UNDERSTANDING C-H BOND ACTIVATION IN HEME PROTEINS: THE IMPORTANCE OF THE FERRYL PKA

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by

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ABSTRACT

The major focus of the Green group involves the study of C-H bond activation by heme proteins and the elucidation of the factors giving rise to this potent chemistry. Because only thiolate ligated heme enzymes such as cytochrome P450 (P450), chloroperoxidase (CPO), and aromatic peroxidase (APO) are capable of difficult hydrocarbon oxidations, we believe that Nature has specifically chosen the thiolate ligand in order to facilitate H-atom abstraction. Current evidence suggests that the strong donating nature of the thiolate elevates the pKa of the compound II (ferryl) species, and could be the reason for the increased driving force. Conversely, it is proposed that a lower ferryl pKa in histidine and tyrosine ligated heme enzymes significantly decreases the reactivity, thus preventing C-H bond activation. In an effort to provide concrete evidence for the magnitude of the proposed driving force, we set out to quantitate the thermodynamic “pKa” parameter in histidine, thiolate, and tyrosine ligated heme systems.

While it is has been shown that the thiolate ligated P450-II and CPO-II intermediates are basic in nature, there is much controversy over the protonation status of histidine ligated ferryls. X-ray crystallographic studies favor long Fe-O bond lengths (~1.85 Å), indicating protonated (Fe$^{IV}$-OH) moieties, while EXAFS report much shorter distances (~1.65 Å), classifying them as authentic Fe$^{IV}$=O intermediates. If histidine ligated ferryls are basic, then theory suggests that they could be able to activate C-H bonds, yet no experimental evidence supports this claim. In order to investigate this controversy, we explored the protonation status of the histidine ligated myoglobin
compound II (Mb-II) intermediate over a wide pH range (9.5 → 3.9). Using a battery of spectroscopic techniques (Mössbauer, Resonance Raman, and EXAFS) our results definitively show that Mb-II is an authentic Fe$^{IV}$ oxo with a pKa ≤ 2.65. We can infer from this study that all histidine ligated ferryls are Fe$^{IV}$ oxos (at physiological pH), and that previous crystallographic reports for protonated ferryls in these systems are a direct result of radiation damage.

Although we could only establish an upper limit for the pKa in histidine ligated proteins, we were able to directly measure the compound II pKa in both thiolate (CYP158A2 with pKa ~ 12) and tyrosine (Helicobacter pylori catalase with pKa ~ 13) ligated systems. These values represent the first ever reported pKas for any Fe$^{IV}$-OH species. Relative to histidine ligated enzymes, the elevated pKa in thiolate and tyrosine ligated ferryls could account for an additional ~ 13 kcals of driving force in a given hydrocarbon oxidation. Unexpectedly, tyrosine ligated hemes have higher ferryl pKas than their thiolate ligated counterparts, suggesting that CPO, APO, and P450 may not be the only heme proteins capable of C-H bond activation.
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Chapter 1

Introduction

1.1 Heme Proteins

Heme proteins are one of the most ubiquitous classes of metalloenzymes in nature. They are primarily involved in, but are not limited to, oxygen transport, catalysis (biosynthesis and detoxification), and electron transfer chemistry. Some heme proteins are multi-functional, but slight structural variations between enzymes typically allow for specialization in one of the aforementioned areas.

The active site is comprised of an iron coordinated tetrapyrrole macrocycle (protoporphyrin IX), Figure 1.1. At least one of the axial coordination sites is occupied by an amino acid ligand (proximal) that tethers the heme prosthetic group to an intricately folded protein superstructure. Depending on the type of heme family, the identity of this ligand is in the form of a histidine (nitrogen), tyrosine (oxygen), or cysteine (sulfur) residue. The chemistry usually occurs at the distal site, but influences from the proximal ligand and heme periphery, including interactions with first sphere amino acid residues, are believed to play a role in tuning the reactivity of each enzyme. Some of the most prominent heme families are globins, peroxidases, catalases, and cytochrome P450 (P450s).
Figure 1.1: Fe bound protoporphyrin IX and UV/visible spectrum of ferric CYP158A2. Porphyrin $\pi \rightarrow \pi^*$ transitions give rise to the Soret and Q band features that make heme proteins rich in color.$^1$

1.1.1 Globins

The most common histidine ligated heme enzymes are globins and peroxidases. Both types of enzymes contain the same active site coordination, but differences in the folds of the protein, as well as the packing of the heme groups, substantially alter their respective function. The differences in the heme environment in globins lead to a higher reduction potential for the active site iron, which favors the stability of an Fe$^{2+}$ porphyrin. This allows for an exceptional binding affinity for dioxygen, as in hemoglobin and myoglobin (Mb), and is otherwise not possible in the Fe$^{3+}$ resting state observed in peroxidases.$^3, 4$ While the role of globins is primarily oxygen transport, in vivo reactions with peroxides are believed to be biological relevant as well, playing a role in reperfusion injuries, arteriosclerosis, and kidney dysfunction.$^5-8$
1.1.2 Peroxidases

Traditional heme peroxidases are histidine ligated and act as metabolizing enzymes in fungal, plant, and mammalian organisms. They play a physiological role in the biosynthesis of hormones, host defense (against infections), and the pathogenesis of some inflammatory diseases. Their natural substrate, hydrogen peroxide, generates high valent peroxidase intermediates that can oxidize/metabolize small molecules such as phenolic derivatives. The two electron oxidized form of the ferric resting state is known as compound I, an Fe$^{IV}$=O porphyrin radical (formally Fe$^{V}$) that acts as the active oxidant in heme reactions. The one electron reduced form of compound I is known as compound II, an authentic Fe$^{IV}$=O species. The major intermediates playing a role in the reaction chemistry are shown below, **Figure 1.2**.

![Figure 1.2: Peroxidase reaction cycle. The ferric resting enzyme is oxidized two electron equivalents by hydrogen peroxide to form the Fe$^{IV}$=O porphyrin radical compound I species. In the presence of substrate compound I is reduced by one electron to form compound II (Fe$^{IV}$=O). Subsequent substrate oxidation regenerates the ferric resting enzyme.](image)

**“Push-Pull” Effect**

There are important features in the active site pocket that are believed to facilitate the formation of the high valent, oxidizing species in peroxidases. This machinery is provided in the form of key residues located on the proximal and distal sides of the heme site. Poulos proposed that these residues allow for a type of “push-pull” mechanism to
help facilitate O-O bond cleavage after binding the natural peroxide substrate, Figure 1.3.

After the ferric enzyme binds hydrogen peroxide, the proximal histidine group provides a “pushing” effect. The source of this push is believed to be from the electron donating ability provided by the strong imidazolate character of the axial histidine. The “push” is enhanced by a hydrogen bond to a nearby carboxylate group. As the “push” effect is being created by the axial side of the heme site during substrate binding, a distal histidine residue acts as a proton acceptor. A proton is “pulled” away from the Fe bound oxygen and transferred to the other oxygen in the hydrogen peroxide molecule, generating the stable H$_2$O leaving group. The resulting heterolytic O-O bond cleavage is facilitated and stabilized by a nearby positively charged arginine residue. This interaction acts to stabilize the negative charge of the outer oxygen during bond cleavage. Together, the histidine and arginine residues on the distal side of heme site provide the “pull” effect.

Globins possess very low peroxidase activity, partially due to a lack of a hydrogen bond to the proximal group, resulting in less imidazolate “push” character. Additionally, the distal histidine in globins is much closer to the active site than in peroxidases (3.79 Å in HRP-II compared to 2.69 Å in Mb-II). It is postulated that this close proximity destabilizes compound I (the active oxidant) by providing a pathway to readily oxidize an active site amino acid residue. This type of mechanism is in accordance with the observation that Compound I is only transiently generated in Mb (wild type), which would also contribute to the diminished peroxidase activity.
Figure 1.3: The “push-pull” mechanism for peroxidase enzymes. The effect is proposed to facilitate O-O bond cleavage in order to generate compound I. Figure adapted from Sono, M.; Roach, M. P.; Coulter, E. D.; Dawson, J. H., Heme-Containing Oxygenases. Chemical Reviews 1996, 96 (7), 2841-2888.

1.1.3 Catalase

Normal metabolic processes can generate hydrogen peroxide as a by-product. To prevent harmful oxidations that can ultimately damage the cell, the tyrosine ligated catalase enzyme facilitates a disproportionation reaction to generate dioxygen and water.\textsuperscript{16}

\[
2\text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2\text{H}_2\text{O}
\]

Eq. 1.1

Although hydrogen peroxide is the natural substrate, catalase is also capable of oxidizing formaldehyde, formic acid, phenol(s), and alcohol(s) in its high valent forms. A similar peroxidase-like “push-pull” effect is believed to facilitate the enzyme’s high reactivity.
In place of the proximal histidine is a tyrosine ligand, which provides the strong “push” to help cleave the di-oxygen bond of the bound peroxide. Distal asparagine and histidine residues are believed to help transfer protons to a single oxygen, generating water as a good leaving group.16

### 1.1.4 Cytochrome P450

P450s are cysteine ligated heme enzymes that act as mono-oxygenases, inserting oxygen into hydrocarbon chains via H-atom abstraction.17-19 No other class of heme enzymes (with axial histidine or tyrosine ligation) is known to perform this demanding chemistry. For this reason, it is generally believed that the axial thiolate plays an influential role in hydrocarbon oxidations. The general form of P450 catalysis is as follows:12

\[
P_{450} + \text{NAD(P)H} + \text{R-H} + \text{O}_2 + \text{H}^+ \rightarrow \text{NAD(P)}^+ + \text{ROH} + \text{H}_2\text{O} + P_{450} \quad \text{Eq. 1.2}
\]

The human genome encodes for 57 different P450 enzymes, 5 of which are present in the liver and are responsible for the phase I metabolism of ~ 75 % of all pharmaceuticals. They also play a role in the biosynthesis of signaling molecules used for control of development and homeostasis.20, 21 While P450s differ from other heme enzymes in many ways, the major differences include the following; 1) They lack the distal residues that provide the “pull” effect during substrate binding 2) Their natural substrate is dioxygen rather than peroxide 3) They possess a proximal sulfur ligand in the form of the strong anionic cysteine residue.12

Until recently, many of the important intermediates in the consensus P450 reaction cycle had yet to be characterized, Figure 1.4. Briefly, the resting ferric enzyme
is in a six coordinate, low spin state with water bound at the distal site. The presence of substrate promotes the loss of the water molecule, generating a 5 coordinate high spin complex. This conversion from low to high spin results in an increase of the active site redox potential, which facilitates electron transfer from the reductase domain. The one electron reduction yields a high spin ferrous state that readily binds dioxygen. A second reducing electron (also from the reductase domain) is accompanied by protonation of an oxygen atom, forming a ferric hydroperoxide complex. An additional protonation of the same oxygen leads to the heterolytic bond cleavage of the dioxygen bond. With water as the leaving group, this generates the “active” oxidant, compound I.\textsuperscript{12,22} In the presence of substrate, compound I abstracts hydrogen (H\textsuperscript{+}) and is reduced by one electron to the protonated compound II species, also known as the \textit{rebound intermediate}. Radical substrate then readily recombines to form hydroxylated product and regenerate the resting ferric enzyme.\textsuperscript{12}

\textbf{Figure 1.4:} The consensus P450 reaction cycle. Figure taken from Behan, R. Spectroscopic Characterization of High-Valent Intermediates in Cytochrome P450s and Other Heme Enzymes. Pennsylvania State University, University Park, PA, 2008.
The Rebound Intermediate

Groves postulated that P450 catalysis proceeds by way of an oxygen rebound mechanism. The idea was based on the observation of large kinetic isotope effects \((K_H/K_D \sim 11)\) during oxidation of norbornane by a reconstituted liver cytochrome P450. These results provided strong indication for an initial H-atom abstraction of substrate and the subsequent generation of a carbon radical intermediate.\(^{17,19,23}\)

Electrons from the Reductase Domain

There are two types of electron transport protein systems in P450s that are used to deliver the pair of electrons in catalysis. One type involves flavin and Fe-S proteins while the other requires only flavins.\(^{17}\) Typically, bacterial and mitochondrial P450s utilize a system where electrons flow from nicotinamide adenine dinucleotide phosphate \([NADP(H)]\) to a flavin adenine dinucleotide (FAD) containing protein to an Fe-S protein to the P450. Conversely, microsomal P450s use the single flavoprotein, NADPH-cytochrome P450 reductase, to transport electrons directly to the protein.\(^{12,24}\)

Peroxide Shunt Mechanism

The high valent intermediates generated in the P450 catalytic cycle can also be generated via an alternate pathway, without dioxygen and a reductase electron transport system. This can be accomplished through the “peroxide shunt” or “short circuit” route which utilizes oxygen atom donors such as alkyl peroxides and peracids.\(^{17}\)
“Push” Effect of the Proximal Thiolate in P450

Because P450s lack the distal machinery required to heterolytically “pull” apart dioxygen in the hydroperoxo complex, this class of enzymes is believed to rely on the powerful internal electron donating ability of the proximal sulfur atom. Sufficient proximal “push” effect, coupled with a distal proton donation, is believed to provide enough driving force to facilitate the heterolytic O-O bond cleavage\textsuperscript{25} to generate the active oxidant, compound I \textsuperscript{22}. Sligar has proposed that the source of protons enabling the outer oxygen in the hydroperoxo complex to leave as water is provided by a charge relay involving nearby Thr and Asp residues\textsuperscript{26}, Figure 1.5.

**Figure 1.5:** The “push” mechanism for cytochrome P450. The effect is proposed to facilitate O-O bond cleavage in order to generate compound I. Figure adapted from Sono, M.; Roach, M. P.; Coulter, E. D.; Dawson, J. H., Heme-Containing Oxygenases. *Chemical Reviews* **1996**, *96* (7), 2841-2888.
1.2 H-atom Abstraction

C-H bonds are generally thought of as being very inert, with bond strengths on the order of ~ 100 kcal/mol, yet P450s are able to functionalize these “unfunctional” groups with ease. They can catalyze a wide variety of reactions in unactivated alkanes, including hydroxylations, epoxidations, and dehydrations (to name a few). Because C-H bonds decorate the structures of biomolecules, pharmaceuticals, and petroleum products, the ability to selectively cleave C-H bonds represents one of the most broadly applicable classes of chemical transformations known. For this reason, the elucidation of the factors giving rise to P450s’ potent catalytic ability has become a major scientific goal.

It is widely believed that a thorough structural characterization of the intermediates involved in catalysis would help provide the insight needed to understand how Nature functionalizes C-H bonds. However, the high valent forms of P450 have, until recently, remained mostly elusive. This is in stark contrast to the intermediates in histidine and tyrosine ligated heme enzymes, which have been captured in quantitative yields. The transient nature of P450s has been attributed to their increased reactivity (relative to histidine and tyrosine ligated systems). As a result, most of what is known about their reactivity comes from the study of a unique analogue, chloroperoxidase (CPO). This structurally similar, thiolate ligated peroxidase is not as potent as P450, but is capable of hydroxylating activated hydrocarbons (89 kcals/mol). Most importantly, the CPO intermediates involved in catalysis (compound I/II) have been structurally characterized by extended X-ray absorption fine structure spectroscopy (EXAFS), electron nuclear double resonance (ENDOR), and resonance Raman spectroscopies.
Because CPO and P450 can perform comparable chemistry, it has generally been assumed that the intermediates playing a role in the hydroxylation chemistry are similar.

**Figure 1.6.**

![Diagram of thiolate ligated cytochrome P450](image)

**Figure 1.6:** Rebound mechanism of the thiolate ligated cytochrome P450. The resting ferric enzyme is oxidized by two electron equivalents to an Fe$^{IV}$ porphyrin radical species (formally Fe$^V$), denoted as compound I (red). In the presence of substrate compound I abstracts hydrogen and is reduced to the protonated compound II ferryl intermediate (blue). The radical substrate then readily recombines to form the hydroxylated product, regenerating the ferric resting species (black).

At this point, it is helpful to turn to the synthetic metal oxo work of Jim Mayer in order to provide a better understanding of the P450 reaction mechanism. Using insight from Polanyi and Bordwell, Mayer sought out to determine what general features allow for H-atom transfer reactions. His work led to the discovery that the ability of a metal oxo to abstract hydrogen generally scales with the energy difference between the O-H bond being formed in the rebounding intermediate (compound II in heme enzymes) and the C-H bond being broken in the substrate. This linear free energy relationship (LFER) between the log of the rate constant (log k) and the strength of the substrate bond being broken suggests that to perform C-H bond activation, the O-H bond strength generated in the rebound intermediate must be of the same or greater magnitude as the C-H bond being cleaved. The O-H bond strength of the hydrogen-abstracting metal oxo
species, D(O-H), can be obtained through a thermodynamic cycle. If written in terms of P450 chemistry, the equation becomes the following.

$$D(O-H) = 23.06 \ E^0_{\text{Comp-I}} + 1.37 \ \text{pKa}_{\text{Comp-II}} + C \ (\text{kcal/mol}) \quad \text{Eq. 1.3}^*$$

From this equation, it can be seen that the ability to abstract hydrogen from substrate depends on the compound I redox potential and the compound II pKa. Because nature is limited by the self destructive nature of extremely high redox potentials, it is postulated that the (donating) axial thiolate ligand may raise the pKa of compound II. Support for this claim stems from the finding that both CPO-II and P450-II are basic ferryl intermediates with pKas ≥ 8.2. This differs from the traditional (histidine ligated) peroxidase compound II intermediate, which features an authentic Fe(IV) oxo moiety (pKa ≤ 3.5) and is not known to activate C-H bonds. If the pKa is high, as found in CPO-II/P450-II, this would allow for H-atom abstraction at a viable compound I reduction potential. By diminishing the driving force for one electron oxidations, thiolate ligation could bias the system towards H-atom abstraction. Evidence for this notion has been provided by experiments that suggest cysteine ligation can lower heme redox potentials by as much as 400 mV relative to histidine ligation. This creates an oxidant capable of cleaving C-H bonds (~ 100 kcal/mol) while avoiding unwanted oxidations of the protein superstructure.

* “C” is a constant that depends on the solvent and reference electrode. Its value is 57 +/- 2 kcal/mol for aqueous solution with E\text{\textsuperscript{0}} versus NHE.
1.3 The Importance of Ferryl Basicity

Synthetic investigations have generally supported this hypothesis, providing a reason for why Nature has chosen a donating ligand to carry out such demanding reactions. Much of the work done by Goldberg and Borovik confirms that synthetic complexes with low reduction potentials are capable of H-atom abstraction. The ability of these low potential oxidants (LPO) to perform H-atom abstraction is credited to the basicity of the oxo ligand. A Goldberg Mn(IV)OH ($E^0 = + 0.19$ V) and a Borovik Mn(II)OH ($E^0 = -1.3$ V) complex, both capable of abstracting hydrogen from dihydroanthracene ($D(C-H) \approx 78$ kcal/mol), have $pK_a \geq 15$ (acetonitrile) and 36 (DMSO) respectively.

Nam and Que complemented these studies by examining the role of the axial ligand in oxygen transfer and H-atom abstraction reactions. They used a series of FeIV(O)(TMC)(L) complexes where the axial ligand (L) was varied in order to determine the effects of electron donation. The series included compounds with L=NCCH$_3$, CF$_3$OO$^-$, N$_3^-$, and SR$^-$ and clearly show a LFER between the rate of H-atom abstraction and the strength of the substrate, $D(X-H)$. The study showed that the rate of abstraction scales inversely with the electrophilicity of the ferryl species, making the thiolate complex most reactive. The thiolate complex also possessed the lowest redox potential ($E^0 = -0.3$ V).

These synthetic experiments parallel the structure and chemistry of CPO/P450, indicating that LPOs with high pKas can perform hydrocarbon oxidations. In Nature, it has been argued that histidine ligated peroxidases have small, restricted active sites that are specifically designed for small molecule oxidations, thus preventing oxygen insertion.
reactions. While it is plausible that a restricted active site prevents substrate binding to the peroxidase’s iron-oxo subunit, synthetic work suggests that regardless of substrate accessibility, the axial ligand is still responsible for tuning reactivity in these systems. This lends credence to the argument that reactivity discrepancies in histidine and thiolate ligated hemes stem from the donating ability of their respective axial ligands (magnitude of the ferryl pKa).

1.4 Ferryl Protonation Status in Heme Enzymes

The equation for D(O-H) suggests that the magnitude of the ferryl pKa plays a major role in the reactivity of heme enzymes. Ferryl intermediates in CPO and P450 have been shown to be protonated (pKa ≥ ~ 8.2), while contradicting structural characterizations have assigned histidine ligated ferryls as either a protonated (X-ray crystallography) or unprotonated (EXAFS) species. If histidine ligated compound II intermediates are protonated at physiological pH, then their potential O-H bond strengths could be similar to CPO/P450. This could allow for C-H bond activation, yet no experimental evidence suggests this possibility.

In order to provide additional evidence that the driving force for C-H bond activation lies heavily on a high ferryl pKa, it is important to resolve the issue of ferryl protonation status in heme enzymes. The following section will discuss the controversial issue, as well as a number of tools we have at our disposal to provide an accurate structural interpretation of the ferryl active site.
Ferryl pKa Controversy: X-Ray Crystallography vs. EXAFS

The controversy surrounding ferryl protonation stems from the different techniques used to structurally characterize heme intermediates: X-ray crystallography and EXAFS spectroscopy. Because of the more transient nature of thiolate ligated ferryl intermediates, only non-thiolate ligated ferryls have been studied using X-ray crystallography. Using this technique, ferryl intermediates in HRP-II (pH 6.5), myoglobin-II (Mb-II, pH 5.2) and cytochrome c peroxidase-I (CCP-I, pH 6) have all been characterized with long Fe-O bond lengths (~ 1.85 Å), indicating protonation. Alternatively, EXAFS studies yield much shorter Fe-O bond lengths (~ 1.65 Å), classifying them as authentic Fe(IV) oxos.58, 59

Badger’s Rule

The contradicting bond distances in ferryl intermediates (X-ray crystallography vs. EXAFS) were evaluated by Green in 2006 using Badger’s rule, a semi empirical correlation (relationship) between Fe-O bond length and stretching frequency. Green initially took a series of heme complexes and used DFT to calculate both $r_e$ (bond length) and $v_e$ (vibrational stretching frequency) for the Fe-O bonds.58 After applying an experimental scaling parameter, the rule (best fit line) was compared to the experimentally determined values (both EXAFS and X-ray crystallography) for heme proteins, Figure 1.7.
Figure 1.7: Badger’s rule for heme proteins. The slope of the best fit represents a semi-empirical correlation between bond distance and stretching frequency. All circles are (scaled) calculated values. The two yellow circles represent calculated values for well known complexes that were used to evaluate the scaling factor of the stretching frequencies. Diamonds are experimental values, black represents resonance Raman and X-ray crystallography while yellow corresponds to resonance Raman and EXAFS experiments. Figure taken from Green, M., Application of Badger's Rule to Heme and Non-Heme Iron− Oxygen Bonds: An Examination of Ferryl Protonation States. J. Am. Chem. Soc 2006, 128 (6), 1902-1906.

Badger’s rule establishes a clear relationship between resonance Raman stretching frequencies and the bond distances obtained from EXAFS. The only outlying EXAFS point is an HRP-II study that was carried out at high temperature. In this study, and in the remaining X-ray crystallographic studies, photoreduction of the samples is suspected. To date, there is no definitive spectroscopic evidence for a protonated histidine ligated compound II intermediate or an unprotonated thiolate ligated compound II intermediate.
1.5 Demonstration of Spectroscopic Methods: Determining the Ferryl Protonation Status in CPO and HRP

In order to provide additional evidence for the different protonation states in heme enzymes (histidine vs. cysteine ligation), it is illustrative to consider a structural comparison between the histidine ligated HRP-II and the thiolate ligated CPO-II intermediates. The spectroscopic arsenal of EXAFS, resonance Raman, and Mössbauer will be paired with DFT calculations to definitively show the ferryl protonation status. These methods serve as the cornerstone for the determination of ferryl pkas determined in later chapters. The importance of multiple structural characterization techniques cannot be overstated, especially when irradiating samples with high energy X-rays to probe redox-active metal sites.

EXAFS of CPO-II and HRP-II

CPO-II was structurally characterized by Green et al. in 2004 by EXAFS spectroscopy. While EXAFS is unable to detect X-ray scattering from protons, the long Fe-O bond of 1.82 Å implied a basic ferryl. A rigorous absorption-edge analysis was used to confirm that the data used for fitting was not photoreduced.37 HRP-II was also characterized by Green in 2004. The Fe-O bond length in the histidine ligated HRP-II intermediate was significantly shorter than in CPO-II, with an authentic oxo bond length of 1.67 Å, Figure 1.8. This distance is in good agreement with the previous EXAFS structural characterization of Penner-Hahn et al.37,64
Resonance Raman of CPO-II and HRP-II

Direct structural evidence for the existence of a protonated ferryl in CPO was later confirmed through resonance Raman spectroscopy. The Fe$^{IV}$-OH stretching frequency at 561 cm$^{-1}$ shifted to 549 cm$^{-1}$ in D$_2$O, in perfect agreement with a two body harmonic oscillator prediction [Fe-(OH)]$^{38}$ Figure 1.9. In HRP-II, Terner performed an extensive Raman study at pHs ranging from 3.5 – 11. Low pH samples showed an Fe$^{IV}$=O stretching frequency at 778 cm$^{-1}$ that shifted to 744 cm$^{-1}$ using $^{18}$O, also in good agreement with oscillator predictions. Deuterium substitution led to small 1-2 cm$^{-1}$ upshifts in the stretching frequency. These upshifts are indicative of hydrogen bonding between the ferryl oxo and a nearby protonated histidine residue and have been ruled out as a possible protonation event.$^{65}$ The Fe-O stretching frequencies for HRP-II and CPO-II are also in good agreement with Badger’s rule predictions for the experimentally determined EXAFS bond distances (763 cm$^{-1}$ and 563 cm$^{-1}$).$^{58}$

![Figure 1.8: EXAFS Fe-O bond distance results for CPO-II (left) and HRP-II (right).](image)
Figure 1.9: Resonance Raman Fe-O stretching frequencies for CPO-II (left) and HRP-II (right). Left figure taken from Stone, K.; Behan, R.; Green, M., Resonance Raman spectroscopy of chloroperoxidase compound II provides direct evidence for the existence of an iron (IV)–hydroxide. *Proceedings of the National Academy of Sciences* **2006**, *103* (33), 12307. Right figure adapted from Terner, J.; Palaniappan, V.; Gold, A.; Weiss, R.; Fitzgerald, M. M.; Sullivan, A. M.; Hosten, C. M., Resonance Raman spectroscopy of oxoiron(IV) porphyrin π-cation radical and oxoiron(IV) hemes in peroxidase intermediates. *Journal of Inorganic Biochemistry* **2006**, *100* (4), 480-501.
DFT Calculations

Compound II in heme enzymes exists in a low spin, $S = 1$ active site environment. In the absence of a strong magnetic field, the Mössbauer spectrum of this intermediate is a single quadrupole doublet, Figure 1.10. The splitting between the doublet, or $\Delta Eq$, is believed to directly reflect the protonation status of ferryl intermediates.\textsuperscript{62}

![Figure 1.10: A generic Mössbauer spectrum of an integer spin system (low field). $\sigma$ represents the isomer shift and gives information about the oxidation state. $\Delta Eq$ is the quadrupole splitting and can yield information about the electric field gradient and shape of the nucleus.](image)

Support for this claim stems from a series of DFT calculations performed on the histidine ligated Mb active site. The oxidation states, spin states, and coordination environments were varied so that comparisons with experimental values could be used to tune the accuracy of the prediction method. Pertinent results are shown in Table 1.1.
Table 1.1: Experimental and calculated Mössbauer parameters for a series of myoglobin complexes with different distal ligands. Table adapted from Behan, R. Spectroscopic Characterization of High-Valent Intermediates in Cytochrome P450s and Other Heme Enzymes. Pennsylvania State University, University Park, PA, 2008.

<table>
<thead>
<tr>
<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>
</tr>
</thead>
<tbody>
<tr>
<td>CO</td>
<td>II</td>
<td>0.31 0.35</td>
<td>0.29 0.50</td>
<td>0.27 0.35</td>
</tr>
<tr>
<td>O₂</td>
<td>II</td>
<td>0.29 -1.94</td>
<td>0.27 -1.97</td>
<td>0.27 -2.31</td>
</tr>
<tr>
<td>None</td>
<td>II</td>
<td>0.81 -2.76</td>
<td>0.73 -2.70</td>
<td>0.92 -2.22</td>
</tr>
<tr>
<td>CN⁻</td>
<td>III</td>
<td>0.23 -2.11</td>
<td>0.22 -2.21</td>
<td>0.16 -1.46</td>
</tr>
<tr>
<td>N₂⁻</td>
<td>III</td>
<td>0.31 -2.07</td>
<td>0.29 -2.21</td>
<td>0.24 -2.25</td>
</tr>
<tr>
<td>None</td>
<td>III</td>
<td>0.39 1.22</td>
<td>0.35 1.33</td>
<td>0.42 1.24</td>
</tr>
<tr>
<td>OH⁻</td>
<td>IV</td>
<td>-0.01 2.78</td>
<td>-0.02 2.73</td>
<td>— —</td>
</tr>
<tr>
<td>O²⁻</td>
<td>IV</td>
<td>0.10 0.98</td>
<td>0.08 1.02</td>
<td>0.11 1.46</td>
</tr>
</tbody>
</table>

Based on the available data, it can be seen that DFT does a relatively good job at predicting experimental parameters for the heme active site. Calculations show a substantial difference in the ΔEq for a Fe(IV)-OH (2.75mm/s) and an authentic Fe(IV)=O (1.00mm/s) moiety, suggesting that the magnitude of the electric field gradient increases considerably as the Fe-O bond elongates due to protonation, Figure 1.11.

Figure 1.11: Theoretical histidine ligated heme models and predicted Mössbauer Spectra for the unprotonated (red, ΔEq = 1.00 mm/s) and protonated (blue, ΔEq = 2.75 mm/s) ferryl intermediates. Figure adapted from Behan, R. Spectroscopic Characterization of High-Valent Intermediates in Cytochrome P450s and Other Heme Enzymes. Pennsylvania State University, University Park, PA, 2008.
Experimental Mössbauer Comparison between CPO-II and HRP-II

While both the CPO-II/HRP-II intermediates have isomer shifts indicative of a Fe\textsuperscript{IV} oxidation state (0.10/0.11 mm/s), their quadrupole splitting values are considerably different (2.05/1.46 mm/s).\textsuperscript{67, 68} Figure 1.12. These parameters follow the same trend that DFT predicts, with protonated ferryls having much larger quadrupole splittings. Additionally, the ΔEq for HRP-II is in accordance with previously prepared synthetic porphyrin oxo species.\textsuperscript{69-72} To date, there are no Mössbauer reports for a synthetic Fe\textsuperscript{IV}-OH complex.

![Figure 1.12: Mössbauer comparison of CPO-II (left) and HRP-II (right). The quadrupole splitting is 2.06 mm/s for CPO-II and 1.61 mm/s for HRP-II. Right figure adapted from Dunford, H. B., *Heme Peroxidases*. Wiley-VCH: New York, 1999.](image)

This spectroscopic comparison has definitively shown the protonation status of the histidine ligated HRP-II (Fe\textsuperscript{IV}=O) and the thiolate ligated CPO-II (Fe\textsuperscript{IV}-OH) intermediates. We believe that the difference in reactivity between these two classes of enzymes can be directly linked to this pKa disparity. Using the O-H bond strength equation, a ΔpKa of ~ 5 units (≥ 8.2 in CPO-II and ≤ 3.5 in HRP-II) results in a ~7 kcal/mol difference in potential driving force for C-H bond activation. This gap in
driving force may potentially be even larger, but to date there have been no quantitative measurements of the ferryl basicity in heme enzymes.

1.6 Goal

The overall goal of this thesis is to quantitate the ferryl pKa in three different heme systems using the methods of UV/visible, resonance Raman, EXAFS, and Mössbauer spectoscopies (in conjunction with DFT). We will explore the relationship between the donating ability of the axial ligand and the ferryl basicity of compound II in the following enzymes; (1) The histidine ligated Mb-II (2) the thiolate ligated CYP158A2-II and (3) the tyrosine ligated *Helicobacter pylori* catalase compound II (HPC-II). By providing a quantitative value for the ferryl pKa, the role of the axial ligand in heme catalysis can be thoroughly evaluated.

1.7 Summary of Chapters

Many of the experiments involved in our studies require ms freeze-quench instrumentation to “trap” and cryogenically preserve transient intermediates so that they may be spectroscopically characterized at low temperature. Chapter 2 will evaluate pertinent factors involved in the millisecond (ms) cryogenic freezing process. Specifically, the “communication” of pH during the (rapid) freeze-quench mixing process and the stability of buffered solutions at low temperature will be discussed.

Chapter 3 will focus on the histidine ligated Mb protein. Recent crystal structures have provided additional controversy for the protonation status of histidine ligated ferryl intermediates. Because the active site of Mb is structurally similar to peroxidases, we can
use it as a model to evaluate the structure and chemistry of this related heme family. Our study of Mb-II at a wide range of pH values provides a definitive answer to the controversy surrounding ferryl protonation status.

Chapter 4 will discuss the ferryl protonation status of the thiolate ligated CYP158A2 enzyme. While we have previously shown that the ferryl forms of CPO and P450 are basic,$^{37,63}$ the limited pH range over which they could be observed prohibited the determination of a pKa. The CYP158A2 enzyme has provided us with a system where the ferryl moiety can be prepared in unprecedented yield (> 90%) over a wide range of pH values, allowing for the direct opportunity to explore the relationship between the axial ligand identity and ferryl basicity. Here, for the first time, we provide direct spectroscopic evidence for the ferryl pKa using stopped-flow, Mössbauer, and EXAFS spectroscopies.

The tyrosine ligated HPC enzyme will be discussed in Chapter 5. Previous reports detailing a pH dependent UV/visible spectral change suggest a pKa of the ferryl intermediate of $\sim 8.4$. The investigation clarifies this result, and provides Mössbauer evidence for a true ferryl pKa of the system. This determination of a second ferryl pKa allows for comparison with the thiolate ligated CYP158A2 system, providing insight for the true role of axial ligand identity in heme proteins.

Chapter 6 will detail the experimental methods used to carry out the experiments discussed in this thesis. Detailed results can be found in each respective chapter.


1.8 References


71. Weiss, R.; Mandon, D.; Wolter, T.; Trautwein, A. X.; Müther, M.; Bill, E.; Gold, A.; Jayaraj, K.; Terner, J., Delocalization over the heme and the axial ligands of one of the two oxidizing equivalents stored above the ferric state in the peroxidase and catalase Compound-I intermediates: indirect participation of the proximal axial ligand of iron in


Chapter 2

Proof of Concept Experiments: the Stability of pH at Low Temperature and the Communication of pH on the Freeze-Quench Timescale

2.1 Significance of “Concept” Experiments

In our effort to quantitate the ferryl pKa in different heme systems, we had to characterize the various intermediates at pH extremes (< pH 4 and > pH 12). Because most heme intermediates are not stable at these pHs, we utilized the pH-jump method. This method involves sample preparation in a stable, low ionic strength buffer, where the intermediate yield is the greatest. The solution is then rapidly mixed (jumped) in a high ionic strength, different pH buffer, so that the final pH of solution is effectively that of the mixing buffer. In order to avoid rapid decay and/or degradation of the intermediate, especially at pH extremes, the use of rapid freeze-quench (mixing) instrumentation is implemented. This technique allows for samples to be mixed and cryogenically trapped in < ~ 15 milliseconds (ms), allowing for preservation and subsequent characterization.

Before a discussion of the spectroscopic results, one must first examine the integrity of the methods used to trap and cryogenically preserve the transient intermediates being studied. The following chapter will discuss two pertinent factors involved in preparing rapid freeze-quenched samples; Part 1) The Stability of pH in Buffered Solutions at Low Temperature and Part 2) The Communication and
Equilibration Time of pH Upon Rapid Freeze-Quench Mixing. The experiments discussed here will serve as a “proof of concept”, assuring that the pH is both equilibrated and stable in any future freeze-quench experiments. This will allow us to accurately track the pH of the transient intermediates being studied in later chapters, thus providing an accurate representation of the proposed driving force/ferryl pKa in heme enzymes.

2.2 Introduction: the Stability of pH in Buffered Solutions at Low Temperature

The importance of pH control in research is universally recognized. It is responsible for the stability and preservation of biological systems. While pH can be directly measured in solution, little is known about the stability of buffered systems in the frozen state. This uncertainty stems from a series of studies illustrating that the pH of buffers can change substantially with temperature, particularly below the freezing point. This can become especially concerning when dealing with low temperature food preservation, or in other biological systems where there is risk for injury during the cooling process.

Colorometric indicators have been used to show that phosphate buffers can drop 2-3 pH units over the temperature range 20°C to -196°C. Measurements in tris-HCl buffer suggest an increase of 2-3 pH units over the same temperature range. In light of this knowledge, most cryogenic enzyme characterizations fail to address this phenomenon. Depending on the buffer, temperature, and pH, there could be considerable alterations or degradation to the system. Structural interpretations including the pKas of pertinent residues hinge on the “true” pH of the frozen solution.
The reasons behind the temperature dependent pH changes in buffered solutions are not completely understood. Major influences can involve salt solubility\textsuperscript{1,2,6,8} and pKa changes of the ionizable groups participating in the buffering process\textsuperscript{4,5}. Other factors could involve solute-solvent interactions and activities\textsuperscript{3}. For sodium phosphate buffer, the change in pH is due to the saturation of the dibasic salt at low temperature\textsuperscript{1,2,6,8}. In buffers where salt solubility is not an issue, the change in pH with temperature is believed to be influenced by the ionization enthalpy of the buffering agent or residue. Depending on the thermicity of this process (endo or exothermic), a change in temperature can significantly drive the reaction towards a protonated or deprotonated form\textsuperscript{9}. The result is a change in the Ka, and hence the pKa of the buffering agent\textsuperscript{4,5}. Depending on how substantial the change of the pKa, the pH of the system can be altered significantly at temperature extremes.

Interestingly, it has been shown that the temperature related changes in pH are suppressed when buffered solutions are supplemented with considerable concentrations of protein. Williams-Smith et al. observed that in the presence of 600 μM albumin, pH changes in tris and phosphate buffers become appreciably smaller, about 0.2 - 0.5 pH units\textsuperscript{7}. However, these suppression effects were only evaluated at liquid nitrogen temperatures (~ -196°C). They were never extended to temperatures at/near the liquid helium range (~ -269°C), where our Mössbauer and EXAFS spectroscopies are performed.

In this study, we look to provide additional insight on the temperature related pH changes in buffered solutions by examining the suppression effects of mM protein concentrations at liquid helium temperatures (~ 4.2K). Horse heart myoglobin (Mb) will
be used because of its unique pH dependent structural change occurring at pKa ~ 8.93. At this pH, the 6 coordinate ferric aqua adduct (Mb\textsuperscript{III}-OH\textsubscript{2}, high spin) deprotonates to an alkaline hydroxide form (Mb\textsuperscript{III}-OH, low spin).\textsuperscript{10} This results in substantial UV/visible and Mössbauer spectral changes that will allow us to accurately track the pH changes in frozen buffered protein solutions (based on the percentage of each form of Mb over the range 20°C to -269°C).

**Sample Preparation**

Briefly, 4 mM ferric Mb (20 mM tris, pH 8.98 at 20°C) was prepared at a pH close to the pKa of the Mb\textsuperscript{III}-OH\textsubscript{2} → Mb\textsuperscript{III}-OH transition. The solution was pipetted into a Mössbauer cup and hand quenched into liquid ethane (~ -160°C). Simultaneously a dilute sample, in the same buffer, was analyzed by UV/Vis (4 - 20°C) so that it could be compared to the low temperature Mössbauer spectrum. The percent composition of each ferric form in the tris buffer was determined by adding a combination of the pure Mb\textsuperscript{III}-OH\textsubscript{2} and Mb\textsuperscript{III}-OH visible spectra together until the reconstructed data overlaid with the experimental, **Figure 2.1.** Percentages from Mössbauer were obtained through spectra subtraction.
2.2.1 Results and Discussion

Figure 2.1: UV/visible spectra of ferrie Mb from pH 6.40 → 11.00. The pure Mb$^{III}$-OH$_2$ form is shown in black and the pure Mb$^{III}$-OH form is in purple. Red and blue traces indicate that the pH of tris buffer is temperature dependent.

Figure 2.2: Mössbauer spectra (4.2 K) of 4 mM ferrie Mb in 20 mM tris buffer (pH 8.98 at 20°C). Spectrum A is the raw data containing Mb$^{III}$-OH$_2$ and Mb$^{III}$-OH in a ~ 22:78 ratio, suggesting a pH of 9.50. Spectrum B is the Mb$^{III}$-OH spectrum obtained by removing the contribution of Mb$^{III}$-OH$_2$ (red) from the raw data.
At 20°C, in pH 8.98 tris buffer, ferric Mb is 47% Mb\textsuperscript{III}-OH\textsubscript{2} and 53% Mb\textsuperscript{III}-OH. Upon cooling this same protein solution to 4°C, the UV/visible spectral changes indicate the pH has risen to 9.13 (38% Mb\textsuperscript{III}-OH\textsubscript{2} and 62% Mb\textsuperscript{III}-OH), Table 2.1. The low temperature Mössbauer study (-269°C) reveals that the 4 mM Mb sample is 22% Mb\textsuperscript{III}-OH\textsubscript{2} and 78% Mb\textsuperscript{III}-OH, Figure 2.2. This ratio suggests a pH of 9.50 at -269°C, a change of 0.52 units from 20°C. The high concentration of Mb (4 mM) has acted to suppress the full effects of temperature change on the pH of tris buffer at liquid helium temperatures. This result is in accordance with the previously reported suppression effects of albumin at liquid nitrogen temperatures.\textsuperscript{7}

In the case of Mb, the suppression of pH change at low temperature may be due to the protein acting to “buffer” the buffer. As the temperature is decreased to -269°C the pKas’ of the 55 ionizable residues in Mb\textsuperscript{11} (32 positively charged side groups and 21 negatively charged side groups) may change, depending on their thermicity. Some increase and others decrease, possibly resulting in a zwitterionic pseudo buffer relative to the tris-HCl. Although not all of these residues may be exposed to bulk solvent (thus being available for buffering), a large concentration of protein could substantially increase the number of residues available. For instance, if all Mb’s 19 lysine residues are ionizable and available for buffering, then a 4 mM sample would have 76 mM lysine available for the buffering of tris.

### Table 2.1: Corrected pH of 20 mM tris-HCl/protein solution (pH 8.98 at 20°C) at various temperatures.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>New pH</th>
<th>% Mb\textsuperscript{III}-OH\textsubscript{2}</th>
<th>% Mb\textsuperscript{III}-OH</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>8.98</td>
<td>47</td>
<td>53</td>
</tr>
<tr>
<td>4</td>
<td>9.13</td>
<td>38</td>
<td>62</td>
</tr>
<tr>
<td>-269</td>
<td>9.50</td>
<td>22</td>
<td>78</td>
</tr>
</tbody>
</table>
2.2.2 Conclusion

It is not clear if the suppression of pH change at low temperature is due to the specific makeup of the protein, *ie.* the pKas and buffering capacity of specific amino acid residues, or if this phenomenon stems mainly from reorganizational effects of high protein concentrations in solution. For the case of tris-HCl buffer, it is clear that the temperature induced pH changes are significantly reduced by high concentrations of Mb and albumin. Further studies, using a variety of different proteins with varying amino acid makeup, concentrations, and buffers, are needed to learn more about these suppression effects. This may help to explain discrepancies between cryogenic and aqueous characterizations on proteins conducted at the “same” pH. 7

2.3 Introduction: the Communication and Equilibration Time of pH Upon Rapid Freeze-Quench Mixing

The characterization of many transient enzyme intermediates has been made possible with freeze-quench instrumentation. Traditional freeze-quench instruments allow for the rapid single/double mixing and freezing of solutions on the ms time scale, Figure 2.3. Even with proper, homogenous mixing one wonders how much time the mixture needs before it is equilibrated. Quite simply, the question posed is, “how fast is too fast?” While the formation and decay of the enzyme intermediate can be monitored by spectroscopic methods, it is much more difficult to determine if the solvent surrounding the protein, as well as inside the active site, is equilibrated. Protonation events, hydrogen bonding, and pKas of the intermediate can be convoluted by questions concerning the conditions of the solvent at the time of freezing. Additionally, the
concern of the pH inside the active site pocket becomes an issue because it presumably takes more time for bulk solvent to diffuse into the pocket of the protein following mixing.

Figure 2.3: Diagram of a two (single mix) and three syringe (double mix) stopped-flow or freeze-quench setup.

In this study, we hope to provide insight on the communication of pH on the ms time scale. We also look to address the issue of how long it takes for the pH inside the active site pocket to be equilibrated with external solvent after mixing. Aside from experimental parameters, the size of the access channel from bulk solvent to the active site, as well as the actual volume of the active site pocket, is highly variable in proteins and most likely plays a large role in this process. In this account, horse heart myoglobin (Mb), which has a very hydrophobic active site with limited accessibility\textsuperscript{12}, will be used as a model for other more accessible proteins. Inference can then be made on the speed
of pH communication in other proteins, based on size and pocket accessibility comparisons relative to Mb.

As discussed in the previous section (Part 1), Mb undergoes a change in the protonation state of the distal ligand (pKa ~ 8.93) whereby the six coordinate ferric aqua adduct (Mb\textsuperscript{III}\textsuperscript{-}OH\textsubscript{2}) deprotonates to an alkaline form (Mb\textsuperscript{III}\textsuperscript{-}OH), Figure 2.1. This structural change has been documented and can be monitored using UV/visible, resonance Raman\textsuperscript{13}, and Mössbauer spectroscopies. In Raman, the Fe-O stretch in the alkaline form is enhanced and can be identified via deuterium substitution (556 cm\textsuperscript{-1} – 543 cm\textsuperscript{-1}), Figure 2.4.

![Figure 2.4](image)

**Figure 2.4:** Resonance Raman of Mb\textsuperscript{III}\textsuperscript{-}OH (pH 10.9, 100 mM borate buffer). Data was collected using the 413 nm krypton laser line.

It can also be seen that the resonance Raman spectra of Mb\textsuperscript{III}\textsuperscript{-}OH and Mb\textsuperscript{III}\textsuperscript{-}OH\textsubscript{2} are substantially different, Figure 2.5. We used this difference to show the communication of the active site pH with bulk solvent after rapid ms freeze-quench.
mixing. This was done by first preparing Mb at pH 10.9 (Mb$^{\text{III}}$-OH), and then rapidly mixing/freeze-quenching the solution against a low pH buffer (pH-jump) so that the equilibration pH was $\sim$ 7 (Mb$^{\text{III}}$-OH$_2$). After mixing, the sample traveled through a 3 ms aging line before being sprayed into liquid ethane (-160°C). We estimate that the sample is completely “frozen” within $\sim$ 10 - 15 ms of entering the cryosolvent. For comparison, a hand-quenched (HQ) experiment was performed using the same protein and buffer solutions. The HQ mixture was equilibrated for $\sim$ 1 min before freezing to ensure that the pH was equilibrated. If pH is communicated on the ms mixing timescale, then the Raman spectra of the freeze-quenched (FQ) and HQ samples should be almost identical, ie. both samples should be equilibrated at pH 7 (Mb$^{\text{III}}$-OH$_2$).

**Figure 2.5:** Resonance Raman comparison of Mb$^{\text{III}}$-OH$_2$ and Mb$^{\text{III}}$-OH. Data was collected using the 413 nm krypton laser line.
2.3.1 Results and Discussion

Figure 2.6: Resonance Raman comparison of HQ and FQ pH-jump experiments from pH 10.9 → 7. Mb$^{III}$-OH (2 mM) was prepared in borate buffer (20 mM, pH 10.9) and mixed/frozen against high strength sodium phosphate buffer so that the equilibrating pH was 7. Data was collected using the 413 nm krypton laser line.

Both the FQ and HQ samples have virtually identical Raman spectra, and demonstrate full conversion (> ~95%) from Mb$^{III}$-OH to Mb$^{III}$-OH$_2$, Figure 2.6. It is clear that there is a change of pH occurring at the active site in the FQ sample, but is the pH completely homogenous both inside the active site of the protein and in the bulk solvent surrounding the protein at the time of freezing? It could be possible that the pH was merely partially communicated, lowering considerably upon FQ mixing, but still requiring more time to fully equilibrate. If the active site pH only reached 8 (~ 90% Mb$^{III}$-OH) at the time of freezing, it would be difficult to discern this Raman spectrum from a pH 7 spectrum (> ~95% Mb$^{III}$-OH).
In order to probe this question, we looked to see if deuterium exchange could occur on the freeze-quench time scale inside the hydrophobic Mb active site, *i.e.* Fe$^{III}$-OH$\rightarrow$Fe$^{III}$-OD. In this experiment, 4 mM Mb$^{III}$-OH (100 mM borate buffer, pH 10.9) was mixed 1:2 with a 99% deuterated solution (100 mM borate buffer, pD 11.3) so that a final, equilibrated solution should be $\sim$ 66% isotopically labeled. A HQ experiment was performed using the same solutions and frozen after 60 seconds of equilibration time.

Again, the Raman spectra of the HQ and FQ sample are almost indistinguishable, Figure 2.7. By adding combinations of the pure Mb$^{III}$-OH and Mb$^{III}$-OD Raman spectra together, we were able to deduce that the experimental samples (HQ and FQ) were both $\sim$ 66% deuterated. If the solution were only $\sim$ 75% equilibrated at the time of freezing, which would be analogous to the previous experiment if the pH-jump from 10.9 $\rightarrow$ 7 only reached pH 8 at the time of freezing, then the final FQ sample would have only been $\sim$ 50% deuterium labeled. This data suggests that the mixing buffer has reached the hydrophobic active site, and has had ample time to equilibrate, as evidenced by the exchange of an active site proton with the deuterated mixing solvent.
Figure 2.7: Resonance Raman comparison of HQ and FQ Mb$_{III}$-OH mixes. Mb$_{III}$-OH (4 mM) was prepared in borate buffer (pH 10.9, 100 mM) and mixed/frozen 1:2 against deuterated borate buffer (pD 11.3, 100 mM) so that the resulting mixture was ~ 66% isotopically labeled. Data was collected using the 413 nm krypton laser line.

2.3.2 Conclusion

Freezing times as fast as < 100 microseconds (μs) have been reported with copper roller freeze-quench devices (including mixing dead time). Using this device, mixing studies with Mb and sodium azide show significant binding after 630 μs (50%), with 100% formation of the azide bound form at ~ 3.3 ms.$^{14}$ While this study shows how fast substrate can reach the active site under homogenous mixing conditions, it provides limited information about solvent equilibration times following mixing.

Our freeze-quench setup has been used to show that pH is fully communicated in Mb on the ~ 10 - 15 ms timescale. While we do not have access to μs quenching devices, the aforementioned Mb azide binding study using copper rollers provides evidence that mixtures may equilibrate much faster than the ms time scale. Our freeze-quench
experiments imply that proteins with more accessible, larger active sites should have pH equilibrations times (after mixing) either faster or on the same time scale as Mb.
2.4 References


Chapter 3

Myoglobin Compound II: Setting an Upper Limit on the Ferryl pKa

3.1 Abstract

There has been much discrepancy in reported Fe-O bond distances for ferryl intermediates in histidine ligated heme proteins. Crystallographic studies report long Fe-O bond lengths (1.84-1.92Å) for horseradish peroxidase-II (HRP-II, pH 6.5), myoglobin-II (Mb-II, pH 5.2) and cytochrome c peroxidase-I (CCP-I, pH 6), suggesting a protonated species, while extended X-ray absorption fine structure spectroscopy (EXAFS) studies best describe these intermediates as authentic iron (IV) oxos (1.65-1.70 Å).\(^1\) It has previously been proposed that the basic ferryl provides the driving force for C-H bond activation in chloroperoxidase (CPO) and cytochrome P450 (P450), and is unique to thiolate ligated systems.\(^2,3\) If histidine ligated ferryl intermediates are also basic in nature, then intuition suggests that they could be capable of similar chemistry. At the center of this controversial protonation topic is Mb-II. We have probed the protonation status of this intermediate over the pH range 9.5→3.9 using the spectroscopic arsenal of EXAFS, Mössbauer, and resonance Raman spectroscopies. Results show no indication of protonation at low pH, setting an upper limit on the ferryl pKa (< 2.65). We believe that previous crystallographic reports for basic ferryls in histidine ligated heme proteins are a direct result of radiation damage, and we can infer from this study that no histidine ligated ferryl is protonated at physiological pH.
3.2 Introduction

High valent ferryl species have been identified as the active oxidant in a number of important biological processes.\(^2\) P450 and CPO are the only known heme enzymes that utilize these intermediates to functionalize C-H bonds.\(^4,5\) P450 can mono-oxygenate unactivated hydrocarbons while CPO can hydroxylate some activated hydrocarbons and oxidize chloride.\(^5,6\) Their potent oxidative power is believed to be linked to their unique axial thiolate coordination.\(^2,3\) A scheme for the intermediates playing a role in the hydroxylation chemistry is shown below, Figure 3.1.

**Figure 3.1:** Rebound mechanism of cytochrome P450. The resting ferric enzyme is oxidized by two electron equivalents to an Fe\(^{IV}\) porphyrin radical species (formally Fe\(^{V}\)), denoted as compound I (red). In the presence of substrate compound I abstracts hydrogen and is reduced to the protonated compound II ferryl intermediate (blue). The radical substrate then readily recombines to form the hydroxylated product, regenerating the ferric resting species (black). Bonds and bond strengths, D(X-H), discussed in the text are indicated. The equation for the O-H bond strength is given in terms of the one-electron reduction potential of compound I and the pKa of compound II. C is a constant that depends upon the solvent and reference electrode. Its value is 57 ± 2 kcal/mol in aqueous solution.

\[
D(O-H) = 23.06 \ E^0_{\text{Comp-I}} + 1.37 \ pKa_{\text{Comp-II}} + C \text{ (kcal/mol)}
\]

Mayer has shown that ground state thermodynamics – the energy difference between the O-H bond formed and the C-H bond broken - are generally sufficient to understand C-H bond activation in such systems.\(^7-9\) In heme enzymes, the energy of this
bond, D(O-H), is dependent on the redox potential of compound I and the pKa of compound II. It has been proposed that the basic ferryl, afforded by thiolate ligation, promotes C-H bond activation at (significantly) reduced one electron reduction potentials by elevating the pKa of compound II. This belief relies on the premise that basic ferryls are a general and unique feature to thiolate ligated hemes.\textsuperscript{2,3}

While it is has been shown that the thiolate ligated P450-II and CPO-II intermediates are basic in nature,\textsuperscript{3,10} there is much controversy over the protonation status of histidine ligated ferryls.\textsuperscript{1} If histidine ligated ferryls are basic, then Mayer’s work (OH bond strength equation) suggests that these systems could be able to activate C-H bonds, however there is no experimental evidence to support this claim. Thus, an accurate ferryl protonation assignment is of significance because it would help to clarify the true role of the axial ligand in heme systems.

3.3 Background

Photoreduction in XAS

The controversy over the protonation status of ferryl intermediates in histidine ligated heme proteins (HRP-II, Mb-II, and CCP-I) stems from a series of X-ray crystallography and EXAFS studies. The nature of the long Fe-O bond assigned by crystallographic studies (\(\sim 1.85 \text{ Å}\)) suggests a protonated ferryl, while the short bond obtained by EXAFS (\(\sim 1.65 \text{ Å}\)) opposes this claim. Green illustrated this disparity through the application of Badger’s rule to ferryl intermediates in various heme proteins, Figure 3.2. The parameterized rule shows good correlation between EXAFS bond distances and the Fe-O stretching frequencies (resonance Raman), while crystallographic
bond distances deviate substantially from the line of best fit. The long Fe-O bond from crystallographic reports are thought to be a direct result of radiation damage. To date, there is no definitive evidence, *ie.* resonance Raman reports of an Fe$^{IV}$-OH stretch, for a protonated ferryl intermediate in histidine ligated heme enzymes.

![Figure 3.2](image)

**Figure 3.2:** (Left) Badger’s rule for heme proteins. The slope of the best fit represents a semi-empirical correlation between bond distance and stretching frequency. All circles are (scaled) calculated values. The two yellow circles represent calculated values for well known complexes that were used to evaluate the scaling factor of the stretching frequencies. Diamonds are experimental values, black represents resonance Raman and X-ray crystallography while yellow corresponds to resonance Raman and EXAFS experiments. (Right) The bottom axis is Fe-O bond distance. The top axis is the parameterized vibrational frequency obtained from Badger’s rule. The top and bottom rows are the results of experimental investigation. Open diamonds are vibrational frequencies obtained by resonance Raman experiments. Blue diamonds are EXAFS measurements while Red correspond to X-Ray crystallography. Experiments were performed on “A” HRP-II, “B” CCP-I, “C” Mb-II, “D” CAT-II, and “E” the ferryl form of TauD. The ellipse highlights the agreement between theory, EXAFS, and resonance Raman experiments. Figures taken from Green, M., Application of Badger’s Rule to Heme and Non-Heme Iron–Oxygen Bonds: An Examination of Ferryl Protonation States. *J. Am. Chem. Soc* **2006**, *128* (6), 1902-1906.

Poulos and Schlichting also investigated the bond distance discrepancy between X-ray crystallography and EXAFS by studying the effects of prolonged radiation exposure on heme crystalline metalloproteins. Specifically, oxidative damage of the
redox-active metals (Fe) and its effect on the structure and coordination of the active site were considered. Photoreduction was monitored by changes in the single crystal absorption spectra (microspectrophotometry) that were taken before and after crystal exposure.11,12

Using CCP-I as an example, Poulos demonstrated a clear trend depicting the elongation of the Fe-O bond with increased radiation dose (from 1.73-1.90 Å).12 Schlichting showed that ferric Mb (Mb\textsuperscript{III}-OH\textsubscript{2}) rapidly reduces to a ferrous state during similar radiation exposure experiments. Her results indicate that the maximum change of the absorption spectrum occurs at 2 Mgy of exposure, just 15 seconds into data collection, and well before the 30 Mgy dose limit determined for cryo-cooled protein crystals. Schlichting also details a compelling figure, indicating substantial changes in the absorption spectrum that occur at radiation doses as small as 0.21 Mgy, just 1.5 seconds after data collection had begun.11 From these studies, the major factors affecting the amount of radiation induced damage were concluded to be % glycerol composition, the temperature of the sample during data collection, the use of free radical scavengers, and the total amount of radiation absorbed by the sample during data collection.11

\textit{Mb-II Protonation Status}

Despite the aforementioned examples of photoreduction in crystallographic studies, Hersleth \textit{et al.} report a long Fe-O bond distance of 1.92 Å for a Mb-II crystal structure at pH 5.2, suggesting a protonated species.13 This contradicts a previous EXAFS study, yielding an authentic oxo distance of 1.69 Å.14 The datasets used to solve the Mb-
II crystal structure were reported to be of short exposure times, and represent only partially photoreduced structures.\textsuperscript{13,*}

Quantum refinement studies on the solved crystal structures concluded either a Fe\textsuperscript{III}-OH or Fe\textsuperscript{IV}-OH intermediate. Both structures fit the data appreciably better than the Fe\textsuperscript{IV}=O model. The Fe\textsuperscript{IV}-OH intermediate was chosen as the best model for the data based on EPR evidence taken prior to irradiating the crystal, and through the observation of a color change in the ferric crystal after incubation in H\textsubscript{2}O\textsubscript{2}. The partially photoreduced structures used to make this structural assignment were also compared to a fully photoreduced structure, called “Intermediate H”. Both yield Fe-O distances of 1.85 - 1.90 Å, and are interpreted as being similar, thereby both existing in Fe\textsuperscript{IV}-OH states.\textsuperscript{15}

Additional support for a protonated Mb-II intermediate may be provided by the recent work of Silaghi Dumitrescu. Using rapid scan stopped-flow, he proposes that the ferryl form of Mb in solution has an optical transition in the Q band region with a pKa ~ 4.7 (no Soret change), indicating an authentic protonation/deprotonation event.\textsuperscript{16} Hersleth suggests that a protonated ferryl is stabilized in crystals, even well above the pKa in solution, and this is why his structures at pH 5.2 reflect a 100% protonated intermediate.\textsuperscript{17}

Magnetic circular dichroism (MCD) studies performed by Foote also suggest an optical transition of the Mb-II intermediate at low pH. This study indicates the existence of two different forms of Mb-II, a basic form at high pH and an acidic form at low pH. Foote offers an alternative interpretation than Silaghi-Dumitrescu, indicating that the

\textsuperscript{*}It is important to note that Hersleth’s Mb-II crystals are composed of ~11% glycerol and are dipped in a cryo-solution containing 20-25% glycerol prior to flash freezing in liquid nitrogen. Crystallographic experiments were performed at 100 - 110K.
transition could result from the protonation/deprotonation of a nearby histidine residue. Protonation of the proximal histidine could result in a trans-effect, while a distal protonation event could result in a hydrogen bond to the ferryl oxygen.\textsuperscript{18} Interestingly, the acidic form is said to closely resemble the typical peroxidase compound II species, which are also known to have protonated distal histidine residues that hydrogen bond to the ferryl oxygen, as in HRP-II, CCP-ES, arthromyces ramosus peroxidase compound II (ARP-II), and myeloperoxidase compound II (MPO-II).\textsuperscript{19-22} Nevertheless, Silaghi-Dumitrescu couples his stopped flow observations with proton affinity calculations to suggest that these changes are irreconcilable with simple changes with the protein environment involving hydrogen bonding.\textsuperscript{16}

Adding controversy to the debate (of the protonation status of histidine ligated ferryls) is a 2010 X-ray crystallography paper by Moody and coworkers. Long Fe-O bond distances (indicating protonation) were determined for both the CCP-II and ascorbate peroxidase compound II (APX-II) ferryl intermediates. Because traditional oxidants could not be used to generate CCP-II, Moody purposefully photoreduces CCP-I in an attempt to produce the intermediate of interest.\textsuperscript{23} Using photoreduction as a “tool”, Moody insists that basic ferryls can exist in histidine ligated heme enzymes. One would think that the nature of this technique begs the question, how can you resolve a ferryl protonation controversy that hinges on bond variations as small as 0.2 Å when the method being used produces unreliable conversion to the species of interest at best?
Ending the Controversy

The evidence provided by Poulos and Schlichting suggests that although X-ray crystallography can be used as a powerful tool for three dimensional analysis, it is limited in its ability to delicately probe redox-active metal sites. We believe that the careless use of this technique has resulted in an inaccurate representation of the ferryl protonation states in histidine ligated systems, and has created considerable debate. In an effort to unify the views of ferryl protonation states in heme enzymes, this report will detail an exhaustive structural characterization of the controversial Mb-II intermediate, Figure 3.3. Because of the recent claims for the observance of a ferryl pKa (~ 4.7), we investigated Mb-II over a wide range of pHs (3.9→9.5). Density functional theory (DFT) calculations and Badger’s rule predictions were used in conjunction with EXAFS, Mössbauer, and resonance Raman spectroscopies to provide insight on 1) the true protonation status of Mb-II and 2) the role of the axial ligand in heme chemistry.

Figure 3.3: UV/visible spectral comparison of Mb^{III}-OH_{2} (pH 6.5), Mb^{III}-OH (pH 10.9), and Mb-II (pH 9.5). While Mb is typically known for its ability to bind oxygen in the ferrous state, ferric aqua/alkaline Mb (Mb^{III}-OH_{2}/Mb^{III}-OH) can react with H_{2}O_{2} to generate a ferryl compound II-like species.\(^2\)\(^4\)
3.4 Results and Discussion

EXAFS

Mb-II at pH 3.9 was the lowest pH sample (prepared via the rapid freeze-quench pH-jump method, 9.5 → 3.9) we could prepare before significant degradation was observed (by Mössbauer). In order to gain significant insight on the ferryl pKa, we elected to take this intermediate to the synchrotron for EXAFS analysis. For comparison, samples of the well characterized Mb\textsuperscript{III}-OH (pH 10.9) were also taken.

The Fe-K edge, which is reflective of the binding energy of the 1s electrons (oxidation states), illustrates that Mb-II (pH 3.9) was not photoreduced to a ferric hydroxide species, Figure 3.4. Additionally, Mb-II exhibits a more intense 1s→3d pre-edge transition than Mb\textsuperscript{III}-OH. The intensity of this transition is extremely sensitive to the M-O bond length,\textsuperscript{25, 26} indicating that the Fe-O distance in Mb-II is considerably shorter than in Mb\textsuperscript{III}-OH, or an analogous Mb\textsuperscript{IV}-OH intermediate.

Figure 3.4: Fe-K edge X-ray absorption edges for Mb\textsuperscript{III}-OH and Mb-II.
Analysis of the EXAFS data and corresponding Fourier transforms yield Fe-O bond distances of 1.87 Å for Mb$^{\text{III}}$-OH and 1.66 Å for Mb-II, Figure 3.5. The Fe-O bond distance for Mb$^{\text{III}}$-OH is significantly longer, and is on the order of what one would expect for a protonated ferryl moiety. Conversely, the much shorter distance obtained for Mb-II is in good agreement with previously reported authentic Fe$^{\text{IV}}$(oxo) intermediates.$^2$ These experimental bond distances are also in good agreement with both DFT (1.85 and 1.65 Å)$^{27}$ and Badger’s rule predictions utilizing previously reported Fe-O stretching frequencies (1.83 and 1.65 Å).$^1$

Figure 3.5: Fe K-edge EXAFS data (left) and Fourier transforms (right) of Mb-II (top) and Mb$^{\text{III}}$-OH (bottom). Black lines show experimental data, and colored lines show the best fits. The fits shown were obtained over the region $k = 3 - 15$ Å$^{-1}$. All EXAFS samples (≈ 3 mM) were analyzed by Mössbauer spectroscopy prior to data collection.
Table 3.1: EXAFS fitting results for Mb-II and MbIII-OH.

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Raw data were fit over the region $k = 3-15$ Å⁻¹. Coordination number $N$, interatomic distance $R$ (Å), mean square deviation in $R$ (the Debye-Waller factor), $σ²$ (Å²), and the threshold energy shift $E₀$ (eV). The fit error $F$ is defined as $[Σk^6(χ_{expt} - χ_{calc})^2/ Σk^6 χ_{expt}^2]^{1/2}$. Best fits are shown in boldface. Alternative fits include the scattering contribution of the nitrogen ($N=5$) from the axial histidine ligand. Coordination numbers, $N$, were constrained during the fits.
Mössbauer samples of Mb-II were prepared at pH 9.5, 4.7, and 3.9 in order to track any changes that may be indicative of the active site ferryl pKa, Figure 3.6. DFT calculations have previously shown that the magnitude of the ΔEq for a protonated ferryl is considerably larger than an unprotonated moiety (2.75 vs. 1.00 mm/s). Based on this insight, our pH 9.5 Mb-II sample, with ΔEq = 1.47 mm/s, is an unprotonated species. This value is also in agreement with previous Mössbauer characterizations of Fe$^{IV}$=O...
The pH 4.7 sample was prepared to investigate the ferryl protonation claim of Silaghi Dumitrescu using stopped flow. Mössbauer clearly shows one species, with $\Delta \text{Eq} = 1.53 \text{ mm/s}$. Similarly, the pH 3.9 reveals a single species with $\Delta \text{Eq} = 1.58 \text{ mm/s}$. Although the pH 4.7 and 3.9 samples have slightly larger quadrupole splittings than the pH 9.5 sample, all values are still in good agreement with authentic oxo intermediates. The slightly larger quadrupole splittings at lower pH could result from the distal histidine protonating and hydrogen bonding to the oxo. This increase in $\Delta \text{Eq}$ is not as large as with an authentic Fe$^{IV}$-OH (CPO/P450, $\Delta \text{Eq} \sim 2.02 \text{ mm/s}$) because, by comparison, a proposed hydrogen bond to an oxo only slightly affects the electric field gradient. If protonation of Mb-II was occurring at low pH, the sensitivity of Mössbauer spectroscopy allows for a contribution as small as $\sim 5\%$ (for integer spin systems using low field) to be seen. There is no indication of the formation of a second species, and thus an upper limit on the pKa of Mb-II can be set at 2.65.

Resonance Raman

In a recent study, Hersleth claims to have found an $^{18}\text{O}$ sensitive Fe-O stretch ($687 \text{ cm}^{-1}$) in Mb-II over the pH range 5.2 - 8.7. This could potentially support the existence of the long Fe-O bond previously observed in his X-ray crystallographic report; however the isotope shift is only about half the value predicted by a diatomic oscillator ($17 \text{ cm}^{-1}$ compared to the expected $28 \text{ cm}^{-1}$ shift). There are also no deuterium shifts to confirm the existence of a proton. Furthermore, if the $687 \text{ cm}^{-1}$ stretch is indicative of an Fe$^{IV}$-OH vibrational stretch, then Badger’s rule predicts the bond length to be $1.72 \AA$.

*Dumitrescu proposes that the ferryl form of Mb in solution undergoes an optical transition in the Q band region with a pKa $\sim 4.7$, indicating an authentic protonation event.
a difference of 0.2 Å from the reported crystallographic value. To further investigate this claim, our own resonance Raman experiments were used to determine the Fe-O stretching frequency in Mb-II over the pH range 9.5 – 4.4, Figure 3.7. Isotope substitutions (¹⁸O and D₂O) were paired with diatomic oscillator predictions to identify features indicative of H-bonding or a protonated ferryl.

**Figure 3.7**: Low frequency resonance Raman of ¹⁶O Mb-II at various pHs. Data was collected using the 501 nm argon laser line.

Using a 501.7 nm laser line, an Fe-O stretching frequency was identified at 805 cm⁻¹ for pH 9.5 and 7 samples. The 36 cm⁻¹ shift (769 cm⁻¹) observed by ¹⁸O isotope substitution is in accordance with harmonic oscillator predictions, Figure 3.8. At pH 5.7 the 805 cm⁻¹ stretch partially shifts to 788 cm⁻¹. This Raman shift coincides with an MCD detected optical transition by Foote *et al.* at the same pH. It is believed to be caused by the protonation and subsequent hydrogen bonding (H-bond) of the distal histidine to the ferryl oxygen, which results in a slightly weakened Fe-O stretch. This
type of phenomenon has been observed by resonance Raman in HRP-II, CCP-ES, ARP-II, and MPO-II.\textsuperscript{18-22} In HRP-II, with pKa $\sim$ 8.6, the $\text{Fe}^{IV}=\text{O}$ stretch shifts from 790 to 778 cm$^{-1}$.\textsuperscript{21} Although the shift is slightly larger in Mb-II, this is most likely due to the closer proximity of the distal histidine.\textsuperscript{*} This could allow for a stronger H-bond and greater weakening of the Fe-O stretch, resulting in a slightly larger shift. It has been shown by Green that this shift in stretching frequency (equivalent to a $\Delta \text{Å}$ of 0.009 using Badger’s rule) is not large enough to suggest an authentic protonation event.\textsuperscript{1} Spectra at pH 4.4 reflect similar results, but indicate that an even larger percentage of Mb-II lies in this alternative, low pH (acidic) conformation.

![Graph of resonance Raman spectra](image)

**Figure 3.8:** Low frequency resonance Raman of $^{16}\text{O}/^{18}\text{O}$ Mb-II at pH 9.5 (100 mM borate buffer). Results at pH 7 were virtually identical. Data was collected using the 501 nm argon laser line.

To convince ourselves that this phenomenon actually was arising from H-bonding to the ferryl oxygen, $^{18}\text{O}/^{16}\text{O}$ (H$_2$O) samples at pH 4.4 were compared to $^{18}\text{O}/^{16}\text{O}$ (D$_2$O) samples.

\textsuperscript{*} The distance from the distal histidine to the ferryl oxygen is 3.79 Å in HRP-II and 2.69 Å in Mb-II.\textsuperscript{13,29}
samples at pD 4.8, **Figures 3.9 and 3.10.** When the exchangeable proton of the protonated distal histidine is substituted with deuterium, it bonds more strongly to the histidine residue and the H-bond to the oxo is weakened. The result of the weakened H-bond is that the Fe-O stretch shifts slightly higher in energy, usually 2-3 cm$^{-1}$.$^{19-22}$ An additional effect of deuterium substitution is the increased intensity of the observable Fe-O stretch in the Raman spectrum.$^{19,22}$

**Figure 3.9:** Low frequency resonance Raman of $^{16}$O/$^{18}$O Mb-II at pH 4.4 (acetate buffer). Data was collected using the 501 nm argon laser line.
Figure 3.10: Low frequency resonance Raman of $^{16}$O/$^{18}$O Mb-II pD 4.8 (acetate buffer). Data was collected using the 501 nm argon laser line.

At pH 4.4 the 805 cm$^{-1}$ stretch appears at the same energy in H$_2$O and D$_2$O, signifying a non H-bonded conformation. The 788 cm$^{-1}$ stretch of the $^{16}$O/H$_2$O spectrum (pH 4.4) appears at 790 cm$^{-1}$ in the $^{16}$O/D$_2$O spectrum (pD 4.8), indicating that a protonated distal histidine is H-bonding to the ferryl oxo in the low pH conformation. Interestingly, the $^{18}$O/D$_2$O spectrum shows that the 790 cm$^{-1}$ peak shifts to 762 cm$^{-1}$, a difference of ~ 28 cm$^{-1}$, and not the full 36 cm$^{-1}$ shift predicted by a diatomic oscillator. This explains why the pH 4.4 $^{16}$O/H$_2$O difference spectrum shows two Fe-O sensitive stretches that shift seemingly into “one” large peak. It is because the Fe-O stretch representing the non H-bonded conformation (805 cm$^{-1}$) shifts 36 cm$^{-1}$ while the Fe-O stretch from the H-bonded conformation (788 cm$^{-1}$) only shifts 28 cm$^{-1}$, creating overlap in the difference spectrum. The smaller $^{18}$O - $^{16}$O shift of the H-bonded conformation may result from the Fe-O stretch no longer being accurately represented by a two body
harmonic oscillator. Studies on the effects of hydrogen bonding on stretching frequencies and the impact on the expected oscillator shifts could support this claim. The proposed destabilization of the H-bonded Fe-O stretch is depicted below, Figure 3.11.

**Figure 3.11:** The proposed distal histidine protonation event that allows for H-bonding to the ferryl oxygen. (A) At low pH (pH < pKa) the Fe=O bond is destabilized by a hydrogen bond from a protonated distal histidine, resulting in a smaller than expected isotope shift. (B) At high pH (pH > pKa) the distal histidine is not protonated and there is no hydrogen bond to the ferryl oxygen. In this scenario the experimental isotope shift is in good agreement with a two body harmonic oscillator prediction. This figure was adapted from Behan, R. K.; Green, M. T., On the status of ferryl protonation. *Journal of Inorganic Biochemistry* 2006, 100 (4), 448-59.

Our Raman studies were limited by the degradation of Mb below pH 4.5. Even small amounts of degradation cause significant fluorescence that can make the assignment of stretching modes difficult. Although spectra could only be obtained as low as pH 4.4, it is reasonable to assume that at lower pH, 100% of the Mb-II oxo would exist in a H-bonding (acidic) conformation.* No new peaks indicative of an Fe^{IV}-OH stretch (500 – 650 cm\(^{-1}\)) were identified in low pH studies.

*Resonance Raman studies by Terner show the complete shift of HRP-II from basic to acidic form (790 – 778 cm\(^{-1}\)) over a pH range of ~ 2.5 units. Our experiments only allowed us to drop 1.3 units below the pKa of the proposed conformational change in Mb-II.
3.5 Conclusion

EXAFS, Mössbauer, and resonance Raman spectroscopies (in conjunction with DFT and Badger’s rule predictions) have shown that Mb-II exists exclusively as an Fe$^{IV}$=O, setting an upper limit on the pka ($< 2.65$). The radiation exposure studies provided by Schlichting and Poulos indicate that the long Fe-O bond length determined for Hersleth’s Mb-II crystal structure is a direct result of radiation damage. This is in agreement with EPR and electron nuclear double resonance (ENDOR) studies by Hoffman suggesting that a cryoreduced Mb-II sample produces an [Fe$^{III}$-O] intermediate at low temperature, which readily generates a ferric hydroxide moiety during low temperature annealing experiments.$^{31}$

To date, there has been no definitive proof for the existence of a histidine ligated Fe$^{IV}$-OH intermediate (no Fe$^{IV}$-OH Raman stretches) in heme proteins. Most of the previous crystallographic characterizations of ferryl intermediates are convoluted by ambiguous product purities (lack of Mössbauer) and poorly discussed radiation effects. This report implies that the proximal histidine ligand in globins and traditional peroxidases is not donating enough to stabilize an Fe$^{IV}$-OH moiety. Using Mayer’s insight on H-atom abstraction in metal oxo complexes, we believe that the pKa of compound II influences the type of chemistry that a particular class of heme enzymes can perform. For illustrative purposes, consider HRP, a well known histidine ligated heme peroxidase that is not known to perform C-H bond activation. EXAFS, Mössbauer, and resonance Raman studies confirm that HRP-II is NOT basic. It is an authentic Fe(IV) oxo, with an upper limit on the pKa of $\sim 3.5$. $^{3, 21, 33}$ Hayashi and coworkers have determined the redox potential of HRP-I to be 0.92 volts.$^{32}$ Using the equation for
D(OH), this yields a value of 83 kcal/mol for a protonated form of HRP-II.\textsuperscript{51}

Theoretically, in order to perform H-atom abstraction of an unactivated hydrocarbon (~100 kcal/mol), which HRP cannot experimentally do, the enzyme would either need to have a compound I redox potential of 1.4 Volts (assuming a pKa ~ 3.5), or a compound II pKa of 14 (assuming a redox potential of 0.92 volts).

It is unlikely that Nature would produce an enzyme with such a high redox potential. Such oxidative potency would lead to damaging self-inflicted oxidations that could destroy the fragile protein superstructure. Instead, it seems more likely that Nature has implemented thiolate ligation as a means to increase the driving force for H-atom abstraction by elevating the pKa of the ferryl species. Previous crystallographic reports for protonated histidine ligated ferryls added controversy to this theory, but our investigation, along with the work of others, strongly implies that these heme systems are all authentic Fe\textsuperscript{IV}=O intermediates.
3.6 References


Chapter 4

Determination of a Ferryl pKa: Understanding C-H Bond Activation in Cytochrome P450

4.1 Abstract

Cytochrome P450 (P450), chloroperoxidase (CPO), and aromatic peroxygenase (APO) are the only heme enzymes known to functionalize C-H bonds.\textsuperscript{1-4} Their unique axial thiolate coordination distinguishes them from other heme proteins, and is believed to play an influential role in reactivity. It has been suggested that Nature uses this donating ligand to promote C-H bond activation at biologically viable reduction potentials by elevating the pKa of the ferryl species.\textsuperscript{5} While we have previously shown that the ferryl forms of CPO and P450 are basic, the limited pH range over which they could be observed prohibited the determination of a pKa.\textsuperscript{5-8} The CYP158A2 enzyme has provided us with a system where the ferryl moiety can be prepared in unprecedented yield (>90%) over a wide range of pH values, allowing for the direct opportunity to explore the relationship between the axial ligand identity and ferryl basicity. Here, for the first time, we provide direct spectroscopic evidence for the ferryl pKa using stopped-flow, Mössbauer, and extended X-ray absorption fine structure (EXAFS) spectroscopy. Our quantitative measure is in agreement with synthetic studies that suggest low potential oxidants (LPO) use strong donating axial ligands as the driving force (via an elevated pKa) in H-atom abstraction and oxygen insertion reactions.\textsuperscript{9-12}
4.2 Background: Trapping the High Valent Intermediates in Cytochrome P450

\textit{P450 Compound I}

For almost 40 years the characterization of a proposed compound I species in P450s has eluded scientists. Only faint glimmers have been observed using stopped-flow spectrophotometry (via the peroxide shunt method), where the compound I spectrum had to be extracted using mathematical techniques (Global Analysis).\textsuperscript{13-16} The intermediate’s constructed UV/visible spectrum resembled the peroxidase analogue, CPO-I, and so was thought to function very similarly, playing the role as the active oxidizing species in catalysis.\textsuperscript{1, 13-16} However, any attempt to prepare the intermediate in quantitatable yield for additional (EPR, EXAFS, Mössbauer, etc.) spectroscopic characterization resulted in failure, even on the extremely fast freeze-quench time scale. Reasons for failure were rationalized by the transient nature of the species, owing to its potent reactivity. As a result of the inability to prepare the intermediate, alternative theories to the identity of the active oxidizing species, such as a perferryl complex, were proposed. To date, there is no evidence for the formation of such a species.\textsuperscript{17}

In 2010, a breakthrough in the field of P450 chemistry occurred when Green and coworkers successfully trapped the elusive compound I species in \textasciitilde 75\% yield. It was prepared in the thermophilic enzyme, CYP119 (mM concentrations), and characterized by Mössbauer, EPR, and stopped-flow spectroscopies. Prior efforts to trap the intermediate were concluded to have failed because of issues with protein purity, and not because the intermediate was too transient to trap. A UV/visible spectral comparison between CPO-I and CYP119-I illustrates the similarities between the green intermediates,\textsuperscript{17} \textbf{Figure 4.1}. 

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\caption{UV/visible spectral comparison between CPO-I and CYP119-I.}
\end{figure}
Figure 4.1: UV\visible spectra of high spin (HS) ferric and compound I for CPO (top) and CYP119 (bottom). Because CYP119-I can only be generated in ~ 75% yield, the pure spectrum was generated using spectra math. The CYP119 figure was adapted from Rittle, J.; Green, M. T., Cytochrome P450 Compound I: Capture, Characterization, and C-H Bond Activation Kinetics. *Science* **2010**, *330* (6006), 933-937.

P450-I is very reactive, and does not accumulate enough to be seen in the presence of substrate. It can only be observed in quantitative yields in the absence of substrate, where auto-oxidation, rather than substrate oxidation, is the sole culprit for the intermediate’s decay. In *E. coli* expression systems, such as the one used to overexpress CYP119, it is very common for small chain fatty acids to be produced during cell growth.
Such fatty acids are substrates for CYP119 and can become bound in the active site pocket.\textsuperscript{18-22} When oxidant is added to generate the reactive compound I species (peroxide shunt), the substrate is readily hydroxylated, regenerating the ferric resting enzyme, and making the observance of any high valent intermediates fleeting at best (slowing the formation and speeding the decay).

However, through purification techniques the Green lab was able to remove a large quantity of the fatty acids in the pocket of the protein that are produced during \textit{E. coli} growth (confirmed by GC analysis).\textsuperscript{19} Once the purified protein was reacted with \textit{meta}-chloroperbenzoic acid (\textit{m}-CPBA), the high valent compound I intermediate was able to build up to unprecedented yields, owing to the absence of available substrate in the enzyme pocket.\textsuperscript{17, 21}

\textit{P450 Compound II}

Up until 2010, P450-II has only been trapped in nominal yields. This was accomplished in 2007 by Green and coworkers through the reaction of peracetic acid (PA) with P450cam (29\%) and P450\textsubscript{BM3} (48\%)\textsuperscript{6}, Figure 4.2. Samples were prepared via rapid freeze-quench and analyzed by Mössbauer spectroscopy. Because the parameters were very similar to the basic CPO-II intermediate, strong inference could be made on the protonation status of P450-II. However, the low yield prevented more definitive spectroscopic characterization methods, \textit{ie.} EXAFS and resonance Raman.
Figure 4.2: Mössbauer spectra of ferric P450 reacted with peracetic acid (PA). (Left) Mössbauer spectrum of P450BM3 PA-generated intermediate (4.2K/54mT). Top spectrum is the raw data for P450BM3-PA overlaid with the ferric contribution (44%). The bottom spectrum is of the raw data after subtraction of the ferric P450BM3 component. The best fit represents 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%). The parameters of the larger component are attributed to compound II. (Right) Mössbauer spectrum of P450cam-PA intermediate (4.2K/54mT). Top spectrum is the raw data for P450cam-PA overlaid with the ferric contribution (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%). Figures taken from Behan, R. Spectroscopic Characterization of High-Valent Intermediates in Cytochrome P450s and Other Heme Enzymes. Pennsylvania State University, University Park, PA, 2008.

We believe that the magnitude of the ferryl basicity could be the key to understanding the driving force for C-H bond activation in heme chemistry. In order to provide insight on this claim, we wondered if the thermodynamic “pKa” parameter could be quantitated in thiolate ligated (CPO-II and P450-II) systems. However, efforts to prepare high pH ferryl intermediates in either system were met with great difficulties. CPO undergoes the formation of an irreversible alkaline species above ~ pH 7.0$^{23}$ and low yield in the previously discussed P450-II systems prevented further
experimentation.⁶ In what follows, we report the identification of a P450 (CYP158A2) in which compound II can be prepared in high yield (>90%) over a wide pH range (7→10), affording us the opportunity to quantitate the long sought after ferryl “pKa” parameter.

4.3 Introduction

The selective functionalization of hydrocarbons through the controlled activation of carbon-hydrogen (C-H) bonds has been called a Holy Grail of chemical synthesis. The C-H bond is a ubiquitous chemical moiety that decorates the structures of biomolecules, natural products, pharmaceuticals, and the various components of petroleum.²⁴, ²⁵ As a result, the ability to selectively cleave a C-H bond would represent one of the most broadly applicable classes of chemical transformations known.²⁶ However, because C-H bonds are ubiquitous, they are also inert.²⁴–²⁶ C-H bond strengths in unactivated hydrocarbons are ~ 100 kcal/mol, making their functionalization a significant challenge.

Nature has produced a number of enzymatic systems that activate C-H bonds. Perhaps the most notable is a class of thiolate ligated heme enzymes known as cytochrome P450 (P450s).¹³, ²⁷, ²⁸ We have argued that the donating thiolate ligand promotes C-H bond activation in P450s through the generation of a basic ferryl (Fe⁴⁺-OH) species.⁵, ²⁹ In metal oxo complexes, the driving force for C-H bond activation is given by the difference between the energies of the O-H bond formed, D(O-H), and the C-H bond broken, D(C-H).³⁰–³⁶ If applied to heme systems, D(O-H) is dependent on the one electron redox potential of compound I, and the pKa of compound II, Figure 4.3.
Figure 4.3: Rebound mechanism of cytochrome P450. The resting ferric enzyme is oxidized by two electron equivalents to an Fe⁴⁺ porphyrin radical species (formally Fe⁵⁺), denoted as compound I (red). In the presence of substrate compound I abstracts hydrogen and is reduced to the protonated compound II ferryl intermediate (blue). The radical substrate then readily recombines to form the hydroxylated product, regenerating the ferric resting species (black). Bonds and bond strengths, D(X-H), discussed in the text are indicated. The equation for the O-H bond strength is given in terms of the one-electron reduction potential of compound I and the pKa of compound II. C is a constant that depends upon the solvent and reference electrode. Its value is 57 ± 2 kcal/mol in aqueous solution.

The compound II pKa is thus a key thermodynamic parameter in C-H bond activation, with a unit increase allowing for a 59 mV drop in the one electron reduction potential of compound I. By diminishing the driving force for one electron oxidations, thiolate ligation biases the system towards H-atom abstraction. This creates an oxidant capable of cleaving C-H bonds while avoiding unwanted oxidations of the protein superstructure.⁵

Results from synthetic models have generally supported this hypothesis,⁹⁻¹² but an experimental parameter that could provide a quantitative measure of the thiolate’s impact on P450 catalysis has proven elusive. Here we report a breakthrough on this front. We have identified the P450 CYP158A2, whose ferryl form can be prepared in unprecedented yield over a wide pH range. This discovery has allowed us to determine
the first ferryl pKa. Its measured value of 11.9 is at least 8.5 units higher than the ferryl pKas in histidine ligated globins and peroxidases.29

4.4 Results and Discussion

Stopped-flow

In our many attempts to characterize the high valent intermediates in the catalytic cycle of P450, we determined that CYP158A2 compound II (CYP158A2-II) could be prepared by reacting the ferric resting enzyme with \( m \)-CPBA (~ 5 equivalents). The intermediate can be made over the pH range 7 – 10, with maximum formation (~ 90 %) at pH 9, Figure 4.4. At this pH the intermediate is relatively stable, decaying back to ferric at a rate of ~ 0.5 s\(^{-1}\) (\( t_{1/2} \approx 1.4 \) s). Because previous experiments with thiolate ligated heme enzymes (CPO and P450s) and organic peracids are known to yield protonated ferryls,6,29 we assigned CYP158A2-II as an Fe\(^{IV}\)-OH.

The UV/visible spectrum of compound II is hallmarked by a split Soret band, with absorption maxima at 370/428 nm and Q bands at 532/565 nm. At first glance the Soret shift from ferric to compound II (416 \( \rightarrow \) 428 nm) is not as considerable as that in CPO to CPO-II (shifting from 400 \( \rightarrow \) 437 nm), but this is because ferric CYP158A2 is low spin, whereas ferric CPO is in a high spin resting state. Binding studies on CYP119 suggest that the high spin form of ferric CYP158A2 should have a Soret max around 390 nm, which would make the compound II shift very similar to CPO-II (Supporting Information).
Figure 4.4: UV/visible spectra obtained from the stopped-flow mixing (1:1) of 20 μM ferric CYP158A2 with 100 μM m-CPBA at 4°C. The blue traces correspond to spectra taken at 5, 200, 500, and 2500 ms after mixing. Maximum yield of P450-II was at 2.5 seconds (90% by Mössbauer).

Given the high yield and stability of CYP158A2-II at pH 9, we wondered if the intermediate could be prepared at even higher pHs, via the pH-jump method. Using rapid sequential mixing, ferric CYP158A2 (pH 9) could first be mixed with m-CPBA and held until maximum formation of compound II. Then, the intermediate could be mixed with basic buffer in order to reach a higher pH. The hope was that this method would allow for access to a ferryl pKa.

The spectra of CYP158A2-II samples prepared from pH 9 to 14 are shown in Figure 4.5. With increasing pH, the CYP158A2-II intermediate converts to a new species. Multiple isosbestic points confirm that the data-set comprises only two components, indicating that the decay of the intermediate is not an issue on the time scale of the experiment. The UV/visible spectrum of the high pH CYP158A2-II intermediate is also hyperporphyrin-like (split Soret), with absorption maxima at 371/440 nm and a
single Q band at 545 nm. Because of the nature of these high pH experiments, our thought was that the Fe$^{IV}$-OH intermediate (pH 9) was converting to an Fe$^{IV}$=O species (pH 14).

![Figure 4.5: UV/visible spectra of CYP158A2-II at various pHs.](image)

30μM ferric CYP158A2 (10 mM Tris-HCl, pH 9.0) was pre-mixed with 5 equivalents of m-CPBA (water) and held until maximum formation of compound II (Fe$^{IV}$-OH) was achieved (2.5 seconds). The solution was then further mixed with a high strength buffer solution (containing 200 mM phosphate, 200 mM carbonate, pH adjusted with KOH). Spectra were collected within 100 ms of mixing with the high-strength pH buffer. Multiple isosbestic points reveal the presence of only two species.

The assignment of the Fe$^{IV}$=O was tentative at first. We worried that a conformational change at such a high pH could lead to the loss of thiolate ligation, and, as a result, the driving force for ferryl protonation. However, this concern can initially be addressed through the close examination of the UV/visible spectrum of the oxo intermediate. It is known that the soret band typically arises from porphyrin $\pi \rightarrow \pi^*$ electronic interactions, but in thiolate ligated systems this band is “split” through interactions with a sulfur$\rightarrow$porphyrin-charge transfer band.$^{37, 38}$ We believe that the
presence of the split Soret band (371/440 nm) in the oxo intermediate indicates the retention of thiolate ligation at high pH. Additionally, the conversion from 2 Q-bands (α and β) to a single broad band is a phenomenon first observed by Dawson and coworkers in their investigation of a 1-propane thiol•Cytochrome P450 complex. It is believed to be as a result of di-sulfur ligation at high pH (two donating ligands). We propose that an oxygen donor ligand in the CYP158A2-II oxo complex could function similarly to a sulfur ligand, producing this same spectral phenomenon.

Mössbauer

The freeze-quench capture of the CYP158A2-II hydroxide intermediate was carried out at pH 9, where the highest yield was obtained (~90%). Mössbauer analysis reveals a large quadrupole splitting (σ = .11 mm/s, ΔEq = 2.02 mm/s), confirming that this species is best described as an Fe(IV)-hydroxide complex. Parameters coincide with the ferryl forms of the previously characterized P450cam, P450BM3, and CPO enzymes.

A three-syringe, double-mix freeze quench setup was required for the quantitative capture of the CYP158A2-II oxo complex. Mössbauer analysis at pH 13.3 reveals a single species, generated in ~ 90% yield. Relative to the hydroxide precursor (pH 9), the quadrupole splitting is reduced substantially (σ = .09 mm/s, ΔEq = 1.30 mm/s). The parameters are typical of an authentic ferryl oxo porphyrin species, confirming our initial assignment from stopped-flow experiments. Mossbauer quenches ranging from pH 9 → 13.3 demonstrate the conversion of an FeIV-OH → FeIV=O species, Figure 4.6.
The Mossbauer spectra confirm that the changes seen in the stopped-flow experiments result only from the low to high pH (hydroxide to oxo) transition. Importantly, analyses of the data reveal that the iron(IV) species are in equilibrium: Plotting the relative concentration of the oxo species as a function of pH results in the pH titration curves shown in Figure 4.7. Fits of these curves provide a ferryl pKa of 11.9.
Figure 4.7 pH titration curves of CYP158A2-II from stopped-flow (blue) and Mössbauer spectroscopies (red). Both methods yield a pKa of 11.9 for the deprotonation of ferryl moiety.

EXAFS

Although stopped-flow and Mössbauer can provide considerable insight, they cannot provide structural information. In order to confirm the structural assignments of the high and low pH CYP158A2-II forms, we elected to perform EXAFS. Fe K-edge absorption edges show that both intermediates lie ~ 1.5 eV higher in energy than the ferric enzyme, consistent with the assignment of an Fe$^{IV}$ oxidation state, Figure 4.8. It can also be seen that the CYP158A2-II oxo exhibits a large 1s->3d pre-edge transition. The intensity of this transition has been shown to be much larger in asymmetric ligand environments, suggesting that the Fe-O bond distance in the CYP158A2-II oxo is considerably shorter than in CYP158A2-II hydroxide intermediate.
Figure 4.8: Fe-K X-ray absorption edges for ferric CYP158A2, CYP158A2-II hydroxide, and CYP158A2-II oxo.

Fits of the EXAFS and Fourier transform data reveal Fe-O/Fe-S bond distances of 1.84/2.27 Å for the hydroxide and 1.68/2.36 Å for the oxo, Figure 4.9. The short Fe-O distance at pH 13.3 is indicative of an authentic Fe$^{IV}$=O species. The Fe-S distance of 2.36 Å is crucial to the fitting of the Fourier transform data. Omission of the sulfur scatterer substantially increases the fit error. Comparisons of fits with and without sulfur scattering (to mimic the loss of cysteine ligation at high pH) indicate that the 6 coordinate heme is intact at high pH (Supporting Information). With decreasing pH, the Fe-O bond length increases by 0.15 Å, consistent with protonation of the ferryl moiety. The 1.84 Å bond distance at pH 9.0 is in good agreement with the 1.82 Å distance previously reported for the Fe$^{IV}$-OH center of CPO-II.$^5$
Figure 4.9: Fe K-edge EXAFS data (left) and Fourier transforms (right) of CYP158A2-II hydroxide (upper) and oxo (lower). Black lines show experimental data, and colored lines show the best fits. The fits shown were obtained over the region $k = 3 – 15 \text{ Å}^{-1}$. All EXAFS samples were analyzed by Mössbauer spectroscopy prior to data collection.
Table 4.1: EXAFS fitting results for CYP158A2-II hydroxide and oxo.

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Raw data were fit over the region \( k = 3-15 \text{ Å}^{-1} \). Coordination number \( N \), interatomic distance \( R \) (Å), mean square deviation in \( R \) (the Debye-Waller factor), \( σ^2 \) (Å²), and the threshold energy shift \( E₀ \) (eV). The fit error is defined as \( \sqrt{\frac{\sum_k (\chi_{\text{exptl}} - \chi_{\text{calc}})^2}{\sum_k \chi_{\text{exptl}}^2}} \). Best fits are shown in boldface. Alternative fits with different coordination numbers are shown also. Coordination numbers, \( N \), were constrained during the fits.

4.5 Conclusion

The role of the thiolate ligand in C-H bond activation has long been a fundamental question in the field of P450 catalysis. While many spectroscopic investigations have provided clear evidence for the donating character of the thiolate ligand,¹ ⁴⁴–⁴⁶ none have provided a quantitative measure of the thiolate’s impact on reactivity. The ferryl pKa reported here provides such a measure.

The ferryl pKa of 11.9 for the CYP158A2 enzyme is significantly higher than the upper limits placed on histidine ligated peroxidases and globins. Using the (O-H) bond strength equation, and assuming a pKa ~ 3.5 for histidine ligated enzymes,³⁴ the (O-H) bond strength could be as much as ~ 12 kcals/mol less than in thiolate ligated systems
based on ferryl basicity alone. In order to make up for this discrepancy, histidine ligated enzymes would need a compound I redox potential of \(~ 1.4 \text{ V}\) to perform inert hydrocarbon oxidations (\(~ 99 \text{ kcal/mol}\)). We have previously reasoned that such a high compound I redox potential could bias the system towards non-productive self oxidations (nearby tyrosine and tryptophan residues), rather than performing H-atom abstraction of substrate.\(^5\), \(^29\) Conversely, if one assumes a C-H bond strength of 99 kcal/mol (cyclohexane), then the pKa determined for CYP158A2-II can be used to estimate the redox potential for compound I in P450s as 1.12 Volts, Figure 4.10. Thus, thiolate ligation may promote C-H bond activation through an attenuation of the driving force for deleterious oxidations.

Figure 4.10: Compound I redox potential necessary to obtain an O–H bond of 99 kcal/mol when the ferryl pKa = 11.9. Figure adapted from Behan, R. K.; Green, M. T., On the status of ferryl protonation. *Journal of Inorganic Biochemistry* 2006, *100* (4), 448-59.
4.6 Supporting Information

Figure 4.11: UV/visible spectral comparison of ferric $\rightarrow$ compound II. (Top) Ferric CPO $\rightarrow$ CPO-II. (Bottom) Ferric CYP158A2 $\rightarrow$ CYP158A2-II hydroxide. A typical ferric high spin P450 spectrum (taken from CYP119) has been overlaid in order to emphasize the similar Soret band shifts.
Figure 4.12: Mössbauer spectra of the ~90% CYP158A2-II hydroxide sample used for EXAFS (4 mM, pH 9.0). Left is raw data and right is after subtracting ~10% ferric.

Figure 4.13: Mössbauer spectra of the ~90% CYP158A2-II oxo sample used for EXAFS (3 mM, pH 13.3). Left is raw data and right is after subtracting ~10% ferric.
Figure 4.14: Fit comparison of the Fourier transform data for the CYP158A2-II oxo intermediate with (left) and without (right) the sulfur scatterer. Black is the raw data and red is the best fit. This comparison demonstrates that the proximal sulfur (cysteine) is ligated to the heme active site at pH 13.3.

Figure 4.15: Fit comparison of the Fourier transform data for the CYP158A2-II hydroxide intermediate with (left) and without (right) the sulfur scatterer. Black is the raw data and blue is the best fit. This comparison demonstrates that the proximal sulfur (cysteine) is ligated to the heme active site at pH 9.0.
Figure 4.16: Mössbauer comparison of 4 mM ferric CYP158A2 at different pHs. (Black) Ferric prepared at pH 9.00. (Red) Ferric prepared by the rapid pH-jump (9.00→13.3) method. The sample was rapidly mixed with high pH buffer and quenched into liquid ethane ~ 3 ms after mixing. This comparison shows that the changes seen in the compound II Mössbauer spectrum at high pH do no arise from ferric conformational changes.
4.7 References


42. Rohde, J.-U.; Torelli, S.; Shan, X.; Lim, M. H.; Klinker, E. J.; Kaizer, J.; Chen, K.; Nam, W.; Que, L., Structural Insights into Nonheme Alkylperoxoiron(III) and


Chapter 5
Determination of the Ferryl pKa in *Helicobacter pylori* Catalase

5.1 Abstract

The elucidation of the factors that govern the selective functionalization of (inert) hydrocarbon substrates has long been a goal of bioinorganic chemists. Synthetic reports suggest that electron donating axial ligands promote C-H bond activation by elevating the pKa of the H-atom abstracting (rebounding) species.\(^1\), \(^2\) This work could parallel the chemistry of cytochrome P450 (P450), where it has been proposed that the role of the (donating) thiolate ligand is to promote H-atom abstraction at viable compound I redox potentials.\(^3\)\textsuperscript{-5} While prior experiments have confirmed the basic nature of these ferryl intermediates,\(^6\) the recent first ever direct quantification of a ferryl pKa (~ 12) in the CYP158A2 enzyme has provided an actual magnitude for this key thermodynamic parameter.\(^5\) Herein we build on this report, as we offer evidence for the determination of a ferryl pKa in a second heme system. Mössbauer spectroscopy (in conjunction with X-ray absorption measurements) has been used to show that *Helicobacter pylori* catalase compound II (HPC-II) undergoes a structural change with a pKa of ~ 13, which we believe corresponds to the deprotonation of the ferryl moiety. This result is surprising; suggesting that an axial tyrosine ligand is sufficiently donating to stabilize a protonated ferryl at high pH. The high ferryl pKa in HPC-II also suggests that tyrosine ligated hemes could be capable of chemistry comparable to their thiolate ligated counterparts.
5.2 Introduction

Through the reduction of dioxygen, normal metabolic processes can produce hydrogen peroxide as a potentially damaging by-product. To combat this reactive oxygen species, cells utilize catalase, a tyrosine ligated heme enzyme, to catalyze the disproportionation of hydrogen peroxide to generate di-oxygen and water.\textsuperscript{7}

\[ 2\text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2\text{H}_2\text{O} \quad \text{Eq. 1.1} \]

The first \( \text{H}_2\text{O}_2 \) molecule oxidizes the ferric heme by two electron equivalents to compound I, an \( \text{Fe}^{IV}=\text{O} \) porphyrin radical species. A second molecule of \( \text{H}_2\text{O}_2 \) is then used to reduce compound I back to ferric catalase, resulting in the release of \( \text{H}_2\text{O} \) and dioxygen. Compound II (the one electron reduced form of compound I) forms at limiting \( \text{H}_2\text{O}_2 \) concentrations\textsuperscript{*}, but it is unclear if this intermediate plays a role in the active catalysis.\textsuperscript{7} In \textit{in vitro} studies utilizing peracetic acid as an oxidant, the intermediates formed in the reaction cycle of HPC are similar to those observed in cytochrome P450 (P450) during oxygen insertion reactions, \textbf{Figure 5.1}.

\*At low substrate concentrations compound I can presumably decay to compound II through a self-oxidation process.
Mayer has shown that the ability of a metal oxo complex to abstract hydrogen from substrate is dependent on the energy difference between the O-H bond being formed, and the C-H bond being cleaved.\(^8\)\(^-\)\(^10\) In heme enzymes, the strength of the O-H bond formed, D(O-H), is dependent on the one electron redox potential of compound I, and the pKa of compound II.

We have previously discussed the importance of the (donating) thiolate ligand in CPO and P450s. It is believed to provide the driving force for C-H bond activation by elevating the pKa of the ferryl species.\(^3\)\(^-\)\(^5\) Conversely, histidine ligated peroxidases and globins are known to have substantially lower pKas, on the order of \(\leq 3.5\).\(^4\)\(^,\)\(^11\)\(^-\)\(^13\) This difference in ferryl pKa results in a 12 kcal/mol drop in the potential D(O-H) bond strength relative to P450s, and could account for the inability of histidine ligated hemes
to activate C-H bonds. In tyrosine ligated enzymes, such as HPC, the ferryl pKa has been proposed to be somewhere in between histidine and thiolate ligated systems (thiolate > tyrosine > histidine). This could suggest the possibility of C-H bond activation. To date however, there are no definitive reports on the protonation status of compound II in catalase.

5.3 Background

In 2007 Jouve et al. detailed a pH dependent UV/visible spectral change in *Proteus mirabilis* catalase compound II (PMC-II) that is believed to coincide with the protonation/deprotonation of the ferryl moiety (pKa ~8.4). The low pH spectrum of PMC-II (LpH-II, pH 6.2) consists of nearly equal alpha (α) and beta (β) Q band intensities while the high pH PMC-II (HpH-II, pH 7.9) spectrum displays a sharp rise in the alpha intensity, roughly doubling the intensity of the beta band, Figure 5.2.

*Jouve reports no difference in the position of the soret max for the LpH-II→HpH-II transition. Stopped-flow experiments with CYP158A2-II show that the soret max shifts from 428→440 nm upon deprotonating the ferryl moiety.*
Mössbauer spectroscopy was used to confirm the identity of two different Fe(IV) species, with ΔEqs of 2.29 and 1.47 mm/s for the low and high pH intermediates respectively. Concomitant extended X-ray absorption fine structure (EXAFS) experiments revealed Fe-O bond distances of 1.80 and 1.66 Å for the low and high pH PMC-II forms. Although the EXAFS and Mössbauer parameters are consistent with the assignment of both an Fe^{IV}-OH (LpH-II) and an Fe^{IV}=O (HpH-II) species, we propose that Jouve’s tentative ferryl pKa assignment warrants reinvestigation. In what follows, we discuss why this is necessary.

Typically, a hallmark of a “pKa” is the reversible nature of the event. When HpH-II (Fe^{IV}=O) was titrated with citric acid down to pH 5.3, the gradual decrease of this
form coincided with the increase of LpH-II (Fe$^{IV}$-OH). Quantifications were made by Mössbauer. However, upon basification of the LpH-II form, there was no generation of the HpH-II intermediate. The irreversibility of this reaction demonstrates the absence of acid base equilibrium that normally accompanies a protonation/deprotonation event.

The PMC-II samples (pH 8, 1.2 mM, $^{56}$Fe) used to determine the Fe-O bond lengths are also concerning. EXAFS samples were not characterized by any direct method, rather they were assumed to be of similar sample composition to previously characterized $^{57}$Fe samples. The assumed composition was 23% ferric, 3% compound I, 30% HpH-II (360 μM), and 44% LpH-II (530 μM). It was reasoned that ferric catalase could be excluded from the data fitting analysis because it is 5 coordinate, and does not have a distal oxygen scattering atom. Compound I could also be excluded based on its negligible percent yield. Under these assumptions, bond distances of 1.66/1.80 Å were determined for HpH-II/LpH-II. The average proximal Fe-O scattering distance (to reflect the iron-tyrosine scatterer) was determined to be 2.27 Å.

The short 1.66 Å oxo distance assigned to HpH-II is the same distance that one would expect for a compound I intermediate. Because the EXAFS sample composition is indefinite, it is possible that the assumption made about the “negligible” contribution of compound I is incorrect. Even if only present in 5-10% yield, the short Fe-O bond in compound I would provide a substantial scattering contribution relative to the 30/44% yield of LpH-II/HpH-II. The 2.27 Å “average” Fe-O proximal tyrosine bond distance is longer than for any calculated model. Density functional theory (DFT) models predict distances on the order of 1.93, 2.04, and 1.97 Å for ferric, compound I, and compound II catalase structures respectively. Additionally, the lack of pure samples convolutes any
possible structural knowledge that could be gained from a pre-edge/absorption edge analysis.*

Finally, Jouve details a resonance Raman investigation that is contradictory to her EXAFS and Mössbauer results. At pH 7.9 and pH 6.2, Fe-O stretching frequencies from an Fe^{IV}=O species are said to be observed (no data is shown). However, there is no evidence of the existence of an Fe^{IV}-OH stretch correlating to the LpH-II form.14 If the pKa of this transition is ~ 8.4, then at pH 6.2 the LpH-II form should be present in ~ 100% yield. While it is possible that an Fe^{IV}-OH stretch may not be enhanced, it is unlikely that one would observe the Fe^{IV}=O stretch from the HpH-II form at this low a pH.

In an effort to reinvestigate the possibility of a ferryl deprotonation event in a tyrosine ligated system, we have prepared (~ 90% yield) and structurally characterized Helicobacter pylori catalase compound II (HPC-II) over a wide pH range (pH 5.3 - 14.2). HPC-II was characterized by UV/visible, Mössbauer, and EXAFS spectroscopies. HPC-I was prepared in ~ 80% yield and structurally characterized by EXAFS for comparison studies. DFT calculations were used in conjunction with these methods to evaluate pertinent results.

5.4 Results and Discussion

UV/Vis

UV/visible spectral analysis reveal the existence of two different ferric HPC forms. The forms are pH and [NaCl] dependent. At pH < 5 (50 mM citrate), with a

*The intensity of the 1s→3d pre-edge transition in heme enzymes can be highly indicative of the distal Fe-O bond length while shifts in the absorption edge can be used to monitor photoreduction.15,16,17
[NaCl] ≥ 250 mM, ferric HPC is green in color. The color of the protein gradually turns brown with increasing pH, with a pKa ~ 6. Additionally, ferric HPC prepared at pH < 5 (50 mM citrate) with a [NaCl] ≤ 100 mM results in a brown colored protein, and has an identical spectrum to the high pH brown form in the presence of ≥ 250 mM NaCl. The different ferric forms are shown in Figure 5.3.

**Figure 5.3:** UV/visible spectra of green (500 mM NaCl) and brown (No Salt) ferric HPC at pH 5 (50 mM citrate buffer).

There is no literature mention of a ferric conformational change for any type of catalase. In fact, many accounts refer to the color of ferric catalase as brown (*Helicobacter pylori* and *Penicillium vitale* catalase), having a virtually identical UV/visible spectrum to our brown HPC form. To test whether this conformational change was unique to our HPC, we dissolved lyophilized (Sigma Aldrich) *Bovine liver* catalase (BLC) into solutions of citrate buffer (50 mM, pH 5.5), one containing ≤ 100 mM NaCl and the other containing ≥ 250 mM NaCl. The salted solution appeared green
while the no salt solution was brown. Similar differences in the UV/visible spectra to the HPC green→brown ferric transition were also observed. Interestingly, when BLC was dissolved into a pH 3.5 citrate buffer (50 mM, no salt), the solution appeared green in color. This did not occur in low pH solutions of HPC with ≤ 100 mM NaCl. “No salt” BLC solutions prepared sequentially higher in pH resulted in the slow transition to a purely brown colored form. This indicates that the green→brown transition is not purely [NaCl] dependent. The addition of ≥ 250 mM NaCl merely raises the pKa of this transition. From this study it appears that a green→brown ferric conformational change could be a general feature to all catalases.

Table 5.1: Color of ferric catalase at different pHs and NaCl concentrations.

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Both green and brown ferric forms make ~ 100% HPC-II in the presence of peracetic acid (PA) at pH 5. However, depending on the ferric form used to generate HPC-II (green or brown), there are distinct spectral differences. For simplicity, HPC-II generated from green ferric will be referred to as GHPC-II and HPC-II generated from brown ferric will be referred to as BHPC-II. Upon addition of PA to green ferric, the resting state immediately converts to GHPC-II. Isosbestic points suggest the clean
conversion from one species to the other, with no visible indication of HPC-I prior to formation, *Figure 5.4*. Conversely, adding PA to brown ferric results in formation of HPC-I, which then slowly decays to BHPC-II, *Figure 5.5*.

To gain insight on the formation of GHPC-II, we performed target testing* to determine if any HPC-I was made prior to compound II formation. Results indicate that an HPC-I spectrum can be extracted from the GHPC-II data points. This could suggest that in the green ferric conformation there is an oxidizeable, or possibly ionizable, residue interacting with the heme active site. Upon formation of HPC-I this residue is readily oxidized, generating HPC-II almost instantaneously. We propose that in the BHPC-II pathway the compound I self-oxidation process occurs more slowly, possibility due to a conformational change that alters the interaction of the proposed residue, *ie.* swinging away from the active site.

*Figure 5.4*: UV/visible spectra of GHPC-II at pH 5 (50 mM citrate buffer, 500 mM NaCl). Ferric HPC was mixed with 12.5 equivalents of PA. No significant buildup of compound I occurred prior to compound II formation. The Q band ratio is ~ 1.1 (α/β).

*Target testing is a mathematical technique that can be used on the UV/visible data sets in order to determine if specific components (intermediates) are present during a reaction. 20*
Figure 5.5: UV/visible spectra of BHPC-II at pH 5 (50 mM citrate buffer, No Salt). Ferric HPC was mixed with 12.5 equivalents of PA. Significant buildup of compound I occurs prior to compound II formation. The Q band ratio is $\sim 1.3 (\alpha/\beta)$.

The GHPC-II spectrum has a soret max at 431 nm and an alpha Q band intensity that is slightly larger than the beta band intensity ($\alpha/\beta \sim 1.1$). BHPC-II has a soret max at 429 nm and has a different ratio of Q band intensities, with the $\alpha$ Q band being noticeably more intense than the $\beta$ band ($\alpha/\beta \sim 1.3$), Figure 5.6. BHPC-II at low pH, formed in the absence of salt, has an identical spectrum to BHPC-II at high pH in the presence of $\geq 250$ mM NaCl. The spectral differences in GHPC-II/BHPC-II as well as green/brown ferric might be attributed to interactions with the proposed nearby active site residue responsible for the rate of formation/decay differences in compound I (green vs. brown pathway).
Figure 5.6: UV/visible spectral comparison of GHPC-II and BHPC-II. There are clear differences in the position of the Soret max and Q band ratios.

Mössbauer

Mössbauer spectroscopy reveals small differences in the green/brown ferric forms of HPC while GHPC-II/BHPC-II have virtually identical spectra, Figure 5.7. Isomer shifts and quadrupole splitting values are 0.02/2.28 mm/s and 0.02/2.27 mm/s for GHPC-II and BHPC-II respectively. Both HPC-II intermediates have parameters that are typical of an Fe$^{IV}$OH species.$^{3, 6, 21}$ Brown ferric at pH 8 (300 mM NaCl) makes about 60% BHPC-II when reacted with PA (Supporting Information). The quadrupole splitting is identical to BHPC-II at lower pH.
Above pH 9 the yield of BHPC-II with addition of PA becomes less than 20%. This made the identification of a possible ferryl pKa very difficult to monitor by Mössbauer. However, we have recently shown that a high pH ferryl intermediate can be prepared by first making the species at a pH where the yield is the highest, and then pH-jumping to the desired higher pH.\textsuperscript{5,13} Rapid freeze-quench techniques are used to mix the solutions so that the decay and/or degradation of the high pH species is minimized.
Using this method, we were able to prepare HPC-II in higher yield (35%) with almost no degradation at pH > 11.

Mössbauer spectra of HPC-II at increasing pH indicate conversion to a new species, Figure 5.8. The isomer shift/quadrupole splitting parameters of the pH ~ 13.8 intermediate are 0.08/1.51 mm/s, suggesting the formation of an Fe$^{IV}$=O moiety. A plot of the percent HPC-II oxo (determined by Mössbauer) as a function of pH results in a pH titration curve, Figure 5.9. A fit of the curve reveals a pKa ~ 13.1. Because this result is very similar to that previously observed in CYP158A2-II at high pH (pKa ~ 12), we strongly believe it represents a ferryl pKa for a tyrosine ligated heme system.

**Figure 5.8:** Mössbauer spectra of HPC-II at increasing pH. Approximately 60% ferric HPC was subtracted from each spectrum. Samples were prepared by a rapid freeze-quench experiment. 4 mM HPC-II (pH 5.3) was prepared by adding 12.5 equivalents of PA (water) at 4°C. Upon compound II formation the mixture was immediately loaded into a freeze-quench syringe and mixed 2:1 with an arginine/NaOH buffer (pH 14). The strength of the arginine/NaOH buffer was varied (54.08 mM – 216.32 mM) to achieve the desired pH. The reaction mixture was quenched in liquid ethane 3.5 ms after the pH-jump. The isomer shift/quadrupole splitting parameters are 0.02/2.26 mm/s and 0.08/1.51 mm/s for the HPC-II hydroxide (blue) and oxo (red) intermediates respectively.
Figure 5.9: Mössbauer pH titration curve for HPC-II obtained by plotting the % oxo as a function of increasing pH. The best fit curve yields a ferryl pKa of 13.1. Blue data points are from real data. The two red data points are not from experiment, but reflect the appropriate % of HPC-II oxo at pH 10.05 (0%) and pH 14.81 (100%). They were added to improve the quality of the fit, but do not change the experimentally determined pKa.

This result is surprising. Experiments have shown that the rate of H-atom abstraction in metal oxo complexes correlates with the donating ability of the proximal ligand, presumably due to an elevated pKa of the abstracting species. Crystal structures of catalase suggest hydrogen bonding of the proximal tyrosine residue to a nearby arginine, effectively neutralizing the axial charge. Intuition would therefore suggest that the pKa of the ferryl species for a tyrosine ligated heme should be lower than in its thiolate ligated P450 counterpart. This however is not the case, as we have previously shown the pKa in CYP158A2-II to be ~ 12.5

Computation provides interesting insight on this matter. Ferryl pKa trends were determined for a series of heme models to evaluate the effect of different axial ligations.
The series included histidine, tyrosine, and thiolate ligations. Calculations indicate the following trend; Histidine < Phenolate + Arginine < Phenolate < S-H < S-Me < O-Me. These results indicate two major possibilities. Either computation does not do a good job at ferryl pKa predictions, or the catalase tyrosine ligand is more donating than originally thought. These results could suggest that the perception of a hydrogen bonded, neutrally charged tyrosine ligand could be an inaccurate representation of the catalase active site.

**EXAFS**

![Figure 5.10: Fe-K X-ray absorption edges for ferric HPC, HPC-I, and HPC-II.](image)

Because Mössbauer is only suggestive of the protonation status of ferryl intermediates in heme proteins, it was important to confirm with XAS. For comparison, HPC-I was prepared (~80% yield, pH 7) and characterized along with HPC-II (~90% yield, pH 5, 500 mM NaCl) and ferric (pH 5, 500 mM NaCl) HPC. Fe K-edge absorption edges show that both HPC-II and HPC-I lie ~2 eV higher in energy than the ferric
enzyme, Figure 5.10. This is consistent with our assignment of both intermediates existing in higher oxidation states (Fe^{IV}).

Changes in X-ray absorption pre-edge intensities (1s→3d) are highly suggestive of protonation differences in heme intermediates. A short metal-oxygen bond can create a highly asymmetric ligand environment, resulting in an intense pre-edge feature. As the metal-oxygen bond lengthens, the intensity of the pre-edge decreases.\textsuperscript{15-17} This type of behavior suggests that HPC-I is unprotonated (Fe^{IV}=O), while HPC-II is better described as a protonated Fe^{IV}-OH intermediate.

Fits of the EXAFS data and Fourier transforms confirm the protonation assignments implied from pre-edge trends, Figure 5.11. The Fe-O/Fe-O'(tyrosine) bond lengths are 1.66/2.05 and 1.77/1.96 Å for HPC-I (pH 7) and HPC-II (pH 5.3) respectively, Table 5.1. These distances are in good agreement with DFT calculations and previously characterized compound I and compound II (protonated) heme intermediates.\textsuperscript{3,28*}

Because the Mössbauer parameters of HPC-II at pH 5.3 are identical to those up until the proposed ferryl pKa (pH 13.1), we can infer that HPC-II is protonated up until this pH. Preparation of the HPC-II oxo intermediate for EXAFS confirmation of the short Fe^{IV}=O bond are currently underway.

\*A previous x-ray crystallography report assigns a long ~1.8 Å Fe-O bond for the compound I intermediate.\textsuperscript{29} It has been previously shown that this type of result is most likely a consequence of radiation damage.\textsuperscript{13,30,31}
Figure 5.11: Fe K-edge EXAFS data (left) and Fourier transforms (right) of HPC-II (upper) and HPC-I (lower). Black lines show experimental data, and colored lines show the best fits. The fits shown were obtained over the region \( k = 3 - 15 \text{ Å}^{-1} \). All EXAFS samples were analyzed by Mössbauer spectroscopy prior to data collection.
Table 5.2: EXAFS fitting results for HPC-II and HPC-I.

<table>
<thead>
<tr>
<th>HPC-II</th>
<th>Fe-N</th>
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<tr>
<td>N</td>
<td>R</td>
<td>σ²</td>
<td>N</td>
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<table>
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<tr>
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<th>Fe-N</th>
<th>Fe-O’</th>
<th>Fe-O</th>
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<tbody>
<tr>
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<td>R</td>
<td>σ²</td>
<td>N</td>
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</tr>
<tr>
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<td>1.995</td>
<td>0.00203</td>
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</tr>
<tr>
<td>4</td>
<td>1.999</td>
<td>0.00192</td>
<td>1</td>
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</table>

Fe-O’ corresponds to the oxygen from the proximal tyrosine residue.

Raw data were fit over the region k = 3-15 Å⁻¹. Coordination number N, interatomic distance R (Å), mean square deviation in R (the Debye-Waller factor), σ² (Å²), and the threshold energy shift E₀ (eV). The fit error is defined as [Σk(χ²exptl - χ²calc)²/Σkχ²exptl]¹/². Best fits are shown in boldface. Alternative fits with and without the axial Fe-O* contribution are also shown. Because the fit resolution was too large (0.12 Å) to distinguish between a porphyrin nitrogen and the axial Fe-O* scattering atom, it is technically not correct to break apart the scatterers. Coordination numbers, N, were constrained during the fits.

5.5 Conclusion

This report provides evidence for a pH and salt dependent conformational change in ferric catalase enzymes (green→brown). It has also been shown that the compound II UV/visible spectrum is influenced by the ferric form being used to generate the intermediate. Mössbauer spectroscopy of GHPC-II/BHPC-II has revealed that these differences are not indicative of a ferryl pKa. Both forms are protonated from pH 5.3 up until, what we believe, is the true ferryl pKa at ~ 13.1, as evidenced by EXAFS/Mössbauer spectroscopy.
This assignment is in accordance with the prior evidence for a ferryl pKa in CYP158A2-II. Much work has been done to suggest that a high ferryl pKa, afforded by the donating ability of the axial ligand, can tune the reactivity of a metal oxo complex. The elevated ferryl pKa in thiolate ligated hemes is believed to bias the system towards H-atom abstraction over sequential one electron oxidations, as in traditional peroxidases, by allowing for a lower compound I redox potential while maintaining an OH bond strength large enough to facilitate the reaction.

Surprisingly, the pKa for HPC-II is higher than that of the thiolate ligated P450-II (∼ 13 vs. ∼ 12). The consequence of this finding is that the basic ferryl intermediate can no longer be thought of as a general and unique feature to thiolate ligated heme enzymes. Instead, it seems that the basic ferryl is a feature common to enzymes with sufficiently donating axial ligands. This result also implies that tyrosine ligated hemes, assuming a compound I redox potential similar to HRP-I (0.92 V), could be capable of H-atom abstraction of C-H bonds > ∼ 90 kcal/mol.

Strong support for this claim has recently been reported by Brash et. al. using the tyrosine ligated Allene Oxide Synthase (AOS) enzyme. AOS has a larger, more open active site than traditional catalases, and allows for some fatty acid substrate accessibility. (Fatty acids are too bulky for that active site pocket of HPC) Brash shows that AOS-I, generated with meta-chloroperbenzoic acid (m-CPBA), is capable of epoxidation/hydroxylation reactions (NMR and GC-MS product analysis) when reacted with a natural substrate derivative (8R/S-HETE). While kinetic isotope experiments need to be conducted to confirm the mechanism of oxygen insertion (H-atom abstraction),

*The O-H bond strength of the natural H₂O₂ substrate is estimated at ∼ 91 kcal/mol.33
the ability of the AOS enzyme to perform this type of chemistry is in agreement with our finding that tyrosine ligated hemes have high ferryl pKas.

The relationship between axial ligand identity and substrate chemistry (oxidation vs. H-atom abstraction) in heme proteins has been a topic of debate for many years. Synthetic investigations strongly suggest that LPOs are biased towards H-atom abstraction through the high pKa of the abstracting species, but a lack of quantitative evidence for the ferryl basicity in heme enzymes made comparisons suggestive at best. Our recent reports for the magnitude of the proposed driving force (ferryl pKa) in histidine, cysteine, and tyrosine ligated systems lend credence to these comparisons, providing substantial support for the ability of an axial ligand to tune the active site chemistry in heme enzymes.
5.6 Supporting Information

**Figure 5.12:** Mössbauer spectra of the ~ 80% HPC-I (4 mM, pH 7) sample used for EXAFS. Left is raw data and right is after subtracting ~ 20 % ferric.

**Figure 5.13:** Mössbauer spectra of the ~ 90% HPC-II (4 mM, pH 5) sample used for EXAFS. Left is raw data and right is after subtracting ~ 10 % degraded HPC-II. The isomer shift and quadrupole splitting values are 0.02/2.28 mm/s.
Figure 5.14: Mössbauer spectra of BHPC-II at pH 8 (100 mM tris). Left is raw data and right is after subtracting ~ 40% ferric. The isomer shift and quadrupole splitting values are 0.02/2.28 mm/s.

Figure 5.15: Mössbauer comparison of high pH ferric (black, pH 13.8) to green (left, red, pH 5.3) and brown (right, blue, pH 9) ferric HPC. (Black) Ferric prepared by the rapid pH-jump (5.3→13.8) method. The sample was first prepared at pH 5.3 (50 mM citrate, 250 mM NaCl) and then rapidly mixed with high pH buffer and quenched into liquid ethane ~ 3 ms after mixing. (Red) Green ferric prepared at pH 5.3 (50 mM citrate, 250 mM NaCl). (Blue) Brown ferric prepared at pH 9.0 (50 mM tris, 250 mM NaCl). This comparison shows that the changes seen in the compound II Mössbauer spectrum at high pH do no arise from ferric conformational changes.
5.7 References


Chapter 6
Experimental Methods

6.1 Reagents

Most reagents were purchased from VWR International and Fisher Scientific. $^{57}$Fe metal was attained from Penwood Chemicals. Protoporphyrin IX and $\delta$-aminolevulinic acid were purchased from Frontier Scientific. IPTG was purchased from Goldbio. Lyophilized horse heart myoglobin (Mb) and horseradish peroxidase (HRP) were obtained from Sigma Aldrich. $^{18}$O hydrogen peroxide was purchased from Icon Isotopes in a 2% solution. All resins were from GE Healthcare.

6.2 Protein Expression and Purification

Procedure for $^{57}$Fe Enrichment

Starter cultures were grown in LB media. At inoculation, ~ 7.5 mL of LB starter were added per Liter of M9 minimal media. At the time of expression, 1-2 mg/L of $^{57}$FeCl$_3$, 0.5 mM $\delta$-aminolevulinic acid, and 1 mL/L of a solution of trace elements (ZnCl$_2$$\cdot$4H$_2$O, 1 g; CoCl$_2$$\cdot$6H$_2$O, 0.2 g; Na$_2$MoO$_4$$\cdot$2H$_2$O, 1 g; CaCl$_2$$\cdot$2H$_2$O, 0.5 g; CuCl$_2$, 1 g; and H$_3$BO$_3$, 0.2 g in 1 L of 10% HCl) were added to the cultures.

P450 CYP158A2

CYP158A2 (pET 17b vector kindly provided by Prof. Michael R. Waterman) was obtained from overexpression in RosettaBlue (DE3) pLysS (Novagen) competent cells. A starter culture was grown overnight (~ 16 hrs, 37°C, 225 rpm) in LB media
supplemented with 50 μg/mL ampicillin and 100 μg/mL of chloramphenicol. The starter was used to inoculate a larger (2-3 L of media) flask. At an O. D. of 0.6-0.8, 0.5 mM IPTG and δ-aminolevulinic acid were added along with an additional aliquot of antibiotics (25 μg/mL ampicillin and 50 μg/mL of chloramphenicol). The temperature and shaker speed were reduced to facilitate proper expression and folding of the protein (28°C, 100 rpm).

After 24 hours, the cells were harvested, redissolved in buffer (50 mM tris-HCl, pH 7.5, 500 mM NaCl, 10% (v/v) glycerol, and 3 mM imidazole), and lysed using a microfluidizer processor (M-110EH-30). The protein was loaded onto a metal (Ni²⁺) affinity column (Qiagen) and washed with 2-3 column volumes of lysis buffer. CYP158A2 was then eluted with buffer containing 50 mM tris-HCl, pH 7.5, 10% (v/v) glycerol, and 80 mM imidazole. Further purification was performed by Q sepharose chromatography (GE Healthcare). Protein was washed with 2 column volumes of buffer containing 20 mM tris-HCl, pH 7.5. CYP158A2 was eluted using a 0-500 mM NaCl gradient of the same buffer. Fractions with an R₂ (A₄₁₆/₂₈₀) greater than 1.8 were pooled for size exclusion (S-100) chromatography (GE Healthcare) in 20 mM tris-HCl buffer, pH 7.5. CYP158A2 with an R₂ greater than 2.0 was used for experiments. To remove fluorescence (resonance Raman), an additional octyl sepharose column can be run (20 mM tris-HCl, pH 7.5). In this case, protein with R₂ > 2.10 should be used.

For ⁵⁷Fe enrichment, cultures were grown in M9 minimal media supplemented with thiamine and proline (1g of each per 3L culture). At induction, 1-2 mg/L of ⁵⁷FeCl₃, 0.5 mM δ-aminolevulinic acid, and 1 mL/L of a solution of trace elements were added to the cultures. The remaining steps are the same as in the rich media procedure.
Helicobacter Pylori Catalase (HPC)

HPC (pSO100 vector kindly provided by Prof. Peter Loewen) was obtained from overexpression in BL21 (DE3) (Novagen) competent cells. A starter culture was grown overnight (~ 16 hrs, 37°C, 225 rpm) in LB media supplemented with 50 μg/mL of kanamycin. The starter was used to inoculate a larger (2-3 L of media) flask. At an O.D. of 0.8-1.0, 0.5 mM IPTG and δ-aminolevulinic acid were added along with an additional aliquot of kanamycin (25 μg/mL). The temperature and shaker speed were reduced to facilitate proper expression and folding of the protein (28°C, 100 rpm).

After 24 hours the cells were harvested, re-dissolved in buffer (50 mM Kphos, 50 mM EDTA, 1% triton x-100 biological surfactant or 1% polyethylene glycol monododecyl ether, pH 7), and lysed using a microfluidizer processor (M-110EH-30). Protein was then initially purified by ammonium sulfate precipitation. Subsequent 40% and 50% fractions were discarded. Fractions between 50-80% were HPC containing. Pellets were resuspended in a minimum amount of 50 mM Kphos buffer (pH 7) and run down a DE-52 column (Whatman), separating a red protein (siderphore) band. Protein was then buffer exchanged into 50 mM citrate (pH 5) and loaded onto a Source S column (Whatman). HPC was washed with 2 column volumes of starting buffer and eluted using a 0-500 mM NaCl gradient of the same buffer. Fractions with R_z > 0.80 were pooled for use.

For ^57Fe enrichment, half of the total aliquot of ^57Fe was added at inoculation and the other half was added at induction. This helped to prevent against siderphore formation. 0.5 mM δ-aminolevulinic acid and 1 mL/L of a solution of trace elements
were also added at induction. The remaining steps are the same as in the rich media procedure.

### 6.3 Preparation of Mb for Mössbauer

#### $^{57}$Fe Heme Synthesis for Mb Mössbauer

$^{57}$Heme synthesis was adapted from the metalloporphyrin synthesis of Adler et al.\(^1\) 10 mg of $^{57}$Fe metal was anaerobically dissolved (60°C) in 1 M HCl to form ferrous chloride. The HCl was evaporated to dryness. The white, powdered, Fe$_2$Cl was combined with 25 mg protoporphyrin IX in a flask containing 50 mL of deaerated DMF. After refluxing the materials for 15 min, the mixture was cooled on ice and exposed to air.

The mixture was then diluted 10x with diethyl ether. Excess ferric salts were separated from the ether layer with 100 mM HCl containing 100 mM NaCl. Excess protoporphyrin IX was separated out with 1 M HCl. The remaining ether phase was then washed to neutrality with H$_2$O and evaporated to dryness. $^{57}$Heme was stored in a -80°C freezer until use.

#### Incorporation of $^{57}$Heme into Mb

1) **Generation of Apo Mb**

Apo Mb was generated using the Teale method.\(^2\)\(^,\)\(^3\) A solution of Mb was acidified to pH 2. $^{56}$Heme was separated from the apo protein by extraction with methyl-ethyl ketone. Several rounds of dialysis, first in H$_2$O and then in Kphos buffer (50 mM, pH 7), were carried out to remove excess methyl ethyl ketone.
2) **Reconstitution**

A slight excess of $^{57}\text{Heme}$ was dissolved in 100 mM NaOH. The solution was then mixed with tris-HCl buffer to a final pH ~ 8.5 and added to the apo Mb (pH 7) solution. The mixture was allowed to stir at 4°C for 30 min. Excess heme was removed by anion exchange chromatography (Whatman De-52 resin). Fractions with $R_z > 5$ were pooled for use.

### 6.4 Generation of the Intermediates Studied

**Ferryl Oxygen Exchange Experiments**

1) $^{18}\text{O}$ Exchange of Ferryl Oxygen in Mb-II (Monitored by Resonance Raman)

1 mL of Mb-II (~ 1 mM) was generated in borate buffer (100 mM, pH 8.5) by mixing with 2.5 x $\text{H}_2^{18}\text{O}_2$. The 1 mL solution of Mb-II was then divided into 200 µL aliquots and frozen in individual Mössbauer cups for resonance Raman. The cups were frozen at $t = 30$ secs, 12 min, 24 min, 36 min, and 60 min.

2) $^{18}\text{O}$ Exchange of Ferryl Oxygen in HRP-II (Monitored by Resonance Raman)

HRP-II (~ 2 mM) was generated in carbonate buffer (10 mM, pH 10) by mixing with 1-2 equivalents of $\text{H}_2^{18}\text{O}_2$. Upon max yield (monitored by UV/Vis), the solution turned bright red. HRP-II was then mixed (pipetted) against a high strength kphos buffer (pH 6.1) as quickly as possible and frozen in liquid nitrogen. This is known as the pH-jump method. The samples were frozen in Mössbauer cups for resonance Raman spectroscopy.
Freeze-Quenched Samples

Freeze-quench methods were used to generate many of the intermediates. A four syringe ram freeze-quench apparatus from Update Instruments (Madison, WI) was used for all freeze-quench experiments. Samples were quenched into liquid ethane (-160°C). A liquid isopentane bath (-144 °C) was used in conjunction with a vacuum line to “pull off” the ethane. The cryo-solvent free sample was then packed in an EPR tube or Mössbauer cup for spectroscopic analysis. All rapid freeze-quench experiments were performed by using the shortest aging line (6.4 μL) at a ram push speed of 8 cm/sec.

Mb-II Preparation at Low pH

Mb-II (4.5 mM) was generated in borate buffer (20 mM, pH 9.5) prior to freeze-quenching by mixing with ~ 2.5 equivalents H₂O₂. Mb-II was then rapidly loaded into the freeze-quench syringe (4°C) and quenched 2:1 against a high strength buffer near the desired pH (Kphos 5.5 - 7 and acetate 3.9 – 5.5). Samples were packed into Mössbauer sample cups for spectroscopic analysis at a final protein concentration of 3 mM. Portions of the quenched samples were set aside to confirm the final pH of the solution.

Aqua (Mb³⁺-OH₂) and Alkaline Ferric Mb (Mb³⁺-OH) Preparation for Mössbauer

Solutions of 4 mM Mb³⁺-OH₂ (100 mM Kphos buffer, pH 7) and Mb³⁺-OH (100 mM borate buffer, pH 10.9) were pipetted into Mössbauer cups and frozen into liquid ethane. Ferric Mb samples were never hand-quenched into liquid nitrogen because of the known formation of hemochrome during the (slower) liquid nitrogen freezing process.
CYP158A2-II Hydroxide and Oxo Preparation

1) Preparation of CYP158A2-II Hydroxide

Ferric CYP158A2 (4.5 mM) was reacted with 5x m-CPBA in a 2:1 mixture to form compound II hydroxide. CYP158A2 was in tris-HCl buffer (100 mM, pH 9) and m-CPBA was in a 30% solution of acetonitrile. Reactants were mixed and quenched into liquid ethane 3.5 ms after mixing. Samples were packed into Mössbauer sample cups for spectroscopic analysis at a final protein concentration of 3 mM. The final concentration of acetonitrile was 10%.

2) Preparation of CYP158A2-II Oxo.

Samples were prepared using a three syringe, double mix setup. Two of the shortest aging lines were used for the double mix. 6 mM ferric CYP158A2 was reacted with 5x m-CPBA in a 2:1 mixture to form compound II hydroxide. CYP158A2 was in tris-HCl buffer (20 mM, pH 9) and m-CPBA was in a 40% solution of acetonitrile/water. Compound II hydroxide was then reacted in a subsequent 3:1 mixture with an arginine HCl/sodium hydroxide buffer (pH 14). The strength of the buffer was varied (10 mM – 108.16 mM) to change the final pH of the solution, thus altering the ratio of compound II oxo/hydroxide. The reaction was quenched in a liquid ethane bath 7 ms after the initial formation of compound II. Portions of the quenched samples were set aside to confirm the final pH of the solution. Samples were packed into Mössbauer sample cups for

* The stock buffer used to make 100% compound II oxo contained 0.570 grams arginine HCl, 10 mL 6M NaOH, and 15 mL deionized water. All other quenches of varying compound II hydroxide/oxo ratios were made with dilutions of this stock to reach the appropriate arginine HCl concentration (10mM – 108.16mM).
spectroscopic analysis at a final protein concentration of 3 mM. The final concentration of acetonitrile was 10%.

**HPC-II Hydroxide and Oxo Preparation**

1) **HPC-II Hydroxide**

Samples were prepared by mixing 3-5 mM ferric HPC with ~20 x peractetic acid (PA). From pH 3.5→5.5 HPC was in citrate buffer (50 mM, with or without 500 mM NaCl) and from pH 5.5→8 HPC was in sodium phosphate buffer (50 mM, with or without 500 mM salt). Tris buffer (50 mM, with or without 500 mM NaCl) was used from pH 8→9. Above pH 7, sequential additions of PA and ascorbate (8.5 x PA, 40 x ascorbate, 8.5 x PA, 40 x ascorbate) were required in order to generate HPC-II in high yield. Once the mixture turned bright red, the solution was pipetted into a Mössbauer cup and hand-quenched into a liquid ethane bath.

2) **HPC-II Oxo**

HPC-II was generated at pH 5.3 (50 mM citrate, 250 mM NaCl) prior to freeze-quenching by mixing with ~12.5 equivalents PA (4°C). HPC-II was then rapidly loaded into the freeze-quench syringe (4°C) and quenched 2:1 against an arginine HCl/sodium hydroxide buffer (pH 14). It is essential that the quench be executed within 1-2 min of initially making HPC-II because of the rapid decay of the intermediate. The strength of the buffer was varied (54.08 mM – 216.32 mM) to change the final pH of the solution,
thus altering the ratio of compound II oxo/hydroxide. The reaction was quenched in a liquid ethane bath 3.5 ms after mixing. Portions of the quenched samples were set aside to confirm the final pH of the solution. Samples were packed into Mössbauer sample cups for spectroscopic analysis at a final protein concentration of 3 mM.

**HPC-I Preparation**

Samples were prepared by mixing 3-5 mM ferric HPC (50 mM sodium phosphate, pH 7, no salt) with 5 x Peractetic Acid (PA). Once the mixture turned from brown to green (1-2 seconds), the solution was pipetted into a Mössbauer cup and hand-quenched into a liquid ethane bath.

**Ferric HPC and HPC-I Preparation for Cryoreduction**

4 mM ferric HPC and HPC-I were prepared at pH 9 (30% glycerol, 100 mM tris buffer) and irradiated by a $^{60}$Co gamma source (4.5 MRad). HPC-I was annealed in an acetone/dry ice bath (-80°C).

**pH Experiments**

1) *Conversion of Mb$^{III}$-OH to Mb$^{III}$-OH$_2$ on the Freeze-Quench Timescale*

Mb$^{III}$-OH (2 mM) was prepared in borate buffer (20 mM, pH 10.9). This protein was then mixed 2:1 against high strength Kphos buffer (pH 7, > 200 mM) in a rapid

* The stock buffer used to make 100% compound II oxo contained 0.570 grams arginine HCl, 10 mL 6M NaOH, and 2.5 mL deionized water. All other quenches of varying compound II hydroxide/oxo ratios were made with dilutions of this stock to reach the appropriate arginine HCl concentration (54.08 mM – 216.32 mM).
freeze-quench setup. The mixture was frozen in liquid ethane 3 ms after mixing. Samples were packed into EPR tubes for resonance Raman spectroscopy.

2) **Conversion of Mb$^{III}$-OH to Mb$^{III}$-OD on the Freeze-Quench Timescale**

Mb$^{III}$-OH (4 mM) was prepared in borate buffer (100 mM, pH 10.9). The protein was then mixed 1:2 against deuterated borate buffer (100 mM, pD 11.3) in a rapid freeze-quench setup. The mixture was frozen in liquid ethane 3 ms after mixing. The 66% deuterated sample was packed into an EPR tube for resonance Raman spectroscopy.

3) **Using the Mb-OH$_2$→Mb-OH (pka = 8.93) Transition to Determine the pH Change of Tris Buffer at Low Temperature**

4 mM ferric Mb (20 mM tris, pH 8.98) was pipetted into a Mössbauer cup and hand-quenched into liquid ethane at the ~ pKa of the Mb$^{III}$-OH$_2$→Mb$^{III}$-OH transition. Simultaneously, a dilute sample (in the same buffer) was analyzed by UV/Vis (4-20°C) so that it could be compared to the Mössbauer spectrum in order to evaluate temperature effects on pH.

**6.5 Synthesis of $^{18}$O $m$-CPBA$^*$**

$^{18}$O $m$-CPBA was synthesized by the method of Jankowski and Kaminski.$^4$ Briefly, $^{18}$O H$_2$O$_2$ from Icon Isotopes (1 mL of a 2% solution) was added to a mixture of sodium hydroxide, magnesium sulfate, and dioxane at 0°C. The solution was stirred vigorously (magnetic stir bar) in an ice bath. Next, 3 chlorobenzoyl chloride was injected

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$m$-CPBA is a potentially flammable oxidizing agent. Be careful!
under the surface of the solution and stirring was continued for 30 min. Then, 20% sulfuric acid was added to the solution as it was transferred to a separatory funnel. *m*-CPBA was extracted with four volumes of chilled dichloromethane. The extract was dried over magnesium sulfate, filtered, and evaporated under vacuum. The dried $^{18}$O *m*-CPBA was stored in a -80°C freezer until use.

### 6.6 Spectroscopic Techniques

1) **Mössbauer Spectroscopy**

A spectrometer from WEB Research (Edina, MN) was used to collect data in constant acceleration mode with a transmission geometry. Spectra were recorded with a 53 mT magnetic field applied parallel to the $\gamma$-beam. All measurements were recorded at 4.2 K using a Janis SVT400 cryostat. Isomer shifts were calibrated relative to the centroid of the spectrum of a metallic foil of $\alpha$-Fe at room temperature. Data analysis was performed using the program WMOSS from WEB research.

2) **Stopped-Flow Spectrophotometry**

Spectral changes were monitored using an SFM-400 stopped-flow spectrometer (Bio-Logic SA, Claix, France). The minimum dead time of the instrument is 1.6 ms. An L7893 light source (Hamamatsu, Tokyo, Japan) and a TIDAS photodiode (J&M GmbH, Essingen, Germany) were used to collect absorption data. All experiments were performed at 4°C. Singular Value Decomposition (SVD) and target testing were performed using a program written by Dr. Jarod Younker in Igor Pro (Wavemetrics, Inc.: Lake Oswego, OR 97035).
3) EXAFS Spectroscopy

XAS data were collected in fluorescence mode at ~ 10 K with a 30-element germanium detector (SSRL, BL7-3) using a Si(220) $\Phi = 90^\circ$ double monochromator with a 9.5 keV cutoff for harmonic rejection. To minimize the effects of photoreduction, samples were moved in the beam so that an unexposed portion of the sample was examined during each set of measurements (exposure time ~ 15min per scan). The effects of photoreduction were monitored via the analysis of data obtained during the second acquisition scan. Background removal and curve fitting were performed with EXAFSPAK (available at http://www.ssrl.slac.stanford.edu/exafspak.html) using \textit{ab initio} phases and amplitudes generated with FEFF version 8.0. Data sets were fit over the range $k = 3 – 15$ Å$^{-1}$. Coordination numbers, $N$, were constrained during the fits. Fits included first and 2$^{nd}$ shell atoms and one multiple scattering component. In all cases, the 2$^{nd}$ shell was comprised of $\alpha$- and meso-carbons and the Fe-$\text{C}_\alpha$–$\text{N}$-Fe multiple scattering paths ($n = 8, 4, \text{ and } 16$ respectively). All distances, $R$, and Debye-Waller factors, $\sigma^2$, were treated as adjustable parameters, and all threshold energy shifts, $E_o$, were linked but allowed to vary. The passive electron reduction factor, $S_o$, was fixed at 0.9. Edge energies were calibrated using $\alpha$-Fe metal foil (7111.3 eV). Edge positions were obtained from the first derivative of the data using EXAFSPAK (1.0 eV smoothing, third order polynomial).
4) Resonance Raman Spectroscopy

Resonance Raman spectra were recorded on a triVista 555 triple monochromator (900/900/2400 gr/mm) equipped with a CCD camera (1340 x 100 pixels). A 501.7 nm line of an argon-ion laser and a 413.1 nm line of a krypton-ion laser were used for excitation. The power was < 25 mW at the sample. Samples were held in a Janis STVP-100 cryostat with a custom holder at 77 K using a ~ 135° back scattering arrangement. Raw Spectra were analyzed using the program Igor Pro for background subtraction. No smoothing procedures were performed on the raw data.


Because of the glass peak contributions from EPR tubes in Raman spectroscopy, we started preparing samples in exposed Mössbauer cups. The cups were mounted in our cryostat sample holder and exposed to the exciting laser (no glass peaks). Hand-quenched samples could be easily prepared; they were pipetted into the Mössbauer cup and frozen in liquid nitrogen. Once frozen, the protein solution adhered to the cup. Conversely, freeze-quenched samples had to be packed into the cup as a frozen powder. The powdered sample floated out of the cup as soon as it was immersed in liquid nitrogen. To combat this issue, we devised the “ethanol-bed” pack method for powdered samples.

Briefly, a Mössbauer cup was filled ~ ½ way with ethanol and frozen in a bath of liquid nitrogen. Excess nitrogen in the cup was flicked away so that the sides of the cup were surrounded with liquid nitrogen (on a packing block), but none was on the surface of the frozen ethanol (inside the cup). The (dry) powdered sample was then scooped from a falcon tube and deposited on the surface of the frozen ethanol-bed. The powdered
protein was then compacted with a packing rod. After the surface of the ethanol-bed had been completely deposited with the powdered protein, the Mössbauer cup was gently placed upright in a liquid nitrogen dewar. From here the cup can be transferred to the Raman cryostat using the appropriate sample holder. Sample integrity is best upheld by using helium vapor to cool the cryostat (rather than liquid nitrogen).
6.7 References


Chapter 7
Summary and Conclusions

The overall goal of my research was to quantitate the ferryl pKa in three different heme systems (with axial histidine, cysteine, and tyrosine ligation). This goal was based on insight from Michael T. Green, who proposed that the pKa of the rebounding metal oxo intermediate in H-atom abstraction has tremendous influence on reactivity. Our hopes were that by determining the magnitude of this thermodynamic parameter, we could provide substantial insight on the driving force behind C-H bond activation in heme proteins. A short summary of each chapter is provided below.

The experiments discussed in chapters 3, 4, and 5 involved the preparation of transient intermediates by rapid pH-jump mixing and freeze-quench techniques. Before spectroscopic analysis of the samples could take place, chapter 2 was used to evaluate the integrity of the preparation methods. Previous reports indicate that the pH of buffered solutions changes substantially at lower temperatures, especially below 0°C. Because we were specifically interested in the ferryl pKa of enzyme intermediates in frozen solutions, it was imperative to determine the “true” pH of the system. By using a pH dependent structural change in ferric Mb (Mb\(^{III-\text{OH}_2}\)→Mb\(^{III-\text{OH}}\) with pKa ~ 8.93), we were able to show that temperature related pH changes (in buffered solutions) are suppressed when in the presence of concentrated protein solutions. From these experiments we could infer that any cryogenically determined pKa reflected the “true” pH of the system.

An additional concern was that we were mixing and freezing our solutions so rapidly (rapid freeze-quench) that equilibration could not take place, \textit{ie.} the pH of our
rapidly jumped solutions was not being communicated on the quench time scale (10-15 ms). This was important because although we knew that the pH was not changing after freezing, we needed to be sure of the exact pH at the time of freezing. To answer this question, we again turned to experiments involving the Mb$^{\text{III}}$-OH$_2$→Mb$^{\text{III}}$-OH transition. By using resonance Raman spectroscopy we were able to confirm that pH is fully communicated on the freeze-quench timescale. The results discussed in chapter 2 ensure the integrity of the techniques used to cryogenically trap and characterize the transient intermediates of later chapters.

Chapter 3 looks at ending the protonation status controversy in histidine ligated compound II intermediates. X-ray crystallographic studies report long Fe-O bond distances (~ 1.85 Å), indicating protonation, while EXAFS studies report shorter, authentic oxo distances (~ 1.65 Å). Clarification of this discrepancy was important; if the driving force for C-H bond activation hinges on a high compound II pKa, then a basic histidine ligated ferryl should be able to do oxygen insertion reactions (which experimentally they cannot). Because the protonation status of Mb-II is perhaps most controversial, we decided to re-evaluate the histidine ligated ferryl protonation claims by starting there.

Through EXAFS, resonance Raman, and Mossbauer/DFT we were able to definitively show that Mb-II is not protonated, setting an upper limit on the pKa of \( \leq 2.65 \). This study implies that all histidine ligated ferryls exist as Fe$^{\text{IV}}$=O intermediates, and that previous crystallographic reports for protonation are a direct result of photoreduction. Extensive reports on radiation damage in heme crystalline proteins by Poulos and Shlichting support our claim.
Chapter 4 introduced the study of the cysteine ligated CYP158A2 enzyme. Until this point, no one had ever prepared a compound II intermediate in P450 in > ~ 48% yield. Previous Mössbauer experiments of P450cam-II (28%) and P450BM3-II (48%) confirmed their basic nature, but low yield precluded further spectroscopic analysis. Remarkably, the CYP158A2 enzyme allowed for the unprecedented buildup (> 90% at pH 9) of this transient species, making the spectroscopic characterization by UV/Vis, Mossbauer, and EXAFS possible. A large $\Delta$E$_{q}$ (2.02 mm/s) determined by Mössbauer suggested a protonated ferryl. This result was later confirmed by EXAFS, yielding an Fe$^{IV}$-OH bond length of 1.84 Å.

At this point, we wondered if it would be possible to prepare CYP158A2-II at higher pH in order to put a lower limit on the ferryl pKa. Previous investigations with the unique thiolate ligated analogue, CPO-II, only allowed for a lower limit of 8.2 to be determined for thiolate ligated systems. Above pH 7.0 an irreversible alkaline transition prohibits the formation of CPO-II. However, in the CYP158A2 system, there appeared to be no such pH limitations on the formation of compound II, especially with the use of rapid pH-jump mixing experiments. By preparing CYP158A2-II at pH 9 (where the yield is the greatest) and then pH-jumping the intermediate to higher pH, we were able to observe a unique transition at a pKa of ~ 12. The new intermediate could be prepared in high yield (>90%) and spectroscopically characterized by UV/visible, Mossbauer, EXAFS, and resonance Raman spectroscopies. Characterized by a narrow $\Delta$E$_{q}$ in Mössbauer (1.30 mm/s), a short Fe-O bond distance (1.68 Å) in EXAFS, and an Fe$^{IV}$=O stretching frequency of 814 cm$^{-1}$ (shifting to 777 cm$^{-1}$ with $^{18}$O) using resonance Raman, we had determined the first ever ferryl pKa.
This determination allowed us to put a quantitative measure on the thiolate's impact on reactivity. Indirectly, it allowed for insight on the compound I redox potential in P450s (1.12 V assuming D(O-H) = D(C-H) ~ 100 kcal/mol). These experiments provided concrete evidence that Nature may be using a high ferryl pKa, coupled with a low compound I redox potential, to bias a system towards C-H bond activation over sequential 1 electron oxidations.

In chapter 5 we moved on to the tyrosine ligated HPC enzyme. We were interested in this system because a previous investigation by Jouve et al suggested a ferryl pKa of 8.4. After careful analysis of the methods used to make this assignment, we decided that a reinvestigation was warranted.

We were able to prepare HPC-II in > ~ 90% yield from pH 4 → pH 7. Mössbauer revealed a large ΔEq (2.28 mm/s) and EXAFS yielded an Fe-O bond distance of 1.77 Å, indicating a protonated ferryl (Fe^{IV}-OH). Similar to the CYP158A2-II system, the yield of HPC-II decreased with increasing pH. To circumvent this problem, we again implemented the pH-jump method and prepared freeze-quenched samples at higher pH. Mössbauer samples of HPC-II at increasing pH revealed a significant structural change with pKa ~13. The new high pH species had a much narrower ΔEq (1.51 mm/s) than the low pH HPC-II form, suggesting the deprotonation of the ferryl moiety (Fe^{IV}=O). While an EXAFS confirmation of the high pH intermediate is still needed, we can infer from the previous investigation on CYP158A2 that this change is indicative of a ferryl pKa.

To our surprise, the ferryl pKa of a tyrosine ligated heme was higher than that of a cysteine ligated system. Basic ferryls can no longer be thought of as a general and unique feature to thiolate ligated systems, rather a feature of systems with sufficiently
donating axial ligands. This result implied that catalases have the potential to perform C-H bond activation, even though their small active sites prohibit fatty acid substrate access.

Supporting evidence for the possibility of C-H bond activation in tyrosine ligated heme enzymes was later provided by Alan Brash of Vanderbilt University. Through experiments with allene oxide synthase (AOS), a catalase-like enzyme with a larger active site pocket, Brash et al. showed that this system is capable of both epoxidations and activated C-H bond hydroxylations. These experiments could provide the first evidence of “true” C-H bond activation in a non-thiolate ligated heme system (excluding experiments with variants). Further kinetic isotope experiments are needed for confirmation of the mechanism of oxygen insertion.

While we were only able to directly observe the ferryl pKa in thiolate and tyrosine ligated heme enzymes, the establishment of an upper limit for the ferryl pKa in the histidine ligated Mb protein provides valuable insight. Assuming that the ferryl pKa in Mb is similar to that of other histidine ligated enzymes, the difference in the magnitude of the ferryl basicity between histidine and thiolate/tyrosine ligated proteins could account for 13-14 kcal/mol of driving force. The extent of this difference could explain the discrepancy in reactivity between these systems, with a unit increase in the ferryl pKa allowing for a 59 mV drop in the compound I redox potential. It seems that through the use of donating ligands, Nature creates a low potential oxidant, biasing a system to perform cleavage of the most inert C-H bonds while avoiding unwanted oxidations of the protein superstructure.
A.1 The Effects of Sample Preparation on Resonance Raman Spectra

Glass Peak Contributions

Most of our Raman experiments were performed in quartz EPR tubes. We noticed early on that there were significant “glass peak” contributions resulting from this surface, Figure A.1. Unfortunately, the major glass contributions are centered over the 500 cm\(^{-1}\) and 800 cm\(^{-1}\) regions, the same areas that we would roughly expect Fe\(^{IV}\)=O/Fe\(^{III}/IV\)-OH stretching frequencies for heme proteins. We worried that any stretches of interest could be potentially “drowned out” by the glass peaks. In order to circumvent this problem, we prepared many of our samples in Mössbauer cups. The cups could be mounted in our cryostat with the surface of the sample directly exposed to the exciting laser. Hand-quenched samples could simply be frozen in the cups while the powdered freeze-quenched intermediates had to be packed using a special “ethanol-bed” pack method (see experimental).
Figure A.1: Low frequency resonance Raman comparison of ferric CYP158A2 in a quartz EPR tube (black) and a Mössbauer cup (red). Differences in the spectra result from the glass peak contributions of the quartz tube. The peak between the blue asterixes is from the laser line. Data was collected using the 458 nm argon laser line.

Fluorescent Backgrounds in P450s

In the initial examination of all P450 species, including ferric, we noticed significant fluorescence when using argon laser lines (≥ 458 nm). Fluorescence was not present when using the 406/413 nm krypton laser lines. The “rising background” either completely drowned out, or significantly convoluted any “real” signal, Figure A.2. We initially thought that this phenomenon was native to the protein, and was unavoidable. However, with extensive purification, we realized that the contaminating species could be removed, affording us the opportunity to probe P450 intermediates with argon laser lines. This is extremely important because the Soret band of P450-II is significantly red shifted (428/440 nm for CYP158A2-II hydroxide/oxo) relative to ferric (416 nm). Intuition
suggests that the closer the exciting laser wavelength is to the Soret band, the better the chance for enhancement of the new intermediate.

Figure A.2: High frequency resonance Raman comparison of ferric CYP158A2 after moderate (blue) and extensive (red) purification. The rising background (fluorescence) in the blue spectrum can be attributed to impurities in the protein. Both samples are ~1 mM. Data was collected using the 458 nm argon laser line.

A.2 Various Resonance Raman Experiments

CYP119-I Raman

CYP119-I was analyzed by resonance Raman (~70% yield by Mössbauer). Using the krypton laser (406/413 nm laser lines), we could only see ferric enhancement. However, the 458 nm argon line enhanced a new set of features, which were very distinguishable from the ferric spectrum, Figure A.3 and Figure A.4. Although there were numerous peaks between 750–815 cm⁻¹, where one would think an Fe⁴⁺=O stretch should be present, none were sensitive to ¹⁸O isotope substitution.
Figure A.3: Low frequency resonance Raman spectra of ferric CYP119 and CYP119-I. We thought that one of the peaks centered at 802 cm$^{-1}$ in CYP119-I could represent the Fe$^{IV}$=O stretch. However, no shifts were observed in $^{18}$O labeling experiments. Data was collected using the 458 nm argon laser line.

Figure A.4: High frequency resonance Raman spectra of ferric CYP119 and CYP119-I. Data was collected using the 458 nm argon laser line.
CYP158A2-II Oxo Raman

While the CYP158A2-II hydroxide intermediate readily photoreduced in the laser (discussed later), the CYP158A2-II oxo intermediate was much more stable, Figure A.5. Using $^{16}$O/$^{18}$O m-CPBA, we were able to locate a Fe-O sensitive stretch at 817 cm$^{-1}$. The 33 cm$^{-1}$ shift (784 cm$^{-1}$) is in good agreement with harmonic oscillator predictions and previously characterized Fe$^{IV}$=O heme stretching frequencies, Figure A.6. Low frequency comparisons between ferric and compound II reveal that there is some slight ferric contamination in our $^{16}$O sample, Figure A.7. This is in agreement with Mössbauer characterizations of both the $^{16}$O (â˜‰ 75%) and $^{18}$O (â˜‰ 90%) intermediate.

Figure A.5: High frequency resonance Raman spectra of ferric CYP158A2 and CYP158A2-II oxo. Data was collected using the 458 nm argon laser line.
Figure A.6: Low frequency resonance Raman spectra of CYP158A2-II oxo. Data was collected using the 458 nm argon laser line.

Figure A.7: Low frequency resonance Raman spectra of ferric CYP158A2 and CYP158A2-II oxo. It can be seen that there is a small amount of ferric enhancement in the $^{16}O$ CYP158A2-II oxo spectrum. Data was collected using the 458 nm argon laser line.
**Synthetic Mn-O Raman**

We collaborated with a number of research groups during the course of my graduate career. Mhadi Abu-Omar, from Purdue University, sent a high valent Mn-oxo complex in which he was hoping for resonance Raman confirmation of the Mn-O coordination. Through the use of isotopic labeling, we were able to confirm an oxygen sensitive stretch at 958 cm\(^{-1}\) that shifted to 920 cm\(^{-1}\) upon \(^{18}\)O substitution, Figure A.8. This stretching frequency is in good agreement with Goldberg’s previously characterized Manganese(V)-Oxo Corrolazine complex with a Mn-O stretching frequency at 979 cm\(^{-1}\) (shifting to 938 cm\(^{-1}\) with \(^{18}\)O substitution).\(^1\)

![Figure A.8](image)

**Figure A.8:** Low frequency resonance Raman spectra of the high valent synthetic manganese oxo complex. Data was collected using the 413 nm krypton laser line.
**Mb-II $^{18}$O Exchange with Bulk Water**

Our $^{18}$O isotopic labeling experiments led to the discovery that the ferryl oxygen in Mb-II is readily exchangeable with bulk water at high pH. This phenomenon has been observed in other histidine ligated heme proteins such as HRP, ARP, and CCP.²⁻⁴ For HRP-II and ARP-II, the exchangeability of the ferryl oxygen coincides with the protonation of the distal histidine residue and subsequent hydrogen bonding to the oxo. Below the pKa of this hydrogen bonding event, rapid oxygen exchangeability with bulk water is observed. The exchange occurs so rapidly that no compound II Fe$^{IV}=O$ stretching frequency is observed by Raman when the enzyme is reacted with $^{18}$O substrate ($H_2^{18}O_2$). Only when the bulk solvent surrounding the active site is $^{18}$O ($H_2^{18}O$) labeled can an oxygen sensitive Raman stretch be detected. Interestingly, above the pKa of the distal histidine protonation, no oxygen exchangeability is exhibited. (This has not been confirmed for CCP-ES) It was concluded that the hydrogen-bonded proton has an essential role in the acid/base catalysis of these enzymes and that their alkaline deactivation can be attributed to the lack hydrogen bonding at high pH.³⁻⁴

The exchange of the ferryl oxygen with bulk water in Mb-II presents a novel circumstance for this phenomenon. At pH 8.5 there is no indication of a proton hydrogen bonding to the ferryl oxygen, yet exchange occurs, **Figure A.9**. Additionally, the exchange occurs so slowly that a time course of the exchange can be followed by Raman spectroscopy, **Figure A.10** and **Figure A.11**. Fits of the area under the Fe-$^{16}$O and Fe-$^{18}$O stretching frequencies as a function of time yield a $\frac{1}{2}$ life of ~ 18 min for this process, **Figure A.12**. Rietjens proposes that the rapid oxygen exchange of HRP and ARP may be due to the reversible formation of compound I.⁵ It seems unlikely that this mechanism
could be used to explain the exchange in Mb-II because of the inability to generate Mb-I in a steady state. The slow exchange in the case of Mb-II provides an opportunity to investigate the mechanism by which this common biological process occurs.

**Figure A.9:** Depiction of the ferryl oxygen exchange phenomenon in Mb-II. Unlike HRP-II, the protonation and H-bonding of the distal histidine to the ferryl oxygen is not necessary to facilitate this process.

**Figure A.10:** Low frequency resonance Raman time sequence of the ferryl $^{18}$O exchange to $^{16}$O (from bulk solvent) where “A,B,C,D,E” represent $t = 30$ secs, 12 min, 24 min, 36 min, 60 min. Data was collected using the 501 nm argon laser line.
Figure A.11: Low (left) and high (right) frequency resonance Raman spectra of the samples from the first (30 sec) and last (1 hr) $^{18}$O Mb-II time points compared to ferric Mb. The enhancement features of the ferric and ferryl states are easily distinguishable. It can be seen that the ferryl oxygen exchange is authentic and is not merely a consequence of contaminating ferric enhancement in a region where the Mb-II Fe-$^{16}$O/Fe$^{18}$O stretch exists. Data was collected using the 501 nm argon laser line. *Mb-II is stable for long periods of time at high pH. Experiment suggests that it takes $\geq 3$ hrs for solutions of Mb-II to decay back to 100% ferric resting state at 20°C (pH 8.5).*

Figure A.12: Plot and exponential fits of the area under the Fe-$^{16}$O/Fe$^{18}$O stretch as a function of time during the exchange process. Analysis reveals a half life of 18 min for this process at $\sim 20^\circ$C.
A.3 Photoreduction in Ferryl Intermediates

One of the underlying goals of this thesis was to determine the protonation status of various ferryl intermediates (histidine, tyrosine, and thiolate ligated systems). The technique of resonance Raman was to be a crucial spectroscopy during this investigation because of its ability to directly probe the Fe-O/Fe-OH vibrational stretch. However, it was determined early on that many of the intermediates prepared were photolabile, making the characterization of an Fe-O/Fe-OH stretch by Raman an extremely daunting task.

Previous groups were able to circumvent this issue by implementing a continuous flow/spinning cell sample apparatus (aqueous 4°C), whereby a fresh (unphotoreduced) solution of the intermediate was constantly in the focus of the excitation laser. It was difficult to incorporate this strategy because most of our intermediates quickly decay/degrade and are only stable for a few seconds at most. The only way to trap them in high yield was through rapid freeze-quench. In order to use a spinning cell, and obtain data with discernable signal to noise, the intermediate must be stable for minutes at a time. In what follows, we discuss a series of resonance Raman experiments that were impeded by photoreduction and/or degradation.

Photoreduction of HRP-II

The photoreduction of ferryl intermediates by resonance Raman spectroscopy was initially noticed when trying to probe HRP-II for the Fe\textsuperscript{IV}-O vibrational stretch at low pH. HRP-II could be prepared in \( > \sim 90\% \) yield and frozen at maximum formation (UV/Vis), yet there was no indication of the ferryl intermediate by Raman. In the many attempts to
locate an Fe$^{IV}$=O stretching frequency, H$_2^{18}$O$_2$ was used. By using isotopic labeling, we were actually able to observe the photoreduction of HRP-II to a partial ferric hydroxide moiety (HRP$^{III}$-OH$^*$).

Under normal conditions, HRP$^{III}$-OH is only formed above pH 11. Below this pH, ferric HRP is 5 coordinate. Both forms can react with H$_2$O$_2$ to form HRP-II, Figure A.13. Additionally, it has been observed that the ferryl oxygen in HRP-II is exchangeable with bulk solvent, but only below pH 8. This event coincides with the protonation and subsequent hydrogen bonding of the nearby histidine residue to the ferryl oxygen.$^3$ Because we did not have H$_2^{18}$O at the time of the experiment, we were able to use this exchange phenomenon to show photoreduction of HRP-II with H$_2^{18}$O$_2$. This was done by comparing our photogenerated HRP$^{III}$-OH$^*$ with the authentic high pH HRP$^{III}$-OH spectrum.

Figure A.13: UV/visible spectra of ferric HRP$^{III}$ (pH 6.1, black), HRP$^{III}$-OH (pH 12, blue), and HRP-II (pH 10, red).
HRP-II was initially prepared at pH 10 with H$_2^{18}$O$_2$, where ferryl oxygen exchange does not occur, thus producing an $^{18}$O labeled intermediate. Next, HRP-II was prepared with H$_2^{18}$O$_2$ at pH 6, where ferryl oxygen exchange does occur, thus producing an unlabeled $^{16}$O intermediate. (We could have used H$_2^{16}$O$_2$ in this portion of the experiment, but we wanted to convince ourselves that the ferryl oxygen exchange phenomenon was real). Samples were then irradiated with a 413 nm krypton laser line in order to probe for the oxygen sensitive Fe-O stretching modes, Figure A.14. In a comparison of the low frequency spectra, an Fe-O sensitive mode can be seen in the exact same place as the Fe$^{III}$-OH stretch in the authentic pH 12 prepared HRP$^{III}$-OH, Figure A.15. The intensity of this stretch is not as large as in the authentic pH 12 HRP$^{III}$-OH, probably because there is not 100% generation of this species from the photoreduction of HRP-II. Additionally, the high frequency data show an oxidation state marker band that is identical to the position of an authentic HRP$^{III}$-OH adduct. The position of the marker band ($\nu_4$) is very sensitive to the expansion/contraction of the heme core (oxidation state) and can be used to track photoreduction in high valent heme intermediates. Our assignment of a photoreduced HRP$^{III}$-OH* from HRP-II is in agreement with the observation that cryoreduced HRP-II intermediates have EPR and ENDOR signals “similar” to HRP$^{III}$-OH.
High pH

Figure A.14: Proposed mechanism to account for ferryl oxygen exchange and the generation of a HRP^{III}-OH* moiety from HRP-II. At high pH (upper), no ferryl oxygen exchange occurs. Laser irradiation (photoreduction from resonance Raman) results in an ^{18}O labeled HRP^{III}-OH* intermediate. At low pH (lower), the protonation ($pK_a \sim 8$) and hydrogen bonding of the distal histidine to the ferryl oxygen facilitates $^{18}O$ exchange with bulk solvent ($H_2^{16}O$). Irradiation from the laser results in an $^{16}O$ labeled HRP^{III}-OH* species.
Figure A.15: Resonance Raman comparison of the authentic HRPIII-OH form (pH 12) and the photogenerated HRPIII-OH* form from HRP-II (pH 6.1). High frequency (left) shows oxidation state marker bands and low frequency (right) details Fe-O stretching frequencies. Because the photogenerated 18O HRPIII-OH* form at pH 6.1 undergoes ferryl oxygen exchange, the low frequency spectrum reflects a Fe-16O stretching frequency. Data was collected using the 413 nm krypton laser line.

The high frequency marker band data combined with the low frequency 18O isotopic labeling experiments provide evidence for the photoreduction of HRP-II. This realization was very important because initially we had thought that the FeIV=O vibrational stretch of HRP-II was simply not enhanced. This study helped make us aware of the sensitivity of heme intermediates and provided reason for why we were unable to locate vibrational stretches in future high valent intermediates that were prepared in high yield (> ~ 80%).
Photoreduction of CYP158A2-II Hydroxide

The CYP158A2-II hydroxide intermediate was prepared in > ~ 90% yield (Mössbauer) and provided a great opportunity to locate the first ever Fe IV-OH stretch in a P450 intermediate. However, unlike the CPO-II analogue, P450-II is very photolabile. 18O isotopic labeling experiments went unrewarded as the low frequency spectrum of compound II looked almost identical to ferric CYP158A2. Analysis of the high frequency oxidation state marker band data revealed that the intermediate of interest was photoreducing in the laser, Figure A.16. Interestingly, the shift of the oxidation state maker band occurs over the course of several minutes, Figure A.17, rather than in an instantaneous fashion.

Figure A.16: High frequency resonance Raman comparison of the CYP158A2-II hydroxide (pH 9) oxidation state marker band before and after photoreduction. The blue spectrum was collected in the first 30 seconds of sample exposure. The red spectrum was collected after the sample had been irradiated for 7 hours. Data was collected using the 458 nm argon laser line.
Figure A.17: High frequency resonance Raman analysis of the CYP158A2-II hydroxide (pH 9) oxidation state marker band as a function of laser exposure time. Data was collected using the 458 nm argon laser line.

We used the knowledge gained from the high frequency time dependent marker band study and designed a set of experiments to try and scan low frequency spots for short 90 seconds increments before moving to a new spot. Even after adding the successive 90 second scans together, the data was too noisy for analysis.

Photoreduction of HPC-II

HPC-II could be prepared in ~ 100% yield (Mössbauer) and was examined by resonance Raman in order to probe for the Fe^{IV}-OH vibrational stretch. Analysis of frozen HPC-II samples revealed immediate photoreduction. HPC-II instantaneously turned from red (compound II) → brown (ferric) after being “burned” by the laser. Because HPC-II is not as transient as P450 intermediates, we could prepare the intermediate at room temperature where it was stable for about 5 min. This allowed us to
make large volumes of the intermediate in a glass beaker, instead of an EPR tube, where rapid stirring (stir plate) could minimize the instantaneous photoreduction of the laser. In a sense we created a “modified” spinning cell. Using this approach we realized that after about 1 min the sample starts to slowly degrade, creating a highly fluorescent background, and drowning out signal. This allowed us a window of about 30 seconds to accumulate data, **Figure A.18** and **Figure A.19**. Even with the stir bar/beaker setup we could only partially observe the formation of HPC-II. No Fe^{IV}-OH sensitive modes were discovered with deuterium substitution.

**Figure A.18**: Low frequency resonance Raman spectra of ferric HPC and HPC-II. HPC-II samples were prepared by adding 12.5 x equivalents of PA to 5mL of 100 μM ferric protein (50 mM citrate, 500 mM NaCl, pH 6.5) in a 20 mL glass beaker with stir bar. The laser was aligned on the beaker (rather than an EPR tube with stagnant sample) as soon as the sample changed from brown (ferric)→red (HPC-II). This technique reduced the amount of photoreduction and allowed for some enhancement of HPC-II. Spectra of HPC-II samples prepared in a stagnant EPR tube were essentially 100% photoreduced. Data was collected using the 413 nm krypton laser line.
Figure A.19: High frequency resonance Raman spectra of ferric HPC and HPC-II. Samples were prepared and analyzed the same as in the low frequency data. Although the HPC-II sample was significantly photoreduced, there is some enhancement of the compound II marker band (~ 1380 cm⁻¹). Data was collected using the 413 nm krypton laser line.

A.4 Mössbauer Experiments

Cryoreduction of HPC-I

In order to determine the ferryl pKa of HPC, we needed to find a way to circumvent the low HPC-II (Fe⁴⁺-OH, ΔEq = 2.29 mm/s) yield at pH > 8. Initially, we implemented the cryoreduction of HPC-I (80% yield at pH 9) as a platform to perform this task, Figure A.20. This method was used prior to preparing HPC-II samples using the pH-jump method. Cryoreduction of HPC-I yielded ~ 45% total HPC-II at pH 9. Because protons are unable to move in low temperature cryoreduction studies, the HPC-II intermediate produced by ⁶⁰Co irradiation has a ΔEq of 1.35 mm/s, classifying it as an Fe⁴⁺=O.¹⁰,¹¹ Upon annealing, the HPC-II oxo species becomes 100% protonated, Figure A.21. If we were near the pKa, we would expect the ratio of uprotonated vs. protonated ferryl to stop changing at some point during the annealing process. This was not the case.
The Mössbauer spectrum of the final annealed HPC-II intermediate showed one clear species with $\Delta E_{q} = 2.29$ mm/s, revealing that at pH 9 HPC-II was fully protonated.$^{10, 12, 13}$ This HPC-I cryoreduction study sets a lower limit on the possibility of a ferryl deprotonation event, with $pK_a \geq 10$.

**Figure A.20:** Depiction of the HPC cryoreduction experiment. HPC-I (black) was irradiated with a $^{60}$Co source at 77 K to generate the HPC-II oxo (red) species. Annealing of the HPC-II oxo produces the HPC-II hydroxide (blue) intermediate.

**Figure A.21:** Mössbauer spectra of the cryoreduced HPC-I (HPC-II oxo) intermediate before and after annealing. Contaminating species comprising $\sim$ 55% (ferric, cryoreduced ferric, uncryoreduced compound I) of the spectra were subtracted out. (A) No annealing (B) 10 min annealing (C) 45 min annealing (D) 70 min annealing (E) 205 min annealing (F) 1165 min annealing. The isomer shift/quadrupole splitting parameters are 0.10/1.35 mm/s and 0.03/2.32 mm/s for the HPC-II oxo (red) and hydroxide (blue) intermediates respectively.
Optimizing the Conditions for Compound II Yield in CYP158A2

In order to maximize the yield of CYP158A2 compound II, we varied buffer compositions, oxidants, and pHs. Ferric samples were mixed with freeze-quench instrumentation (3 ms dead time) and frozen in liquid ethane (-160°C). After much trial and error, we determined that the combination of tris-HCl buffer and $m$-CPBA gave good preliminary results. In experiments at ~ pH 7, we noticed a large contribution from several unknown species. However, upon increasing the pH, the contribution from these “contaminants” gradually dwindled. The final, optimized conditions (100 mM pH 9 tris-HCl buffer, 5 x $m$-CPBA) produced intermediate yields of $\geq 90\%$. The decrease of the “contaminating” species at high pH is shown below, Figure A.22.

**Figure A.22:** Mössbauer spectra of ferric CYP158A2 reacted with 5 x equivalents of $m$-CPBA at various pHs. (Red) Ferric, (Green) Unknown species with $\sigma/\Delta E_q = 0.14/0.62$ mm/s, (Purple) Unknown species with $\sigma/\Delta E_q = 0.20/2.70$ mm/s, (Blue) CYP158A2 compound II with $\sigma/\Delta E_q = 0.12/2.04$ mm/s.
A.5 References


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Publications


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