction potential, figure 1.8. It was determined that this effect can be broken down into two components: a classical electrostatic effect due to the negative charge of the thiolate ligand and a quantum chemical effect as a result of the σ- and π- donor abilities of the thiolate ligand. Initially, the ‘push’ effect was determined by comparing the energy of the second protonation of the distal oxygen and the formation of compound I in the P450 model with the same processes with no proximal ligand. These calculations
determined that the spontaneous formation of compound I from Fe^{III}-OOH is significantly more favorable in the thiolate-ligated model because both the electronic and quantum mechanical factors play a significant role in breaking the O-O bond. The quantum mechanical factor was attributable to the $\sigma$-donor capability of the thiolate and its ability to mix with the $a_{2u}$ orbital on the porphyrin. Conversely, the effect of the thiolate ligand on the Fe^{III}/Fe^{II} redox couple was found to be mainly electrostatic in nature. The negative charge on the thiolate disfavored the reduction of Fe^{III} by close to 100 kcal/mol.

**DFT and compound I.** Compound I in a P450 (P450-I) has yet to be trapped in high enough yield to characterize the iron-porphyrin center; however, there are three examples in the literature where compound I has been observed by stopped-flow UV/Visible spectroscopy utilizing the peroxide shunt mechanism.\textsuperscript{67-69} Compound I was generated in yields of less than 5% by mixing the P450s with peracetic acid (P450cam) or m-chloroperbenzoic acid (P450cam and CYP119). The resulting spectrum resembled the UV/Vis spectrum of CPO compound I (CPO-I).\textsuperscript{1} This result was promising because it meant that the electronic structure of CPO-I and P450-I are similar. Since CPO-I can be generated in almost 100% yield, insight into the reactive intermediate of cytochrome P450 can be drawn from experimental studies on CPO-I.

In light of work done on CPO-I, many computational studies concerning the electronic structure of P450-I have been performed. These studies include the determination of the structure and electronic ground state, the calculation of experimental details such as EPR, Mössbauer and UV/Vis spectra, and the mechanism and transition states of P450’s hydroxylation and epoxidation reactions.
The electronic structure of P450-I has been studied using a variety of methods and models.\textsuperscript{51, 70-73} The consensus from these investigations was that the best description of P450-I is an antiferromagnetically coupled doublet ground state. Two unpaired electrons were delocalized over the iron-oxo unit and one electron was distributed in the \(a_{2u}\) orbital of the porphyrin ring and the sulfur \(\pi\)-orbital, Figure 1.9. Since the amount of unpaired spin density on the sulfur ligand is a result of the mixing of the \(a_{2u}\) porphyrin orbitals and the sulfur \(\pi\)-orbitals in the HOMO, it follows that the amount of delocalization of the spin on the sulfur ligand would be a consequence of the energy ordering of the molecular orbitals. The extent of delocalization of this electron on the sulfur ligand is thus highly dependent on the DFT method and basis set, model system, and dielectric chosen.

Figure 1.9: Electronic structure of P450-I from DFT calculations. Natural orbitals of the S=1/2 thiolate. Occupancies are: a) 1.10, b) 1.0, and c) 0.90. The natural orbitals show significant radical character on the thiolate sulfur atom in the gas phase. Taken from Figure 1 in Green, M.T. Evidence for Sulfur-Based Radicals in Thiolate Compound I Intermediates. \textit{J. Am. Chem. Soc.} \textbf{1999}, 121, 7940.
An EXAFS study on CPO-I by Stone et al. gave us some insight into the geometry of P450-I.\textsuperscript{7} Using the raw EXAFS data, the Fe-N bonds were fit to 2.0 Å, the Fe-O to 1.65 Å, and the Fe-S bond was fit to 2.47 Å. An Fe-S bond of 2.47 Å is quite long for this type of bond, the ferric CPO Fe-S bond length is 2.24 Å, which suggests that the thiolate may possess some radical character in CPO-I. Since these Fe-ligand bond lengths are the closest we can get to the expected bond lengths of P450-I, we will refer back to these bond lengths throughout the section.

The major debate concerning P450 compound I is the location of the radical.\textsuperscript{74} There was one \textsuperscript{1}H-ENDOR experiment published in which the location of the radical hole in CPO compound I was examined. In this study, the amount of sulfur spin density was estimated from the spin coupling of the cysteine β-protons. Setting an upper limit for the proton hyperfine coupling to the S=1/2 radical, the maximum possible sulfur spin density was 23%. This value is consistent with QM/MM studies of P450cam-I, which determined that most of the spin density is localized on the porphyrin ring and ~25-30% on the sulfur.\textsuperscript{32}

Figure 1.10: Fe-O and Fe-S bond lengths for ferric CPO and its high-valent intermediates.
Using the unrestricted B3LYP functional and 6-311+G basis set, Green calculated the geometry and spin densities of a P450-I model.\textsuperscript{70} The porphine-methyl mercaptide model had a doublet ground state, in agreement with EPR measurements on CPO-I. The optimized Fe-S bond length for the P450-I model was 2.69 Å, which is significantly longer than the EXAFS bond length in CPO-I. The lengthening of this bond was attributed to the participation of the sulfur ligand in compound I formation. It was, therefore, estimated that \( \sim 80\% \) of the radical spin density was sulfur in character and \( \sim 20\% \) porphyrin \( a_{2u} \) character, figure 1.9.

The electronic spectrum for P450-I has also been calculated from the lowest energy multiconfigurational doublet ground state.\textsuperscript{71} The resultant spectrum exhibited a split-Soret and one Q-band. The features (contour and relative shifts) of the excitations for P450-I, along with those of the ferric resting state, match the experimental spectra for P450cam.\textsuperscript{67, 68} The major difference between the calculated and experimental spectra is that the calculated spectra are blue-shifted by \( \sim 60\text{nm} \) compared to the experimental absorption spectra. The Q-bands for the compound I and ferric models also reproduce experimental trends in that the compound I Q-band is red-shifted in comparison to ferric. The characteristic 690 nm peak in compound I was determined to be a mixture of the \( 3a_{2u} (\pi)\rightarrow 4e_g (\pi^*) \) and \( a_{1u} (\pi)\rightarrow 4e_g (\pi^*) \) excitations.

QM/MM methods, along with DFT and correlated \textit{ab initio} methods, were used to calculate EPR and Mössbauer parameters for P450cam-I.\textsuperscript{75, 76} DFT and \textit{ab initio} calculations were first performed on compound I in smaller porphyrins, tetramesitylporphyrin and tetrakis-(2,6-dichlorophenyl)porphyrin (along with a non-heme
oxo and an iron-oxo with four equatorial ammonias and an axial water ligand) to compare the computational results to experimental EPR and Mössbauer parameters. A comparison of calculated and experimental parameters for known complexes is imperative for the calibration of these methods prior to calculation of P450-I, especially since P450-I has yet to be trapped in sufficient yield for EPR and Mössbauer measurements.

The Heisenberg exchange coupling constants were calculated using the TZVP basis set for P450cam-I. Single point calculations on the doublet ground state from QM/MM calculations resulted in \( J = -16 \text{ cm}^{-1} \), using the following Hamiltonian:

\[
H = -2JS_{FeO}S_{Por}
\]

In the gas phase, a coupling constant of \( J = -27 \text{ cm}^{-1} \) was calculated, which is in good agreement with CPO-I (\( J = -37 \text{ cm}^{-1} \)). Mössbauer parameter calculations on the doublet state of P450-I in the gas phase and in a protein environment resulted in \( \Delta E_Q = 1.34 \text{ mm/s} \) and \( \delta = 0.09 \text{ mm/s} \) and \( \Delta E_Q = 0.67 \text{ mm/s} \) and \( \delta = 0.13 \text{ mm/s} \), respectively. The experimental parameters for CPO-I are \( \Delta E_Q = 1.02 \text{ mm/s} \) and \( \delta = 0.14 \text{ mm/s} \). Calculations in both the gas phase and protein environment resulted in Mössbauer parameters that were within the previously determined error of these calculations (\( \Delta E_Q = \pm0.3-0.6 \text{ mm/s} \) and \( \delta = \pm0.1 \text{ mm/s} \)).

The QM/MM method was most recently used to study the electronic structure of compound I in human isoforms of cytochrome P450. All prior QM/MM studies had been performed on P450cam; therefore, it was of interest to determine if similar environmental factors play a roll in other P450s. With the recent ability to crystallize mammalian,
membrane-bound P450s differences in the size and shape of the enzyme active sites, as well as the number of flexible regions enabling the enzymes to change conformation depending on the substrate have frequently been observed. The spin densities and the Fe-S bond length from these calculations were very similar to P450cam, which suggests that there are negligible changes in the electronic structure of P450 intermediates across this family of enzymes.

The Rebound Mechanism. Compound I is believed to be the active oxidant in P450 chemistry. Compound I abstracts a hydrogen from the hydrocarbon substrate forming an Fe(IV)-OH species and a substrate radical. The OH radical and the substrate radical recombine to generate hydroxylated product and the heme iron returns to resting state ferric, Figure 1.11. This mechanism is referred to as the rebound mechanism and is the generally accepted mechanism for substrate hydroxylation by P450s.

The rebound mechanism was first proposed by Groves and coworkers in 1976 stemming from their work on heme models of P450, as well as the actual enzyme, in which they initially proposed several possible mechanisms for the interaction of the ferryl intermediate with cyclohexene. The use of specifically deuterated cyclohexene substrates cleared up some of the questions involving the oxygen insertion mechanism utilized by P450s. A substantial kinetic isotope effect was observed when deuterated cyclohexene was used, meaning that all potential mechanisms must support a large isotope effect. The most feasible mechanism for cyclohexene oxidation, therefore, involved allylic hydrogen atom abstraction followed by geminate radical recombination.
Groves and coworkers also performed measurements on different P450s. They found that similar products resulted when three different oxidants were used. That P450s that utilize dioxygen, peroxide, and iodosylarene as oxidants oxidized cyclohexene with similar product distributions using their native oxidant. The independence of product distribution on the oxidant used suggests a common reactive intermediate and mechanism of these reactions.

Ortiz de Montellano and Stearns were the first to use radical clock substrates to study the P450 hydroxylation mechanism. These experiments measured the lifetime of the substrate radical to be 50 ps. Subsequent radical clock experiments performed by Newcomb and coworkers used substrates in which it was possible to determine whether the oxidation reaction undergoes a radical or cationic mechanism. Unrearranged products would indicate a “ring-closed” mechanism in which a cation intermediate is invoked, whereas rearranged products would result from a ring-opening reaction involving a radical substrate intermediate. According to the ratio of rearranged to unrearranged products, the rate constants calculated for the oxygen rebound step in P450s were deemed too fast for a ring-opened mechanism, which supports the formation of a cationic intermediate. These results were in opposition to Groves’ rebound mechanism.

Figure 1.11: Consensus rebound mechanism for P450 hydroxylations.
Newcomb’s work suggested that a ferric-peroxo intermediate inserts an OH$^+$ moiety into the substrate rather than a Fe(IV)-porphyrin radical inserting an OH radical as proposed by Groves. Nevertheless, both groups provide compelling evidence supporting both mechanisms, but without confirmation (trapping P450 compound I), this part of the P450 reaction mechanism remains an open question.

Figure 1.12: Two-state reactivity model for a generic FeO$^+$ oxidant illustrating the possibility of the use of both the low-spin and high-spin transition states in the hydrogen atom abstraction mechanism. Taken from Figure 2 in Shaik, S., Filatov, M., Schröder, D., Schwartz, H. Electronic Structure Makes a Difference: Cytochrome P450 Mediated Hydroxylations of Hydrocarbons as a Two-state Reactivity Paradigm. Chem. Eur. J. 1998, 4(2), 196.

Shaik and coworkers have proposed a “two state reactivity” (TSR) scheme for this process, Figure 1.12. Previous DFT calculations have shown that the doublet and quartet states for P450-I are nearly degenerate (~4 kcal/mol). In the proposed TSR mechanism, the low-spin compound I transition state lies about 2 kcal/mol lower in energy than the high spin transition state, indicating that both transition states are available to participate in C-H bond activation.$^{83-85}$ The reaction coordinate for the hydroxylation mechanism in these calculations consisted of hydrogen atom abstraction, reorganization of the substrate radical and positioning of the rebound intermediate, and the rebound process in which the
substrate radical recombines with the hydroxide radical leaving resting state P450. The compound I transition state (prior to H-atom abstraction) was the highest energy state, meaning that the rate-determining step is H-atom abstraction. In these DFT calculations, Shaik and coworkers found that the doublet state resulted in a substrate radical transition state with essentially no barrier to H-atom abstraction and a slight barrier for radical rearrangement. Conversely, the quartet state had two barriers, a smaller barrier for radical rearrangement and a transition state barrier for radical rebound. The rearranged product was favored in this pathway. This same reaction pathway has been suggested for substrates such as methane, ethane, propane, propene, benzene, toluene, ethyl benzene, camphor, and two radical clock substrates as well.

**Conclusion.** The availability of high-resolution X-ray crystal structures and advancements in computers and computational methods provide great tools for understanding P450 reactions. Through comparing calculations to experimental results, we have gained a deep understanding of previously characterized intermediates/species involved in alkane hydroxylation by P450s, as well as insight into the intermediates not yet trapped experimentally.

1.4 The Role of the Axial Ligand in P450s

1.4.1 Ferryl Protonation

Recent high resolution crystal structures of high-valent intermediates in horseradish peroxidase, cytochrome c peroxidase (CCP), catalase (CAT), and myoglobin
(Mb) and X-ray absorption measurements in chloroperoxidase and HRP have indicated long Fe-O bonds for these intermediates.\(^{10, 87-92}\) Though crystallography and X-ray absorption measurements cannot prove the presence of protons, the long Fe-O bonds relative to an authentic Fe-O double bond (1.65 Å) implied that these intermediates were protonated. The Fe\(^{IV}\)-O bond lengths measured for these intermediates were 1.84 Å and 1.93 Å (HRP-II; crystal structure and XAS, respectively), 1.92 Å (Mb-II), 1.87 Å (CCP-ES), 1.87 Å (CAT-II), and 1.82 Å (CPO-II).

These protonated ferryl species were unexpected for two reasons: 1) ferryl intermediates are believed to be electrophilic species due to their Fe(IV) oxidation state and 2) experimental evidence from X-ray absorption, resonance Raman, and Mössbauer spectroscopies for HRP-II, Mb-II, CCP-ES, and CAT-II have indicated the presence of authentic Fe\(^{IV}\)oxo species. The experimental parameters for the four histidine-ligated heme proteins in question can be found in Table I.1.

DFT calculations on a histidine-ligated protonated ferryl model (B3LYP functional and 6-311G basis set) gave an Fe-O bond length of 1.76 Å.\(^{93}\) DFT-calculated bond lengths tend to match experimental results quite well; for instance, the calculated Fe-O bond length for a CPO Fe\(^{IV}\)-OH was 1.81 Å and the bond length from EXAFS was 1.82 Å.\(^{10}\) The crystallographic Fe-O bond lengths, ranging from 1.84-1.92 Å, are actually closer to what was calculated for ferric (1.81-1.85 Å) or ferrous (1.85-1.92 Å) imidazole-ligated hydroxides by DFT.
Important to the discussion of protonated ferryls in histidine-ligated heme proteins is that, although X-ray crystallography measurements have reported long Fe-O bonds in HRP-II, Mb-II, CCP-ES, and CAT-II, resonance Raman, EXAFS, and Mössbauer (indirectly) measurements have indicated that these intermediates are authentic Fe\textsuperscript{IV}oxo species. Fe-O stretching frequencies for these intermediates were determined at a variety of pHs and for the lowest pHs reported the ν(Fe-O)s were 780 cm\textsuperscript{-1}, 753 cm\textsuperscript{-1}, 797 cm\textsuperscript{-1}, and 775 cm\textsuperscript{-1} for HRP-II, CCP-ES, Mb-II, and CAT-II, respectively.\textsuperscript{94-99} These Fe-O stretching frequencies are similar, although slightly red-shifted, to those of synthetic Fe(IV)oxo porphyrins (807-852 cm\textsuperscript{-1}).\textsuperscript{47, 100-103}

EXAFS provides a structural measure of the local environment surrounding a central atom. The problematic outlier in the EXAFS measurements is one HRP-II measurement. The 1.93 Å bond distance found for HRP-II is in disagreement with two other measurements performed on the same intermediate.\textsuperscript{92} The two experiments performed at pH 7 yielded Fe-O bonds of 1.64 ± 0.03 Å and 1.93 ± 0.02 Å and at pH 6.0

---

Table 1.1: Experimental parameters for histidine-ligated compound II species.

<table>
<thead>
<tr>
<th>System</th>
<th>X-ray crystal Fe-O bond (Å)</th>
<th>rR Fe-O stretch (cm\textsuperscript{-1})</th>
<th>EXAFS Fe-O bond length (Å)</th>
<th>Mössbauer parameters</th>
<th>δ (mm/s)</th>
<th>ΔE\textsubscript{Q} (mm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRP-II</td>
<td>1.84</td>
<td>787</td>
<td>1.64 ± 0.03</td>
<td>0.03</td>
<td>1.61</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.77</td>
<td>776</td>
<td>1.93 ± 0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mb-II</td>
<td>1.92</td>
<td>804</td>
<td>1.69 ± 0.02</td>
<td>0.09</td>
<td>1.44</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>790</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>797</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCP-ES</td>
<td>1.87</td>
<td>753</td>
<td>1.67 ± 0.02</td>
<td>0.05</td>
<td>1.55</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>767</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAT-II</td>
<td>1.87</td>
<td>786</td>
<td>—</td>
<td>0.07</td>
<td>1.47</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>775</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
the HRP-II Fe-O bond was $1.70 \pm 0.02 \text{ Å}$.

Clearly there is some discrepancy between these measured bond lengths. The 1.64 and 1.70 Å bond lengths are close to the bond lengths found in synthetic Fe$^{IV}$oxo complexes ($\sim 1.65$ Å) and therefore are reasonable bond lengths for HRP-II. The 1.93 Å bond length, however, is close the crystallographic bond length (1.87 Å), which has led to increasing confusion concerning the existence of protonated ferryls in heme enzymes.

Chance and coworkers performed EXAFS measurements on Mb-II at an unspecified pH. The edge energy for ferryl myoglobin was blue shifted $\sim 1$eV relative to ferric Mb, as was expected. The Fe-O bond distance determined for ferryl Mb from these measurements was 1.69 Å, which is in the range of an authentic Fe$^{IV}$oxo. However, like the results from resonance Raman, this measurement is in serious disagreement with the crystal structure of ferryl Mb at pH 5.2 (1.92 Å).

CCP-ES was also examined by X-ray absorption spectroscopy at pH 6.0. As expected, the CCP-ES edge was 1.2eV higher in energy than ferric CCP. The shells used to fit Mb-II were also applied to the CCP-ES data (3 shells: 1.98, 2.11, and 1.69 Å). The fits for the three shells in CCP-ES were 2.02, 1.91, and 1.67 Å for the porphyrin nitrogens, the axial nitrogen, and the ferryl oxygen, respectively. Again, an Fe-O bond length of 1.67 Å is indicative of an authentic Fe(IV)oxo.

Finally, on the topic of ferryl protonation states, our group has determined that Mössbauer spectroscopy can serve as an indirect measure of the protonation state of ferryl species. Using DFT calculated Mössbauer parameters for Fe$^{IV}$oxo and Fe$^{IV}$OH models of imidazole-ligated hemes, we determined that the quadrupole splitting parameter ($\Delta E_Q$) is drastically affected by protonation (the isomer shift does not change
however).\(^\text{107}\) The theoretical $\Delta E_Q$ changes by 1.75 mm/s simply by protonation of the ferryl unit (unprotonated ferryl $\Delta E_Q = 1.00$ and protonated ferryl $\Delta E_Q = 2.75$ mm/s). If the calculated Mössbauer parameters for an unprotonated ferryl (1.00 mm/s) are compared to those obtained experimentally for HRP-II, Mb-II, CCP-ES, and CAT-II, according to Mössbauer spectroscopy these intermediates are not protonated. The $\Delta E_Q$ parameters for these intermediates are 1.61, 1.44, 1.55, and 1.47 mm/s,\(^\text{108-111}\) respectively, which is closer to the calculated $\Delta E_Q$ for the unprotonated oxo and supports the EXAFS and resonance Raman results.

![Figure 1.13: Predicted Mössbauer spectra (c) for the ferryl (A, $\Delta E_Q = 1.00$ and $\delta = 0.08$ mm/s) and protonated ferryl (B, $\Delta E_Q = 2.75$ and $\delta = 0.10$ mm/s) histidine-ligated heme models.\(^\text{107}\)](image)

1.4.2 Protonation state of Chloroperoxidase Compound II: Mössbauer and EXAFS spectroscopy

EXAFS measurements were performed on CPO-II at pH 6.5.\(^\text{10}\) As expected, the Fe K-edge lies about 1 eV higher in energy than ferric CPO due to its Fe(IV) oxidation state. The metal-ligand bond distances for CPO-II were determined from the EXAFS
The Fe-O bond length was best fit to 1.82 Å, which is 0.17 Å longer than a typical Fe$^{IV}$ oxo bond. As was mentioned above, DFT calculations on a CPO-II model agreed quite well with the EXAFS bond distance with an Fe-O bond length of 1.81 Å. The long Fe-O bond in CPO-II can be understood because the thiolate axial ligand is a good π-donor (exerting a trans effect on the oxo ligand) and the heme ferryl complex is anionic. Protonation of the ferryl unit would result in a neutral complex.

The protonation state of CPO-II was inferred from the long Fe-O bond; therefore, further verification of the protonation state of CPO-II was necessary. As with crystallography, EXAFS utilizes synchrotron radiation during data collection meaning that photoreduction is a concern with EXAFS data as well. The necessity of a technique that does not suffer from photoreduction was apparent; therefore, Mössbauer spectroscopy was used to verify the protonation state of CPO-II (in the capacity discussed in the preceding section).

Mössbauer spectroscopy, coupled with DFT calculations, supported the existence of a protonated ferryl unit in CPO-II. Prior to generating the intermediate in CPO, it was determined that the quadrupole splitting parameter would be drastically affected by protonation of the ferryl unit (>0.5 mm/s). When CPO-II was prepared for Mössbauer measurements, two ferryl species in a 70:30 ratio were generated. Both intermediates had small isomer shifts indicative of an Fe(IV) oxidation state (0.10 and 0.11 mm/s, respectively); however, the quadrupole splitting for the majority species (2.06 mm/s) was quite a bit larger than the minor component and the $\Delta E_Q$ for authentic heme Fe(IV)oxos (∼1.4 mm/s). The remarkable agreement between the calculated and experimental Mössbauer parameters for the majority species (2.05 mm/s was calculated, 2.06 mm/s
from experiment) confirmed the EXAFS result that the majority species in CPO-II is a protonated ferryl species.

1.4.3 Badger’s Rule

Badger’s rule is an empirical formula that relates bond length to vibrational frequency using the following equation:

\[ r_e = \frac{C_{ij}}{\nu_e^2} + d_{ij} \]  \hspace{1cm} 1.2

where \( r_e \) is the equilibrium nuclear bond distance, \( \nu_e \) is the vibrational frequency, and \( C_{ij} \) and \( d_{ij} \) are the empirical constants determined for a pair of atoms by fitting a variety of \( r_e \) and \( \nu_e \)'s.\(^{112}\) Green studied the applicability of Badger’s Rule to heme Fe-O bonds.\(^9\) He examined over 30 compounds, with different proximal ligands, iron oxidation states, and oxygen protonation states, in which he used DFT to calculate their theoretical Fe-O bond lengths and stretching frequencies. It was determined that Badger’s Rule performs quite well in predicting Fe-O stretching frequencies to within 9 cm\(^{-1}\) for a 17-molecule training set and 19 cm\(^{-1}\) for a set of heme and non heme systems that were previously unexamined (14 molecules).
If one plots $r_{\text{Fe-O}}$ versus $\nu_{\text{Fe-O}}$ for the imidazole-ligated heme intermediates in question and the compounds from the training set (figure 1.14), one can see an immediate correlation between the theoretical data and that of the resonance Raman and EXAFS data. These data points lie on a straight line indicating that there is a correlation between bond lengths and stretching frequencies for the heme ferryl intermediates. The points that do not agree with Badger’s Rule are the bond lengths from X-ray crystallography measurements. The overall disagreement between spectroscopy and crystallography indicates that there may be an issue with determining Fe-O bond lengths using X-ray crystallography. Either there is systematic error in determining ferryl bond lengths using this technique (~0.2 Å), or the crystals have been reduced to ferric or ferrous hydroxides by the high energy X-ray beam.

Figure 1.14: Fe-O bond distance versus $1/\nu^{2/3}$. The circles represent the calculated bond lengths and frequencies (scaled). The best-fit line through the theoretical data is Badger’s rule. The yellow diamonds represent the EXAFS and resonance Raman data and the black diamonds are from resonance Raman and X-ray crystallography. Figure adapted from reference 93.
If Badger’s rule is used to calculate the expected $\nu_{\text{Fe-O}}$ for the crystallographic Fe-O bond lengths we would observe Fe-O stretches of 543 cm$^{-1}$ for HRP-II, 473 cm$^{-1}$ for Mb-II, and 515 cm$^{-1}$ for CCP-ES and CAT-II. These stretching frequencies are on the order of 250-300 cm$^{-1}$ lower than what was measured for these species. According to this Badger’s Rule analysis, we believe that these bond lengths are likely a consequence of photoreduction of the Fe$^{IV}$oxo unit to ferric or ferrous hydroxides. Compiling all of the available experimental data, Mb-II (pKa $\leq$ 4), HRP-II (pKa $\leq$ 4), CCP-ES (pKa $\leq$ 4), and CAT-II (pKa $\leq$ 7) are not protonated down to the pHS indicated in parentheses.

We have made the claim that the crystals for the high-valent intermediates in HRP-II, Mb-II, CCP-ES, and CAT-II have been reduced by the high intensity radiation used for X-ray data collection (~10keV). There is precedence for the photoreduction of heme protein crystals due to synchrotron radiation, even at low temperature (100 K). A systematic study of the effects of radiation dose and radiation energy on crystals of myoglobin, P450cam, and CPO as it relates to changes in their absorption spectrum was performed. These studies examined the ferric form of the enzymes. Photoreduction was observed in the UV/vis spectra almost instantaneously in all three proteins. The immediate changes in the oxidation state of the crystals is understandable if one considers the number of free electrons generated in the crystals as a consequence of synchrotron radiation. This was estimated based on the energy of the incoming photon and the size of the crystal. For the crystals in this study, 34 mM of trapped electrons are generated per second of data collection, meaning that within seconds there are more electrons than protein molecules in the sample.
If the ferric form of myoglobin is so easily reduced by synchrotron radiation, then it follows that the high-valent ferryl species in myoglobin should also be easily reduced. The results of this photoreduction study demonstrate the importance of monitoring photoreduction using techniques like microspectrophotometry, as well as the necessity of using multiple spectroscopies to characterize the intermediate in question.

_Badger’s Rule and the CPO-II protonation state._ Parameterization of Badger’s Rule for Fe-O bonds in heme complexes enables one to determine a theoretical stretching frequency for the Fe-O bond in CPO-II. The Fe-O bond length determined from EXAFS experiments was 1.82 Å. Using Badger’s Rule we would expect an Fe-O stretch around 563 cm⁻¹. Previous attempts to locate the Fe-O stretch in CPO-II were unsuccessful, in which it was explained that the Fe-O resonance was not enhanced.¹²,¹¹⁵ A typical ferryl Fe-O stretch would be located around 750-850 cm⁻¹, which is presumably why Kitagawa and coworkers recorded data from 655-875 cm⁻¹.¹² Their work was done prior to the EXAFS report on CPO-II.

![Figure 1.15: Resonance Raman spectrum of CPO-II prepared with m-CPBA, right, and the corresponding Mössbauer spectrum, left. The Fe-O stretching frequency for this intermediate is at 561 cm⁻¹. Upon D₂O substitution, the Fe-O stretch shifts to 549 cm⁻¹.](image-url)
With the theoretical Fe-O stretching frequency known, resonance Raman data was collected on CPO-II using a 458 nm excitation energy (figure 1.15). A peak at 561 cm\(^{-1}\) was present at pH 6.5 that was sensitive both to deuterium and \(^{18}\)O substitution and was assigned to the Fe-O stretch in CPO-II. The 561 cm\(^{-1}\) peak was red-shifted 13 cm\(^{-1}\) with deuterium and 22 cm\(^{-1}\) with \(^{18}\)O-hydrogen peroxide, both of which agree quite well with a Fe-OH diatomic harmonic oscillator (23 and 12 cm\(^{-1}\), respectively). In the same work, ENDOR spectroscopy on cryoreduced compound II (to generate the Fe(III)OH complex) was performed to provide further verification of the ferryl proton. Cryoreduction techniques were necessary because CPO-II is an S=1 species and is EPR silent. The X-band continuous wave ENDOR spectrum revealed the presence of a strongly coupled proton resonance of 13.4 MHz at 3100 Gauss. This coupling is similar to that of the hydroxide complex in myoglobin (11.4 MHz, 3100 Gauss), which is why the 13.4 MHz resonance was assigned to the CR-CPO-II hydroxide.

1.5 The Importance of the Ferryl pKa

Hydrogen atom abstraction is required in a plethora of biochemical processes. As was briefly discussed above, P450s are suspected to utilize a hydrogen atom abstraction mechanism in their metabolism and detoxification roles. Hydrogen atom transfer has also been implicated in the catalytic cycles of other metalloenzymes involved in, for example, the synthesis of natural products and antibiotics, the biosynthesis of dopamine, and DNA repair.
Mayer and coworkers were interested in the mechanism of hydrogen atom abstraction by metal oxo compounds. In their quest to understand the requirements that enable metal-oxo complexes to abstract hydrogen, they determined that this ability was not related to the spin density on the metal-oxo, but rather had to do with reaction thermodynamics. CrO$_2$Cl$_2$ was the first compound to be investigated for its ability to abstract hydrogen as it relates to reaction thermodynamics.$^{118, 119}$ CrO$_2$Cl$_2$ oxidized isopropylcyclopropane via H-atom abstraction, resulting in a variety of products; however, the amount of unrearranged and ring-opened products was consistent with CrO$_2$Cl$_2$ trapping the tertiary radical at $10^{8.1}$ M$^{-1}$s$^{-1}$. The mechanism observed for the oxidation of isopropylcyclopropane by CrO$_2$Cl$_2$ was very similar to the proposed P450 rebound mechanism.

It was determined that the rate of the initial hydrogen atom transfer step was equal to the disappearance of CrO$_2$Cl$_2$ times the number of CrO$_2$Cl$_2$ consumed per substrate radical formed. The $\Delta H^\ddagger$ and $\Delta G^\ddagger$ for H-atom abstraction correlated well with the strength of the C-H bond cleaved, Figure 1.16. It also follows that the strength of C-H bond being cleaved is equal to the O-H bond formed. The Polyani correlation for rate constants with bond strengths has shown to work quite well with H-atom transfer reactions and also translates well to activation enthalpy and free energy of the H-atom transfer reaction.$^{120, 121}$
Interestingly, CrO$_2$Cl$_2$ and MnO$_4^-$ are $d^0$ closed shell species and, therefore, according to the previously used spin density arguments, these complexes should not abstract H, yet they do. There must be some explanation for why these closed-shell systems can perform H-atom transfer reactions. To fully understand the correlation of rate constants with C-H bond strength, Mayer and coworkers performed a study of the H-OMnO$_3^-$ bond strength.$^{122-126}$

CrO$_2$Cl$_2$ and MnO$_4^-$ form very strong O-H bonds to the “abstracted hydrogen”. According to the Polanyi correlation, the reactivity of the complex is related to the strength of the substrate C-H bond and the $\Delta H^\circ$ for H-atom transfer. The strength of the O-H bond formed in MnO$_4^-$ can be calculated using a thermodynamic cycle similar to the thermodynamic cycle proposed by Bordwell and coworkers for organic X-H bond strengths.$^{127}$ The thermochemical cycle as it relates to H-atom abstraction by transition metal oxo complexes involves the following steps (figure 1.16, right); 1) reduction of the

Figure 1.16: H-atom abstraction reactions performed by CrO$_2$Cl$_2$ and MnO$_4^-$. Left. Correlations of $\Delta H^\circ$ and $\Delta G^\circ$ with C-H bond strength for oxidations by CrO$_2$Cl$_2$. Right. Scheme used to calculate O-H bond strength in HMnO$_4$. Figures from Figure 1 (left) and Scheme 5 (right) in Mayer, J.M. Hydrogen Atom Abstraction by Metal-oxo Complexes: Understanding the Analogy with Organic Radical Reactions. Acc. Chem. Res. 1998, 31, 441-450.
metal center, 2) the oxidation of H$_2$ to H$_3^+$, 3) the pKa of the H-addition process, 4) a constant related to bond cleavage and solvation of H$_2$. This mechanism implies that the strength of the O-H bond formed is related to the one-electron redox potential of the oxidizing metal complex and the pKa of the complex following H-atom transfer.

Assuming the rebound mechanism is operative in P450s, the thermodynamic cycle proposed by Mayer and coworkers for the ability of metal oxos to abstract hydrogen can be applied to the proposed intermediates in P450 chemistry. Figure 1.17 shows the correlation between Mayer’s work and the P450 intermediates. In this case, the strength of the O-H bond formed during the H-transfer process is related to the one-electron reduction potential of compound I and the pKa of the rebound intermediate (compound II).

![Diagram of compound I, protonated compound II, hydroxylated product](image)

Figure 1.17: Strength of the O-H bond as it relates to P450 chemistry. The rebound mechanism is summarized at the top and below it is the equation used.

\[
D(O-H) = 23.06 \times E^\circ_{\text{compound I}} + 1.37 \times \text{pKa}_{\text{compound II}} + 57 \pm 2 \text{ (kcal/mol)}
\]

Only thiolate-ligated heme enzymes have shown the ability to hydroxylate hydrocarbons using the H-atom abstraction mechanism. P450s for example can abstract hydrogen from cyclohexane, which has a C-H bond strength of 99 kcal/mol. The relationship between Mayer’s work on inorganic complexes and that of P450 chemistry is
important. Because the strength of the C-H bonds that can be broken by P450s is related to both the redox potential of compound I and the pKa of compound II, it poses an interesting explanation for how P450s are capable of abstracting hydrogen from unactivated C-H bonds at viable redox potentials. For instance, HRP-II has a pKa ≤ 4, which means that to abstract hydrogen from cyclohexane an extremely large compound I redox potential of >1.5 eV is required. We know from experiment that the pKa of CPO-II is greater than 8.2; therefore, the same reaction performed by a ferryl intermediate with a pKa of 8 would require a compound I reduction potential of 1.3 eV, which is more biologically feasible.

Mayer’s work suggested a role for the compound I reduction potential and the ferryl pKa in promoting C-H bond hydroxylation reactions by oxidative heme proteins. Until the structure of CPO-II was obtained from EXAFS measurements, the role of basic ferryl intermediates in P450 chemistry was not much of a concern. Following the CPO-II work, however, the importance of basic ferryls was evident. Basic ferryls allow for H-atom abstraction and hydroxylation of strong C-H bonds at reasonable compound I redox potentials.

1.6 Peroxynitrite

Peroxynitrite (PN) is a potent biological oxidant that is formed by the diffusion-controlled combination of superoxide and nitric oxide radicals. The rate of PN generation in vivo can reach up to 50-100 μM per min. The lifetime of PN at physiological pH (pKa = 6.8) and temperature is only about 10 ms; however, because PN
can easily pass through cell membranes its potential targets can be up to one to two cell diameters away (~5-20 µm).\textsuperscript{130,131} This property makes this molecule a significant threat to many biomolecules.

Many biomolecules are oxidized (either by one or two electrons) or nitrated by PN. These include tyrosine residues, thiols, DNA, and fatty acids. Tyrosine residues can undergo hydroxylation, nitration, and oxidation to form dimers as a result of PN attack.\textsuperscript{132-136} Thiols are oxidized by one electron by PN-derived radicals to produce higher oxides of sulfur, which can have disastrous effects on protein tertiary structure.\textsuperscript{137,138} PN can cause deoxyribose oxidation and DNA strand breaks, as well as the formation of 8-oxo and 8-nitroguanine species.\textsuperscript{139-142} Lipids are particularly susceptible to oxidation by PN. Reaction of lipids with PN causes lipid peroxidation and nitration.\textsuperscript{143} Other examples of how PN can disrupt biological processes include antioxidant enzyme inhibition, antioxidant depletion, cytosolic enzyme inhibition, protein aggregation, activation of enzymes involved in the pro-inflammatory response, membrane channel inhibition, modification of cell signaling molecules and receptors, and calcium dysregulation.\textsuperscript{129}

There is a large amount of evidence supporting a pathological role for PN in humans.\textsuperscript{144} For instance, PN is believed to be involved in circulatory shock because an increase in plasma nitrotyrosine levels in patients with chronic renal failure and septic shock was observed.\textsuperscript{145} Interestingly, the level of plasma nitrotyrosines correlated with the severity of the septic shock. Chronic heart failure and Parkinson’s disease have also been linked to PN nitrations.\textsuperscript{146-148} Cardiomyocyte and skeletal muscle nitrotyrosine
levels were increased in instances of heart failure; also, diabetic islets have showed elevated levels of 3-nitrotyrosine.\textsuperscript{149, 150}

Another danger that PN poses is its ability to react with cofactors. It has been found that PN can oxidize tetrahydrobiopterin (BH\textsubscript{4}) to quinoid 5,6-dihydropterin.\textsuperscript{151} BH\textsubscript{4} is an essential cofactor for nitric oxide synthase (NOS). NOSs are thiolate-ligated heme proteins, like Cytochrome P450, that utilize NADPH and O\textsubscript{2} to catalyze the two-step oxidation of L-arginine to nitric oxide and citrulline (passing through a stable substrate intermediate, N\textsuperscript{ω}-hydroxy-L-arginine).\textsuperscript{152} NO is known to be involved in vasodilation and the tumor cytotoxicity of macrophages, as well as many other physiological and pathological processes and thus is an essential molecule.

When BH\textsubscript{4} is not present in sufficient concentrations, NOS dysfunction results. The oxidation of BH\textsubscript{4} by PN results in low levels of the cofactor, which sends NOS into a cycle of its own destruction where NOS effectively becomes a PN-synthase. Low levels of BH\textsubscript{4} results in the uncoupling of O\textsubscript{2} reduction by NOS and the formation and release of superoxide.\textsuperscript{153, 154} Thus, NOS is capable of synthesizing both “reagents” necessary for PN synthesis \textit{in vivo}. It is believed that this mechanism may contribute to vascular dysfunction that is induced by oxidative stress in various diseases.

\textbf{1.6.1 Reactions of PN with Metalloproteins}

Since metalloporphyrins have been shown to be quite efficient PN-decomposition catalysts, there was an effort to understand how these compounds function in order to
develop better catalysts. Both manganese and iron porphyrin complexes have been investigated for this purpose.\textsuperscript{155-158}

Figure 1.18: Reactions of metal porphyrins with PN. \textit{Left}. Mn pophryins can react with PN via two pathways. Heterolytic cleavage of the PN O-O bond results in the ability to perform DNA strand scission reactions, whereas homolytic cleavage results in the generation of oxidants capable of the nitration of added phenols. \textit{Right}. Proposed mechanism for the initial reaction of stoichiometric amounts of PN with Fe\textsuperscript{III}TMPyP. Reactions of excess PN with FeTMPyP appeared to generate the ferryl radical intermediate. The figure on the left was taken from Scheme 1 in Groves, J.T., Marla, S.S. Peroxynitrite-induced DNA Strand Scission Mediated by a Manganese Porphyrin. \textit{J. Am. Chem. Soc.} 1995 117(37), 9579. The figure on the right was taken from Scheme 2 in Lee, J.B., Hunt, J.A., Groves, J.T. Mechanisms of Iron Porphyrin Reactions with Peroxynitrite \textit{J. Am. Chem. Soc.} 1998, 120(30), 7499.

Manganese(III) porphyrins, like Mn(III) tetra-N-methylpyridylporphyrin (Mn(III)TMPyP), have been shown to rapidly react with PN to generate an Mn(IV)oxo species, figure 1.18.\textsuperscript{155} The speed with which Mn-porphyrins can reduce PN implies that these complexes can be used to prevent the oxidation and nitration of biological substrates. Development of Mn(III) catalysts led to the development of amphiphilic analogs, which allowed the porphyrin complex to pass through membranes.\textsuperscript{130} Iron(III)
porphyrins were also shown to be capable of PN decomposition and are the complexes most often chosen for therapeutic purposes.

Determining the mechanism of PN decomposition by these metalloporphyrin complexes is of importance not only for understanding whether these compounds could be used as drugs, but also to understand the mechanism of metalloprotein inactivation in biological systems. PN is known to react with many metalloenzymes such as myeloperoxidase, Cu, Zn-superoxide dismutase, Mn-SOD, and heme enzymes like Cytochrome P450s, Cytochrome c, and prostacyclin synthase.136, 159-163

A mechanism has been proposed for the decomposition of PN by FeTMPyP (a PN decomposition catalyst) in which the PN molecule initially binds to the Fe(III) center through the peroxy portion of the molecule.156 Heterolytic cleavage of the O-O bond in the peroxynitrite molecule by FeTMPyP would lead to the formation of compound I (Fe(IV)oxo porphyrin cation radical) and a nitrite anion, whereas homolytic cleavage would generate compound II (Fe(IV)oxo) and a nitrite radical, figure 1.18. Evidence for the formation of both high-valent intermediates has been observed. In FeTMPyP, the intermediate generated appeared to be dependent on the concentration of PN used. According to stopped-flow spectrophotometry, when concentrations of PN were less than FeTMPyP, a species with behavior resembling compound I was generated; however, with an excess of PN, the Fe(IV)oxo intermediate was observed. Similarly, the ability of the peroxynitrite-generated intermediate in MnTMPyP to cause DNA strand scission led to the conclusion that the Mn(V)oxo species was the active oxidant.164 It should be noted that the mechanism of the reaction of MnTMPyP with PN is less complex, and therefore better understood, than the FeTMPyP reaction.
It is not clear which factors are involved in the preferred method for O-O bond cleavage during PN decomposition by metalloporphyrin complexes; however, what is known is that the mechanism involves the generation of high-valent transition metal intermediates. This mechanism was also tested in several peroxidases. When PN was reacted with horseradish peroxidase, compound I was the first observed intermediate.\(^{162}\) This indicated that HRP decomposes PN via heterolytic cleavage of the PN O-O bond. On the other hand, when myeloperoxidase and lactoperoxidase reacted with PN, the first and only intermediate observed was compound II.\(^{162}\) Formation of compound II in these enzymes with PN was surprising because this was different behavior than when H\(_2\)O\(_2\) is the oxidant, compound I is formed. The authors of this work believe that compound I is indeed generated in myeloperoxidase, but the excess nitrite in solution (from the PN preparation) can act as a reducing agent and compound I was quickly reduced. Compound I without reductant was very short lived in this enzyme (t\(_{1/2}\) = 100ms) and adding reductant significantly sped up this process. HRP-I, however, is a long-lived intermediate in comparison, which may be why HRP-I was observable.

1.6.2 P450s and PN

Ullrich and coworkers have studied the reaction of PN with several P450s and thiolate-ligated heme proteins.\(^{161,165-168}\) This work began when the group discovered that prostacyclin synthase (PGI\(_2\) synthase) was inactivated by submicromolar concentrations of PN.\(^{161}\) PGI\(_2\) synthase is a thiolate-ligated heme enzyme involved in platelet aggregation and vasodilation in humans. Inactivation of this enzyme by peroxynitrite
was paralleled with the formation of 3-nitrotyrosine residues. Because PGI\(_2\) synthase is difficult to obtain in yields necessary to study the mechanism of inactivation by PN, several other thiolate-ligated heme proteins were chosen. These included P450\(_{BM3}\), P450cam, P450nor, and CPO.

The mechanism for the formation of nitrotyrosine residues by PGI\(_2\) synthase was postulated from experiments involving the nitration of phenols by heme proteins and iron complexes with PN.\(^{156, 157, 168}\) In this mechanism, homolytic cleavage of the O-O bond in PN would result in the formation of an Fe(IV)oxo intermediate with release of a nitrite radical. The Fe(IV)oxo could oxidize a nearby tyrosine residue and the resulting tyrosyl radical could then recombine with the nitrite radical resulting in nitrotyrosine formation.

It is believed that the thiolate ligand may play a role in the efficient decomposition of PN by these enzymes. The decay of PN was measured in HRP, microperoxidase (MP-11), hemoglobin, P450nor, P450\(_{BM3}\).\(^{161}\) From these experiments, it was apparent that thiolate-ligated heme proteins are better catalysts for PN decomposition. P450nor was the best catalyst of the five. Whether there is biological significance for the increased reactivity of the thiolate-ligated heme proteins towards PN has yet to be determined.
Mechanistic studies on P450\textsubscript{BM3} and CPO determined that compound II was the intermediate that was formed when these enzymes were reacted with peroxynitrite.\textsuperscript{165} The stopped-flow data is shown in figure 1.19. When wild-type P450\textsubscript{BM3} reacted with PN, the Soret absorbance for low-spin ferric P450\textsubscript{BM3} shifted from 418nm to 430nm after 300ms. This intermediate was stable as long as an excess of PN was available in solution.

Figure 1.19: Time course of the reactions of P450\textsubscript{BM3} and CPO with a large excess of PN. Left. Top. P450\textsubscript{BM3} spectra over time: 1) 5ms, 2) 320ms, 3) 640ms, 4) 960ms, 5) 1.6s, 6) 3.84s, 7) 15.84s, 8) 27.84s. Bottom spectrum is the kinetic traces for 302nm (PN, circles), 418nm (ferric P450\textsubscript{BM3}, squares), and 435nm (ferryl P450\textsubscript{BM3}, triangles). Right. Top. CPO reaction with PN: 1) 5ms, 2) 16ms, 3) 32ms, 4) 128ms, 5) 1.41s, 6) 3.65s, 7) 6.89s, 8) 27.01s. Bottom spectrum shows the kinetic traces of the reactions. The PN absorbance (302nm, circles), ferric CPO Soret (397nm, squares) and ferryl CPO Soret (434nm, triangles) were followed over the 10s reaction. These figures were taken from Figures 6 and 7 in Daiber, A., Herold, S., Schoneich, C., Namgaladeze, D., Peterson, J.A., Ullrich, V. Nitration and Inactivation of Cytochrome P450BM-3 by Peroxynitrite. Stopped-flow Measurements Prove Ferryl Intermediates. Eur. J. Biochem. 2000, 267(23), 6735.
(\(\lambda = 302\text{nm}\)). Once two-thirds of the original PN concentration was consumed, the intermediate decayed back to ferric P450. In order to determine the identity of this intermediate as an Fe(IV)oxo intermediate, the analogous reaction was performed with CPO. The absorption spectra of compounds I and II in CPO are known. When CPO was reacted with a large excess of PN, an intermediate with a Soret maximum at 434nm was formed and had similar decay characteristics as P450_{BM3}. The similarity between the absorption spectrum for intermediate generated in both P450_{BM3} and CPO with PN to that of CPO-II led to the characterization of both PN-generated intermediates as Fe(IV)oxo species.

These investigations led to considerable interest in utilizing PN to generate compound II in P450s as the generation of high-valent intermediates had not yet been achieved in high yield in these enzymes. Newcomb and coworkers sought to use the PN-generated intermediate in CYP119 as a platform from which compound I could be generated.\(^{169}\) Previously it was shown that compound I could be generated from laser flash photolysis (LFP) of compound II in HRP and synthetic heme compounds.\(^{170}\) The same method was utilized with CYP119 in this study. It was determined that compound II was generated in CYP119 through reaction with PN at pH 7.4 with a Soret maximum at 429nm, figure 1.20. The PN intermediate in CYP119, like P450_{BM3}, resembled that of CPO-PN as well as CPO-II. Interestingly, according to these studies “compound II” generated in CYP119 with PN was more stable than compound II in CPO. This is counterintuitive since there has been much difficulty in trapping the high-valent intermediates in P450s, including CYP119. Nevertheless, CYP119-PN was subjected to laser flash photolysis and a product in 5% yield was generated.
Drawing analogies from the work on HRP and heme complexes, it was suggested that the intermediate generated in 5% yield was P450 compound I. The difference spectrum for this product had a Soret maximum between 400 and 410 nm and a Q-band absorbance around 640-670 nm. These peaks were likened to the spectrum of CPO-I, which has a Soret at 367 nm and a significant Q-band at 688 nm with a smaller peak at 610 nm.\footnote{Prior to this work, compound I was generated in CYP119 using m-CPBA as an oxidant.} The spectrum of this intermediate had a Soret at 370 nm and Q-bands at 610 and 690 nm. One would expect the LFP-generated product to have the same absorption spectrum as the species generated with m-CPBA, yet it does not. Another interesting result from the CYP119-PN experiments was that the LFP intermediate was significantly more stable than CPO-I. When substrates were introduced to the LFP-CYP119-PN intermediate they had no effect on the lifetime of the intermediate. This was unusual since P450cam was shown to hydroxylate camphor during low temperature studies.

Figure 1.20: Reaction intermediates for the reaction of PN with CPO (left) and CYP119 (right). The inset shows the kinetic traces for the decay of the ferryl intermediate (\(\lambda_{\text{max}}\) for the intermediate) at 40ms (CPO) and 40s (CYP119). This figure was taken from Figure 1 in Newcomb, M., Zhang, R., Chandrasena, R.E., Halgrimson, J.A., Horner, J.H., Makris, T.M., Sligar, S.G. Cytochrome P450 Compound I. \textit{J. Am. Chem. Soc.} \textbf{2006}, 128(14), 4580.
without the buildup of a detectable intermediate.\textsuperscript{52} Therefore, under LFP conditions with the CYP119-PN intermediate, compound I should also show some hydroxylation activity.

We have also contributed to the topic of the reaction of thiolate-ligated heme enzymes with PN with our recent work on P450\textsubscript{BM3}, CPO, CYP119, P450cam, CYP154, and gsNOS. This work will be presented in Chapters 4 and 5 and will discount the currently accepted mechanism for tyrosine nitration by thiolate-ligated heme enzymes.

1.7 Summary of Chapters

Chapter 2 concerns the extensive characterization of the ferryl intermediate in myoglobin (Mb-II). The protonation state of this intermediate has been under some scrutiny over recent years due to results from a recent crystal structure. We will present Mössbauer and resonance Raman results on Mb-II over a large pH range that disprove the existence of a protonated ferryl in this protein.

Chapter 3 will focus on Mössbauer experiments that have proven the general existence of protonated ferryls in thiolate-ligated heme proteins. Protonated ferryl intermediates have been trapped in three cytochrome P450s: P450\textsubscript{BM3}, P450cam, and CYP158.

Experiments characterizing the intermediate generated when P450\textsubscript{BM3} is reacted with peroxynitrite are presented in Chapter 4. Previous stopped-flow investigations on this reaction have suggested the existence of a ferryl intermediate. We used Mössbauer and resonance Raman spectroscopies to characterize the PN intermediate in which we
determined that the intermediate previously believed to be a ferryl species is in fact a ferric nitrosyl complex.

Chapter 5 will focus on reactions to further investigate the reaction of peroxynitrite with heme proteins. We examined the reaction with five other thiolate-ligated heme proteins and two histidine-ligated heme proteins. Interestingly, it appears as though the mechanism of peroxynitrite breakdown in heme proteins is dependent on the axial ligand.

Chapter 6 describes the experimental materials and methods used for the experiments described throughout this thesis. Detailed descriptions of the results of these experiments are found in the respective chapters.

Appendix A describes work done on the reaction of CYP154 with peracetic acid. The intermediate has been characterized by EPR and Mössbauer spectroscopies to date.

1.8 References


Chapter 2

Resonance Raman and Mössbauer Spectroscopy of Myoglobin Compound II: Setting an Upper Limit on the Ferryl pKa

2.1 Abstract

A recent X-ray structural investigation of myoglobin compound II (Mb-II) has suggested that this species is best described as an Fe^{IV}OH species at pH 5.2, 6.8, and 8.7. Using density functional calculations and resonance Raman and Mössbauer spectroscopies we have assessed this claim. This chapter presents the first low pH spectroscopic measurements on ferryl myoglobin. We find no evidence of a hydroxide intermediate over a large pH range. Our investigation sets an upper limit of 3.5 for the pKa of the Fe-O moiety.

2.2 Introduction

X-ray crystallographic reports have suggested that a number of enzymatic iron(IV)oxo (ferryl) species may be protonated. The protonation state of these intermediates was inferred from their Fe-O bond lengths, which are longer than expected for authentic ferryl species. Bond lengths of 1.84, 1.92, 1.87, and 1.87 Å have been reported for horseradish peroxidase-II (HRP-II, pH 6.5), myoglobin-II (Mb-II, pH 5.2),
cytochrome c peroxidase-I (CCP-I, pH 6), and catalase-II (CAT-II, pH 5.2), respectively.\textsuperscript{1-4}

The long bond lengths obtained from these crystallographic investigations do not agree with previous spectroscopic characterizations of these intermediates. Bond lengths from resonance Raman and EXAFS spectroscopies resemble those of ferryl Fe-O bond lengths in synthetic heme compounds, $r_{\text{Fe-O}} = 1.65$ Å.\textsuperscript{5-7} This was quite puzzling. In general, crystallographic studies have yielded Fe-O bonds that are \( \approx 0.2 \) Å longer than the bonds obtained from spectroscopy.

It is well known that synchrotron radiation generates potent reducing equivalents in the form of solvated electrons.\textsuperscript{8,9} The concentration of free electrons generated from X-ray irradiation during data collection at 10keV are on the order of 34 mM per second (estimated from the photon energy, photon flux, the absorption probability of the photons). Typical protein concentrations in crystals are about 50-60 mM.\textsuperscript{9} Photoreduction is therefore a significant concern when collecting structural data on high-valent intermediates. This process may explain why the Fe-O bonds observed in the crystal structures of HRP-II, Mb-II, CCP-I, and CAT-II are closer to those expected for ferric and ferrous hydroxides.

One consideration that should be noted is that in some cases the spectroscopic investigations have been performed at a higher pH than the crystallization conditions; therefore, the issue of ferryl protonation in these systems remains an open question. Resonance Raman measurements on myoglobin compound II (pH 8.6), for example, have provided an Fe-O stretching frequency ($\nu_{\text{Fe-O}} = 797$ cm$^{-1}$) that is typical of an authentic
(i.e. unprotonated) ferryl unit. Also, EXAFS experiments (pH not given) have yielded an Fe-O bond length of 1.69 Å,\textsuperscript{10, 11} in good agreement with the ≈ 1.65 Å Fe-O bonds calculated for ferryl porphyrins,\textsuperscript{12} but significantly shorter than the 1.92 Å Fe-O bond obtained from crystallography at pH 5.2.\textsuperscript{3}

**QM/MM investigation of HRP-II protonation states**

The issue of ferryl protonation, namely the discrepancies between the Fe-O bond distances measured experimentally, has sparked computational studies on the identity of compound II in histidine-ligated heme proteins. Shaik and coworkers recently published a QM/MM study on HRP-II in which they considered the participation of different protonation states, spin states, and electromers of HRP-II during the HRP reaction cycle.\textsuperscript{13} In addition to the previously identified ferryl and protonated ferryl species, Shaik and coworkers have proposed the existence of Fe(III)-porphyrin radical species.

QM/MM calculations on HRP-II determined a pathway for the conversion of the Fe\textsuperscript{IV}oxo intermediate to a protonated ferryl. This occurred through proton donation from a nearby water molecule (W\textsubscript{427}) positioned perfectly in the distal pocket to shuttle a proton to the oxo moiety by way of His42. However, when they attempted to generate the Fe(III)-OH porphyrin radical species from the authentic Fe\textsuperscript{IV}oxo and vice versa, they were unsuccessful. Starting with the ImPor\textsuperscript{\textsuperscript{[Fe\textsuperscript{III}OH]}, the lowest energy intermediate was a doubly protonated Fe(III)-porphyrin radical intermediate. This species was referred to as the “surprise species”.

An Fe(III)-porphyrin radical aquo species was formed following protonation of the hydroxide species by Arg38 in the distal pocket. This process showed no significant
barrier to formation. This $[\text{ImPor}^{\text{III}}\text{Fe}^{\text{III}}(\text{OH}_2)]^{2+}$ species had the same electronic structure as the $S = 0$ and $S = 1$ Fe(III)-OH porphyrin radical species and thus was considered to be another isomer of HRP-II with similar oxidative power. Interestingly, the Fe-O bond length in the “surprise” species was 1.92 Å, which is exactly the Fe-O bond length found for the Mb-II crystal structure and close to that from one of the HRP-II EXAFS measurements.

Mössbauer parameter calculations were performed on all of the potential intermediates as well. The only theoretical parameters that matched the experimental results were those for the Fe$^{\text{IV}}$oxo model. Although the theoretically determined parameters for the other species that were considered to be biologically relevant by Shaik and coworkers did not agree with the Mb-II experimental parameters, they still insinuated that the two Fe(III)-porphyrin radical species were not simply computationally derived species. The reasons for this conclusion were: 1) Fe(III)-porphyrin radical species were previously reported for synthetic heme complexes,\textsuperscript{14} 2) Fe(III) or Fe(IV) oxidation states could not be ruled out from previous experimental reports on the oxidation state and spin state of HRP-II,\textsuperscript{15, 16} and 3) Mössbauer spectroscopy missed both the Fe(IV)OH and Fe(III)-porphyrin radical isomers. Our thorough computational, Mössbauer, and resonance Raman investigations on horse heart Mb-II will clear up some of these discrepancies.

\textit{Biological Significance of Ferryl Myoglobin}

Although we used ferryl Mb as a model for ferryl protonation states in peroxidases, interest in studying ferryl Mb is more than just academic. Myoglobin is a
protein found in high concentrations in cardiac and skeletal muscle tissues. The biological role of myoglobin is O$_2$ storage and diffusion within cells. To bind O$_2$, Mb must be present in the ferrous oxidation state; however, during O$_2$-binding the ferrous iron can be autooxidized to ferric Mb (also referred to as metMb). The O$_2^-$ released can disproportionate to H$_2$O$_2$ and O$_2$.$^{17}$

The reaction between Mb and H$_2$O$_2$ can be quite harmful.$^{18}$ Though myoglobin is not a peroxidase, and thus in its normal function does not perform oxidation reactions, the ability of metMb to generate high valent intermediates with H$_2$O$_2$ is believed to play a role in reperfusion injuries, arteriosclerosis, and kidney dysfunction.$^{19-22}$ Therefore, understanding how myoglobin (and hemoglobin) contributes to these pathological disorders is of great importance.

Efforts to understand the reactivity of ferryl myoglobin were undertaken by Wilson and coworkers.$^{18,23,24}$ Ferryl myoglobin and hemoglobin are both stable at high pH, but their stability drops off as the pH decreases. A report on Mb-II utilized stopped-flow spectrophotometry to monitor changes in the UV/visible spectrum of Mb-II from pH 8 to pH 2.$^{18}$ The spectra were pH-dependent with a pKa ~5. Interestingly, this was consistent with the pH-dependent reactivity of Mb-II in oxidative reactions.$^{18}$ Because pH plays a role in determining the reactivity of Mb-II, it was believed that protonation of the ferryl group occurred below pH 5. The pH-dependence of Mb-II could be pathophysiologically significant because environments such as the kidneys and cerebral spinal fluid are acidic.$^{23,24}$ The authors suggest that Nature has chosen to use ferryl protonation to gate the reactivity of ferryl species.
Revisiting the Mb-II Crystal Structure

Hersleth et al. have recently revisited the issue of the protonation state of ferryl myoglobin.\textsuperscript{25} In this most recent analysis, a pH study of the crystal structures, single crystal microspectrophotometry spectra during data collection, resonance Raman, and Mössbauer data were provided. In this report, the crystal structures of Mb-II were solved at pH 5.2, 6.8 and 8.7. As was the case in the original paper,\textsuperscript{3} the Fe-O bond lengths from the respective crystal structures were too long to be labeled authentic Fe\textsuperscript{IV}oxo species (1.86 – 1.90 Å). Interestingly, there was no pH trend for the length of the Fe-O bond in these crystal structures. The Fe-N\textsubscript{His} bond length did change slightly with pH. This bond was elongated 0.1 Å as the pH increased from 5.2 to 8.7.

For quality control purposes, the absorption spectrum of the crystals was monitored during data collection (typically 4-6hrs) to determine if any major changes were observable. Immediate changes in the UV/vis spectrum were detected. Mb-II has significant Q-bands at 542, 580, and 595 nm. Upon irradiation with the X-ray beam, the peaks at 580 and 595nm decreased and a new peak at 567nm grew in. The authors found that if the crystal was annealed for 1s at room temperature, the Mb-II spectrum was regenerated. It appears as though the formation of the photoreduced product is reversible, yet does not resemble any known Mb complex.

Spectroscopic characterization of Mb-II by resonance Raman and Mössbauer were also performed. Resonance Raman spectra of Mb-II at pH 6.8 were collected in both solution and the crystal form from 600-1600 cm\textsuperscript{-1} using a 413nm excitation wavelength. A $\nu_4$ stretch at 1375 cm\textsuperscript{-1} was present in the resonance Raman spectra in Mb-II in the
solution and crystal forms. When samples were generated using D$_2$O, there were no changes in the spectra greater than 1 cm$^{-1}$ (Note: 1 cm$^{-1}$ was smaller than the resolution of the monochromator). Also, though the data was not shown, a peak at 690 cm$^{-1}$ was identified as the Fe-O stretch for ferryl Mb because this peak shifted with $^{18}$O-hydrogen peroxide. The 690 cm$^{-1}$ peak was believed to be at a reasonable energy for the Fe(IV)-OH stretch because it was between that of a Fe$^{III}$-OH and an Fe$^{IV}$oxo. The authors, however, question their assignment of the 690 cm$^{-1}$ peak because the $^{18}$O-shift was smaller than expected and there was no deuterium-related shift for this peak.

Mössbauer measurements were performed on $^{57}$Mb-II at pH 5.2 and pH 6.8 at 77K. The parameters for the pH 5.2 intermediate were $\Delta E_Q = 1.46$ mm/s and $\delta = 0.07$ mm/s. Mb-II at pH 6.8 showed the existence of two quadrupole doublets in an 89:11 ratio. The majority species had a $\Delta E_Q = 1.48$ mm/s and $\delta = 0.07$ mm/s and the minority species had a $\Delta E_Q = 1.96$ mm/s and $\delta = 0.42$ mm/s. The Mössbauer parameters at pH 5.2 and for majority species at pH 6.8 agreed with each other and with previous measurements on ferryl Mb ($\Delta E_Q = 1.44$ mm/s and $\delta = 0.09$ mm/s).$^{26,27}$ The 11% species was assigned as unreacted high spin ferric Mb (previously reported parameters for ferric Mb at 77K were $\Delta E_Q = 1.24$ mm/s and $\delta = 0.42$ mm/s$^{28}$). The Mössbauer results from this paper will be discussed below.

*Determining Ferryl Protonation States with Resonance Raman and Mössbauer Spectroscopies.*
It has recently been shown that resonance Raman spectroscopy (via the application of Badger’s rule) can provide accurate Fe-O bond distances for oxo and hydroxo heme complexes.\(^\text{12}\) Using Badger’s rule parameterized for Fe-O bond distances and stretching frequencies, we can extrapolate Fe-O bond distances for compound II intermediates from resonance Raman stretching frequencies. The application of Badger’s rule to the \(v_{\text{FeO}} = 797\ \text{cm}^{-1}\) (pH 8.6) observed in sperm whale Mb-II provides an Fe-O bond length of 1.65 Å, which is in good agreement with previous EXAFS experiments, 1.69 Å.\(^\text{11}\)

Mössbauer spectroscopy is particularly suited for determining the composition and oxidation state of iron containing samples, but it has not generally allowed for structural characterization of the iron center. An important aspect of Mössbauer spectroscopy, as it relates to our discussion of ferryl protonation, is that it does not suffer from photoreduction like EXAFS and X-ray crystallography. Recently it was shown that density functional methods are capable of determining Mössbauer parameters with good accuracy.\(^\text{29-31}\) In general, quadrupole splittings, \(\Delta E_Q\), can be obtained to within 0.3 mm/s, although errors on the order of 0.3-0.5 mm/s are not uncommon. Isomer shifts, \(\delta\), can be predicted to within 0.1 mm/s. The accuracy with which these parameters can be determined suggests that theoretical methods can be used to obtain structural information from Mössbauer experiments. Our calculations suggest that protonation of the ferryl unit should significantly affect the Mössbauer parameters of Mb-II.

Though resonance Raman, Mössbauer, and NMR\(^\text{32}\) data corroborate the existence of an Fe(IV)oxo species for compound II in the four histidine-ligated heme proteins we
have discussed, it is clear that further verification is necessary. The combination of spectroscopic and computational methods provides a straightforward means to assign the protonation state of ferryl myoglobin. Due to the large changes expected in both Mössbauer ($\Delta E_Q \approx 2.75$ mm/s) and resonance Raman spectra ($\nu_{\text{Fe-O}} \approx 550$ cm$^{-1}$) with ferryl protonation, we believe that the data reported in this chapter provide indisputable evidence for the protonation state of Mb-II.

Using density functional calculations and Mössbauer and resonance Raman spectroscopies we have determined that ferryl myoglobin is not protonated at any pH thus far examined. Our measurements set an upper limit of 3.5 on the ferryl pKa of Mb-II. The importance of this study is twofold: 1) measurements to determine the protonation state of Mb-II were performed over a large pH range and 2) both the Mössbauer and resonance Raman measurements were performed on identical samples at pH 8.5 and 5.2. The second point is particularly important because it allows us to rule out the existence of any “hidden” species and/or oxidation states in these spectroscopies.

2.3 Computational Procedures

In the myoglobin DFT calculations either an imidazole ligand was used (computationally derived model), or the model was taken from the Mb-II crystal structure. In the model taken from the crystal structure, the axial histidine residue was truncated at the C$\alpha$ position and replaced with a methyl group. Mössbauer parameters were determined at optimized geometries.$^{33}$ During optimizations, the positions of all atoms were constrained to their position in the ferric crystal structure except Fe, the distal
ligand, the porphyrin-nitrogens, alpha-carbons, meso-carbons, meso-hydrogens, and the proximal histidine. Geometry optimizations were performed at the B3LYP/6-311G level. Quadrupole splittings were also determined at the B3LYP/6-311G level. Isomer shifts were determined 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.

2.4 Results and Discussion

Resonance Raman

Previous resonance Raman investigations of Mb-II were performed with sperm whale myoglobin at pH 8.6. These experiments obtained an Fe-O stretching frequency of 797 cm\(^{-1}\), which shifted to 771 cm\(^{-1}\) with \(^{18}\)O substitution. The oxidation state marker band (\(\nu_4\)) for Mb-II was found at 1380 cm\(^{-1}\), which is indicative of an iron(IV) center.

We have chosen horse heart myoglobin for our investigation of ferryl Mb as this was the enzyme used for the X-ray crystal structures that reported the long Fe-O bonds. We recorded resonance Raman spectra on horse heart Mb-II from pH 4.5 to pH 8.5. High frequency data are shown in Figure 2.1. The position of the oxidation state marker band was independent of pH. It was located at \(\nu_4 = 1379\) cm\(^{-1}\), consistent with the assignment of Mb-II as an iron(IV) species over the entire pH range.
Low frequency data were collected at 501.7 nm for Mb-II at pH 7.0 and 8.5. These spectra are shown in figure 2.2. The spectra at these two pHs are identical. Spectrum A reveals a band at $\nu_{\text{FeO}} = 804$ cm$^{-1}$ that shifted to 769 cm$^{-1}$ (spectrum B) upon $^{18}$O substitution. This 35 cm$^{-1}$ shift is almost identical to the calculated Fe-O diatomic harmonic oscillator, 36 cm$^{-1}$. This shift is highlighted in the difference spectrum, C. The application of Badger’s rule to the $\nu_{\text{FeO}} = 804$ cm$^{-1}$ stretching frequency yields a 1.65 Å Fe-O bond distance. This bond length is in good agreement with the results of EXAFS measurements on horse heart Mb-II (pH not given).
Interestingly, spectra recorded at pH 5.0 and 6.0 were different from those at higher pH. The pH 5.0 data (using a 501.7 nm excitation wavelength) is shown in figure 2.3. The pH 8.5 spectra have been included in this figure for illustrative purposes. In addition to the 804 cm\(^{-1}\) stretch, these spectra exhibited a new stretch at 790 cm\(^{-1}\). This stretching frequency is within the range for an authentic Fe(IV)oxo Fe-O stretch. Upon \(^{18}\)O substitution, both peaks shifted. The 804 cm\(^{-1}\) peak shifted to 769 cm\(^{-1}\) and the 790 cm\(^{-1}\) peak shifted to 754 cm\(^{-1}\). These isotopic shifts (35 cm\(^{-1}\) and 36 cm\(^{-1}\), respectively) are in excellent agreement with the predicted shifts.
The resonance Raman spectrum at pH 4.5, however, only exhibited an Fe-O stretch at 790 cm\(^{-1}\), figure 2.4. The pH 4.5 data was collected using a 431nm excitation wavelength because the Fe-O stretch was better enhanced using Soret excitation compared to the 501nm excitation. As with the pH 5.0 and 6.0 samples, the 790 cm\(^{-1}\) peak shifted to 754 cm\(^{-1}\) with \(^{18}\)O substitution. No other peaks were sensitive to \(^{18}\)O substitution from 400 to 1100 cm\(^{-1}\). Our resonance Raman results indicate that the \(\nu_{\text{FeO}}\) in Mb-II is pH-dependent with a pKa \(\approx 5.5\).

We have also performed resonance Raman experiments at pD 4.5, 5.0, and 8.5. There were no observable changes in the spectra with deuterium substitution from 400-900 cm\(^{-1}\) (data not shown). This observation agrees with the resonance Raman results reported by Hersleth et al.
Distal or Proximal Protonation Event?

A magnetic circular dichroism (MCD) study published by Foote et al. observed a pH-dependence for the spectroscopic parameters of ferryl myoglobin as well. These studies, performed at pH 3.5 and 8.5, determined that the Mb-II had a triplet (S = 1) ground state at both pHs, but the zero-field splitting parameters, \( D \), were slightly different. The pH-dependence of the MCD data has frequently been used to argue that the low-pH species from the Mb-II crystal structure was a protonated ferryl intermediate. The authors of this MCD study, however, attribute the difference in \( D \) values to a trans-effect caused by the deprotonation of the axial histidine at high pH. Though the pKa of the axial histidine in ferryl myoglobin has not been measured, the authors believe that the pKa is close to 6. It should be stressed that the pH-dependent

Figure 2.4: Resonance Raman spectrum of Mb-II at pH 4.5 using a 431 nm excitation wavelength. In this spectrum A) is the \( ^{16}\)O spectrum, B) is the \( ^{18}\)O spectrum, and C) is the \( ^{18}\)O-\( ^{16}\)O difference spectrum.
shift of the Fe-O stretch (14 cm\(^{-1}\)) is not large enough to be attributed to ferryl protonation, but would be consistent with a trans effect of < 0.01 Å (Badger’s rule).

We believed that there were two potential explanations for the pH-dependent shift in ν\(_{\text{Fe-O}}\). One is the mechanism described above in which the deprotonation of the axial histidine at high pH invokes a trans-influence, weakening the Fe-O bond. To test this effect, we performed DFT calculations on the imidazole and imidazolate models of Mb. A trans influence was observed in these calculations, figure 2.5. As a result of the negatively charged imidazolate, the Fe-N\(_{\text{His}}\) bond was shortened by 0.11 Å and the Fe-O bond was lengthened by 0.025 Å. These calculations effectively rule out this mechanism since the DFT calculations indicate that the Fe-O stretching frequency should increase with decreasing pH, which is opposite to what was seen experimentally.

The second mechanism involves the pKa of the distal histidine. pH-dependent shifts in ν\(_{\text{FeO}}\) have previously been observed in HRP-II and CAT-II. Both proteins exhibit an 11 cm\(^{-1}\) red-shift (pKa ≈ 8) with decreasing pH, figure 2.6. The decrease in stretching frequency has been attributed to the formation of a hydrogen bond between the ferryl oxygen and a distal histidine residue.\(^{40,41}\) A similar phenomenon could also be at work in Mb-II, for the pKa of the distal histidine in Mb has been reported to range from 4 to 6.\(^{42-44}\)
Regardless of the origin of the frequency shift in $\nu_{FeO}$, it is clear that it is not attributable to a ferryl protonation event. Only two isotope-sensitive stretches were observed for Mb-II from pH 4.5 to pH 8.5. According to Badger’s rule, the 1.92 Å Fe-O bond reported in the Mb-II crystal structure would have $\nu_{FeO} \approx 473$ cm$^{-1}$. For illustrative
purposes we examined the Fe-O stretch in alkaline myoglobin. The Fe-O stretch in alkaline myoglobin was located at $v_{\text{Fe-O}} = 557$ cm$^{-1}$ (shifting to 544 cm$^{-1}$ with deuterium substitution, Figure 2.7). The Mb-OH stretch was also reported by Feis et al.$^{45}$ Similarly, they observed an Fe-O stretch at 556 cm$^{-1}$ that shifted to 543 cm$^{-1}$ in D$_2$O. Badger’s rule equates this Fe-O stretching frequency to a 1.83 Å Fe-O bond. This distance is in very good agreement with XAS measurements on alkaline myoglobin (1.84 Å)$^{46}$ and DFT calculations on an imidazolate-ligated ferric hydroxide heme (1.85 Å)$^{12}$.

![Figure 2.7: Resonance Raman spectrum of Mb$^{III}$-OH (431 nm excitation). Top spectrum is of Mb-OH and bottom is Mb-OD.](image)

**Mössbauer Calculations.**

We also sought to determine the protonation state of Mb-II using Mössbauer spectroscopy coupled with DFT calculations, figure 2.8. The accuracy of these calculations was determined by comparing the theoretical Mössbauer parameters to the experimental parameters for the ferric and ferrous (5-coordinate) forms of myoglobin, as well as the following Mb complexes: Fe$^{II}$CO, Fe$^{II}$O$_2$, Fe$^{III}$CN, Fe$^{III}$N$_3$. Two active-site
models were chosen for this investigation. The first model was an imidazole-ligated porphine (47 atoms). All atom geometry optimizations were performed for this model. The second model was taken from the Mb-II crystal structure. It contained a porphine unit, distal ligand, and proximal histidine (truncated as imidazole-CH\textsubscript{2}CH\textsubscript{3}). During geometry optimizations of the second model, 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 (imidazole-CH\textsubscript{2}CH\textsubscript{3}) were constrained to their position in the crystal structure.

Figure 2.8: Models used for DFT calculations on myoglobin complexes. The model on the left is a generic porphine with a imidazole ligated to the heme iron and on the right is the structure taken from the myoglobin crystal structure.

The results of these calculations are found in Table 2.1. In contrast to our investigation of chloroperoxidase compound II, we find no significant difference in the accuracy of the results obtained with either the full- or constrained-optimization model. In both treatments the calculated Mössbauer parameters were in reasonable agreement with experiment. The magnitude and sign of $\delta$ and $\Delta E_Q$ were reliably predicted,
and differences between theory and experiment were within previously reported accuracy limits, 0.1 mm/s for $\delta$ and 0.3-0.6 mm/s for $\Delta E_Q$.$^{29-31}$

<table>
<thead>
<tr>
<th>Distal Ligand</th>
<th>Oxidation State</th>
<th>Spin State</th>
<th>Optimized Porphine [mm/s]</th>
<th>Constrained Optimization [mm/s]</th>
<th>Experiment [mm/s]</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO</td>
<td>II</td>
<td>0</td>
<td>0.31</td>
<td>0.35</td>
<td>0.27</td>
<td>0.35</td>
</tr>
<tr>
<td>$O_2$</td>
<td>II</td>
<td>0</td>
<td>0.29</td>
<td>-1.94</td>
<td>0.27</td>
<td>-2.31</td>
</tr>
<tr>
<td>none</td>
<td>II</td>
<td>2</td>
<td>0.81</td>
<td>-2.76</td>
<td>0.92</td>
<td>-2.22</td>
</tr>
<tr>
<td>CN$^-$</td>
<td>III</td>
<td>—</td>
<td>0.23</td>
<td>-2.11</td>
<td>0.16</td>
<td>-1.46</td>
</tr>
<tr>
<td>$N_3^-$</td>
<td>III</td>
<td>—</td>
<td>0.31</td>
<td>-2.07</td>
<td>0.24</td>
<td>-2.25</td>
</tr>
<tr>
<td>OH$^-$</td>
<td>IV</td>
<td>1</td>
<td>-0.01</td>
<td>2.78</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>$O_2^-$</td>
<td>IV</td>
<td>1</td>
<td>0.10</td>
<td>0.98</td>
<td>0.08</td>
<td>1.02</td>
</tr>
</tbody>
</table>

Once we had demonstrated that our DFT methods (B3LYP/6-311G) were sufficient to provide reliable Mössbauer parameters, it was possible to assess the effect of ferryl protonation on $\delta$ and $\Delta E_Q$ in a histidine-ligated heme model. The parameters calculated for the ferryl form of myoglobin are in reasonable agreement with experiment (Table 2.1).$^{26, 27}$ Calculations revealed that the $^{57}$Fe quadrupole splitting increases dramatically upon protonation of the ferryl moiety. The constrained optimizations provided $\Delta E_Q = 2.73$ mm/s for the Fe$^{IV}$OH versus 1.02 mm/s for the Fe$^{IV}$oxo. This difference is sufficiently larger than the average error associated with theoretically determined $\Delta E_Q$, which indicates that it is possible to use Mössbauer spectroscopy to determine ferryl protonation states.

The Mössbauer calculations presented in this chapter are also in agreement with those calculated by Shaik and coworkers on HRP-II.$^{38}$ Using a QM/MM model that
included several distal amino acid residues deemed to be important to peroxidase chemistry. They found a significant increase in $\Delta E_Q$ with protonation of the Fe(IV)oxo, from 1.72 mm/s to 2.43 mm/s, with little effect on the isomer shift (0.07 mm/s and 0.064 mm/s, respectively) in their calculations.

**Mössbauer Spectroscopy.**

To determine the protonation state of Mb-II, Mössbauer experiments were performed at pH 8.5, 5.2, 4.5, and 3.5. If the Mb-II crystal structure is correct, a dramatic increase in $\Delta E_Q$ as the pH is decreased to pH 5.2 should be observed.

At pH 8.5, the measured Mössbauer parameters for Mb-II were $\delta = 0.09$ mm/s and $\Delta E_Q = 1.43$ mm/s (Figure 2.9). An isomer shift of 0.09 mm/s was indicative of an Fe$^{IV}$ center and both the isomer shift and the quadrupole splitting of Mb-II at pH 8.5 agreed with previous Mössbauer studies of Mb-II at pH 8.7 and pH 7.0. These parameters were also similar to those of several other compound II intermediates in heme proteins. To ensure that we were examining the same intermediate in both the resonance Raman and Mössbauer experiments, we prepared Mb-II in a volume sufficient for both spectroscopies. The $^{16}$O resonance Raman spectrum for $^{57}$Fe-Mb-II at pH 8.5 was identical to the $^{56}$Fe-Mb-II spectrum with a $\nu_{FeO}$ of 804 cm$^{-1}$, figure 2.10.
Figure 2.9 also shows the Mössbauer spectrum of $^{57}\text{Fe}$-enriched Mb-II at pH 5.2 (the pH of the Mb-II crystal structure). The Mössbauer parameters for Mb-II at this pH were $\delta = 0.08$ mm/s and $\Delta E_Q = 1.53$ mm/s. Also included with the Mössbauer data is the simulated spectrum of an imidazole-ligated protonated ferryl heme ($\delta = -0.02$ mm/s and $\Delta E_Q = 2.73$ mm/s). The lack of a significant change in the Mössbauer parameters between pH 8.5 and 5.2 indicated that Mb-II was not protonated at pH 5.2. This hypothesis was also confirmed by the $^{16}\text{O}$ resonance Raman spectrum, figure 2.10. There are two Fe-O stretches present at this pH, one at 804 cm$^{-1}$, identical to the pH 8.5 Fe-O stretch, and one at 790 cm$^{-1}$ in agreement with the previously discussed $^{16}\text{O}$-$^{18}\text{O}$ measurements.
The lowest pH where myoglobin is reasonably stable is pH 4.5; therefore, we prepared Mössbauer samples of Mb-II at this pH. A UV/vis spectrum from the same reaction mixture was recorded after the Mössbauer sample was frozen to verify the formation Mb-II. Mb-II at pH 4.5, spectrum shown in figure 2.11, had Mössbauer parameters that were identical to the parameters at pH 5.2 (δ = 0.08 mm/s and ΔE_Q = 1.53 mm/s). In this spectrum, the hash marks correspond to the experimental data and the blue line to the fit. The quadrupole doublet shown in red once again corresponds to the DFT-determined Mössbauer parameters for a protonated ferryl in an imidazole-ligated heme.

Figure 2.10: Mb-II generated for Mössbauer (top) and 16O resonance Raman (bottom) spectroscopies. These samples were generated to confirm the individual experimental results that were reported at these pHs. In the Mössbauer spectra, the black hash marks represent the raw data and the red line is the fit for the data. The extra species in the pH 5.2 (black solid line) is an oxyferrous Mb contamination.51
The differences between these spectra clearly indicates that Mb-II at pH 5.2 is not a protonated ferryl species.

Figure 2.11: Mössbauer spectrum of Mb-II at pH 4.5. Raw data is represented by the black hash marks, the blue line is the fit of the experimental data, and the red line is the theoretical spectrum from DFT calculations on a protonated ferryl in Mb.

Because myoglobin is not stable below pH 4.5, we used the pH-jump method to circumvent the stability issues to obtain a Mössbauer sample for Mb-II at pH 3.5. This method could not be used for resonance Raman because we observed significant fluorescence background in these samples. The Mössbauer spectrum for Mb-II at this pH is shown in Figure 2.12. For this experiment, Mb-II was first generated at pH 5.2, where it is relatively stable, and then mixed with low pH buffer. The final pH of the solution was 3.5. The hash marks represent the experimental data and the blue solid line is the experimental data at pH 5.2. The quadrupole doublet of Mb-II at pH 5.2 matches the quadrupole doublet of Mb-II at pH 3.5 exactly (\( \delta = 0.08 \text{ mm/s} \) and \( \Delta E_Q = 1.53 \text{ mm/s} \)). Again, we have included a simulated spectrum of the calculated parameters for a
protonated ferryl in myoglobin (red line). The other species in the raw spectrum have yet to be identified; however, they are most likely due to the degradation of myoglobin known to occur around pH 3.5. The small quadrupole splitting obtained from our Mössbauer measurements at low pH indicate that the pKa of ferryl Mb must be less than 3.5.

Figure 2.12: Mössbauer spectrum of Mb-II at pH 3.5. Raw data is shown in the black hash marks, the blue line is the experimental data from pH 5.2, and the red line is the theoretical spectrum from DFT calculations on a protonated ferryl in Mb.

2.5 Conclusion

In this chapter we have investigated the ferryl intermediate in horse heart myoglobin using density functional theory and resonance Raman and Mössbauer spectroscopies. Our investigations reveal that the ferryl form of Mb is not protonated at
any pH thus far examined, pKa < 3.5. Mössbauer measurements at pH 5.2 provide $\delta = 0.08$ mm/s and $\Delta E_Q = 1.53$ mm/s, both of which are in good agreement with those previously reported for 6-coordinate ferryl porphyrins.\textsuperscript{47} According to DFT calculations, imidazole-ligated Fe(IV)OH porphyrins are expected to display much larger quadrupole splittings than those observed to date.

Although resonance Raman measurements revealed that the ferryl stretching frequency was pH-dependent, shifting from 804 cm$^{-1}$ to 790 cm$^{-1}$ with decreasing pH (pKa $\approx 5.5$), we stress that the pH dependence of $\nu_{FeO}$ in Mb-II is not related to the pKa of the ferryl moiety. The application of Badger’s rule to the observed $^{16}$O ferryl stretching frequencies provides Fe-O bond distances of 1.647 and 1.654 Å. These stretching frequencies and bond distances are representative of authentic iron(IV)-oxos, not iron(IV)-hydroxides. If this were a protonation event we would expect a shift in $\nu_{FeO}$ on the order of 150 cm$^{-1}$ ($\nu_{FeO} \approx 631$ cm$^{-1}$ using an Fe-O bond length of 1.76 Å from the geometry optimized Fe$^{IV}$OH structure).\textsuperscript{12}

In light of our experimental results, we can also rule out the existence of an Fe(III)-porphyrin radical species as was postulated by Shaik and coworkers.\textsuperscript{38} The conclusion from their work was that because previous measurements on HRP-II resulted in long Fe-O bonds matching the Fe-O bond lengths from their calculations and Fe(III)-porphyrin radical species were observed in model complexes, the hydroxide species (Fe(IV) or Fe(III)-porphyrin radical) had been missed by Mössbauer spectroscopy. Prior to our investigation of Mb-II at low pH, this could have indeed been the case. The formation of the hydroxide species was thought to require acidic pH and, because
previous Mössbauer studies on compound II in histidine-ligated heme proteins were performed near pH 7, the possibility of a protonated species could not be ruled out.

We have reported the first Mössbauer study of compound II in a histidine-ligated heme protein at low pH. Not only did we observe the formation of solely Fe(IV)oxo species, but the sensitivity of Mössbauer spectroscopy to the oxidation state of an iron center allows us to rule out the formation of an Fe(III)-porphyrin radical species. Also, because these Mössbauer measurements were performed on the same samples as were the $^{16}$O resonance Raman measurements (pH 5.2 and 8.5, figure 2.10), there is no doubt that Mb-II is an Fe(IV)oxo between pH 3.5 and 8.5.

2.6 References


13. Derat, E.; Kumar, D.; Hirao, H.; Shaik, S., Gauging the relative oxidative powers of compound I, ferric-hydroperoxide, and the ferric-hydrogen peroxide species of


Chapter 3

Evidence for Basic Ferryls in Cytochrome P450s


3.1 Abstract

We have used Mössbauer spectroscopy and density functional calculations to prove the existence of protonated ferryl intermediates in three Cytochrome P450s. Using density functional theory we have shown that there is a correlation between Mössbauer parameters and ferryl protonation states in heme complexes. This trend is independent of the axial ligand. These calculations determined that the quadrupole splitting parameter, $\Delta E_Q$, is significantly larger for protonated ferryl species as compared to the identical unprotonated ferryl model. In Chapter 2 we showed how this trend was used to determine the protonation state of compound II in myoglobin. In the present chapter we will use the same theoretical and experimental techniques on ferryl intermediates in P450$_{BM3}$, P450cam, and CYP158A1. The experimentally determined quadrupole splittings for these intermediates were 2.16, 2.06, and 2.13 mm/s, respectively, which is in good agreement with the values calculated for protonated ferryl species in the respective enzyme models. These results suggest that basic ferryls are a general feature of thiolate-ligated hemes.
3.2 Introduction

P450s are found throughout nature. So far, 57 active P450 genes and 58 pseudogenes have been identified in the human genome. The majority of human P450s are located in the liver, which is the organ responsible for filtering the blood of exogenous compounds (among many other functions). Several P450s have also been found in the respiratory and gastrointestinal tracts. P450s are involved in the catabolism of natural products and the synthesis of steroids, drug metabolism (examples are CYP1A2, CYP2C9, CYP2C19, 2D6, and 3A4), and the metabolism of most toxins and carcinogen precursors. Since these enzymes have proven to be critical to a variety of different processes across biology, understanding the mechanistic details of P450 reactions is important.

The active oxidant in P450 chemistry is believed to be an intermediate called compound I (P450-I). Presently, there is no definitive structural or magnetic data on P450 compound I. Through analogy to the reactive intermediates observed in peroxidases, like HRP, compound I is described as an Fe(IV)oxo (ferryl) intermediate with a porphyrin cation radical. Experiments on chloroperoxidase (CPO) compound I, a thiolate-ligated heme protein capable of performing P450-like reactions, and DFT calculations on thiolate-ligated heme models have suggested that some of the spin density from the porphyrin radical may be delocalized over the axial thiolate ligand in these enzymes. Three stopped-flow spectrophotometry investigations have reported the build up of small amounts of P450-I in P450cam (peracetic acid and mCPBA) and CYP119 (mCPBA). P450-I formation was confirmed through spectral similarities to CPO-I.
blue-shifted Soret and a peak at ~690nm. Efforts to generate P450-I at concentrations sufficient for other spectroscopies, like EPR and Mössbauer, have not been as successful. Schünemann and coworkers attempted to generate P450-I in P450cam, P450_{BM3}, and the inducible and neuronal nitric oxide synthases (iNOS and nNOS).\textsuperscript{11-15} The reaction of P450cam and P450_{BM3} with peracetic acid (PA) resulted in the formation of high-valent radical species. iNOS and nNOS also formed radical intermediates; however, the Mössbauer parameters for nNOS (none were reported for iNOS) more closely resembled those of a P450 oxyferrous complex.\textsuperscript{16} These radical species were subsequently examined by high-frequency EPR.\textsuperscript{13, 14} The $g = 2$ EPR signal generated was attributed to either tyrosine or tryptophan radicals. An examination of the crystal structures for these proteins confirmed the location of tyrosine and tryptophan residues close to the active site. These EPR results led the authors to propose that the prototypical compound I intermediate was initially formed in these enzymes; however, with no substrate present, nearby amino acids were rapidly oxidized instead.

Though compound I was not observed in any of these enzymes by Mössbauer or EPR spectroscopies, this was the first time a high-valent intermediate was trapped in a P450. The Mössbauer parameters for the PA intermediates in P450cam (13\% yield) were $\Delta E_Q = 1.94 \pm 0.01$ mm/s and $\delta = 0.13 \pm 0.01$ mm/s and for P450_{BM3} (12\% yield) $\Delta E_Q = 1.94 \pm 0.03$ mm/s and $\delta = 0.13 \pm 0.03$ mm/s.\textsuperscript{12, 14, 15} These isomer shifts are indicative of an Fe(IV) oxidation state ($\delta \sim 0.1$ mm/s). Even more interesting, however, the parameters for P450cam and P450_{BM3} matched the Mössbauer parameters for CPO compound II quite well ($\Delta E_Q = 2.06$ mm/s and $\delta = 0.10$ mm/s).\textsuperscript{17}
Green et al. performed the first X-ray absorption spectroscopy measurements on CPO-II.\(^\text{18}\) The authors expected bond lengths typical of other heme ferryl intermediates like HRP-II and Mb-II.\(^\text{19-21}\) The Fe-O bond length for CPO-II at pH 6.5 was 1.82 Å, which is over 0.1 Å longer than any other previously characterized ferryl intermediate in an enzyme or model compound (~1.65 Å).\(^\text{20}\) This long Fe-O bond was attributed to protonation of the ferryl unit in CPO-II. The protonation state of CPO-II was also supported by DFT calculations on a thiolate-ligated protonated ferryl model; the calculated Fe-O bond length was 1.81 Å.

This result was linked to P450 chemistry. The structure of CPO-II was similar to the proposed “rebound intermediate” in the P450 hydroxylation mechanism. The consensus mechanism for P450 hydroxylation reactions is known as the rebound mechanism.\(^\text{22}\) In the rebound mechanism, figure 3.1, compound I abstracts hydrogen from a hydrocarbon substrate, which yields a substrate radical and a neutral Fe(IV)OH intermediate. The substrate radical then recombines with the OH radical from the “rebound intermediate”, followed by the release of hydroxylated product and the return of the enzyme to the resting state. This mechanism has been supported by work from Groves and coworkers in both model systems and P450 enzymes. Subsequently, several other groups have confirmed the feasibility of the rebound mechanism in P450 chemistry.\(^\text{23-27}\)
Experimental evidence has suggested that the ability of metal oxo complexes to abstract hydrogen from a substrate scales with the strength of the O-H bond formed during this step.\textsuperscript{25-30} If we relate Mayer’s work on hydrogen atom abstraction to the intermediates in heme enzymes, the strength of the O-H bond formed is determined by the one electron reduction potential of compound I and the pKa of compound II.

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

Equation 3.1 highlights the importance of the ferryl pKa and suggests that this property can play a role in P450 oxidation reactions, namely in promoting H-atom abstraction at biologically viable redox potentials. This hypothesis rests on two assumptions: that the rebound mechanism is operative in P450 hydroxylations and that basic ferryls are a general and unique feature of thiolate-ligated hemes. The first assumption has been supported by several experimental and computational investigations. The second assumption also appears to be reasonable since, to date, thiolate-ligated heme enzymes are the only heme enzymes capable of H-atom abstraction. Thus, it follows that basic ferryls should only be an attribute of these enzymes.
It is apparent that protonated ferryls could be important to heme enzyme chemistry. Confirmation of the basicity of CPO-II was necessary because X-rays are known to generate potent reducing equivalents, which could be problematic when studying high-valent intermediates. To ensure that CPO-II had not been reduced to a ferric or ferrous hydroxide a new spectroscopic tool was necessary that would not suffer from photodamage. Recently, our lab has employed the use of Mössbauer spectroscopy and DFT calculations to determine ferryl protonation states. Initially, DFT calculations on a thiolate-ligated porphine model were performed. These calculations determined that a protonated ferryl should have an enlarged quadrupole splitting compared to a ferryl species, $\Delta E_Q = 1.84$ mm/s (protonated) versus $\Delta E_Q = 0.63$ mm/s (unprotonated). When the axial helix was included in these calculations (structure taken from X-ray structure of CPO), the parameters were $\Delta E_Q = 2.05$ mm/s and $\Delta E_Q = 1.00$ mm/s.

Mössbauer measurements on CPO-II confirmed the results of the calculations. The Mössbauer spectrum of CPO-II showed the presence of two distinct ferryl species in a 70:30 ratio. The major component had an enlarged quadrupole splitting and a characteristic Fe(IV) isomer shift ($\Delta E_Q = 2.06 \pm 0.03$ mm/s and $\delta = 0.10 \pm 0.03$ mm/s). The calculated Mössbauer parameters, and the results of the EXAFS investigation, led to the assignment of the majority species in CPO-II as an Fe$^{IV}$OH intermediate. This CPO-II result also supported the notion that DFT can be used in combination with Mössbauer spectroscopy to obtain structural information about a species, in this case the ferryl protonation state.
Though the basicity of CPO-II supports the concept that the ferryl pKa is important to P450 hydroxylation chemistry, CPO is not a P450. For instance, CPO cannot hydroxylate unactivated C-H bonds\textsuperscript{34, 35} and P450s cannot chlorinate substrates.\textsuperscript{3} Because there are slight differences in the oxidative power of these two enzymes, there may also be a difference between the reactive intermediates in CPO and P450. This prompted our investigation of compound II in P450s. The results from Schünemann and coworkers on the P450cam-PA reaction served as a starting point for studying this intermediate in P450s. The intermediate that was trapped during these experiments had a $\Delta E_Q = 1.94$ mm/s and $\delta = 0.13$ mm/s.$^{12}$

In this chapter we present the results of experiments on the ferryl forms of P450\textsubscript{BM3}, P450cam, and CYP158A1. We determined that all three ferryl intermediates have Mössbauer parameters typical of protonated ferryl hemes. Our results suggest that basic ferryls are a natural consequence of thiolate-ligation.

### 3.3 Computational Methods

Theoretical Mössbauer parameters were determined by performing DFT calculations on large active-site models of the protonated and unprotonated ferryl forms of P450cam, P450\textsubscript{BM3}, and CYP158A1. In each case the starting structures were taken from available crystal structures of ferric P450\textsubscript{BM3},\textsuperscript{36} P450cam,\textsuperscript{37} and CYP158A1 (PDB accession code 2DKK). The models employed contained a porphine, the appropriate distal ligand, and a portion of the proximal helix (86, 80, and 80 atoms, respectively, for the ferryl species). Inclusion of the proximal helix allowed for the consideration of
important hydrogen-bonding between the helix and the axial-thiolate, while the geometry constraints allowed us to examine structures that more closely resembled those found in the enzyme. The helix in the P450_{BM3} model contained residues Cys400-Gln403, the P450cam model contained Cys357-Gln360, and the CYP158 model contained Cys356-Ala359. In each model all residues except Cys and Gly were converted to Ala. The CYP158A1 model is shown in figure 3.2.

Figure 3.2: DFT model for a protonated ferryl intermediate in CYP158A1.

Mössbauer parameters were determined at optimized geometries. During optimizations, the positions of all atoms were constrained to their position in the ferric crystal structure except Fe, the distal ligand, the porphyrin-nitrogens, alpha-carbons, meso-carbons, meso-hydrogens, and the proximal SCH_{2}CH. Geometry optimizations were performed at the B3LYP/6-311G level. The iron-ligand bond distances and spin densities obtained for the optimized structures are listed in Table 3.1. Quadrupole splittings were determined at the B3LYP/6-311G level and isomer shifts were determined
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.  

Calculated Mössbauer parameters can be found in Table 3.2.

3.4 Results and Discussion

Mössbauer spectroscopy

We have used Mössbauer spectroscopy coupled with DFT calculations to determine the protonation state of the PA-generated intermediates in P450BM3, P450cam, and CYP158A1. The spin densities and Fe-ligand bond lengths are found in Table 3.1. The computationally determined Mössbauer parameters for the ferryl and protonated ferryl models are shown in Table 3.2. Like the histidine-ligated systems in Chapter 2, we observed a trend in the predicted quadrupole splitting parameters upon ferryl protonation.

Table 3.1: Spin densities and bond lengths for the P450 intermediates studied.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Distal Ligand</th>
<th>Oxidation/Spin State</th>
<th>Distances</th>
<th>Spin Densities</th>
</tr>
</thead>
<tbody>
<tr>
<td>P450BM3</td>
<td>OH−</td>
<td>IV (S = 1)</td>
<td>Fe-N_{avg} 2.01</td>
<td>Fe-S 2.39</td>
</tr>
<tr>
<td></td>
<td>O2−</td>
<td>IV (S = 1)</td>
<td>2.01 2.56 1.65</td>
<td>1.25 0.88</td>
</tr>
<tr>
<td>P450cam</td>
<td>OH−</td>
<td>IV (S = 1)</td>
<td>2.03 2.38 1.80</td>
<td>2.01 0.25</td>
</tr>
<tr>
<td></td>
<td>O2−</td>
<td>IV (S = 1)</td>
<td>2.03 2.51 1.66</td>
<td>1.23 0.90</td>
</tr>
<tr>
<td>CYP158A1</td>
<td>OH−</td>
<td>IV (S = 1)</td>
<td>2.03 2.37 1.80</td>
<td>1.98 0.25</td>
</tr>
<tr>
<td></td>
<td>O2−</td>
<td>IV (S = 1)</td>
<td>2.03 2.50 1.66</td>
<td>1.23 0.89</td>
</tr>
<tr>
<td>CPO</td>
<td>OH−</td>
<td>IV (S = 1)</td>
<td>2.02 2.38 1.81</td>
<td>1.95 0.21</td>
</tr>
<tr>
<td></td>
<td>O2−</td>
<td>IV (S = 1)</td>
<td>2.03 2.57 1.66</td>
<td>1.25 0.88</td>
</tr>
</tbody>
</table>
The 4.2K/54mT Mössbauer spectrum of the P450BM3 intermediate (5:1 PA to protein, pH 7, and 3.4ms reaction time) is shown in figure 3.3. There are three species in this spectrum. 44% of the spectrum was attributable to ferric P450BM3 and was subtracted from the raw data. The residual spectrum was fit with two symmetric quadrupole doublets. The largest component was present in 48% overall yield with ΔEQ = 2.16 mm/s and δ = 0.13 mm/s. The second component was a small oxyferrous contaminant in 8% yield (ΔEQ = 2.41 mm/s and δ = 0.33 mm/s). The oxyferrous contaminant was believed to be a consequence of the reaction of compound II with excess PA, as was observed in HRP-II in the presence of a large excess of hydrogen peroxide (compound III). We have found this contamination in varying yields in every P450-II sample that we have prepared.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Distal Ligand</th>
<th>Theory</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔEQ</td>
<td>δ</td>
<td>ΔEQ</td>
</tr>
<tr>
<td>P450BM3</td>
<td>OH⁻</td>
<td>2.17</td>
<td>0.09</td>
</tr>
<tr>
<td>P450cam</td>
<td>OH⁻</td>
<td>1.84</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>O²⁻</td>
<td>0.66</td>
<td>0.14</td>
</tr>
<tr>
<td>CYP158A1</td>
<td>OH⁻</td>
<td>1.87</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>O²⁻</td>
<td>0.66</td>
<td>0.13</td>
</tr>
<tr>
<td>CPO</td>
<td>OH⁻</td>
<td>2.06</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>O²⁻</td>
<td>1.00</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Table 3.2: P450 compound II Mössbauer parameters (mm/s).
The experimental parameters for P450\textsubscript{BM3}-II agreed very well with the calculated parameters for the P450\textsubscript{BM3}-OH model ($\Delta E_Q = 2.17$ mm/s and $\delta = 0.09$ mm/s). It was the agreement between the theoretical and experimental parameters that allowed us to confirm the formation compound II in P450\textsubscript{BM3} and that it was protonated at pH 7. P450\textsubscript{BM3}-II was further characterized using high field Mössbauer spectroscopy. The sample was analyzed in a 7T external magnetic field, which enabled us to determine the sign of the quadrupole splitting parameter and the spin state of the intermediate. The raw data is shown in figure 3.4, top. Removal of the ferric P450\textsubscript{BM3} component yielded the

![Mössbauer spectrum of P450\textsubscript{BM3} PA-generated intermediate (4.2K/54mT). Top spectrum, in purple, is the raw data for P450\textsubscript{BM3}-PA and in black is ferric P450\textsubscript{BM3} that was subtracted from the raw data (44%). The bottom spectrum is of the raw data after subtraction of the ferric P450\textsubscript{BM3} component. The black line is the fit for two quadrupole doublets with $\Delta E_Q = 2.16$ mm/s and $\delta = 0.13$ mm/s (48%) and $\Delta E_Q = 2.41$ mm/s and $\delta = 0.33$ mm/s (8%).]
bottom spectrum in figure 3.4. This spectrum was modeled using a spin Hamiltonian formalism in the slow relaxation limit. Because sufficient experimental data was not available to determine the spin Hamiltonian parameters, the following parameters typical of ferryl hemes were assumed: zero-field splitting, \( D = +23 \text{ cm}^{-1} \) and \( E/D = 0 \), \( g = (2.1, 2.1, 2.0) \), asymmetry parameter, \( \eta = 0 \), \( A/g_\text{N}^\beta_\text{N} = (-19, -19, -7) \) T. The Mössbauer parameters were taken from the 54 mT data. Simulations using the preceding parameters determined that the \( \Delta E_Q \) was positive and the P450_{BM3}-II intermediate had an \( S = 1 \) ground state, both of which agreed with the DFT calculations. \textit{Note}: The slight difference between the experimental data and the fit \(~0\) mm/s was likely due to the 8% oxyferrous contamination.

Figure 3.4: High-field Mössbauer spectrum of P450_{BM3}-PA at 7T and 4.2K. The top spectrum is the raw data and the bottom spectrum is the data after subtraction of 44% the 7T spectrum of ferric P450_{BM3} (top spectrum, solid black line). The fit for the PA intermediate is shown as the solid black line overlaid on the bottom spectrum.
Experiments on P450cam and CYP158A1 found similar results. P450cam-II was generated under the same conditions as P450\textsubscript{BM3}-II, figure 3.5.\textsuperscript{41} An intermediate with a quadrupole doublet spectrum was formed in 29% yield that had parameters similar to P450\textsubscript{BM3}-II and CPO-II, $\Delta E_Q = 2.06$ mm/s and $\delta = 0.13$ mm/s. These parameters were in agreement with the theoretically determined parameters, as well as the parameters previously reported by Schünemann and coworkers (13% yield, $\Delta E_Q = 1.94$ mm/s and $\delta = 0.13$ mm/s).\textsuperscript{11,12}

![Mössbauer spectrum of P450cam-PA intermediate (4.2K/54mT). Top spectrum, in green, is the raw data for P450cam-PA and in black is ferric P450cam that was subtracted from the raw data (71%). The bottom spectrum shows the raw data following subtraction of the ferric P450cam component. The black line is the fit for a single quadrupole doublet $\Delta E_Q = 2.06$ mm/s and $\delta = 0.13$ mm/s (29%).](image)

The best yield for a protonated ferryl intermediate in a P450 was obtained in CYP158A1. Previous yields for the ferryl species in P450\textsubscript{BM3} was 48% and P450cam
was 29%. The CYP158A1-PA reaction was quenched using a 3.4ms delay line with a 5-fold excess of PA at pH 7, figure 3.6. Under these conditions a quadrupole doublet was formed in 65% yield with a $\Delta E_Q = 2.13$ mm/s and $\delta = 0.09$ mm/s. Ferric CYP158A1 comprised 21% of the total spectrum. Again, a second quadrupole doublet was present in 14% yield ($\Delta E_Q = 2.42$ mm/s and $\delta = 0.39$ mm/s), which is likely due to the oxyferrous contamination.

Figure 3.6: Mössbauer spectrum of CYP158A1-PA intermediate (4.2K/54mT). The raw data for CYP158-PA is shown with the hash marks and the black solid line is the spectrum of ferric enzyme (21%). The bottom spectrum is the raw data after subtraction of the ferric component. The blue line is the fit for two quadrupole doublets with the following parameters: $\Delta E_Q = 2.13$ mm/s and $\delta = 0.09$ mm/s (65%) and $\Delta E_Q = 2.42$ mm/s and $\delta = 0.39$ mm/s (14%).

$P450_{BM3-II}$.
We have shown that structural information can be inferred from Mössbauer data with the aid of DFT calculations, but this method still falls short of techniques that can provide a direct measure of the structure of P450-II. For this reason, we wanted to study this intermediate using EXAFS and resonance Raman spectroscopies. The CYP158A1 results were a fairly recent discovery; therefore, at the time we chose to use P450_{BM3} for measurements on P450-II. P450_{BM3}-II could be generated in close to 50% yield.

The original conditions used to generate P450_{BM3}-II were 0.1M KPhos, pH 7, 5-fold excess of PA, and the fastest quench time possible on our apparatus (3.4ms delay line, ~8ms including freeze time) at 4°C. The P450-II yield using these conditions was always around 45%. Both EXAFS and resonance Raman spectroscopies require the preparation of homogeneous samples for reliable measurements; therefore, we tried over twenty different conditions in an effort to increase the yield of P450_{BM3}-II. The conditions that were varied included the pH, the concentration of peracetic acid, the oxidant, the freeze-quench time, reaction temperature, and the use of a reductant/substrate.

The first condition that was tested was the yield of compound II at different pHs. The P450_{BM3}-PA reaction was performed from pH 6.5 to pH 8. We found that the yield of P450_{BM3}-II was unaffected from pH 6.5 to 7.5, but the yield decreased significantly at pH 8. One possible explanation for this behavior is that at high pH the reaction time for compound II formation is slightly longer because there are less protons available at pH 8 than at pH 7 (to protonate the peroxo intermediate leading to O-O bond heterolysis). We did not attempt any other reaction times at this pH, so we can only speculate on this observation. The amount of PA used to generate compound II was tested next. We tried
a 20-fold, 10-fold, 2-fold, and equimolar amount of oxidant. At PA concentrations greater than 5x, the amount of the ferryl intermediate remained constant, but the amount of ferrous-O_2 contaminant increased. When a 2-fold excess and an equimolar concentration of PA were used, a decrease in the yield of the ferryl intermediate was observed. Based on these results we continued testing reaction conditions using a 5-fold excess of peracetic acid at pH 7.

We next considered that reducing the reaction temperature from 4°C to -10°C would increase the yield of P450_{BM3}-II. The assumption here was that the ferric component from the reaction was a consequence of the decay of P450_{BM3}-II by the time the reaction was quenched. Lowering the reaction temperature would slow the decay of the intermediate. The P450_{BM3}-PA reaction at -10°C generated even less compound II (~35% ferryl, 60% ferric) than at 4°C. To test the opposite effect, we performed the reaction at room temperature. This assumes that at 4°C and 8ms we observed the formation of 50% compound II (and not the decay). Following this assumption, quenching the reaction at room temperature would speed up the reaction and increase the yield of P450_{BM3}-II at 8ms. The room temperature quench at 3.4ms with a 5-fold excess of PA resulted in 100% ferric P450_{BM3}. These experiments determined that the best reaction temperature for the P450_{BM3}-PA reaction was 4°C.

We next quenched the P450_{BM3}-PA reaction at different time points. We had previously performed the reaction using a 3.4 ms delay line (freeze time has not been accounted for in these reaction times; typically around 5-10ms). The new time points we tried were 10.6 and 21.1 ms. The amount of compound II generated decreased with increasing freeze quench time. At 10.6 ms the ferryl yield was similar to the 3.4 ms yield
(~45%), whereas at 21.1 ms the P450-II yield was 35%. It was apparent from these results that the 3.4 ms was the best quench time.

The best conditions for the formation of compound II in P450\textsubscript{BM3} were a 5-fold excess of PA, pH 7, 4ºC, and the fastest freeze quench time possible on our instrument. In a last ditch effort to increase the yield of compound II we tried using a different oxidant. Using a 20-fold excess of m-CPBA, we generated the same overall amount of oxygenated species (~50% of the total spectrum), but interestingly, there was an equal amount of ferryl and oxyferrous P450\textsubscript{BM3}. Typical ferryl-to-ferrous ratios in previous P450-II quenches were about 6:1. A similar ratio was obtained when the PA reaction was performed with an excess of lauric acid, a substrate for P450\textsubscript{BM3}.\textsuperscript{45} Lastly, using an equimolar amount of PA and ascorbic acid, in conditions similar to those used to generate CPO-II,\textsuperscript{17} only ferric P450\textsubscript{BM3} was observed.

After attempting over twenty different reaction conditions we determined that the best yield of P450\textsubscript{BM3}-II was obtained using the original Mössbauer conditions. We used these conditions to generate the EXAFS and resonance Raman samples that will be discussed in the next sections.

\textit{P450\textsubscript{BM3}-II EXAFS}

We have obtained preliminary EXAFS data on P450\textsubscript{BM3}-II. These samples were always characterized by Mössbauer spectroscopy prior to the EXAFS measurements. The yield for P450\textsubscript{BM3}-II was typically around 40%. The contributions of the two other components were around 50% ferric P450\textsubscript{BM3} and 10% ferrous P450\textsubscript{BM3}. Importantly,
Our knowledge of the exact composition of the EXAFS samples allowed for the subtraction of the ferric contribution from the XAS absorption spectrum.

Since these samples were not homogeneous and the structure of P450-II had not previously been reported, reproduction of the EXAFS data was deemed necessary in order to confirm the iron-ligand bond distances. Only one SSRL trip was devoted to studying P450$_{BM3}$-II; therefore, we have not reported the Fe-ligand bond distances here. We did, however, determine the XAS edge energies for ferric, the P450$_{BM3}$-PA sample, and P450$_{BM3}$-II (after subtracting the ferric contribution, 50%). In each set of data the edge energies were determined using the derivative of the XAS edge. The edge energy for ferric P450$_{BM3}$ was 7125.06 eV, the P450$_{BM3}$-PA mixture was 7125.66 eV, and the PA intermediate with the ferric component subtracted was 7126.18 eV. This $\sim$1 eV blue-shift in the XAS absorption edge in P450$_{BM3}$-II reflects the increased binding energy of the 1s electrons in the Fe(IV) intermediate and is in agreement with the XAS edge shift observed between ferric CPO and CPO-II.$^{18}$ An interesting point is that the edge energy from the P450$_{BM3}$ raw data was about 0.5 eV higher in energy, which is reasonable since ferric P450$_{BM3}$ constitutes about 50% of the sample. The edges for ferric P450$_{BM3}$ and the P450$_{BM3}$-PA raw data are shown in figure 3.7.
We also attempted to generate P450BM3-II for resonance Raman analysis. These experiments began after the successful identification of the Fe-O stretch in CPO-II ($\nu_{\text{Fe-O}} = 561 \text{ cm}^{-1}$). This stretch was best enhanced using a 458 nm excitation wavelength from an Ar$^+$ ion laser. When P450BM3-II was generated for resonance Raman studies, a significant fluorescence background was observed. Several different purification procedures were tried to eliminate the source of the fluorescence. Unfortunately, no purification procedure was found that effectively reduced the fluorescence background. One possibility for the origin of the fluorescence was that 6-His tag that was engineered onto the P450BM3 heme domain was the source of the fluorescence. This hypothesis,
however, was not tested. A similar problem occurred when compound II was generated in CYP158A1 for resonance Raman. The CYP158A1 expression system also exhibits a His tag for purification purposes (4-His sequence).

### 3.5 Conclusion

We have trapped high-valent intermediates in three P450s. In each case, using a combination of Mössbauer spectroscopy and density functional theory, the intermediates were basic at physiological pH. Attempts to structurally characterize these intermediates by XAS and resonance Raman, however, were unsuccessful.

Though further confirmation is necessary, the results presented in this chapter provide an important clue to the understanding of P450 hydroxylation chemistry. The existence of basic ferryls in P450s supports the idea that Nature is using protonated ferryls to promote H-atom abstraction. These results also lend credence to the participation of the rebound mechanism in P450 hydroxylations.

### 3.6 References


Chapter 4

The Reaction of P450_{BM3} with Peroxynitrite


4.1 Abstract

Peroxynitrite has come into the spotlight in recent years. Its effects on proteins have been implicated in several diseases such as acute lung injury, rheumatoid arthritis, implant rejection, artherosclerosis, Parkinson’s disease, and Alzheimer’s disease. Peroxynitrite is thought to inactivate a variety of proteins including thiolate-ligated heme proteins such as cytochrome P450 2B1 and PGI2 synthase, through the nitration of tyrosine residues. In previous studies it was reported that thiolate-ligated heme enzymes react with peroxynitrite to form a ferryl intermediate. In an effort to spectroscopically characterize this species in P450_{BM3}, we discovered that the peroxynitrite generated intermediate is not an Fe^{IV}Oxo, but rather an iron-nitrosyl \{FeNO\}_6 complex. In this chapter, we present Mössbauer, resonance Raman, and stopped-flow data, as well as density functional calculations to support this assignment.
4.2 Introduction

Prostacyclin (PGI₂) synthase, a protein involved in the inflammatory response and platelet accumulation in humans, is a thiolate-ligated heme protein that is inactivated in the presence of low amounts of peroxynitrite (PN).¹ In general, thiolate-ligated heme proteins have proven to be a significant target for PN nitrations, including proteins like cytochrome P4502B1 and nitric oxide synthase. Experimental evidence suggests that these enzymes are inactivated as a result of tyrosine nitration.²⁻⁵ These nitrosylated residues are of pathological significance having been detected in Parkinson’s and Alzheimer’s diseases as well as other neurodegenerative, chronic inflammatory, gastrointestinal tract, and cardiovascular disorders.⁶⁻¹⁰

In an attempt to understand the mechanism by which PN inactivates PGI₂ synthase, Ullrich and coworkers studied the reaction of PN with P450cam, P450nor, chloroperoxidase (CPO), and P450BM3.¹¹⁻¹⁵ Stopped-flow experiments identified spectroscopically similar intermediates during the reaction between PN and these thiolate-ligated heme enzymes. Based on previous reports of PN generated oxos in histidine-ligated peroxidases and comparisons with the UV/Visible absorption spectrum of chloroperoxidase compound II (CPO-II), it was concluded that an Fe⁴⁺oxo (ferryl) species was a common intermediate in all four reactions. From these stopped flow experiments, it is currently believed that PN can be used to generate P450-II in high yield and that under certain conditions P450-II is more stable than CPO-II.¹¹,¹⁶ Moreover, it has recently been reported that the relatively stable P450-PN intermediate can serve as a platform from which P450 compound I can be generated by laser flash photolysis.¹⁶
These reports have stirred considerable interest in the use of peroxynitrite as an alternative oxidant to aid in the study of P450 chemistry.

Inspection of the previous experimental results for the reaction of heme proteins with PN, especially those of the thiolate-ligated heme enzymes, indicates that this reaction is unusual. Using peracetic acid, P450-II is more reactive than CPO-II;\textsuperscript{17-19} however, according to the experiments performed by Ullrich and coworkers, the ferryl intermediate in P450\textsubscript{BM3} is over twice as stable as CPO-II when PN is used.\textsuperscript{11} In Chapter 3 we presented experimental results on P450-II. We found that the highest obtainable yield of P450-II was 65\% (CYP158A1), which was only possible at the fastest freeze quench time (~8 ms). CPO-II, on the other hand, can be formed in 100\% yield and is stable for over two seconds.\textsuperscript{19} The stability of the PN-generated intermediates in P450s versus CPO suggests that there is a difference between these two intermediates, and therefore, characterization of the P450-PN intermediate is not yet complete.

The PN-intermediate in P450\textsubscript{BM3} was chosen for further characterization for two reasons: 1) the P450\textsubscript{BM3} intermediate was formed in the highest yield in this enzyme according to the reports from Ullrich and coworkers and 2) we had previously characterized compound II in P450\textsubscript{BM3} by Mössbauer spectroscopy.\textsuperscript{11,17} In this chapter we present Mössbauer and DFT results on the P450\textsubscript{BM3}-PN intermediate. This approach was believed to be useful for this application because of the previous success in obtaining structural information for the ferryl intermediates in P450s, CPO, and myoglobin. Resonance Raman spectroscopy was subsequently used to confirm the Mössbauer characterization of the P450\textsubscript{BM3}-PN intermediate.
Our prior characterization of P450-II and CPO-II revealed that these intermediates were protonated near physiological pH. However, unlike the thiolate-ligated heme proteins, ferryl myoglobin was unprotonated down to pH 3.5 (unpublished). The distinguishing property of thiolate-ligated ferryl intermediates from other S = 1 ferryl intermediates was an enlarged quadrupole splitting. The ΔE_Q for a protonated ferryl is ~2.1 mm/s, whereas the ΔE_Q for typical 6-coordinate ferryl species is about 1.4 mm/s. The assignment of an Fe(IV)OH structure for P450-II by Mössbauer spectroscopy was also supported by Mössbauer, EXAFS, and resonance Raman measurements on CPO-II. EXAFS on CPO-II revealed an Fe-O bond distance of 1.82 Å, while resonance Raman experiments identified a resonance that was sensitive to both 18O and deuterium substitution at 561 cm⁻¹. According to Badger’s rule, parameterized for Fe-O bonds, an Fe-O stretching frequency at 561 cm⁻¹ would be equal to an Fe-O bond length of 1.82 Å.

Our investigations have elucidated the experimental parameters for ferryl intermediates in thiolate-ligated heme proteins. If the PN-intermediate in P450BM3 is a ferryl species, we would expect it to be an Fe(IV)OH with ΔE_Q ≈ 2.1 mm/s and an S = 1 ground state by Mössbauer spectroscopy. Using resonance Raman spectroscopy, we would expect an Fe-O stretch around 560 cm⁻¹ that is sensitive to 18O and deuterium substitution. This was not the case. We found that PN reacted with P450BM3 to generate an {FeNO}₆ nitrosyl complex, which is an S = 0 diamagnetic species. Resonance Raman measurements corroborated our Mössbauer characterization of this intermediate. These
experiments identified an Fe-N and an N-O stretch, both of which were sensitive to $^{15}$N substitution.

4.3 Computational Procedures

DFT calculations were performed on several P450$_{BM3}$ complexes to compare theoretical Mössbauer parameters to the experimentally determined parameters for the P450$_{BM3}$-PN intermediate. These calculations were performed on a large active site model of P450$_{BM3}$. The starting structure for these calculations was taken from the ferric P450$_{BM3}$ crystal structure (PDB accession code 1POV), which included a porphine and the first four residues in the axial helix (Cys400-Gln403). All residues except Cys and Gly were converted to Ala. An example of the model used for these calculations can be found in Figure 4.1.

Figure 4.1: Example of P450$_{BM3}$ computational model. This figure represents the nitro-complex.
Geometry optimizations were performed using Gaussian 03 (B3LYP/6-311G). During geometry optimizations the positions of the distal ligand, iron atom, porphyrin nitrogens, alpha carbons, meso carbons, meso hydrogens, and the axial SCH₂CH were allowed to fluctuate. Mössbauer parameters were determined at the optimized geometries. Quadrupole splittings were determined at the B3LYP/6-311G level and isomer shifts were calculated using Neese’s core properties (CP) basis set. An integration grid of 199 radial shells with 590 angular points was used to determine the theoretical isomer shifts. The electron density at the nucleus was obtained using the Atoms In Molecules (AIM) option in Gaussian 03.

4.4 Results and Discussion

Stopped-flow of the P450BM3-PN reaction.

Initially, we performed stopped-flow experiments on the P450BM3-PN reaction (Figure 4.2). Our results were similar to those obtained by Ullrich and coworkers. We observed the formation of only one transient intermediate during the reaction. This species had an absorbance maximum at 432 nm, but as with the previous investigation of this reaction, we report single wavelength data at 435 nm. It was the perceived similarity of the 432 nm absorbance to the absorption maximum of CPO-II ($\lambda_{\text{max}} = 437$ nm) that led to the initial assignment of the P450BM3-PN intermediate as a ferryl species. As will be shown, however, the PN intermediate has almost identical spectral features (both Mössbauer and UV/Visible) to the ferric-nitrosyl complex of P450BM3.
Mössbauer spectroscopy on P450\textsubscript{BM3}-PN.

Using the stopped-flow spectrophotometric results, we determined that an appropriate quench time for the P450\textsubscript{BM3}-PN reaction was 450ms. The resulting 4.2K/54mT Mössbauer spectrum of the P450\textsubscript{BM3}-PN intermediate is shown in figure 4.3. The EPR spectrum for this sample is also shown in figure 4.3. According to the EPR and Mössbauer spectra, the P450\textsubscript{BM3}-PN intermediate was formed in about 80% yield. These measurements also determined that this species was either a diamagnetic or integer spin species, as evidenced by the quadrupole doublet Mössbauer spectrum and the lack of an EPR signal.

Figure 4.2: Stopped-flow spectrophotometry experiments on the P450\textsubscript{BM3} reaction. The blue spectrum corresponds to ferric P450\textsubscript{BM3} and the red spectrum is the fully-formed P450\textsubscript{BM3}-PN intermediate (300ms). The time points of the spectra are 10, 20, 40, and 70ms. These spectra correspond to the decreasing absorbance at 302nm. The inset shows single-wavelength data at 302, 417, and 435nm.
The Mössbauer parameters for the P450BM3-PN intermediate were $\Delta E_Q = 1.15$ mm/s and $\delta = 0.15$ mm/s. The isomer shift was similar to those reported for Fe$^{IV}$ centers in P450cam-II, P450BM3-II, and CPO-II. The quadrupole splitting, however, was not as expected. A $\Delta E_Q$ of 1.15 mm/s was significantly smaller than the quadrupole splitting previously measured for P450BM3-II generated with peracetic acid ($\Delta E_Q = 2.16$ mm/s, pH 7).

It was clear from the difference in the quadrupole splittings that the reaction of P450BM3 with peracetic acid and peroxynitrite yielded different reaction intermediates. Reaction of P450BM3 with peracetic acid resulted in an $S = 1$ intermediate with a $\Delta E_Q = +2.16$ mm/s, whereas the $\Delta E_Q$ of the PN-intermediate, $\Delta E_Q = 1.15$, better resembled...
those of authentic 6-coordinate Fe$^{IV}$oxo intermediates from the histidine-ligated heme systems. Based on these Mössbauer results, the characterization of the P450$_{BM3}$-PN intermediate as an Fe(IV)oxo was reasonable; however, it seemed unlikely that protonation of the ferryl moiety would be oxidant-dependent.

Figure 4.4: Mössbauer spectra of P450$_{BM3}$-PN (red) and P450$_{BM3}$-II (blue, generated with peracetic acid). The $\Delta E_Q$ for the PN intermediate is 1.15 mm/s, whereas for P450$_{BM3}$-II the $\Delta E_Q = 2.16$ mm/s.

It was apparent that a simple comparison of the Mössbauer parameters for the PN- and PA-generated intermediates in P450$_{BM3}$ was not sufficient for the characterization of the P450$_{BM3}$-PN intermediate. The inconsistency between the Mössbauer parameters of the two intermediates prompted the calculation of possible species that could conceivably result from the reaction of P450$_{BM3}$ with PN at pH 6.8. Only diamagnetic or integer spin species were considered because those species would agree with the experimental Mössbauer and EPR results. A summary of these theoretical Mössbauer parameters can be found in Table 4.1.
We began our theoretical analysis of the PN reaction by determining several possible Fe(IV)/S = 1 species that could result from the reaction of P450BM3 with either byproducts of the initial P450BM3-PN reaction, or the breakdown products of PN at pH 7. These complexes included the two previously mentioned ferryl species, nitro- and nitrito-nitrite complexes, and nitrate and nitroxyl species. Of the six complexes that were originally calculated, only the parameters for the Fe(IV)oxo P450BM3 model were in reasonable agreement with experiment (ΔE_Q = 1.05 mm/s and δ = 0.11 mm/s).

Optimization of the Fe(IV) nitrite, nitrate, and nitroxyl complexes revealed that their ground state electronic structures were best described as an FeIII coupled to a porphyrin radical. The ferric character of these species was reflected in the isomer shifts (0.18-0.36 mm/s).

Based on our previous investigations on P450-II and CPO-II, we felt that the presence of an authentic ferryl species in P450BM3 with PN was unlikely. We calculated the Mössbauer parameters for two more P450BM3 complexes, the ferric superoxide and ferric nitrosyl P450BM3 complexes (Table 4.1). Both of these species are S = 0

<table>
<thead>
<tr>
<th>Distal Ligand</th>
<th>Formal oxidation/ spin state</th>
<th>Theory (mm/s)</th>
<th>Experiment (mm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O2^-</td>
<td>IV (S = 1)</td>
<td>0.11</td>
<td>1.05</td>
</tr>
<tr>
<td>NO2^-</td>
<td>IV (S = 1)</td>
<td>0.28</td>
<td>2.87</td>
</tr>
<tr>
<td>NO3^-</td>
<td>IV (S = 1)</td>
<td>0.21</td>
<td>-2.07</td>
</tr>
<tr>
<td>HNO</td>
<td>IV (S = 1)</td>
<td>0.31</td>
<td>-2.19</td>
</tr>
<tr>
<td>O2•-</td>
<td>III (S = 0)</td>
<td>1.32</td>
<td>0.09</td>
</tr>
<tr>
<td>NO+</td>
<td>II (S = 0)</td>
<td>0.31</td>
<td>1.15</td>
</tr>
</tbody>
</table>

Table 4.1: Mössbauer parameters for P450BM3 species in mm/s
complexes; the ferric superoxide complex is a resonance form of the oxyferrous species (Fe^{II}O_2) and the linear coordination geometry of the nitrosyl ligand in the ferric nitrosyl complex yields an Fe^{II}NO^+, or \{FeNO\}^6, complex.

Interestingly, the ferric-nitrosyl had theoretical Mössbauer parameters that matched the experimental parameters well (\(\Delta E_Q = 1.32 \text{ mm/s} \) and \(\delta = 0.09 \text{ mm/s} \)). At first glance, the small isomer shift for the \{FeNO\}^6 complex was unexpected. One would expect an isomer shift that reflects the ferrous oxidation state (\(\delta = 0.4-0.8 \text{ mm/s} \)) for the Fe^{II}NO^+ species. However, the linear geometry of the strongly \(\pi\)-accepting NO\(^+\) ligand causes the delocalization of the 3d electrons between the metal and the ligand, which results in an Fe(IV)-like isomer shift, figure 4.5.\(^{32}\)

Figure 4.5: P450\(_{BM3}\) \{FeNO\}\(^6\) structure from DFT calculations. Calculated Fe-N (1.65 Å), N-O (1.18 Å), and Fe-S (2.38 Å) distances and the Fe-N-O (170.3°) bond angle are shown.
DFT calculations of Mössbauer parameters led us to two possibilities for the P450_{BM3}-PN intermediate: the Fe(IV)oxo species, or the \{FeNO\}_{6} complex. The obvious difference between these two species is the spin state, thus we used variable-field Mössbauer spectroscopy to determine the spin state of P450_{BM3}-PN, figure 4.6. These measurements were performed in 6T and 8T external fields. The experimental data (hash marks) for both fields were fit well using a spin Hamiltonian formalism. For these simulations, the isomer shift and quadrupole splitting were taken from the 54mT data and an asymmetry parameter \( \eta = 0 \) was assumed. The simulations demonstrated that the effective magnetic field at the \(^{57}\)Fe nucleus equaled the externally applied field. This indicated that there was no internal magnetic field for this intermediate. The P450_{BM3}-PN intermediate is diamagnetic. These measurements also determined that the \( \Delta E_{Q} \) was positive. Attempts to simulate a paramagnetic center (\( S = 1 \)) did not fit the experimental data, figure 4.6, in red. The \( S = 1 \) fit was simulated in the slow relaxation limit assuming parameters for a typical heme ferryl species: \( g = (2.1, 2.1, 2.0) \), \( D = 23 \text{ cm}^{-1} \), \( E/D = 0 \), \( A/g_{N}\beta_{N} = (-19, -19, -7) \text{ T} \), \( \eta = 0 \), and the \( \Delta E_{Q} \) and \( \delta \) from the 54mT data.\(^{17}\) This variable-field Mössbauer result was our first indication that the P450_{BM3}-PN intermediate was an \{FeNO\}_{6} complex.
Confirmation of the P450BM3-PN intermediate as an \( \{ \text{FeNO} \}_6 \) complex.

The first spectroscopic tool we used to support our assignment of the P450BM3-PN intermediate as an \( \{ \text{FeNO} \}_6 \) complex was UV/visible spectroscopy. We compared the UV/vis spectrum for the PN intermediate from stopped-flow (300ms) to the UV/vis of the \( \{ \text{FeNO} \}_6 \) complex. These spectra are shown in figure 4.7. The spectra for these two species are almost identical with absorption peaks at 432, 541, and 572 nm. The increased absorbance around 300 nm in the spectrum for the PN intermediate was due to excess PN in the reaction (\( \lambda_{\text{max}} = 302 \) nm).
We next generated the nitrosyl complex for Mössbauer measurements at 4.2K/54mT. Figure 4.8, right, shows a direct comparison of these two species. The Mössbauer parameters for the P450_{BM3}-NO complex were identical to the PN-intermediate, $\Delta E_Q = 1.15$ mm/s and $\delta = 0.15$ mm/s. The similarities between the UV/vis and Mössbauer spectra in figure 4.9 support our assignment of the P450_{BM3}-PN intermediate as an $\{\text{FeNO}\}^6$ complex.
Resonance Raman spectroscopy of P450\textsubscript{BM3}-PN and P450\textsubscript{BM3}-NO.

Mössbauer spectroscopy and the calculation of Mössbauer parameters with DFT led us to the conclusion that P450\textsubscript{BM3}-PN was an \{FeNO\}\textsuperscript{6} complex. It was the remarkable agreement between the DFT calculations on an \{FeNO\}\textsuperscript{6} model of P450\textsubscript{BM3} and the experimental Mössbauer parameters that enabled us to proceed in our characterization of P450\textsubscript{BM3}-PN. Determination of the spin state and the comparison of Mössbauer parameters and UV/vis absorption spectra of the P450\textsubscript{BM3}-PN intermediate and the \{FeNO\}\textsuperscript{6} complex provided strong evidence for the identity of the PN intermediate. Finally, we utilized resonance Raman spectroscopy to obtain structural data to providing inarguable evidence for the identity of P450\textsubscript{BM3}-PN.

P450\textsubscript{BM3}-PN for resonance Raman analysis was prepared under identical conditions as our previous Mössbauer investigation. The high-frequency region in P450\textsubscript{BM3}-PN contains a $\nu_4$ stretch typical of thiolate-ligated ferric-nitrosyls, 1378 cm\textsuperscript{-1}.\textsuperscript{33,35} In ferric P450\textsubscript{BM3} this peak appears at 1376 cm\textsuperscript{-1}. It was previously demonstrated that, in heme proteins, a blue-shift in the $\nu_4$ stretch is indicative of an increase in the oxidation state of the iron.\textsuperscript{36} The $\nu_4$ stretch is sensitive to the occupancy of the porphyrin $\pi^*$ orbitals. As the number of $d_e$ electrons changes, the energy of the $\nu_4$ stretch changes as well. For instance, HRP-II has a $\nu_4$ stretch at 1380 cm\textsuperscript{-1} and the ferric HRP $\nu_4$ stretch is at 1376 cm\textsuperscript{-1}.

It is well known that $\pi$-acidic ligands, like CO, NO and O\textsubscript{2}, cause the withdrawal of $d_e$ electrons from the porphyrin $\pi^*$ orbitals. This effect leads to a higher energy $\nu_4$ stretch. The axial NO$^+$ ligand in P450\textsubscript{BM3}-PN, being a good $\pi$-acceptor, reduces the
electron density at the iron nucleus causing a blue-shift in the \( \nu_4 \) peak. The effect of a linear NO\(^+\) ligand on the resonance Raman spectrum of P450\(_{BM3}\)-PN is also in agreement with the small isomer shift from Mössbauer spectroscopy.

Figure 4.10 shows the low frequency region for P450\(_{BM3}\)-PN. Using a 413 nm excitation wavelength we identified two isotope-sensitive peaks, one at 535 cm\(^{-1}\) and another at 550 cm\(^{-1}\). When P450\(_{BM3}\)-PN was prepared with \(^{15}\)N-PN, these peaks shifted to 525 cm\(^{-1}\) and 543 cm\(^{-1}\), respectively. Using the previously reported values for the Fe-N stretch in P450cam and P450\(_{BM3}\), and our own measurements on ferric P450\(_{BM3}\)-NO, the 535 cm\(^{-1}\) peak in the PN intermediate was assigned as the \( \nu(\text{Fe-NO}) \) stretch.\(^{34,37}\) The 550 cm\(^{-1}\) peak in the P450\(_{BM3}\)-PN spectrum was assigned to the \( \delta(\text{Fe-N-O}) \) bending mode due to its similarity to the bending mode observed in the \{FeNO\}\(^6\) complex in camphor-bound P450cam.\(^{34}\)

![Figure 4.10: Resonance Raman spectra of the P450\(_{BM3}\)-PN intermediate using a 413nm excitation wavelength. Left. Low frequency data indicating the Fe-N stretch for the PN intermediate. Two peaks shifted with \(^{15}\)N substitution, 535 and 550 cm\(^{-1}\). Right. High frequency data in the N-O stretch region. Upon \(^{15}\)N-substitution, the N-O stretch shifted from 1850 to 1815 cm\(^{-1}\).](image)
Upon further examination of the high-frequency region we identified the ν(N-O) for the P450\textsubscript{BM3}-PN intermediate. A peak at 1850 cm\textsuperscript{-1} was present in the P450\textsubscript{BM3}-PN spectrum, figure 4.10, which was absent from ferric P450\textsubscript{BM3} in this region. This peak shifted to 1815 cm\textsuperscript{-1} upon \textsuperscript{15}N-substitution. The location of the ν(N-O) stretch and its \textsuperscript{15}N-isotopic shift were similar to those published for the ferric P450\textsubscript{nor} N-O stretch (1853 cm\textsuperscript{-1} peak shifts to 1816 cm\textsuperscript{-1} with \textsuperscript{15}N).\textsuperscript{35}

\textit{Summary of P450\textsubscript{BM3} reactions.}

The results of our P450\textsubscript{BM3} investigations using the two different oxidants and several different reaction conditions are summarized in figure 4.11. The top section of the figure shows the conditions used to generate P450\textsubscript{BM3}-II (see Chapter 3) and the Mössbauer parameters for this intermediate. We have shown, using Mössbauer spectroscopy, that this intermediate is described as an Fe\textsuperscript{IV}OH complex. The middle section of figure 4.11 shows the reaction conditions and results for the P450\textsubscript{BM3} and PN reaction. Using variable-field Mössbauer and UV/vis spectroscopies we have determined that the intermediate in this reaction is a ferric nitrosyl complex (path B), not a ferryl species (path A) as was suggested previously for P450-PN intermediates.\textsuperscript{11, 12, 16} The bottom of figure 4.11 shows the reaction of P450\textsubscript{BM3} with nitric oxide gas. The P450\textsubscript{BM3}-PN intermediate and nitrosyl complex have identical spectroscopic features.
Figure 4.11: Reactants, reaction conditions, and observable iron-containing products for the reaction of P450BM3 with the following: (Top) Peracetic acid: the first spectroscopically characterizable intermediate during the reaction of P450BM3 with PA is compound II, an FeIVOH species. (Middle) Peroxynitrite: the reaction of PN with P450BM3 results in the formation of a nitrosyl complex (path B). No ferryl intermediates are observed (path A). (Bottom) Nitric oxide: the ferric P450BM3-NO complex and the P450BM3-PN intermediate have identical spectroscopic features.
4.5 Conclusion

Using Mössbauer and resonance Raman spectroscopies, stopped-flow spectrophotometry, and DFT calculations, we determined that the PN intermediate in P450$_{BM3}$ is a ferric nitrosyl complex, not a ferryl species. Its spin state, UV/visible, and Mössbauer parameters are consistent with an $\{\text{FeNO}\}$ complex. Even more convincing was the resonance Raman data in which the Fe-NO and NO stretches were identified. The peaks representing $\nu$(Fe-N), $\nu$(N-O), and $\delta$(Fe-N-O) shifted with $^{15}$N-substitution, in agreement with the predicted shifts for a diatomic harmonic oscillator.

Many times, spectroscopically detectable intermediates in enzymatic reactions are characterized with only one spectroscopic method, usually UV/vis spectroscopy. Characterization of the P450$_{BM3}$-PN intermediate with only one spectroscopy has led many research groups off course. The investigations reported in this chapter emphasize the importance of employing multiple spectroscopic tools to characterize transient intermediates. Several other PN intermediates in heme enzymes have been described as ferryl intermediates by stopped-flow spectrophotometry. We believe that it is necessary to re-examine these intermediates using multiple spectroscopies, similar to those employed in our P450$_{BM3}$-PN investigations. There is the potential for the formation of nitrosyl complexes with PN not just in P450$_{BM3}$, but in all heme proteins. Investigations on this matter are the subject of Chapter 5.
4.6 References


Chapter 5

The Unusual Reaction Between Heme Proteins and Peroxynitrite is Driven by Heme Axial Ligation

5.1 Abstract

Using variable-field Mössbauer spectroscopy in concert with density functional calculations, we have recently shown that a nitrosyl complex is generated during the reaction of Cytochrome P450\textsubscript{BM3} with peroxynitrite (PN). We now show that under the same conditions nitrosyl complexes are generated in five other thiolate-ligated heme proteins (P450cam, Chloroperoxidase, CYP119, CYP154, and a thermostable Nitric Oxide Synthase). We believe that nitrosyl complexes are formed when any thiolate-ligated heme protein reacts with PN. This is not the case in all heme systems. We show that horseradish peroxidase and cytochrome c peroxidase react with PN to form ferryl intermediates, not nitrosyl complexes.

5.2 Introduction

Peroxynitrite is a potent oxidant and, when formed \textit{in vivo}, is a threat to many types of biological molecules. PN can react with a wide variety of endogenous and exogenous substrates including amines, thiols, sulfides, DNA, lipids, ascorbate, and other reducing agents.\textsuperscript{1} The direct reaction between PN and several amino acids has also been
established. Tyrosine is particularly susceptible to nitrosylation by PN, the product being 3-Nitrotyrosine (3-NT). 3-NT residues have been identified in diseased tissues resulting from human atherosclerosis, pulmonary and heart disease, acute and chronic kidney rejection, Alzheimer’s disease, Parkinson’s disease, and Lou Gehrig’s disease, suggesting a role for PN in these conditions. Interestingly, the amount of nitration in diseased tissues can be quite high: 1-3% of the tyrosine residues in the spinal cords of patients with Lou Gehrig’s disease are nitrated and a significant amount of free nitrotyrosine has been found in septic patients who are in renal failure.

Prostacyclin (PGI₂) synthase, a thiolate-ligated heme protein involved in the inflammatory response and platelet accumulation, is directly affected by PN. This enzyme is rapidly inactivated in the presence of submicromolar concentrations of PN. Enzymatic inactivation was linked to the nitration of a tyrosine residue near the active site. These findings lead to an investigation of autocatalytic nitration and inactivation of thiolate-ligated heme proteins by peroxynitrite. In these studies, the ability of thiolate-ligated heme proteins to nitrate added phenol was established. Further analysis of these reactions showed that P450BM3, P450cam, and Chloroperoxidase (CPO) not only catalyzed the nitration of added phenol, but also nitrated their own tyrosine residues. It was also found that both phenol nitration and tyrosine nitration were affected by the addition of substrates in P450BM3, P450cam, and CPO, suggesting that catalysis occurs at the heme iron.

UV/visible spectroscopic characterization of the P450BM3, P450cam, P450nor, and CPO reactions with PN showed the formation of a transient intermediate in all four reactions. Interestingly, these intermediates persisted as long as PN was present in
solution. The intermediate formed during these reactions had an absorption spectrum similar to the ferryl intermediate in CPO (437, 542, 572 nm). The similarity between the absorption spectra of the PN intermediates to CPO compound II (CPO-II), as well as the discovery of ferryl intermediates during the reaction of PN with histidine-ligated peroxidases,\(^9\) led to the conclusion that PN generates an Fe(IV)oxo intermediate in all four proteins.

The mechanism proposed for this reaction begins with the binding of PN to the heme iron. An Fe(IV)oxo species (compound II) would be generated through homolytic cleavage of the PN ONO-O bond, accompanied by the release of an NO\(_2\) radical. Compound II would then oxidize a nearby tyrosine residue and the oxidized tyrosine would recombine with the NO\(_2\) radical forming nitrotyrosine.\(^1\) Not only does the formation of a ferryl intermediate in these reactions agree with the results of previous experiments on heme peroxidases, it is also consistent with the formation of nitrotyrosine residues.

Our recent work on P450\(_{\text{BM3}}\) refutes this mechanism. We characterized the P450\(_{\text{BM3}}\)-PN intermediate as a ferric nitrosyl complex using Mössbauer and resonance Raman spectroscopies and DFT calculations. This result was extremely surprising because all previous experimental evidence suggested formation of high-valent oxo intermediates during the reactions of heme proteins and porphyrin complexes with PN.\(^1\)\(^-\)\(^4\) We were puzzled by the presence of 3-NT residues in P450\(_{\text{BM3}}\) following its reaction with peroxynitrite since our characterization of the P450\(_{\text{BM3}}\)-PN intermediate did not agree with the mechanism proposed. We have reconciled the detection of nitrated
tyrosine residues with the formation of a nitrosyl complex in P450BM3. This mechanism will be discussed in the Conclusion section.

Using the knowledge that P450BM3-PN is a ferric nitrosyl complex, we have expanded our investigation into the reaction between PN and heme proteins to further evaluate the reaction intermediate in both histidine- and thiolate-ligated systems. We have discovered very different reactivities between PN and the histidine- and thiolate-ligated heme proteins, which appears to be dependent on the axial ligand. In this chapter, we provide spectroscopic evidence in support of the formation of a ferric nitrosyl species when PN reacts with thiolate-ligated heme proteins. However, we will also show that PN reacts with histidine-ligated hemes to generate the originally proposed Fe(IV)oxo species.

5.3 Results and Discussion

Examination of P450BM3-PN reaction under various conditions

Using UV/vis, Mössbauer, and resonance Raman spectroscopies we have shown that when P450BM3 is mixed with a large excess of PN a nitrosyl complex is formed. This result was surprising since it contrasted a large body of literature concerning the reaction of heme proteins and porphyrin complexes with PN.20-26 Though we have definitively shown that P450BM3 forms a nitrosyl complex at 450 ms when reacted with a 16-fold molar excess of PN at pH 6.8, we still could not rule out the existence of an FeIVoxo intermediate during this reaction. To further investigate the reaction between P450BM3 and PN we varied the reaction time and the concentration of PN in an attempt to trap
intermediates that were not previously observed. These experiments are summarized in Table 5.1.

Using freeze-quench Mössbauer spectroscopy we performed a reaction time course using a 16-fold excess of PN at 12°C. The reaction was quenched at 8ms, 215ms, and 450ms. Over this large time range we identified only two species in the Mössbauer spectra: ferric P450\textsubscript{BM3} and the {FeNO}\textsuperscript{6} complex. This evidence, along with stopped-flow UV/vis spectroscopy, supported our conclusion that only one intermediate was formed during the P450\textsubscript{BM3}-PN reaction with excess PN and it was the ferric nitrosyl complex.\textsuperscript{27}

Since the sheer excess of PN under the previous reaction conditions could influence the reaction mechanism, we varied the concentration of PN. In the first set of conditions, we used a 1:1 ratio of P450\textsubscript{BM3} to PN and freeze-quenched the reaction 450ms after mixing. We observed the formation of 25% of the ferric-nitrosyl complex by Mössbauer spectroscopy. The rest of the spectrum was attributed to ferric P450\textsubscript{BM3}. We also performed the P450\textsubscript{BM3}-PN reaction with a slight excess of PN and quenched the reaction as fast as possible. These reaction conditions were a close match to the conditions used to generate P450\textsubscript{BM3} compound II using peracetic acid.\textsuperscript{28} Using a 5-fold

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Molar excess of PN</th>
<th>Quench time (ms)</th>
<th>NO yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 6.8, 12°C</td>
<td>16:1</td>
<td>450</td>
<td>85 ± 5%</td>
</tr>
<tr>
<td>pH 6.8, 12°C</td>
<td>16:1</td>
<td>215</td>
<td>72%</td>
</tr>
<tr>
<td>pH 6.8, 12°C</td>
<td>16:1</td>
<td>8</td>
<td>75%</td>
</tr>
<tr>
<td>pH 6.8, 12°C</td>
<td>1:1</td>
<td>450</td>
<td>25%</td>
</tr>
<tr>
<td>pH 6.8, 12°C</td>
<td>5:1</td>
<td>8</td>
<td>36%</td>
</tr>
</tbody>
</table>
excess of PN and freeze-quenching the reaction at ~8ms, once again, we saw the formation of solely the P450_{BM3}-nitrosyl complex.

**PN reaction with thiolate-ligated heme proteins**

Our work definitively showed that over a large time range (8-450ms) and range of PN concentrations (1 molar equivalent to 16 molar equivalents), reactions of P450_{BM3} with PN yielded only an \{FeNO\}^6 nitrosyl complex. The question that follows is: Does this reaction occur similarly in all thiolate-ligated heme proteins? The results from a recent paper suggested that when PN was reacted with CPO and CYP119 (a thermophilic P450) an intermediate that, according to stopped-flow spectrophotometry, was spectroscopically similar to CPO-II was formed.\(^{29}\)

Using our knowledge of the P450_{BM3} reaction, we re-examined the PN reaction in CPO and CYP119 using stopped-flow spectrophotometry. We compared the UV/Vis spectrum of the time point with the highest yield of the PN-intermediate in CPO and CYP119 to the UV/Vis spectrum of the respective ferric-nitrosyl complex. The UV/Vis spectra for the PN-intermediates and the nitrosyl complexes in CPO and CYP119 were nearly identical. This data are found in **Table 5.2**.

We made an interesting observation during the stopped-flow spectrophotometric investigation of the reaction of CPO with PN. As was mentioned, the first detectable intermediate in this reaction was the \{FeNO\}^6 nitrosyl complex (436, 543, 574nm). Interestingly, as the PN-intermediate decayed a 690nm peak, presumably from CPO-I, was detected (figure 5.1). Hydrogen peroxide was usually present as a contaminant in PN preparations from the acidified H\textsubscript{2}O\textsubscript{2} and nitrite synthesis procedure (~20%).
Following treatment of the same PN solution with MnO₂, to remove the H₂O₂ impurity, CPO-I formation was not observed. Although the formation of CPO-I proved to be unrelated to the CPO-PN reaction, it did show that PN may react with CPO even faster than its natural oxidant, H₂O₂.

Figure 5.1: Kinetic data for the CPO-PN reaction with (left) PN not treated with MnO₂ ([PN] = 6mM, [H₂O₂] = 1.2mM) and (right) after treatment with MnO₂. The yellow trace is the PN decay at 302nm, the blue trace is ferric CPO monitored at 395nm, the red trace is CPO-PN monitored at 436 nm, and CPO-I monitored at 690nm.
It should again be stressed that the intermediate formed during the P450BM3-PN reaction was only elucidated once Mössbauer spectroscopy and resonance Raman spectroscopy were performed. Prior to these experiments only the similarities in UV/Vis spectra were used to characterize these intermediates, which has shown to be problematic as of late. In light of this discovery, we also analyzed CPO-PN and CYP119-PN by Mössbauer spectroscopy. Both reactions contained quadrupole doublet species (in varying yields) with Mössbauer parameters similar to P450BM3-PN, see Table 5.2. CYP119-PN had a ΔE_Q = 1.13 mm/s and δ=0.15 mm/s and CPO-PN had a ΔE_Q = 1.33 mm/s and δ = 0.14 mm/s. The CYP119-PN stopped-flow and Mössbauer data are presented in Figure 5.2.

Table 5.2: PN versus {FeNO}_{6} in thiolate-ligated proteins.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Absorbance Maxima (nm)</th>
<th>Mössbauer parameters (mm/s)</th>
<th>PN Quench time/ FQ Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PN intermediate</td>
<td>{FeNO}_{6} complex</td>
<td>PN intermediate</td>
</tr>
<tr>
<td>P450BM3</td>
<td>432,542,572</td>
<td>433,542,574</td>
<td>1.18</td>
</tr>
<tr>
<td>CPO</td>
<td>436,543,574</td>
<td>436,545,577</td>
<td>1.38</td>
</tr>
<tr>
<td>CYP119</td>
<td>431,542,572</td>
<td>433,543,575</td>
<td>1.13</td>
</tr>
<tr>
<td>P450cam</td>
<td>433,542,573</td>
<td>433,542,573</td>
<td>1.19</td>
</tr>
<tr>
<td>CYP154</td>
<td>427,541,572</td>
<td>432,542,573</td>
<td>1.28</td>
</tr>
<tr>
<td>gsNOS</td>
<td>437(s),548,582</td>
<td>439,549,582</td>
<td>1.39</td>
</tr>
</tbody>
</table>
If we compare the Mössbauer parameters for CPO-PN to those of the major component in CPO-II, we see that the quadrupole splittings are strikingly different. The isomer shifts for the two intermediates are similar because the strongly π-accepting NO⁺ ligand withdraws electron density from the dₓ-orbitals. This effect causes the isomer shift of the Fe(II) complex to resemble an Fe(IV) center.³⁰,³¹ The quadrupole splitting for CPO-II was ΔE₀ = 2.06 mm/s, which is significantly larger than ΔE₀ = 1.33 mm/s measured for the CPO-PN intermediate.³² Our CPO Mössbauer results once again emphasize the importance of employing multiple spectroscopies to characterize reaction
intermediates, since the absorption spectrum of CPO-PN (436, 543, and 574 nm) and CPO-II (437, 542, and 572 nm) are strikingly similar.

Once we had data in support of the formation of nitrosyl species in P450BM3, CYP119, and CPO, we performed the same reaction on all of the thiolate-ligated heme proteins that were available to us at the time. These enzymes included P450cam, CYP154 and gsNOS. Ullrich and coworkers previously studied the reaction between P450cam and PN. They observed the autocatalytic nitration of tyrosine residues in this enzyme, but were unable to build up a large amount of the PN-intermediate under the conditions employed (as evidenced by the Soret shift). We examined the P450cam-PN reaction as well. Under identical stopped-flow conditions, 100-fold excess PN at 12°C, an intermediate was formed in high yield. The UV/Vis spectrum for P450cam-PN was identical to its ferric-NO complex. P450cam-PN was also prepared for Mössbauer analysis using a 16-fold excess of PN at 12°C. The sample was quenched at 110 ms. Under these conditions P450cam-PN was generated in 40% yield and had Mössbauer parameters that, like CPO and P450BM3, clearly did not match those previously measured for P450cam compound II ($\Delta E_Q = 2.06$ mm/s and $\delta=0.13$ mm/s).²⁸

*Mössbauer measurements on thiolate-ligated {FeNO}_6 complexes*

Mössbauer samples of the ferric-nitrosyl complexes were prepared for all six enzymes. These Mössbauer parameters for the {FeNO}₆ complexes and the PN intermediates are listed in Table 5.2. We found very good agreement between the PN intermediates and their ferric-nitrosyl complexes ($\pm0.05$ mm/s for $\Delta E_Q$ and $\pm0.02$ mm/s

---

28. Mössbauer measurements on thiolate-ligated {FeNO}₆ complexes

Mössbauer samples of the ferric-nitrosyl complexes were prepared for all six enzymes. These Mössbauer parameters for the {FeNO}₆ complexes and the PN intermediates are listed in Table 5.2. We found very good agreement between the PN intermediates and their ferric-nitrosyl complexes ($\pm0.05$ mm/s for $\Delta E_Q$ and $\pm0.02$ mm/s.
for δ). The agreement between the Mössbauer and UV/Vis spectra for the thiolate-ligated heme proteins, as well as the similarity of these values to the extremely well characterized P450\textsubscript{BM3}-PN intermediate, facilitated our assignment of these PN-generated intermediates as ferric-nitrosyl complexes. The UV/Vis data for the PN reaction and the ferric nitrosyl species in each of the thiolate-ligated heme proteins discussed can be found in Table 5.2. We have also included the freeze-quench times for the PN intermediates. The Mössbauer spectra for the thiolate-ligated heme enzyme PN-intermediates and their nitrosyl complexes are presented in figure 5.3.
Figure 5.3: Mössbauer spectra of PN intermediate (top), spectrum following subtraction of ferric component (middle), and \(\{\text{FeNO}\}_6\) complex (bottom). A) CYP119, B) P450cam, C) CPO, D) CYP154.
Peroxynitrite reaction with histidine-ligated hemes: Stopped-flow spectrophotometry

Studying the reaction of the six thiolate-ligated heme proteins with PN has allowed us to make a general statement about these reactions: *The reaction between PN and thiolate-ligated heme proteins yields a nitrosyl complex.* However, is the reaction the same in all heme proteins? That is, does the thiolate ligand influence the preference of P450s to form an $\{\text{FeNO}\}^6$ complex versus a ferryl intermediate? Previous experiments suggested that high-valent oxo species were generated during the reaction of porphyrin complexes and imidazole-ligated heme proteins with PN. For instance, HRP reacts with PN to form an intermediate capable of oxidizing 2-methoxyphenol (a common peroxidase substrate) and a Mn-porphyrin complex, MnTMPyP, could initiate DNA strand scission using PN as the oxidant.\textsuperscript{19, 22} These results supported the existence of high-valent oxo intermediates in non-thiolate ligated systems; however, we were cautious of these results because their assignment was solely based on UV/Vis spectroscopy.

To answer these questions we wanted to spectroscopically characterize the PN intermediates in horseradish peroxidase (HRP) and cytochrome c peroxidase (CCP), both of which are histidine-ligated heme proteins. Using multiple spectroscopies our goal was to determine whether PN reacts with these proteins to produce an $\{\text{FeNO}\}^6$ complex or a ferryl intermediate.

Initially, we monitored these reactions using stopped-flow spectrophotometry. The first protein examined was HRP. During this reaction, compound I (HRP-I) was formed within 15ms, decayed to compound II (HRP-II), and then back to ferric.\textsuperscript{33} Figure
Figure 5.4 (left) shows the spectra of HRP-I and HRP-II generated with peroxynitrite. This result was quite unexpected. We had clearly established that peroxynitrite reacted with thiolate-ligated heme proteins to generate nitrosyl complexes and that ferryl intermediates were not observed at any point during the reaction; however, the formation of HRP-I and HRP-II did agree with previous studies of the HRP-PN reaction. Curiously, the formation of high-valent intermediates in HRP implies that PN reacts with heme proteins differently and it is dependent on the axial ligand.

In accordance with the HRP-PN reaction, the reaction between CCP and PN also resulted in the rapid formation of an intermediate in high yield (~40 ms). The absorption spectrum for this intermediate (420, 530, 560, 635 nm) was nearly identical to that of CCP compound ES, an Fe(IV)oxo with a nearby tryptophan radical. It appeared as though a similar mechanism was at work in CCP as well.
Figure 5.4 shows a direct comparison of the UV/Vis spectra for the ferryl, PN intermediate, and ferric nitrosyl complex in both HRP and CCP. In HRP, all three species have nearly identical Soret peaks (420nm); however, the Q-bands for HRP-PN and HRP-II were significantly different than HRP-NO. The inset highlights the Q-band region and shows clear differences between the nitrosyl complex versus the PN-intermediate and compound II. The energies of the Q-bands for HRP-PN and HRP-II were 525 and 556 nm, whereas HRP-NO had peaks at 533 and 568nm. Also, the absorptivities were very different between compound-II and PN-intermediate versus the ferric-nitrosyl complex.

The Soret maxima were also very similar for the ferryl, PN-intermediate, and ferric NO complex in CCP (420nm). The Q-bands, however, were significantly different in energy and appearance (CCP-ES and CCP-PN: 530, 560 and 635 nm; CCP-NO: 535 and 571nm), which made assignment of the PN-intermediate as a ferryl species relatively straightforward. The most prominent difference between compound ES, PN-intermediate, and the ferric-nitrosyl complex was the existence of an extra peak at 635nm in compound ES and PN-intermediate.

This result raises some interesting questions in terms of how PN reacts with heme proteins. The similarity of the UV/Vis spectra of the HRP and CCP PN intermediates to their respective ferryl species implies that a similar mechanism for O-O bond cleavage occurs with hydrogen peroxide and PN. PN likely binds to the heme iron via the peroxo-portion of the molecule. The O-O bond of PN (O-ONO) is then heterolytically cleaved via the “push-pull” mechanism to generate compound I. This reactivity appears to be entirely different than what occurs in thiolate-ligated heme proteins. We have shown in
six thiolate-ligated heme proteins that addition of PN results in a nitrosyl complex. Our experiments thus far suggest that the axial ligand influences the manner by which PN reacts with heme proteins.

Peroxyinitrite reaction with histidine-ligated hemes: resonance Raman spectroscopy

Though it was apparent that we could assign the PN-intermediate in HRP and CCP as ferryl species simply from UV/Visible spectroscopy, we recognized the importance of studying these intermediates using multiple spectroscopies. To this end, we proceeded in our characterization of HRP-PN and CCP-PN to include a resonance Raman investigation of these histidine-ligated PN intermediates. We did not choose Mössbauer spectroscopy to examine these intermediates because the Mössbauer parameters for compound II and ferric nitrosyl complexes are quite similar. For example, the ferric-nitrosyl complex in HRP has a $\Delta E_Q = 1.53$ mm/s and $\delta = 0.06$ mm/s (Figure 5.5) and HRP-II has $\Delta E_Q = 1.61$ mm/s and $\delta = 0.03$ mm/s,\textsuperscript{35} which makes definitive assignment of these two species difficult.
We examined the low-frequency (475-900 cm\(^{-1}\)) and high-frequency (1075-1700 cm\(^{-1}\)) regions of the resonance Raman spectra for HRP-II, HRP-PN, HRP-NO, and ferric HRP using a 413nm excitation wavelength, figure 5.6. There are striking differences between these spectra. HRP-PN, HRP-II, and HRP-NO had similar oxidation state marker bands (\(\nu_4\)) at 1382 cm\(^{-1}\), which were blue-shifted in comparison to ferric HRP, 1376 cm\(^{-1}\). In comparing HRP-II to HRP-NO, the first noticeable difference was that the peak at 1592 cm\(^{-1}\) in HRP-II shifted to 1586 cm\(^{-1}\) in HRP-NO and ferric HRP\(^{36, 37}\). Also, HRP-II exhibited a peak at 1478 cm\(^{-1}\) that was absent in HRP-NO and ferric. As for the rest of the high-frequency spectrum, the peaks were nearly identical.
In the low-frequency region, there were several differences between HRP-II and HRP-NO. The most notable difference was the presence of peaks at 574 and 628 cm\(^{-1}\) in HRP-NO, which were absent in HRP-II. We assigned the 574 cm\(^{-1}\) stretch to the Fe-N-O
bending mode because this band was previously identified as an isotope-sensitive band related to the Fe-N-O bending mode.\textsuperscript{38}

In the past, Terner and coworkers identified a pH-sensitive stretch in HRP-II at 775 cm\(^{-1}\) (pH 6.9) that shifted to 747 cm\(^{-1}\) with \(^{18}\)O substitution. This peak was assigned as the HRP-II Fe-O stretch.\textsuperscript{39} In our experiments, HRP-II was prepared at pH 6.8. We did not resolve a peak at 775 cm\(^{-1}\), but did identify a shoulder at \(~\)780 cm\(^{-1}\). Two independent resonance Raman studies, with an excitation wavelength of 406.7 nm, determined that the intensity of the Fe-O stretch was pH-dependent with the maximum intensity observed around pH 11 (pKa \(~\)8.8). This observation likely explains our inability to resolve the Fe-O stretch in HRP-II at pH 6.8.\textsuperscript{36,39} Also, \(^{18}\)O-labelling of HRP-II showed no change in the resonance Raman spectrum at this pH. Previous experiments have indicated that the ferryl oxygen in HRP-II is readily exchangeable with bulk solvent at neutral pH, which explains why our \(^{18}\)O-HRP-II resonance Raman spectrum was identical to the \(^{16}\)O-HRP-II spectrum.\textsuperscript{36} For the abovementioned reasons, \(^{18}\)O-PN was not synthesized for resonance Raman characterization of the PN intermediates.

Despite our inability to definitively assign the Fe-O stretch in HRP-II and HRP-PN, the HRP-PN and HRP-II spectra were identical throughout both the low-field and high-field regions.\textsuperscript{36,37} Clear differences that ruled out that HRP-PN was a ferric-nitrosyl complex were the peaks at 574, 628, and 1478 cm\(^{-1}\), which were present in HRP-NO, but not present in HRP-PN or HRP-II. The most important difference was the lack of a 574 cm\(^{-1}\) peak, the \(\delta\)(Fe-N-O) mode, in the HRP-II spectrum.\textsuperscript{38} Combining our UV/Vis results with the resonance Raman, data we have confirmed our assignment of HRP-PN as a ferryl species.
Resonance Raman spectroscopy was also used to analyze the four CCP species. In the high-frequency region the oxidation state marker bands were at 1379 cm\(^{-1}\) for CCP-PN, CCP-ES, and CCP-NO, whereas the ferric CCP \(\nu_4\) stretch was 1377 cm\(^{-1}\). The CCP-NO spectrum contained two peaks in this region that were not present in CCP-ES and ferric CCP, one at 1246 cm\(^{-1}\) and the other at 1572 cm\(^{-1}\). Also, the strong peak in CCP-ES at 1555 cm\(^{-1}\), circled in Figure 5.6, was not present in either CCP-NO, or ferric CCP.\(^{40}\)

The low-frequency spectra of the four CCP species show striking differences between CCP-PN, CCP-ES, CCP-NO, and ferric CCP spectra. When we examined the CCP-NO spectrum versus that of CCP-ES, one obvious difference was that the peak at 680 cm\(^{-1}\) in CCP-ES was split into two peaks in CCP-NO (675 and 683 cm\(^{-1}\)). Other peaks present in CCP-NO that were not present in CCP-ES were the 574, 603, 709, 719, and 842 cm\(^{-1}\) peaks. The peaks at 574 and 603 cm\(^{-1}\) were nearly identical to the isotope-sensitive bands previously identified in ferric HRP-NO. In HRP, using \(^{15}\)N and \(^{18}\)O labeled NO, these bands were assigned to the Fe-N-O bending and Fe-N stretching modes, respectively.\(^{38}\) Importantly, the lack of these two peaks in CCP-PN confirms our assignment of CCP-PN as compound ES.

The CCP-ES resonance Raman spectrum also exhibited peaks that were not present in CCP-NO. These peaks were 552, 693, 754, and 813 cm\(^{-1}\). The Fe-O stretching frequency was previously identified in CCP-ES (753 and 767 cm\(^{-1}\)).\(^{40,41}\) In this region we saw significant differences between CCP-ES and CCP-NO; however, our attempt to observe an \(^{18}\)O-shift for CCP-ES at pH 6.8 was inconclusive (like HRP-II, the ferryl oxygen was found to be exchangeable with bulk solvent at neutral pH\(^{36}\)). This region of
the spectrum has been circled in figure 5.6 to highlight the differences between the spectra. As with HRP-PN and HRP-II, the resonance Raman spectra for CCP-ES and CCP-PN were identical.

Using resonance Raman spectroscopy, we have shown that, like HRP-PN, CCP-PN is not a ferric nitrosyl, but rather CCP-ES. In this example, resonance Raman spectroscopy was a straightforward means to assign the PN intermediate as many of the CCP-NO peaks were significantly enhanced compared to those of CCP-ES. We were also able to identify, through comparison with previously published data for ferric HRP-NO, the Fe-N-O bend and Fe-N stretch in CCP. Importantly, these peaks were completely lacking in the CCP-PN spectrum.

**CCP-PN EPR spectroscopy**

Because CCP-ES was characterized as an Fe(IV)oxo intermediate with a tryptophan radical, the possibility of observing a protein radical allowed us to use EPR in our characterization of CCP-PN as well. If the CCP-PN intermediate is in fact compound ES, we would expect to see a radical signal at \( g = 2 \). If, however, CCP-PN is a ferric nitrosyl species we would see no EPR signal because \( \text{FeNO}^6 \) complexes are diamagnetic and thus EPR silent. At 77K and 8mW power, CCP-PN exhibits a large radical signal around \( g = 2.00 \), which was identical to the tryptophan radical in CCP-ES (figure 5.7). This EPR evidence provided additional support for our assignment of CCP-PN as compound ES.
When we began studying the reactions of P450BM3 with PN, it was clear that the best yield of the intermediate was obtained when the reaction was performed exactly at pH 6.8. The pKa of PN is 6.8, which meant that the PN intermediate was only generated in high yield at pHs close to the pKa of PN. Additionally, it was previously shown that the yield of o- and p-nitrophenol in camphor-free P450cam at pH 7 was twice the yield of the identical reaction performed at pH 7.5, indicating that tyrosine nitration is also pH-sensitive. For both of these reasons our experiments were performed at pH 6.8.

Preliminary experiments pointed toward the fact that the PN reactions in P450BM3 and CCP were pH-dependent. This property provides insight into how PN reacts with thiolate-ligated hemes versus histidine-ligated hemes. We observed that the formation of a nitrosyl complex in the thiolate-ligated hemes was only favorable at or below the pKa

Figure 5.7: EPR spectra of CCP-PN and CCP-ES at 77K and 8mW power.
of PN. This suggests that PN must be present in the acidic form to react with the thiolate-ligated hemes proteins. Recent work on the kinetics of CPO and PN has come to a similar conclusion. They observed that both the rate of PN decay and the rate of CPO-PN formation increased as the pH decreased from 7.1 to 5.1. This work implied that peroxynitrous acid reacts with CPO. In the CCP, however, the best yield of compound II was found at or above the pKa of PN, which suggests that PN reacts as the anion in histidine-ligated heme systems.

In an attempt to explain these results, we performed density functional calculations on the protonated and unprotonated forms of peroxynitrite (B3LYP/6-311G). The optimized geometries for these structures showed that the ON-OOH bond (in both the cis and trans isomers) in peroxynitrous acid was lengthened by over a tenth of an angstrom compared to the anionic isomers. The lengthening of the ON-OOH bond in PN favors the formation of ferric nitrosyl complexes by weakening the ON-OOH bond in the thiolate-ligated heme proteins at the pKa of PN.

5.4 Conclusion

Proposed Tyrosine Nitration Mechanism

Protein inactivation as a result of tyrosine nitration has been recognized across biology. Tyrosine nitration and subsequent enzyme inactivation has been observed in several P450s when they are exposed to PN, including PGI2 synthase, P450BM3, CPO, and P450cam. The generally accepted mechanism for tyrosine nitration in P450s involved the formation of high-valent iron intermediates; however, in a recent report we
determined that the intermediate believed to be compound II in P450BM3 was actually a ferric nitrosyl complex.\textsuperscript{27}

Though the P450BM3 intermediate was not assigned properly in this work,\textsuperscript{12} there is precedence for the formation of nitrated tyrosines with nitric oxide. An extremely in depth study on the interaction of P450BM3 with nitric oxide provided clear evidence that the P450BM3 \{FeNO\}\textsuperscript{6} complex, when formed aerobically, resulted in the nitration of tyrosine 51.\textsuperscript{44} Nitration of Tyr51 caused the inactivation of wild type protein (45\% inhibition). The inactivation of P450BM3 by NO is strikingly similar to the inactivation of P450BM3 by PN; therefore, nitration of Tyr51 could be the source of inactivation in the PN reaction as well.

Quaroni \textit{et al.} claimed that because the spontaneous reoxidation of ferric P450BM3-NO occurs over the same time frame as the nitration of tyrosine residues, NO released from the heme iron could be responsible for tyrosine nitration.\textsuperscript{44} This study can also be used as a model for what has been reported for the P450BM3-PN reaction. The formation of the ferric nitrosyl complex in P450BM3 would spontaneously reoxidize to ferric P450BM3 with release of nitric oxide. All of the previously described PN reactions were performed aerobically. Therefore, the nitrotyrosines identified in the thiolate-ligated heme enzymes may be formed via a higher oxide of nitrogen (N\textsubscript{2}O and N\textsubscript{2}O\textsubscript{3}) from NO released from the heme, as was proposed for the aerobic oxidation of ferric P450BM3-NO.\textsuperscript{44}

These studies connected our observation that P450BM3, P450cam, and CPO form ferric nitrosyl complexes with PN and the fact that 3-NT residues were detected in these proteins. It is likely that our hypothesis can be extended to explain the reaction between
PN and cytochrome P450 2B1 as well. P450 2B1 is yet another thiolate-ligated heme protein that was inactivated in the presence of PN.\textsuperscript{45,46} The loss of enzymatic activity in P450 2B1 was correlated with the formation of nitrotyrosine residues; therefore, tyrosine nitration in P450 2B1 is likely accomplished via the same mechanism proposed for tyrosine nitration in P450\textsubscript{BM3}, P450cam, and CPO. The inactivation of mammalian enzymes like PGI\textsubscript{2} synthase and P450 2B1 by tyrosine nitration suggests that the inactivation of thiolate-ligated heme proteins by PN could be a significant biological concern.

Summary

In this chapter, we have demonstrated that the intermediate formed when thiolate-ligated heme proteins are reacted with PN is an \{FeNO\}\textsuperscript{6} complex. This reactivity has now been observed in six different thiolate-ligated heme proteins, indicating that this is a general phenomenon. PN instead reacts with histidine-ligated heme proteins to form high-valent oxo intermediates. Scheme 5.8 summarizes our findings for the reaction of PN with heme proteins. Our work suggests that the intermediate formed with PN is dependent on the identity of the axial ligand.
To the best of our experimental capabilities we have shown that thiolate-ligated heme proteins prefer to react with PN to form a nitrosyl complex. Reactions performed over a range of reaction times and PN concentrations have shown only the formation of a \( \{\text{FeNO}\}_6 \) complex from 8 ms to 450 ms; however, because cytochrome P450s are extremely reactive, the binding of NO to form the \( \{\text{FeNO}\}_6 \) complex could be a secondary reaction with the generation of a high-valent oxo intermediate preceding it. What we do know, however, is that a nitrosyl complex is generated in high yield as fast as \(~8\) ms after mixing P450\(_{\text{BM3}}\) with peroxynitrite suggesting that P450s catalyze the break down of PN to NO and superoxide.

There are many questions still to be addressed concerning the reaction of PN with heme proteins. Because there is differential reactivity between the histidine- and thiolate-ligated heme proteins with PN, we presume that the reaction is strongly influenced by heme axial ligation. Thiolate ligation is proposed to be the driving force in the ability of P450s to hydroxylate unactivated hydrocarbons. Thus, it is possible that the donating
nature of the thiolate-ligand, and the \( \pi \)-acidity of the \( \text{NO}^+ \) ligand, in P450s influences the binding of PN to the iron so that breaking the OO-NO bond is more favorable. A second interesting point is that if P450s cause the breakdown of PN to NO and superoxide, why would the two molecules not recombine to form PN again? Reactivity similar to this has been suggested in nitric oxide synthases (NOSs). Under uncoupling conditions, NOSs generate superoxide. If NO from normal NOS function is still in the area, then NOS will effectively become a PN synthase. Efforts to understand more about the reactivity of PN with heme proteins, as well as the products from the PN reaction with thiolate-ligated heme enzymes is necessary.

5.5 References


33. Dunford, H. B., 1982; Vol. 4, p 41-68.


38. Benko, B.; Yu, N.T., Resonance Raman studies of nitric oxide binding to ferric and ferrous hemoproteins: detection of Fe(III)--NO stretching, Fe(III)--N--O bending,


Chapter 6

Experimental Methods

6.1 Reagents

Most reagents were purchased from VWR International. Hemin, protoporphyrin IX, and δ-aminolevulinic acid were purchased from Frontier Scientific. $^{57}$Fe metal was procured from Pennwood Chemicals. Horse heart myoglobin and horseradish peroxidase were obtained as a lyophilized powder from Sigma Aldrich. $^{18}$O hydrogen peroxide was purchased as a 2% solution from Icon Isotopes. The TALON Metal Affinity resin was bought from BD Biosciences. All other resins were purchased from GE Healthcare.

6.2 Protein Expression and Purification

General Procedure for $^{57}$Fe enrichment

Cultures were grown in M9 minimal media using a 25mL starter culture. At the time of induction 1-2 mg/L of $^{57}$FeCl$_3$, 0.5 mM δ-aminolevulinic acid, and 1 mL/L of a solution of trace elements (ZnCl$_2$ • 4H$_2$O, 1g; CoCl$_2$ • 6H$_2$O, 0.2g; Na$_2$MoO$_4$ • 2H$_2$O, 1g; CaCl$_2$ • 2H$_2$O, 0.5g; CuCl$_2$, 1g; H$_2$BO$_3$, 0.2g in 1 liter of 10% HCl) were added to the cultures.
**P450\textsubscript{BM3}**

Cytochrome P450\textsubscript{BM3} (pCWori\textsuperscript{+} vector conferring ampicillin resistance) was obtained from overexpression in *E. coli* BL21 CodonPlus RIL competent cells (Stratagene) in 2xYT media. An overnight, 10mL, starter culture (37\textdegree C, 200rpm) was used to inoculate 2L culture. At an O.D. of 0.8, protein expression was induced with 0.5 mM IPTG, the temperature was reduced to 28\textdegree C, and the shaking speed reduced to 100rpm.

After 18 hours the cells were harvested and lysed using a French press. P450\textsubscript{BM3} was purified using a Co\textsuperscript{2+}-affinity column (Clontech). The protein was loaded using buffer containing 0.1M Kphos, pH 8, 300mM NaCl, and 20mM imidazole. P450\textsubscript{BM3} was eluted with the same buffer, except the concentration of imidazole was increased to 200mM. The bound imidazole was later removed using size exclusion chromatography. The fractions of P450\textsubscript{BM3} with an Rz (A\textsubscript{418}/A\textsubscript{280}) greater than 1.4 were used for Mössbauer spectroscopy.

**P450\textsubscript{cam}**

P450cam (pCWori\textsuperscript{+} vector conferring ampicillin resistance) was expressed using the same procedure as P450\textsubscript{BM3} in BL21 CodonPlus RIL competent cells. Purification of P450cam was accomplished by first loading the protein onto a DEAE sepharose column. The column was washed with 50mM KPhos pH 7.2, 500µM camphor, 2mM DTT and the protein was eluted with a 0-500mM KCl gradient of the same buffer. P450cam should elute around 150-200mM KCl. The red fractions, containing P450cam, were pooled. The pooled fractions were then brought to 60% ammonium sulfate saturation. Following
centrifugation, the resulting pellet was resuspended in 50mM KPhos, 30% saturated ammonium sulfate, pH 7.2, 500µM camphor, 2mM DTT and loaded onto a Sephacryl S200 size exclusion column. P450cam was eluted with buffer containing 0.1M KPhos, pH 7.0. P450cam used for Mössbauer experiments had an Rz (A_{417/280}) greater than 1.4.

**Streptomyces coelicolor P450s**

Both CYP154C1 and CYP158A1 were expressed and purified using the same procedure. CYP154C1 and CYP158A1 from *Streptomyces coelicolor* were overexpressed in the BL21(DE3) pLysS strain of *E. coli*. Briefly, cells were cultured overnight in LB media supplemented with 50µg/mL ampicillin (37ºC, 200rpm). The overnight culture was then diluted 1:100 in TB broth containing ampicillin and grown to an O.D. of 0.6-0.7. At this point, 0.5 mM IPTG and δ-aminolevulinic acid were added to the cultures and the temperature and shaking speed was reduced (28ºC and 100 rpm). The cultures were shaken for another 24 hrs. The cells were harvested and resuspended in lysis buffer (50mM Tris(HCl), pH 7.5, 500mM NaCl, 0.5mM EDTA, and 10% glycerol).

CYP154C1 and CYP158A1 were purified using a Ni^{2+} column (GE Healthcare). The column was equilibrated and the protein loaded using the lysis buffer from above. The column was then washed with the lysis buffer containing 3mM imidazole. The protein eluted with buffer containing 20mM Tris(HCl), pH 7.5, 10% glycerol, and 80mM imidazole. Following the metal chelating column, the protein was applied to a Q-sepharose column washed with 20mM Tris-HCl, pH 7.5 with 0.5mM EDTA and 10%
glycerol. The protein was eluted with a linear gradient of 0-500mM NaCl in 20mM Tris-HCl, pH 7.5.

For $^{57}$Fe-enrichment, CYP154 C1 and CYP158 A1 were expressed in RosettaBlue BL21(DE3) pLysS cells. Cultures were grown in M9 minimal media that was supplemented with 0.4% w/v glucose, 5mM MgSO$_4$ • 7H$_2$O, and 0.01% thiamine (for proline biosynthesis). When the cultures reached an O.D. of 0.6-0.7, 1 mg/L of $^{57}$FeCl$_3$, 1mM IPTG, 1mM δ-aminolevulinic acid, and a solution of trace elements were added. The remaining steps were identical to the rich medium.

*Thermophilic CYP119 from* Sulfolobus solfataricus

The CYP119 (pCWori vector from the Ortiz de Montellano lab) plasmid was transformed into BL21 competent cells. An overnight culture was grown in 2xYT medium with 100µg/mL ampicillin (37°C, 200rpm). The overnight culture was used to inoculate 2L of 2xYT medium. The cells were grown to an OD of 0.8-1.0 at which point the temperature and shaking speed (100 rpm) were reduced and 1mM IPTG was added. After shaking for 40hrs at 30°C the cells were harvested and lysed by sonication with lysozyme.

CYP119 was purified first by heating the cells at 60°C for 1 hour. After centrifugation of the denatured proteins, the supernatant was applied to a Q-sepharose column (GE Biosciences) equilibrated with 50mM Tris-HCl, pH 7.0. The protein was eluted with a 0-250mM gradient of NaCl. Following the anion exchange column, an S200 size exclusion column was run to further purify CYP119.
The general procedure for the production of $^{57}$Fe-enriched CYP119 in M9 minimal medium is identical to P450$_{BM3}$ and can be found above.

**Thermophilic NOS (gsNOS)**

The plasmid for gsNOS was provided by the Crane lab at Cornell University. The gsNOS gene was cloned into the pET28 vector, which confers kanamycin resistance. The protein was expressed in BL21(DE3) competent cells supplemented with 15µg/mL kanamycin. An overnight culture, in 2xYT medium, was used to inoculate a 2L culture of the same media. This culture was grown to an O.D. of 0.7-0.8 at which point 100µM IPTG and 25mg/mL δ-ALA were added. The cultures continued to grow at 37°C for another 8-10 hrs and then the cells were harvested. The cells were resuspended in lysis buffer (25mM HEPES, pH 7.5, 500mM NaCl, 10% glycerol) and lysed with lysozyme followed by sonication.

Purification of gsNOS was accomplished by metal affinity chromatography. The protein was loaded on to a Co$^{2+}$ metal affinity column and washed with 25mM HEPES, pH 7.5, 500mM NaCl, 10mM imidazole. The protein was eluted with 25mM HEPES, pH 7.5, 500mM NaCl, and 300mM imidazole. Imidazole was removed by size exclusion chromatography using 50mM Tris-HCl, pH 7.5, 200mM NaCl buffer. The molar absorptivity that was used to determine protein concentration was 120 mM$^{-1}$cm$^{-1}$ at 403nm.

Once again, $^{57}$Fe enriched gsNOS was synthesized using the procedure described above.
Yeast Cytochrome c Peroxidase

Yeast Cytochrome c Peroxidase was expressed in the BL21(DE3) Star strain of E. coli in 2xYT media with 100µg/mL ampicillin. At an O.D. of 1, the cells were induced with 0.4mM IPTG. After 6-15 hrs of vigorous shaking at 33°C, the cells were harvested and lysed by sonication. Yeast CCP is expressed as the apoprotein, thus the protein must be reconstituted with hemin. Prior to reconstitution, the apoprotein was purified by gel filtration. The apoprotein (55 mM⁻¹cm⁻¹ at 280nm) was then mixed 1:1 with hemin. The mixture was stirred on ice for 2 hours and washed with 50mM KPhos, pH 5.8. Reconstituted CCP was further purified by anion exchange (Whatman DE-52) with a 50-500mM NaCl gradient.

⁵⁷Fe-enriched CCP was generated using the same reconstitution procedure as described, but replacing the ⁵⁶Fe-hemin with ⁵⁷Fe-hemin.

6.3 Preparation of Myoglobin and HRP for Mössbauer Spectroscopy

Preparation of ⁵⁷Hemin

The metallloporphyrin synthesis of Adler et al. was adapted for the ⁵⁷Fe enrichment of hemin. ⁴ 10mg of ⁵⁷Fe metal was oxidized anaerobically to ferrous chloride at 60°C in 1M HCl. The HCl was evaporated to dryness. The FeCl₂ and protoporphyrin IX (25mg) were added to a flask containing 50mL of deaerated DMF. The starting materials were refluxed for 10-15 minutes. When the reaction was complete, the mixture was cooled on ice and exposed to air.
The reaction mixture was then diluted 10x with ether. Excess ferric salts were removed from the ether layer with 0.1M HCl containing 0.1M NaCl and excess protoporphyrin IX was removed with 1M HCl. The ether phase was then washed to neutrality with H$_2$O and evaporated to dryness. $^{57}$Fe-hemin was stored at -20ºC until use.

Before use, the hemin solution was assayed via the pyridine hemochromogen method. Hemin was used if the A$_{557}$/A$_{534}$ was greater than 1.97. Hemin was quantified based on A$_{557}$ = 34.4 mM$^{-1}$cm$^{-1}$.5

*Myoglobin Preparation*

Horse heart myoglobin, purchased from Sigma Aldrich, was used without further purification. Buffers chosen for the various pHs were 0.05M Tris(HCl) for pH 8.5, 0.1M KPhos for pH 7.0, and 0.05M sodium acetate for pH 5.2, 5.0, 4.5, and 3.5. Ferryl Mb was generated at pH 3.5 by the pH-jump method in which Mb was dissolved in low concentration buffer (5mM KPhos, pH 6) and mixed with a higher strength buffer near the desired pH. $^{57}$Fe-enriched myoglobin was prepared by reconstitution of apo-myoglobin and $^{57}$Fe-hemin as described below.

*Preparation and Purification of Reconstituted Myoglobin*

Apo-myoglobin was generated using the Teale method.6 The pH of the apoprotein solution was adjusted to pH 7. A slight excess of hemin was added to the apoprotein and stirred at 4ºC for 10 minutes. Following incubation of the apoprotein with hemin, excess hemin was removed by anion exchange chromatography (Whatman DE-52).
**Preparation and Purification of Reconstituted Horseradish Peroxidase**

HRP was purchased from Sigma Aldrich and was purified prior to use. HRP was purified to an Rz of greater than 3.0 by cation exchange chromatography (Whatman CM-52). The cation exchange column was equilibrated with 5mM sodium acetate buffer, pH 4.4. The protein was eluted with a 0-150 mM NaCl gradient. Pure HRP elutes around 50mM NaCl.

Hemin incorporation into HRP was accomplished similar to myoglobin (using the Teale method). For HRP, special attention had to be paid to the pH of the apoprotein. Efficient incorporation of hemin was only possible at pH 7. Hemin was added to the apoprotein and stirred on ice overnight. Again, excess hemin was removed from HRP by anion exchange chromatography.

**Peracetic acid synthesis**

We synthesized peracetic acid (PA) for two reasons: 1) to minimize the hydrogen peroxide contamination in the PA solution and 2) to generate $^{18}$O-peracetic acid for resonance Raman labeling. To synthesize peracetic acid 1 mole of NaCO$_3$ (8.48 g) was dissolved in a solution of 1 mole of H$_2$O$_2$ (10mL H$_2$O$_2$ in 125mL H$_2$O) and cooled to room temperature. 1 mole of acetic anhydride (7.56 mL) was then added and stirred on ice for 45min. At the completion of the reaction, 10mL of cold ether was added followed by 1 mole (4.4 mL) of concentrated H$_2$SO$_4$. The aqueous layer was saturated with ammonium sulfate and then the peracetic acid was extracted 6x with cold ether. The ether was evaporated under a stream of nitrogen. Typical hydrogen peroxide
contamination was < 5%. The amount of PA synthesized and H₂O₂ contamination was
determined using the total peroxide and hydrogen peroxide assays described below.

*Hydrogen Peroxide Assay*

The amount of hydrogen peroxide in solution was measured using an oxytitanium
was added to ~3mL of 2M H₂SO₄. 0.5mL of oxytitanium sulfate was added to the
peroxide solution, which turns the solution orange. The reaction was then diluted to
50mL with water and the absorbance at 406nm was measured. The calibration curve,
generated from plotting A₄₀₆ for the peroxide/oxytitanium sulfate assay solution versus
known concentrations of peroxide, used for the hydrogen peroxide assay was y = 932.41x
+ 0.1135; R² = 0.984. Hydrogen peroxide concentration was determined using this curve.

*Total Peroxide Assay*

The concentration all peroxides in a solution was determined by taking solution 1
(20mL of 2M H₂SO₄, 10mL of 1g/10mL potassium iodide) plus 5mL of the peroxide
solution. Solution 1 and the peroxide were reacted in the dark for 60-90 min. After
completion of the reaction, 3mL was taken out and titrated with a solution of 10mM
sodium thiosulfate (0.079g in 50mL) until the solution was clear. The concentration of
peroxide was determined from the concentration of thiosulfate titrated: 1 equivalent of
peroxide to 2 equivalents thiosulfate. Taken from Daly, R.E., Lomner, J.J., Chafetz, L.
6.4 Peroxynitrite

_Synthesis of Peroxynitrite_

PN was synthesized via the reaction of acidified hydrogen peroxide and sodium nitrite, similar to previously published procedures.\(^8\) Briefly, two syringes, one containing 0.6 M NaNO\(_2\) and the other containing 0.6 M H\(_2\)O\(_2\) in 0.7 M HCl, were mixed 1:1 via a T-junction. The reaction was quenched with 1M NaOH at 4°C. Using this method, the concentration of H\(_2\)O\(_2\) was determined to be about 3mM by the titanium sulfate spectrophotometric assay (which amounts to <5% of the PN concentration). Residual H\(_2\)O\(_2\) was removed by incubating with MnO\(_2\) on ice for 15min and the H\(_2\)O\(_2\) concentration was assayed again.

Nitrite contamination was determined spectrophotometrically using the Greiss reagent (see below). It is known that about 30% of PN decomposes to nitrite at pH 7.4.\(^9\) Once the total nitrite concentration was determined using the Griess assay, we could determine the concentration of the original nitrite contamination by subtracting the nitrite concentration originating from the decay of PN from the total nitrite measured. Using this method, there was 20-30% nitrite in the PN solution.

_Griess Assay_

The Griess assay was used to determine the amount of residual nitrite following PN synthesis. The Griess reagent consisted of 1 part 0.1% napthylethyldiamine HCl in H\(_2\)O and 1 part of 1% sulfonylamide in 5% phosphoric acid. The nitrite solution was mixed in a 1:1 volumetric ratio with the Griess reagent and the absorbance at 590nm
measured. The concentration of nitrite was determined using the following calibration
curve: \( y = 0.0154x + 0.0224, R^2=1 \). This calibration curve was generated using stock
solutions of NaNO\(_2\) and plotting the absorbance of the Griess assay product versus the
known concentration of nitrite.

6.5 Generation of Transient Intermediates Studied

*Ferryl Myoglobin for Mössbauer and resonance Raman Spectroscopies*

Mb-II, at the appropriate pH, was generated using a 10x molar excess of H\(_2\)O\(_2\), or
H\(_2\)\(^{18}\)O\(_2\) (Icon Isotopes) for the rR experiments. For the pH 8.5 and 5.2 samples,
resonance Raman and Mössbauer samples were prepared from the same Mb-II reaction
mixture enriched with \(^{57}\)Fe. These samples were prepared by generating Mb-II in the
appropriate volume for both rR and MB spectroscopies (~700\(\mu\)M) and then aliquoting the
mixture into a Mössbauer cup and an EPR tube. These samples were frozen in liquid
nitrogen after verification by UV/visible spectroscopy.

*HRP-II for resonance Raman Spectroscopy*

HRP compound II was generated according to previously published methods.\(^{10}\) HRP (300 \(\mu\)M in the appropriate buffer) was mixed with an equimolar amount of
potassium ferrocyanide. This solution was then mixed with 300\(\mu\)M H\(_2\)O\(_2\) and rapidly
frozen in an EPR tube. The protein, reductant, and oxidant were used in equimolar
quantities.
**CCP-ES for resonance Raman Spectroscopy**

CCP compound ES was generated by mixing ferric CCP (at the desired pH) with a 5-fold excess of H$_2$O$_2$. Peracetic acid was also used to generate CCP-ES with similar success.

**Ferric Nitrosyl Complexes**

Ferric nitrosyl complexes for all proteins were prepared similarly. For UV/Visible measurements, ~10 μM protein in 0.1 M KPhos, pH 7.0 was degassed and mixed with NO-saturated buffer to give a final protein to nitric oxide ratio of about 1:10. Samples for resonance Raman were generated by mixing 300μM protein in a 1:1 (v/v) ratio with NO-saturated buffer under anaerobic conditions. For Mössbauer spectroscopy $^{57}$Fe protein (1.4mM) was mixed in a 1:1 (v/v) ratio with NO-saturated buffer (1.4mM NO) in an inert atmosphere and frozen. A UV/Visible spectrum was recorded to ensure full conversion to the ferric-nitrosyl complex.

**Formation of P450 Compound II for Mössbauer Spectroscopy**

Freeze quench methods were used to generate the ferryl intermediates in P450$_{BM3}$ and P450cam. Ferryl intermediates were prepared in P450$_{BM3}$ and P450cam by reacting a solution of ferric protein (4 mM) with a 5-fold excess of peracetic acid in a 1:1 ratio for a final protein concentration of 2mM. All reagents were in 0.1M Kphos, pH 7. Reactants were mixed through the shortest aging line (5.3 μL) at 4°C and sprayed into a bath of cold isopentane, −145°C, ~4 ms after mixing. Samples were packed into a Mössbauer sample holder for analysis.
PN intermediates

All PN intermediates, with the exception of CYP119, were prepared using freeze-quench methods. In these experiments, ferric protein was mixed with a 16-fold excess of PN. Because PN is only stable at high pH (pHs above 7), the reaction conditions had to be adjusted so that the PN solution remained at high pH until immediately before the reaction was initiated. Therefore, in these experiments, the protein was exchanged into 1M Kphos, pH 6.4 and was mixed with PN in 10mM NaOH. The final pH of the reaction was pH 6.8. The syringes were kept in a bath at 12°C and the reaction was quenched in a bath of cold isopentane, −145°C. The quench times for these reactions were determined from stopped flow spectrophotometry experiments.

The CYP119-PN intermediate was fairly stable, allowing us to freeze the intermediate by hand. The experimental conditions described above were identical in this reaction, except that the reaction was frozen in liquid nitrogen immediately after mixing.

6.6 Spectroscopic and Computational Techniques

Resonance Raman spectroscopy

Our resonance Raman system consists of three lasers and a triple monochromator (Acton Research). The table setup is found in figure 6.1. We have a Coherent I-308 Argon ion, Coherent I-302 Krypton ion, and a Coherent 599 Dye Laser. Two laser tables, 4' x 6' from TMC, were co-joined to make an L-shape. The laser light was
directed from the lasers to the sample using several mirrors and a diffraction grating. The diffraction grating was used to reduce the plasma lines from laser emission.

![Diagram of laser setup](Image)

**Figure 6.1: Resonance Raman table set up.**

The 599 Dye Laser was purchased with the appropriate optics to be used over a large wavelength range that would fill in the energy gaps left by the ion lasers. The stilbene dyes were used for wavelengths between 420 and 440nm, coumarin from 530 to 560nm, and DCM Special from 620 to 670nm. Each dye was pumped by the I-308 Ar\(^+\)-ion laser (multiline UV for stilbene and multiline visible for coumarin and DCM special).

The scattered light from the sample was dispersed by the triple monochromator and detected with a back-illuminated CCD camera (Princeton Instruments, 1340 x 100...
pixels). The triple monochromator from Acton Research was most often used in a configuration in which two 900 and one 2400 gr/mm gratings were utilized in subtractive mode. However, each monochromator was equipped with three diffraction gratings for a wide energy range.

The samples were placed in an EPR finger-dewar filled with liquid N\textsubscript{2} (T = 77K) in a 135° backscattering arrangement. Light coming from the sample was collected using a collimating lens and focused into the monochromator by a second lens, figure 6.2.

![Diagram of sample configuration](image)

**Figure 6.2:** Sample configuration used for low temperature resonance Raman measurements.

Though wavenumber shifts were generally only a problem when the dye laser was used, wavenumber calibration was accomplished using the liquid nitrogen vibration (2326.5 cm\textsuperscript{-1}).\textsuperscript{11} Also, due to fluorescence background, resonance Raman spectra were often interpolated with a 20-point spline to remove this background.

*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). A magnetic field of 54 mT was applied parallel (or perpendicular) to the γ-beam. The quoted isomer shifts were relative to the centroid of the spectrum of a metallic foil of α-Fe at room temperature. Data analysis was performed using the program WMOSS from WEB research.

High field Mössbauer measurements were performed in the Krebs lab at Penn State. These measurements were performed with the sample housed in a 12SVT dewar (Janis), which also contained a superconducting magnet for the application of variable magnetic fields from 0 to 8 T parallel to the γ-beam. High field data was again fit using WMOSS.

DFT calculations

All density functional calculations discussed were performed using Gaussian 03. Large active site models were most often employed for geometry optimizations and Mössbauer calculations because they consistently gave geometries and theoretical parameters that were close to experimental values. The starting structures were taken from X-ray crystal structures of the enzymes. The models employed contained a porphine, the appropriate distal ligand, and a portion of the proximal helix. Inclusion of the proximal helix allows for the consideration of important hydrogen-bonding between
the helix and the axial-thiolate for the P450s, while the geometry constraints allowed us to examine structures that more closely resemble those found in the enzyme. For these calculations, all proximal residues except Cys, His, and Gly were converted to Ala.

Mössbauer parameters were determined at optimized geometries. During optimizations, the positions of all atoms were constrained to their position in the ferric crystal structure except Fe, the distal ligand, the porphyrin-nitrogens, alpha-carbons, meso-carbons, meso-hydrogens, and the proximal $\text{SCH}_2\text{CH}$ (P450s) and histidine residue (myoglobin). Geometry optimizations were performed at the B3LYP/6-311G level. Quadrupole splittings were also determined at the B3LYP/6-311G level. Isomer shifts were determined 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.

Models for myoglobin were slightly different than the thiolate-ligated heme proteins because myoglobin does not form a helical structure following the axial histidine residue. In the myoglobin calculations, either an imidazole ligand was used (computationally-derived model), or the histidine residue from the myoglobin compound II crystal structure was kept and truncated at the C$\alpha$ position and replaced with a methyl group. In general, the parameters were calculated using the myoglobin crystal structure model because in this model the position of the axial histidine was frozen according to its position in the crystal structure.
6.7 References


Chapter 7

Summary and Conclusions

The overriding goal of the research described in this thesis was to trap high-valent intermediates in cytochrome P450 and compare the geometric and electronic structures of those intermediates to the analogous intermediates in peroxidases, especially chloroperoxidase. These experiments eventually moved into the area of NO chemistry and peroxynitrite reactivity with heme proteins. A summary of the experimental chapters and some concluding remarks are presented below.

Chapter 2 concerned experiments on ferryl myoglobin. Recent X-ray crystal structures on Mb-II, as well as three other histidine-ligated systems, indicated that these ferryl intermediates had long Fe-O bonds, while resonance Raman, EXAFS, and Mössbauer spectroscopies on the same intermediates pointed to the formation of authentic ferryl species ($r_{Fe-O} = 1.65$ Å). The long Fe-O bonds in Mb-II, HRP-II, CCP-ES, and CAT-II signify that these intermediates are protonated. The discrepancy between the spectroscopically-determined and crystallographically-determined Fe-O bond lengths therefore was a concern.

Previous measurements on CPO compound II indicated that this intermediate was protonated at pH 6.5. This result was unexpected, since no Fe$^{IV}$OH species had been observed in an enzyme or model system. To explain this result, Green et al. turned to work performed by Mayer and coworkers on the ability of metal oxo complexes to
perform H-atom abstraction reactions. If Mayer’s work is applied to P450 chemistry, it follows that the strength of the MO-H bond formed (and C-H bond cleaved) is related to the redox potential of compound I and the pKa of compound II. Connection of the CPO-II EXAFS results to the thermochemical cycle proposed from Mayer’s work highlights the importance of the ferryl pKa in thiolate-ligated enzymes as these are the only heme proteins capable of H-atom abstraction.

To prove that protonated ferryl intermediates are indeed a unique feature of thiolate-ligated heme proteins, we first set out to determine the protonation state of Mb-II. We utilized resonance Raman and Mössbauer spectroscopy to determine whether Mb-II was protonated down to pH 3.5. We did not observe either an Fe-OH stretch, or an enlarged quadrupole splitting both of which are diagnostic of a protonated ferryl heme. Our experiments determined that the pKa of ferryl myoglobin must be less than pH 3.5, and therefore, the crystal structures have likely suffered from photoreduction (the bond lengths are actually more in line with ferric or ferrous hydroxides).

The ferryl myoglobin work also allowed us to calibrate the DFT methods used to calculate Mössbauer parameters. As was the case with the CPO models, the Mössbauer parameters calculated for the myoglobin models, which included 5- and 6-coordinate complexes, matched experiment very well. This indicated that DFT calculations could be used to obtain structural data on previously uncharacterized complexes, for instance, a protonated ferryl in a histidine-ligated heme protein. This strategy was also used in subsequent investigations of P450 intermediates.

In chapter 3 we discussed experiments in which compound II was trapped in cytochrome P450. As was mentioned in chapter 2, this characterization was based on
two observations: 1) compound II in CPO is a protonated ferryl as evidenced by EXAFS and Mössbauer spectroscopies and DFT calculations and 2) Mössbauer calculations on heme protein models revealed that the quadrupole splitting parameter is significantly larger for a protonated ferryl compared to an authentic ferryl species. The similarity between the Mössbauer parameters for P450BM3-II, P450cam-II, and CYP158-II to the well-characterized CPO-II intermediate, as well as the agreement between the calculated Mössbauer parameters and the experimental parameters, led to the assignment of this species as a protonated ferryl. The formation of a ferryl species in P450BM3 was further confirmed by high-field Mössbauer spectroscopy in which it was determined that the intermediate had an $S = 1$ ground state. Preliminary EXAFS data on P450BM3-II was presented in which the Fe K-edge was blue-shifted by about 1eV relative to ferric P450BM3. A blue-shift is indicative of an increase in the oxidation state of the iron, which is another piece of evidence in support of the generation of compound II in these P450s.

While attempting to generate compound II in a P450 in sufficient yield for spectroscopic characterization, we switched oxidants from peracetic acid to peroxynitrite. It had previously been reported that the reaction of P450BM3 with peroxynitrite resulted in the formation of an intermediate with a UV/vis absorption spectrum similar to CPO-II. The absorption spectrum for the CPO-PN intermediate was also very similar to CPO-II, therefore, it was assumed that compound II was generated in both enzymes. According to stopped-flow UV/vis, it appeared as though compound II could be generated in P450BM3 in close to 100% yield.

Because we knew the Mössbauer parameters for P450BM3-II, we could confirm the formation of compound II with peroxynitrite using Mössbauer spectroscopy. We
found that the parameters for P450BM3-PN were quite different than P450BM3-II. The intermediate did appear to be an Fe(IV) center ($\delta = 0.15$ mm/s), but the quadrupole splitting was about 1 mm/s smaller than P450BM3-II ($\Delta E_Q = 1.15$ mm/s versus 2.17 mm/s). Using DFT Mössbauer calculations, we assessed several possible reaction intermediates from the P450BM3 PN reaction. We determined that the $S = 1$, Fe(IV)oxo and the $S = 0$, $\{\text{FeNO}\}^6$ complexes were reasonable candidates for this intermediate. High-field Mössbauer spectroscopy determined that this intermediate had a diamagnetic, $S = 0$ ground state. This was our first indication that the P450BM3-PN intermediate was a ferric nitrosyl complex. This result was confirmed through a direct comparison of the UV/vis and Mössbauer spectra for the $\{\text{FeNO}\}^6$ complex and the PN intermediate in P450BM3. Indeed, the spectra were nearly identical. Resonance Raman spectroscopy was also utilized to confirm the identity of P450BM3-PN. Using $^{15}$N-labelled peroxynitrite we found two isotope-sensitive stretches in the low frequency region that were assigned to the Fe-NO stretching and Fe-N-O bending modes. On examination of the high-frequency region we identified the N-O stretch in this intermediate. This stretch also shifted with $^{15}$N-substitution. It was clear from this work that the P450BM3-PN intermediate was an $\{\text{FeNO}\}^6$ complex and not a ferryl intermediate.

Next, in chapter 5, we examined the reaction of peroxynitrite with five other thiolate-ligated heme proteins (3 P450s, CPO, and NOS). We were curious as to whether $\{\text{FeNO}\}^6$ complexes were generated in all thiolate-ligated heme proteins, since several previous investigations on this reaction suggested that these intermediates were ferryl species by UV/vis spectroscopy. Using stopped-flow UV/vis and Mössbauer
spectroscopy, we determined that PN reacts with all thiolate-ligated heme proteins to generate ferric nitrosyl complexes.

A significant amount of literature concerning the reaction of heme proteins with PN is available. As was mentioned, in each case these intermediates were characterized as ferryl species solely by UV/vis spectroscopy. Although insufficient spectroscopic data was presented to support the assignment of these intermediates, there was other experimental data to support this claim. We were skeptical of these results. We decided to re-examine the PN intermediates in cytochrome c peroxidase and horseradish peroxidase using UV/vis and resonance Raman spectroscopies. We compared the UV/vis and resonance Raman spectra for compound II, the PN intermediate, and the \( \{\text{FeNO}\}^6 \) complex in these two proteins. Our results confirmed that ferryl intermediates are generated in histidine-ligated heme proteins using PN. This result was interesting because it indicated that the PN reaction in heme proteins is dependent on the axial ligand. This was not the first time that P450 reactions have been influenced the axial ligand; recall that under similar conditions authentic ferryls are generated in histidine-ligated heme proteins while protonated ferryls were generated in thiolate-ligated hemes. At this time we are still unsure of what factors are involved in the reaction of PN with heme enzymes.

The role of the thiolate ligand in P450s has been a topic of investigation for several decades. Experiments suggest that the thiolate ligand could be involved in O-O bond cleavage to facilitate compound I formation, stabilization of the Fe(IV)oxo intermediates, and controlling the redox potential of the enzyme. The work presented in this thesis has shown that the axial ligand is involved in determining the ferryl pKa in
compound II as well as influencing the reaction of heme enzymes with peroxynitrite. It is clear that the role of the axial ligand in heme enzyme chemistry still remains an open question.
Appendix

Spectroscopic Characterization of the Intermediate Generated in CYP154 with Peracetic Acid

A.1 Results and Discussion

The spectroscopic characterization of compound I in a P450 was the underlying goal in many of the experiments that were attempted during my PhD career. In yet another attempt to trap P450-I we reacted CYP154 C1, a P450 enzyme from Streptomyces coelicolor, with peracetic acid (PA). CYP154 C1 will be referred to as CYP154 throughout the rest of this section. Using the same conditions from our analysis of compound II in P450BM3, 5-fold excess of PA at pH 7, we generated an intermediate that was partially green in color. This was exciting to us because CPO-I is also green, due to its radical character, which prompted the characterization of this intermediate by EPR and Mössbauer spectroscopies.

We began the characterization this intermediate using EPR spectroscopy. The spectrum of the intermediate quenched at 8ms is shown in figure A.1. The asterisks represent peaks from a ferric CYP154 contamination (~40%). The CYP154-PA spectrum shows the appearance of three new peaks (\(g = 2.33, 2.21,\) and 1.94) and a large radical peak \(~g = 2.0\). The spectrum of the new intermediate, however, did not resemble that of CPO-I \((g = 2.008\) and 1.73)\).
We next annealed the CYP154-PA sample to monitor changes that to the EPR spectrum. The annealing temperatures and times are reported for each successive annealing step. The spectra are shown in Figure A.2 (top). Interestingly, annealing of the sample resulted in the disappearance of ferric CYP154 when the sample was kept at −80°C for a total of 45min. Annealing the sample at -35°C for 15min resulted in a similar spectrum. When CYP154-PA was further annealed at -10°C for 15min, the new intermediate decayed and ferric CYP154 grew back in. Finally, when the sample was thawed and refrozen a spectrum identical to ferric CYP154 resulted. This indicated that there were no covalent modifications to the heme during the reaction.

The bottom spectrum in Figure A.2 shows the radical signal during the annealing process. Interestingly, the maximum intensity of the radical spectrum occurred when the amount of ferric CYP154 was a minimum and the new intermediate a maximum.

This annealing experiment indicated that there was a chemical reaction taking place at −80°C. Ferric CYP154 was consumed at this temperature. Changes in the EPR spectrum were observed within 15min of annealing at −80°C. No more changes occurred after 45 min at this temperature. Above about −50°C, the intermediate began to decay and ferric CYP154 was regenerated. These preliminary results suggest that the ferric CYP154 that disappeared during the annealing experiment was converted to the CYP154-PA intermediate. This is interesting because it appears as though a chemical reaction is occurring at −80°C; however, more experiments are necessary to confirm this hypothesis.
Figure A.1: EPR spectrum of the CYP154-PA intermediate (20K and 2mW power). The asterisks represent the peaks due to ferric contamination.
Figure A.2: Top: EPR spectrum of annealed CYP154-PA sample. The annealing conditions are indicated next to the spectra. Bottom: Radical signal during the annealing of CYP154-PA. Maximum intensity occurred on annealing the sample for 45min at –80ºC.
We examined the CYP154-PA intermediate by Mössbauer spectroscopy, figure A.3. Both spectra shown in figure A.3 have had the ferric contribution subtracted, about 40%. We found that the intermediate displayed a magnetic spectrum as indicated by the multiline spectrum. We placed the sample in a perpendicular magnetic field to identify changes in the spectrum as a result of the orientation of the magnetic field. If, on the other hand, a species has an integer spin ground state, the spectrum should be unaffected by the direction of the magnetic field. Figure A.3 shows the Mössbauer spectra in both parallel and perpendicular fields. These measurements revealed that the CYP154-PA spectrum was only slightly affected by switching the magnetic field.

Figure A.3: Mössbauer spectra (4.2K/54mT) of CYP154-PA after subtraction of ~40% ferric CYP154. The top spectrum is in a parallel magnetic field and the bottom is in a parallel magnetic field.
We also annealed a CYP154-PA Mössbauer sample. The EPR annealing indicated that chemistry was occurring as we warmed up the sample. Because EPR cannot follow diamagnetic species, we also performed a similar annealing experiment using Mössbauer spectroscopy. The temperatures with the largest changes were chosen for this investigation. The Mössbauer spectra are shown in figure A.4. Annealing of the original sample at –80°C for 30min resulted in significant changes to the Mössbauer spectrum (figure A.4, middle). We then annealed the same sample for 15min at –72°C. This annealing step resulted in the appearance of a quadrupole doublet (in about 10% yield) that was fit to $\Delta E_Q = 0.77$ mm/s and $\delta = 0.10$ mm/s. We are still unsure of the identity of the annealed species at this point.

The last experiment that we performed was the cryoreduction (CR) of the CYP154-PA sample. If this is a high-valent paramagnetic species we would expect the formation of a quadrupole doublet upon cryoreduction. In these experiments, ferric CYP154 would be reduced to ferrous CYP154. Figure A.5 shows the spectra of CYP154-PA prior to CR, following CR, and the subtraction of the two spectra. After subtracting the CYP154-PA spectrum from the CR-CYP154-PA spectrum we saw the formation of a quadrupole doublet with parameters similar to a ferrous heme, but did not agree with those of CR-ferric CYP154. No Fe(IV)-like species were generated, which implies that we did not generate compound I in this reaction. Further experiments probing the identity of CYP154-PA are necessary.
Figure A.4: 4.2K/54mT Mössbauer spectra of annealed CYP154-PA reaction. Top is the original sample, middle is the spectrum of the species following annealing at –80°C for 30min, and the bottom is the spectrum following annealing at –72°C for 15min.
A.2 References


Figure A.5: 4.2K/54mT Mössbauer spectra of CYP154-PA (top) and CR-CYP154-PA (middle). The bottom is the species generated upon cryoreduction. Approximate parameters for this quadrupole doublet are \( \Delta E_Q \sim 2.2 \text{ mm/s} \) and \( \delta \sim 0.6 \text{ mm/s} \).

VITA

Rachel Koren Behan

Education:
Providence College, Providence, RI 02918, **B.S. Biochemistry**, May 2002.
The Pennsylvania State University, University Park, PA 16802, **Ph.D. Chemistry**, 2008.

Research:
Advisor: Michael T. Green
Thesis Title: “Spectroscopic Investigations of High-Valent Intermediates in Cytochrome P450s and Other Heme Enzymes”.

Publications: