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STRUCTURE AND DYNAMICS OF C-H-BOND-CLEAVING
HIGH-VALENT IRON INTERMEDIATES

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by
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ABSTRACT

C-H bond cleavage is one of the primary tenets of the chemical world. Industry has long searched for more efficient commercial processes to functionalize C-H bonds to activate such molecules as hydrocarbons. Current processes require large amounts of energy and often expensive reagents and often produce a number of increasingly less desirable pollutants such as carbon dioxide. Discovering a process that requires less energy input or is more environmentally neutral is still a very desirable research goal.

The natural world already has generated a number of enzymatic processes that can activate C-H bonds at ambient temperatures and pressures. These oxygenase enzymes typically operate at temperatures less than 37 °C and atmospheric pressure. Both heme and non-heme containing oxygenases as well as certain DNA cleaving anti-cancer compounds activate C-H bonds via high valent iron-oxo compounds.

A C-H bond activating organic molecule of interest to this document is bleomycin. Bleomycin is a glycopeptide macrocycle used in clinical situations as Blenoxane® for treatment of a variety of cancers. It coordinates an iron with five nitrogen (or four nitrogen and one oxygen) ligands allowing a sixth coordination site to bind molecular oxygen in a manner similar to heme oxygenases. The clinical function of bleomycin is to bind DNA and activate the C4 hydrogen to generate single and double strand breaks in the chain and ultimately cause the death of the cancerous cell. Here, samples of the activated form of bleomycin have been cryoreduced in an attempt to observe a Fe(IV)=O containing species that has not been previously characterized.

The non-heme, taurine:α-ketoglutarate (αKG) dioxygenase (TauD) has been found to activate C-H bonds by generating an Fe(IV)=O species in order to hydroxylate substrate. The ferryl species of TauD has already been well characterized, but recent observations suggested that in the ferryl state, TauD may be able to release and rebind the taurine substrate. Here, substrate exchange during the ferryl and ferrous states of TauD is one main focus of this study. The primary difference between the αKG dependent oxygenase family of enzymes and the halogenase SyrB2 is that SyrB2 contains an alanine rather than a carboxylate ligand in the iron coordinating facial triad. Recent studies have shown that under certain conditions SyrB2 is able to hydroxylate its substrate. The carboxylate residue of TauD is D101, and the D101A variant was generated for studies to determine if TauD D101A is able to bind Cl⁻ and potentially halogenate its substrate.

P450s have been studied for more than 50 years and until recently, the Fe(IV)=O intermediate in their reaction had not been characterized. The novel P450 fatty acid hydroxylating peroxygenase from Bacillus subtilis P450BSβ was believed to be a good candidate to observe P450 Compound I, the Fe(IV)=O species that is coupled to a π-cation radical. Characterization of a P450 Compound I from Sulfolobus acidocaldarius rendered the search for a P450 Compound I much less novel. Studies of P450BSβ never produced spectra of a Compound I-like species. However, other observations made through the course of study yielded further insight into the reaction and capabilities of P450BSβ that have not been previously reported.
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Chapter 1

Introduction

C-H Bond Activation

The use of reduced transition metals (most commonly iron) to activate O$_2$ or its partially reduced forms (superoxide, peroxide) for cleavage (activation) of strong C-H bonds is a common strategy of enzymes and some DNA-cleaving drug compounds. Often, it is desirable to activate C-H bonds at certain molecular positions to replace the hydrogen with a more “activated” functional group (-OH, -Cl, etc) to allow for successive reactions downstream, such as the degradation of xenobiotics carried out by a number of P450 enzymes. It is relatively easy to activate a C-H bond that is already low in electron density, such as that of formaldehyde (bond strength = 87 kcal/mol) due to the electronegativity of the oxygen atom pulling electron density away from the C-H bond. (1) Conjugated systems also allow for relative ease in removing the hydrogen due to the ability of the conjugation to distribute and stabilize any negative charge that may be left on the carbon. However, the more covalent C-H bonds such as those in methane (bond strength = 105 kcal/mol) are much more difficult to break for activation purposes. (1)

Disadvantages of Strictly Chemical Processes for C-H Bond Activation

A major target for bond activation in much of the industrial world, particularly the natural gas and petroleum industries, is alkanes. These chains of methyl units are often referred to as paraffins and are fairly inert due to the lack of empty low energy orbitals (LUMOs) or filled high energy orbitals (HOMOs) that will readily participate in chemical reactions. (2) The molecules themselves house large amounts of energy that can be released via combustion, the complete
oxidation of the molecule, but the only physical products generated by this process are water and the greenhouse gas, carbon dioxide since partially oxidized forms do not accumulate. Of more industrial interest is using alkanes as precursors to generate more reactive species such as olefins, but all large scale processes to this point are “energy intensive” and highly unselective in terms of the C-H bond being targeted. Even relatively “mild” conditions used in synthesis reactions to activate C-H bonds still require temperatures in excess of 100 °C and expensive and/or hazardous reagents. Because of this, a large scale method for producing a more uniform product is still highly sought after. Recent efforts have focused on using transition metals as part of the catalyzing force to generate even milder conditions for bond activation. The primary impetus behind this is the ever increasing number of metallo-enzymes that have been shown to activate C-H bonds.

Advantages the Natural World Has Developed for C-H Bond Activation

Nature has developed a number of strategies for activating substrate C-H bonds at ambient conditions (i.e. 1 atm pressure, and temperatures typically- but not exclusively- between 5 and 40 °C). Several main classes of C-H bond activating enzymes include non-heme mono- and di-iron oxygenases, heme oxygenases, and copper oxygenases. The most prominent strategy couples the four electron reduction of O₂ to substrate oxidation. In some cases a co-substrate is necessary to provide reducing equivalents. The reduced reactant(s) must be able to react with O₂, which exists in a triplet state, without violating the laws of conservation of spin. The enzymes capable of C-H bond activation have evolved highly specialized active sites that position the substrate molecule so that only one of potentially many C-H bonds of the substrate is able to be activated. The mechanisms of C-H bond activating enzymes typically utilize one or
more “potent” intermediates to abstract the hydrogen from the C-H bond being activated. The identities of these intermediates will be discussed in greater detail below.

**Reactive Intermediates and Why They are Interesting**

The race to be the first to characterize a novel intermediate has always held great interest for the scientific community. Many of the now characterized intermediates were originally deemed too reactive to ever be observed. For example, the potent Fe(IV)-oxo and Fe(III)-superoxo intermediates have created many challenges along the path to their discoveries, so one may wonder, why bother to try and observe these species at all? The root of this answer lies often in therapeutic or industrial applications. Knowledge of the exact reaction mechanism of an enzyme can generally be applied to the entire family of enzymes. This often reveals other useful routes of study such as mutagenesis to further clarify what residue in the enzyme is responsible for a certain aspect of the reaction or what substrate analog may be useful in inhibiting the reaction altogether.

Industrially, the intimate knowledge of the reaction mechanism can lead to strategies for maximizing output of useful products such as fuel components or consumption of hazardous materials such as greenhouse gasses. Relative to current industrial processes such as the Haber process, enzymes typically do not require large amounts of expensive (and often rare) reagents to complete their reaction.

**Trapping Reactive Intermediates**

Use of rapid kinetic methods (e.g. stopped-flow spectrophotometry, freeze quenching) to detect and ultimately trap intermediates for structural characterization by multiple spectroscopic
methods has contributed greatly to our understanding of how these systems work and the Bollinger and Krebs research group has been at the forefront of this research area.

**Our Approach to Understanding Mechanisms: Mapping Intermediate States**

As previously mentioned, an important method to understanding enzymatic reactions is to understand the mechanism the enzyme uses to carry out its reaction. This requires more than just a theoretical presumption of what intermediate states the enzyme likely goes through, it requires as much detailed spectroscopic information as can be gleaned from an enzyme in motion. Certain states are typically easy to characterize, such as a resting state or a substrate bound state (obtained with one component such as oxygen absent). Many enzymes have quick catalytic rates that make observing intermediates along the reaction pathway difficult. The P450 family of enzymes provides a prime example where for almost 50 years scientists believed they knew the steps of the reaction mechanism but until very recently were unable to characterize the Compound I species. (11) Our laboratory group has generated a “road map” for characterizing these difficult, highly reactive, and short lived species.

**Hallmarks of Trappable Intermediates**

Typically, the main barrier to being able to trap and characterize a reactive intermediate is the unfavorable kinetics of the reactive species. The moniker “reactive species” is used to imply that the intermediate will not exist for very long once it is formed since it will rearrange or interact with other constituents to further the reaction. Generally, the first order rate constant for formation of the species needs to be greater than or at least comparable to the first order rate constant of its decay in order to be able to accumulate an observable amount of the
In many cases the native kinetics must be perturbed to either increase the rate of formation or decrease the rate of decay to obtain intermediate accumulation. One method to ensure intermediate formation is as fast as possible is to initiate the reaction with whichever substrate or cofactor binds and reacts the fastest. In most cases relevant to this work, the small apolar O₂ molecule is able to bind and react (much) faster than larger, often bulky substrates. (12)

If simply maximizing formation of the intermediate does not yield sufficient accumulation, a popular strategy to slow the decay of the intermediate is to use a modified substrate. In a number of cases, providing substrate isotopologs in which the hydrogen of the target C-H bond is replaced with deuterium slows the decay to generate a kinetic isotope effect (KIE) on $k_{cat}$ to allow for substantial intermediate accumulation. This indicates that the chemical modification of the substrate is at least partially rate limiting. One potentially significant drawback to substituted substrates is the possibility of the reaction uncoupling, where the reaction proceeds but via an undesired path to generate an alternative product. This may shunt some protein into an unproductive path to release the new product and try again, or it may actually inactivate the protein by causing the iron center to remain in an inactivated oxidation state as in taurine:α-ketoglutarate dioxygenase. (12)

To be able to characterize a reactive intermediate, it is often imperative to be able to observe the transient state of the reaction, ideally during the initial turnover of the reaction once the sample has been exposed to its final necessary reactant. The initial turnover phase provides maximal accumulation of the various species in the reaction as all reacting enzymes in the sample are proceeding through the same reaction intermediate at essentially the same time. Steady state observations do not provide this convergence of states. One major consideration in generating transient kinetics is the solubility of O₂. Typically oxygenated buffer can only be generated in concentrations up to about 2 mM O₂ in solution at 5 °C. (13) This means that typically the enzyme needs to be less concentrated than the O₂ solution to ensure saturation of the
enzyme:substrate complex with O$_2$ for reaction initiation. This limitation does not affect a number of spectroscopies, especially UV-Visible based techniques, but those such as Mössbauer spectroscopy are aided by higher concentrations of enzymes. One strategy to overcome the solubility of O$_2$ is to utilize H$_2$O$_2$ when possible such as in the P450 peroxide shunt reaction which will be discussed in greater detail in later chapters.

Another positive indicator that a reactive intermediate may be trappable is if the enzyme is substrate triggered. (12) This means that the iron center becomes much more reactive to O$_2$ once all other substrates are bound. It is likely this is a defensive mechanism to prevent formation and subsequent release of reactive oxygen species when there is no substrate to act upon thereby preventing damage to the protein. Triggering by the substrate often also means that the reaction of the enzyme to O$_2$ in the absence of substrate is sluggish or may proceed via an entirely different (uncoupled) mechanism altogether. (12) This is yet another reason to initiate the reaction with O$_2$ and not with another substrate. These strategies cannot guarantee the characterization of a reactive intermediate but thus far have had a high rate of success. (14-16)

**Spectroscopic Methods for Trapping and Observing Reactive Intermediates**

**Stopped-Flow Spectroscopy**

Since typical half lives of reactive intermediates can easily be less than 1 or 2 seconds, techniques that operate with greater speed and accuracy than the human hand are required. (12) One extremely useful technique is stopped-flow (SF) spectrophotometry that utilizes rapid mixing of two to three reagents (or mixtures of reagents) in individual syringes to monitor absorption changes of a single UV-Visible wavelength or the entire UV-Visible spectrum as a function of time after a dead time of approximately 3 ms. Mixing two reagent solutions is referred to as a
single mixing scheme and mixing three reagent solutions is a double or sequential mixing scheme, both of which are represented in Figure 1-1. Typically a sequential mixing scheme is employed when the order of addition of multiple reagents is time sensitive or multiple reagents are not compatible long term with each other or other reaction components (i.e. reducing agents and O$_2$). A fourth solution (syringe F), typically just a buffer solution, is used in the double mixing scheme to flush the reagents A and B mixed in the first step to the second mixer for combination with the third reagent solution C. Exact details of the SF device used in the experiments in this document can be found in the Materials and Methods appendix.

![Diagram of mixing schemes](image)

**Figure 1-1:** Representation of single and double mixing schemes available for stopped-flow spectrophotometry. Black squares represent four way T mixers.

Typically one begins the search for a transient intermediate by monitoring the UV-Visible spectrum and any changes with time that occur during the turnover reaction. One then identifies representative wavelengths that change with time, especially complimentary wavelengths where the absorbance of one decreases while the other increases and vice versa at a
slightly later time. This is a very good indicator of a transient, potentially reactive intermediate. There is software that can be used to aid in the analysis of how many intermediates may be forming in any reaction time.

Often the time of maximal change in an absorbance versus time trace (i.e. a kinetic trace), which may present as a minimum or maximum, indicates the time of maximal species accumulation. In order for this information to be of use, one needs to attempt to ensure that this maximum is “catchable” by the quenching methods that will be discussed shortly. The methods utilized in this document generally allow for minimum quench times of approximately 10 ms, so if the time of maximal species accumulation is quicker than this, the reaction needs to be slowed further. The easiest way to attempt to slow the reaction is to decrease the temperature. An operating temperature between 0 and 5°C is often sufficient for quenching and does not require the use of freezing point depressants that often are not amenable to protein solutions. Low temperatures in conjunction with previously discussed methods of maximizing intermediate accumulation provide the basis for generating a spectrophotometrically observable species that can be further characterized by other spectroscopies. Some hyperquenching methods provide quench times on the μs timescale so may be of use in particularly quick systems. These methods have not been utilized here so will not be discussed further.
Figure 1-2: Comparison of Stopped-flow (A), Freeze-quench (B), and Chemical-quench (C) techniques and the types of spectroscopies typically used by each method.

Quenching reactions

The SF kinetic traces can be very useful in determining kinetics of potential transient species but further analysis needs to be done with other spectroscopic methods such as Mössbauer, EPR, EXAFS, Resonance Raman, etc in order to obtain oxidation state and structural data. The rapid freeze-quench (FQ) or rapid chemical-quench (CQ) technique typically is used to generate samples for these spectroscopies. The kinetic traces from SF experiments are typically the basis for deciding what times to quench the reaction. A minimal kinetic timecourse is usually the first quench experiment and involves a starting material (often anaerobic) control, a sample at the maximum of the potential intermediate, samples halfway up and halfway down the growth and decay phases of the intermediate and finally a sample where the reaction is believed to have
proceeded to completion. Mixing during a quench occurs in essentially the same manner as with SF mixing but instead of going to a UV-Visible flow cell, the solution is sprayed into a concentrated acid or base (CQ) or into a cryosolvent such as isopentane at -150 °C (FQ) (Figure 1-2). This stops the progress of the reaction within a few milliseconds of entering the quenching solution and, in the case of freeze quenching, allows for packing of the frozen sample into a holder appropriate for the spectroscopy of interest.

Mössbauer Spectroscopy

Once appropriate samples are prepared, they can then be processed on instruments that can confirm or refute the assumptions made about the transient intermediate in the sample. Mössbauer spectroscopy can yield insight into the oxidation and spin states of any \(^{57}\)Fe containing species in a sample as well as information on the concentrations of different species in the sample. (17) There are actually 43 elements containing sufficiently low-lying excited states for which the Mössbauer transition has been observed but \(^{57}\)Fe is by far the most utilized. (17) A 14.4 KeV photon emitted by a \(^{57}\)Co containing source is then absorbed by \(^{57}\)Fe in a solid or frozen sample and the transition from the nuclear ground state \(I_n=1/2\) to the nuclear excited state \(I_e=3/2\) is observed. (17) A simple Mössbauer spectrum of a quadrupole doublet is seen in Figure 1-3 with the two primary parameters, quadrupole splitting (\(\Delta E_Q\)) and isomer shift (\(\delta\)) specified. Iron centers with a spin state of an integer value (s= 0, 1, 2), no coupling to other species, and exposure to no or small external magnetic field will generate spectra comprised of quadrupole doublets. Iron centers with half-integer spin ground states may electronically couple to form an overall integer spin state and will also generate a quadrupole double spectrum.
Figure 1-3: Simple Mössbauer spectrum of a quadrupole doublet with quadrupole splitting ($\Delta E_Q$) and isomer shift ($\delta$) specified.

The origin of $\Delta E_Q$ and $\delta$ can be seen in Figure 1-4. When no magnetic interactions are present, $I_e$ splits into two degenerate states $M_I = \pm 1/2$ and $M_I = \pm 3/2$. These states are separated in energy by the magnitude of the quadrupole splitting. The isomer shift arises from interactions between nuclear charges and electrons at the nucleus that shift $I_e$ and $I_c$ without changing the magnitude of $\Delta E_Q$. Incidentally, the isomer shift is reported relative to a standard, typically either an iron metal foil or sodium nitroprusside dihydrate spectrum collected at 298 K. (17) The isomer shift can reveal much about the structure and chemistry of the iron center(s) in the sample. It is dependent on oxidation, spin state, coordination environment and degree of covalency of bonds to the iron center. (17) As a result of these factors, the higher the oxidation state of the iron center the lower the resulting isomer shift.
Figure 1-4: Origin of quadrupole splitting parameters in Mössbauer spectroscopy. The transitions from $I_g = 1/2$ to $I_g = 3/2$ result in the peaks observed in the experimental spectrum.

$I_g$ and $I_e$ have magnetic moments that can interact with an external magnetic field ($B$) to remove the degeneracy of the nuclear states, subsequently splitting them into more states with more observable transitions. As seen in Figure 1-5, each $M_I$ state splits in two and transitions observable in a magnetic Mössbauer spectrum follow $\Delta I = 1$ and $\Delta M_I = 0 \pm 1$. The splitting of the six line spectrum corresponds to the strength of the magnetic field at the nucleus of the iron center. (17)
Figure 1-5: Splitting of nuclear states of the iron center in the presence of a magnetic field.

Individual iron centers with half integer spin oxidation states ($s = 1/2, 3/2, 5/2$) or coupled iron centers with half integer spin states will yield magnetically split spectra when exposed to a small magnetic field (e.g. approximately 50 mT). All iron centers with non-zero spin states will exhibit splitting of the quadrupole doublet species in higher external magnetic fields (e.g. 8 T) as seen in Figure 1-6.
Figure 1-6: Example of how the Fe(IV)=O spectrum of TauD splits when exposed to increasing magnetic fields (0.053 T to 8 T). (18)

Electron Paramagnetic Resonance Spectroscopy

Electron paramagnetic resonance (EPR) spectroscopy is a complementary method to Mössbauer spectroscopy as it detects species containing unpaired electrons. Species that can be monitored include organic radicals or the paramagnetic Fe(III) low spin (s=1/2) and high spin species (s=5/2) (or Fe(V) if one is fortunate enough to observe it) or any spin coupled system where the total spin ($s_{\text{total}}$) is half-integer. EPR utilizes the splitting of the spin states in a varied magnetic field that is bombarded with a single microwave frequency. (17) The separation of the states is proportional to the magnetic field applied and acquisition of resonance determines the position of features in the resulting spectrum. Resonance is achieved when the separation of the states due to the magnetic field is proportional to the applied frequency in Equation 1-1.
Equation 1-1: Resonance condition for generation of EPR signal. \( g = \) g factor, \( \beta = \) Bohr magneton, \( B_0 = \) intensity of magnetic field, \( h = \) Planck’s constant, \( v = \) microwave frequency of spectrometer.

\[
\Delta E = g\beta B_0 = hv
\]

Figure 1-7: Graphic representing the resonance condition which generates features of an EPR spectrum.

When \( hv = g\beta B_0 \) the energy of the microwave quantum matches the energy separation of the splitting of the spin states as seen in Figure 1-7. (17) Absorption of the microwave energy occurs based on \( \Delta m_l = 0 \) and \( \Delta m_s = \pm 1 \). Generally one knows the microwave frequency (9.5 GHz for X-band) and magnetic field value so the g value (spectroscopic splitting factor) for features of the spectrum can be calculated. The g value is anisotropic which means there is orientation dependence of the magnetic moment of the free electron being evaluated. (17) It may take up to nine values to define the orientation dependence of the g value however there should be a condition in which only 3 principal values \( (g_x, g_y, g_z) \) have non-zero magnitudes. Typically, three types of spectra result from the presence of a paramagnetic species, an isotropic, an axial or a rhombic spectrum. For increased clarity of spectral features and ease of g value determination,
EPR spectra are typically reported as the derivative of the absorption spectra as shown below in Figure 1-8. Isotropic spectra arise when $g_x=g_y=g_z$ and present as derivative spectrum A in Figure 1-8. Axial spectra arise when $g_x=g_y<g_z$ or $g_x=g_y>g_z$ as in derivative spectra B and C of Figure 1-8. Rhombic spectra are generated when $g_x\neq g_y\neq g_z$ and appear as derivative spectrum D of Figure 1-8. The $g$ values generated by paramagnetic species are indicative of the spin and oxidation state of the species. For example, an isotropic signal at $g=4.3$ is typical of a high spin ferric species while an isotropic signal at $g=2$ may be an organic radical.

![EPR spectra and absorption spectra](image)

**Figure 1-8**: Representations of isotropic (A), axial (B) and rhombic (C) EPR signals. From (17).

The primary spectroscopies relevant to this discussion are Mössbauer and EPR so other spectroscopies will not be discussed in detail here. However, a number of resources exist with
more information on all of the spectroscopies mentioned. Once the presence of a novel intermediate (e.g. an Fe(IV) or an Fe(III) believed to be attached to a superoxo) is confirmed, spectroscopies such as EXAFS and Resonance Raman are used to determine more structural details including Fe-O stretching distances and frequencies.

**Strategies of Enzymes Using O₂ and Iron**

Reactive intermediates of interest in this document are all high valent iron-oxo containing species. Iron and oxygen both provide unique advantages for facilitating the C-H bond activating reaction.

**Oxygen**

One key feature of the C-H bond activating reaction is that all of the enzymes utilize some form of oxygen in their reaction mechanism. Most often the oxygen donor is molecular oxygen, but in some cases may also be hydrogen peroxide. O₂ is a thermodynamically potent oxidant but the fact that the molecule exists as a triplet electronic state (instead of a singlet state) generates a high kinetic barrier to reactivity. As previously mentioned, this requires a reduced substrate with the ability to react with a triplet O₂. Typically, in the presence of substrate(s), a transition metal center bound to the enzyme then binds oxygen in order to generate the potent intermediate that will perform the hydrogen abstraction. The bound molecular oxygen then undergoes a 4-electron reduction to, in many cases, be inserted into and thereby complete the activation of the substrate C-H bond.
Iron

Many proteins have evolved to utilize iron preferentially or exclusively over other prevalent metals such as copper and manganese. Typically, C-H bond activating enzymes are broken into two major groups—heme containing and non-heme enzymes. Thousands of heme containing proteins exist, each with a porphyrin ring structure that coordinates an iron via four nitrogens in the ring. A few of the other numerous iron containing non-heme enzymes capable of C-H bond activation include taurine:α-ketoglutarate dioxygenase (TauD), prolyl-4-hydroxylase (P4H), halogenases CytC3 and SyrB2, isopenicillin-N-synthase (IPNS), myo-inositol oxygenase (MIOX), ribonucleotide reductase (RNR), P450s, and some peroxidases. (5-8, 10)

The availability of iron combined with its ability to change oxidation state by relatively easily accepting and donating electrons in redox reactions undoubtedly influenced the evolution of enzymatic families to prefer iron over most other metals. Iron’s flexible valence shell allows it to coordinate more than four (typically five or six) atoms. Heme containing proteins generally coordinate the iron with a ligand from the protein, either a histidine or cysteine, in addition to the four ligands of the porphyrin ring. This leaves one coordination site available for oxygen binding and subsequent reaction. A common motif in a number of non-heme mononuclear (one iron center) oxygenase proteins (e.g. TauD) is the presence of a facial triad that coordinates the iron to the enzyme. This is generally comprised of two histidine and one carboxylate amino acid (aspartate or glutamate) residues that coordinate iron through three bonds. This leaves three other coordination sites for substrates and co-substrates to bind during the reaction. The facial triad coordination scheme allows for more flexibility in the reaction mechanism, e.g. the halogenation reaction is not possible in a heme system since it requires a coordination site for the anion in addition to a site for oxygen.
“Potent” Reaction Intermediates

The reactive intermediates of most interest generally include a high valent iron-oxo species. This document deals with members of the heme as well as non-heme mononuclear monooxygenase families. In heme containing enzymes, the intermediate most stalked for characterization is Compound I, a formally Fe(V)=O species, but spectroscopically is observed as an Fe(IV)=O coupled to a π-cation radical.

Heme enzymes utilize a 3/5 mechanism to generate their potent intermediate Compound I. This means that the resting enzyme exists in a ferric [Fe(III)] state and the potent intermediate of the cycle that carries out hydrogen abstraction is formally an Fe(V)-oxo. Non-heme systems follow a similar reaction path to their heme cousins, however they operate in the 2/4 manifold. Here, resting enzyme is present in the ferrous form [Fe(II)] and the active oxidant is a ferryl species (Fe(IV)=O with no conjugated radical).

The presence of the ferryl species in the non-heme enzymes was postulated long before it was characterized. This postulation was based on the knowledge of bond strengths of C-H bonds that were known to be cleaved in non-heme reactions and the fact that simple ferric-oxo species would not be strong enough to cleave the bond. In some enzymes such as MIOX and IPNS and the anticancer drug bleomycin, a ferric-superoxo or ferric-hydroperoxo species is suitably potent for C-H bond cleavage, but for enzymes like TauD, P4H and the halogenases, it does not have the reactivity to break the substrate’s C-H bond. (6)
Bleomycin, Taurine:α-Ketoglutarate Dioxygenase and P450

All C-H bond activating enzymes include at least one “potent” intermediate comprised of an Fe(IV)-oxo or an Fe(III)-superoxo species (or both in the case of IPNS) in their reaction mechanism. The Bollinger and Krebs labs have characterized a number of these intermediates including ferryl species in the mononuclear enzymes TauD, P4H, SyrB, and IPNS and dinuclear enzymes of ribonucleotide reductase in both *Escherichia coli* and *Chlamydia trachomatis*. (5, 14, 19, 20) Ferric superoxide species have been characterized in MIOX and IPNS. (6, 7) The reactions of the mononuclear non-heme enzymes will be discussed in greater detail in subsequent chapters.

This dissertation deals with three O$_2$ or peroxide activating transition metal enzymes/complexes. These are the DNA-cleaving anticancer drug, bleomycin, taurine:α-ketoglutarate dioxygenase, and a novel bacterial (*bacillus subtilis*) cytochrome P450-like fatty acid hydroxylating enzyme which has been characterized as a peroxygenase. The bleomycin reaction has many similarities to the reaction of P450 enzymes and because of this, the presence of a Compound I or Compound II like species in the reaction has long been postulated. The activated form of bleomycin is actually a ferric hydroperoxo species that is believed to pass through a ferryl intermediate during its reaction to cleave DNA. (21) As with P450s, characterization of the ferryl species in the bleomycin reaction has proven elusive. In an effort to observe a ferryl species in the reaction of bleomycin, a sample of activated bleomycin generated in the absence of DNA was exposed to γ-irradiation in an attempt to homolytically cleave the O-OH bond via cryoreduction.

As already mentioned, a ferryl species has previously been characterized in the TauD reaction. A former laboratory technician, Eric Barr, believed that TauD may allow the taurine
substrate to exchange out of the active site even while the enzyme was generating the reactive ferryl species. This is in contrast to observations made in the halogenase SyrB which locks its substrate in place throughout its reaction and crystal structure studies of TauD that indicate conformational changes occur upon substrate binding to close a “lid” over the active site. Spectrophotometry based experiments have been used to determine kinetic parameters of taurine exchange in the ferryl and ferrous states. Additionally, studies of SyrB have shown the halogenase is able to hydroxylate under certain conditions. The primary difference between the SyrB and TauD active sites is an Ala replaces the carboxylate ligand of the iron binding facial triad in SyrB. Here, the TauD D101A variant, which replaces the aspartic acid of the facial triad with alanine, is studied for its ability to bind chloride.

The third system that has been investigated is that of the P450 from Bacillus subtilis, P450<sub>bsi</sub>. The characterization of Compound I from a variety of heme containing enzymes, including chloroperoxidase and horseradish peroxidase, almost immediately led to speculation of the presence of a Compound I species that should develop in the P450 enzyme family. (22) Until recently however, only hints of Compound I could be observed, generally via UV-Visible methods that did not allow further characterization. (23) The species decays quickly, and likely due to the fact that electron injection into the active site is often rate limiting, does not accumulate for characterization. Recently, Jonathan Rittle in the laboratory of Professor Michael Green here at the Pennsylvania State University experimented with a thermophilic P450 enzyme from Sulfolobus acidocaldarius that yielded a Compound I species suitable for spectroscopic characterization that confirmed the presence of a ferryl intermediate in the reaction cycle of P450s. (11) Published information suggested the novel peroxygenase P450 fatty acid hydroxylating enzyme from Bacillus subtilis should also be a good candidate for employing the strategies discussed above to trap Compound I. During experimentation to attempt to trap
Compound I, several additional observations that had not previously been reported yielded additional insight into this enzyme.
References


Chapter 2

Bleomycin

Bleomycin Background

Cancer and Bleomycin

In 2008, approximately 12.7 million people were diagnosed with cancer and approximately 7.6 million cancer caused deaths occurred worldwide. (1) In fact, cancer accounts for one in every four deaths in the United States and one in every eight deaths worldwide; more than malaria, tuberculosis, and HIV/AIDS combined. (1) The United States congress has allotted nearly $5 billion dollars each year since 2005 to the National Cancer Institute to fund the search for cancer treatments and cures. (2) The American Cancer Society adds an additional $130 million dollars a year for research and there are many other private foundations providing more funding as well. (1) One natural product that has emerged as a useful anticancer agent is the family of bleomycins.

Bleomycins, specifically the therapeutic form called Blenoxane ® (Bristol-Myers Squib), have been used to treat a variety of germ cell tumors, cervical cancers, testicular cancer, squamous cell carcinomas of the head and neck and Kaposi’s sarcoma. (3, 4) They are glycopeptide molecules (Figure 2-1) originally isolated from Streptomyces verticillus that have been well documented to generate both single strand and double strand DNA breaks in cells. (5) The DNA damage ultimately leads to tumor necrosis, but unfortunately this sometimes is accompanied by pulmonary complications that, rarely, can even be fatal. (4) As such, there are still many groups investigating the mechanism of action of bleomycin in hopes of developing even more efficient delivery systems and less toxic versions of the molecules.
Relationship of Bleomycin to P450s

Thus far, studies indicate that bleomycin generates its activated form through a reaction similar to that of the P450 family of enzymes. (7) As seen in Figure 2-2, bleomycin can generate activated bleomycin (ABLM), a low spin (LS) ferric hydroperoxide (OOH-Fe(III)$_{LS}$•BLM) complex, through multiple pathways, but two in particular are the most utilized for experimental purposes. In one pathway, bleomycin binds Fe(II) and oxygen and then obtains an exogenous electron to generate ABLM in approximately a 50% yield. An alternate pathway allows bleomycin to bind Fe(III) and H$_2$O$_2$ to generate ABLM without the need for an extra electron.
Figure 2-2: Possible pathways of formation of Activated BLM (ABLM). Figure kindly provided by Dr. Richard M. Burger.

The pathway to generate ABLM can be directly compared to the P450 cycle in Figure 2-3. (8) P450s contain a ferric resting state as seen in species 1 of Figure 2-3 where the iron is coordinated by four nitrogens of the heme macrocycle, a cysteine or histidine from the protein, and a water molecule. (8) When substrate binds in the active site it displaces the water molecule and also causes the iron to leave the plane of the heme, causing a spin state shift of the iron center from LS to HS. (8) The enzyme in this ferric, substrate bound state (2) can then either pass through the peroxide shunt reaction or can utilize an exogenous reductase domain that shuttles an electron to the iron allowing the enzyme to bind O$_2$. The reductase domain then provides a second electron and a solvent proton is obtained to generate a ferric hydroperoxo state (7) homologous to ABLM. (8)
Figure 2-3: General reaction of P450 enzymes. Modified from (8).

Figure 2-4: Possible reaction intermediates observable in the decay of ABLM. (9)

Early studies theorized BLM molecules would pass from the ABLM species through a P450 Compound I like (formally Fe(V)=O, spectroscopically an Fe(IV)=O coupled to a π-cation
radical) species (Figure 2-3, species 8; Figure 2-4 top) or a Compound II like (Fe(IV)=O) species during the decay of ABLM (Figure 2-4). (7) More recent studies have provided evidence that suggests the Fe(V)=O species is energetically unfavorable and therefore less likely to form, but the generation of an Fe(IV)=O containing species is still a plausible reaction intermediate. (6, 10) It is also now widely believed that the Fe(IV)=O species does not attack DNA but is a byproduct of a hydrogen abstraction by the ABLM form to remove the C-4’ hydrogen of DNA and generate a C-4’ radical (Figure 2-5). (9)

![Figure 2-5: Currently accepted mechanism of hydrogen abstraction by ABLM from DNA. Adapted from (6).](image)

**Relationship of Bleomycin to Isopenicillin-N-Synthase**

While BLM seems to follow the same type of reaction pathway as that of the P450s to generate the ABLM species, BLM chemistry once the hydroperoxo species is formed may be more closely related to that of the non-heme mono-iron enzyme isopenicillin-N-synthase (IPNS). (7, 11, 12) This enzyme is closely related to the α-ketoglutarate dependent family of enzymes in that it contains an iron center coordinated by two histidine residues and one aspartic acid residue. However IPNS does not require α-ketoglutarate (αKG) to generate the two rings of isopenicillin-N from the linear tripeptide \( L-\delta\)-aminoadipoyl-L-cysteinyl-D-valine (ACV). (11) In generating the β-lactam and thiazolidine rings, IPNS is believed to utilize a ferric-superoxo (species I), a
ferric-hydroperoxo (species II), and a ferryl (species IV) intermediate to carry out multiple hydrogen abstraction steps (see Figure 2-6). (12)

Figure 2-6: Currently accepted mechanism of isopenicillin-N-synthase (IPNS). Adapted from (12).

Despite decades of study, the BLM intermediates between ABLM and the final ferric product(s) have not been observed. As already discussed, in the presence of DNA it is now believed that hydrogen abstraction from DNA will yield a DNA radical and an Fe(IV)=O intermediate (Figure 2-5). In order to attempt to observe an Fe(IV) intermediate in the BLM reaction, it was hypothesized that the process of cryoreduction may yield such an intermediate. During cryoreduction, γ-radiation bombards a sample of ABLM maintained at 77 K in liquid nitrogen. This allows for one electron reduction of susceptible reaction centers while preventing any molecular motion in the system. Theoretically, the O-OH bond of ABLM could be
homolytically cleaved during the cryoreduction process to form the Fe(IV)=O species seen in Scheme 2-1.

Scheme 2-1: Possible O–OH bond cleavage mechanism that occurs during the cryoreduction of ABLM.

In this study, samples of ABLM have been exposed to cryoreduction in an attempt to homolytically cleave the O–OH bond in ABLM and generate an Fe(IV) containing species. In addition to ABLM, a variety of control samples for spectral deconvolution have been exposed to the cryoreduction process as well.

Bleomycin Results and Discussion

Details of sample preparation can be found in the Materials and Methods Appendix.

Since ABLM is an unstable species, even frozen reaction mixtures containing it will also contain its products. The spectra of such mixtures require deconvolution for interpreting and quantitating their various constituent spectra. For this, the spectra of any constituents that are available individually, and in relatively simple mixtures, must first be characterized for generation of reference spectra. This allows subtraction of the reference spectra from spectra containing any new spectral constituent(s), such as the product(s) of cryoreduction. Incidentally, these new spectral species are expected to be present only in partial yield. Further minor complications occur: when ABLM is regenerated, as it is below, in the steady-state reaction of
(LS)-Fe(III)•BLM with peroxide, not all of the LS precursor is consumed leaving “contamination” of LS species in the sample. (7) Also, during ABLM decay, particularly in the absence of DNA, ABLM may decay into “suicide” products that are EPR active but can no longer generate ABLM when exposed to the appropriate oxygen donor (O₂ or H₂O₂). (7, 13) Finally, as previously discussed, the bleomycin metal ligands are identical for all bleomycins, but peripheral substituents that vary could affect the spectral homogeneity of Blenoxane® (60% bleomycin A₂, 30% B₂, and 10% other bleomycins). (4)

The EPR and Mössbauer parameters of iron-bleomycins from prior published studies are included in Table 2-1 for comparison to those of new species presented here. (13, 14) The spectra addressed and discussed below will progress from those of stable iron-bleomcins (high-spin (HS)-Fe(II)•BLM; LS- and HS-Fe(III)•BLM), to their cryoreduction products, to preparations of the reactive activated bleomycin, and its novel Fe(IV) cryoreduction product.
Figure 2-7: Summary of BLM species and spectroscopic parameters observed in this study. BLM species that have been previously characterized (green) as well as new species characterized in this study (red) and possible decay intermediates that could form during decay of ABLM (grey).
Table 2-1: Table of Mössbauer and EPR parameters for BLM states. Species in bold italics have been previously characterized. (7, 14)

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<th>ΔE_Q (mm/s)</th>
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<th>E/D</th>
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<th>g_y</th>
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**HS-Fe(II)•BLM and its Cryoreduced Form**

The 4.2-K/53-mT Mössbauer spectrum of a sample of BLM, to which 0.75 equivalents $^{57}$Fe(II)$_{aq}$ was added anaerobically, displays two prominent lines, which are assigned to a quadrupole doublet with parameters typical of high-spin Fe(II) [isomer shift, δ, of 1.13 mm/s, ΔE_Q, of 2.62 mm/s, Figure 2-8A, Table 2-1 “HS-Fe(II) Ref”]. The large linewidths of $\Gamma_L \approx 0.60$ mm/s and $\Gamma_R \approx 0.66$ mm/s suggest significant conformational heterogeneity of HS-Fe(II)•BLM. Again, previous studies and this study provide clear evidence for severe conformational heterogeneity of many forms of BLM. (14) The Mössbauer parameters of Fe(II)•BLM differ from those previously published for this form (δ = 1.22 mm/s and ΔE_Q = 2.83 mm/s Table 2-1 “HS
Fe(II) pub”). (14) We tentatively attribute this discrepancy to sample heterogeneity. Previous studies have shown that Fe(II) containing species will not cryoreduce to an Fe(I) species. (15)

Figure 2-8: 4.2-K/53-mT Mössbauer spectra of HS-Fe(II)•BLM before (A) and LS-Fe(III)•BLM control samples before (B) and after (D) cryoreduction. All raw and reference spectra are represented by the vertical error bars in the figure and italicized text in figure legends. All simulations or spectra used for subtraction are represented by solid or dashed lines. The spectra are as follows: A) HS-Fe(II)•BLM reference; HS-Fe(II)•BLM reference fit, light blue; B) LS-Fe(III)•BLM raw; 78% LS-Fe(III)•BLM published, light blue dashed; 8% HS-Fe(III)•BLM theory, magenta; 15% HS-Fe(III)•BLM + P, raw, black dashed; C) LS-Fe(III)•BLM reference; 78% LS-Fe(III)•BLM theory, spring green; D) CR LS-Fe(III)•BLM raw; 39% LS-Fe(III)•BLM reference (1C), spring green; 14% HS-Fe(II)•BLM reference (1A), light blue; E) CR LS-Fe(III)•BLM reference; Simulation of CR LS-Fe(III)•BLM species, red; new species 1- HS-Fe(II)•BLM, green; new species 2- LS-Fe(II)•BLM, dark blue.
**LS-Fe(III)•BLM and its Cryoreduced Form**

Addition of one equivalent of $^{57}$Fe(III)$_{aq}$ to a solution of BLM under aerobic conditions results in a sample containing primarily the LS-Fe(III)•BLM complex. The X-band EPR-spectrum (Figure 2-9A) reveals the rhombic signal with g-values of 2.45, 2.18, and 1.89, which were reported previously for LS-Fe(III)•BLM. (13) In addition, the spectrum displays a signal with an effective g value of 4.3. This feature is typical of the middle Kramers doublet of a HS-Fe(III) complex ($S = 5/2$) in the near-rhombic limit ($E/D \approx 1/3$). (16) All EPR spectra are reported from 1300 G to 3800 G because there are no signals that arise outside of this range.
Figure 2-9: EPR spectra of BLM samples run at 10-K, with 20 μW power, 10 G modulation amplitude, and 9.51 GHz microwave frequency. A) LS-Fe(III)•BLM; B) HS-Fe(III)•BLM; C) 1.5 mM ABLM; D) 1.5 mM CR ABLM; E) 3.5 mM ABLM; F) 3.5 mM CR ABLM.

Consistent with the EPR spectrum of Figure 2-9A, the 4.2-K/53-mT Mössbauer spectrum (Figure 2-8B) displays the typical features of a magnetically split LS-Fe(III) complex. A simulation with parameters previously published for the LS-Fe(III)•BLM complex (Table 2-1 “LS Fe(III)””) is shown as a dashed blue line in Figure 2-8B. The LS Fe(III) spectrum accounts for ~78% of the total intensity. The remaining ~22% can be attributed to two impurities. The mismatch between the experimental spectrum and the simulation of LS-Fe(III)•BLM (see black
solid arrows) at 0.0 mm/s and 2.5 mm/s suggests that the sample also contains a HS-Fe(II) complex. The spectral properties of this HS-Fe(II) complex and its relative amount were evaluated by removing the simulation for LS-Fe(III)•BLM shown in Figure 2-8B from the raw data (transformation not shown). The remaining features of the HS-Fe(II) impurity match reasonably well with those of the HS-Fe(II)•BLM reference (Figure 2-8A) and account for 14% of the total area. The second impurity displays features at −7.0 mm/s and + 8.5 mm/s (red arrows) and these peak positions are typical of the magnetically split spectral features of HS-Fe(III) complexes.

Because it is known that Fe(III)•BLM in phosphate buffer exists mainly in the HS configuration and some decay products of ABLM exist in the HS configuration as well, we have also prepared and studied a HS-Fe(III)-containing sample (the spectral features of this sample are presented in more detail below). (13, 14) Its 4.2-K/53-mT Mössbauer spectrum is shown as a dashed black line in Figure 2-8B (scaled to 15% of the intensity of the experimental spectrum for clarity of line position). Although the features of this spectrum are qualitatively typical of HS-Fe(III), they do not match those of the HS-Fe(III) impurity in the sample. Again, we attribute this discrepancy to conformational heterogeneity. We model the small contribution of the HS-Fe(III) impurity in the spectrum shown in Figure 2-8B by a simulation with parameters typical of HS-Fe(III) (see Table 2-1 “Theory HS Fe(III) A”). Removal of the spectral features of the two impurities (8% of the HS-Fe(III) simulation and 14% of the experimental spectrum of HS-Fe(II)•BLM) results in the reference spectrum of LS-Fe(III)•BLM (Figure 2-8C). This reference spectrum matches reasonably well to the simulation using the published parameters of LS-Fe(III). Refinement of these parameters by least-square fitting improves the quality of the simulation slightly (green line in Figure 2-8C). The new simulation parameters (Table 2-1 “LS Fe(III) Ref”) are similar to the published values (LS Fe(III)). (14)
The 4.2-K/53-mT Mössbauer spectrum of a duplicate sample containing LS-Fe(III)•BLM as the main component, and which was subsequently subjected to CR (total radiation dose was 4 Mrad), is shown in Figure 2-8D. The features of LS-Fe(III)•BLM are clearly detectable, but have been reduced in intensity. Using the reference spectrum of LS-Fe(III)•BLM, 2-8C, we estimate that the sample contains ~39% of LS-Fe(III)•BLM (Figure 2-8D, green line), which in turn suggests that the CR yield of LS-Fe(III)•BLM is ~50%. Because HS-Fe(II)•BLM is not cryoreduced (see above), the 14%-contribution of HS-Fe(II)•BLM from before CR (Figure 2-8D, blue line) remains unchanged in the post CR Mössbauer spectra. Removal of the latter two components results in the spectrum displayed in Figure 2-8E as vertical bars and represents the 39%-contribution of CR LS-Fe(III)•BLM, as well as the features of the HS-Fe(III)•BLM impurity and its cryoreduced form (the added contribution of the latter two components is 8%). We estimate that the CR yield of the HS-Fe(III) impurity is ~50%, based on the diminished intensity of the diagnostic Mössbauer features of the HS-Fe(III) control discussed below, therefore the contribution of CR HS-Fe(III)•BLM is expected to be ~4%. Furthermore, we speculate that CR HS-Fe(III)•BLM contains a HS-Fe(II) center based on the conservation of spin. The spectrum in Figure 2-8E displays four distinct lines, suggesting that CR LS-Fe(III)•BLM yields two species. The fact that quadrupole doublet features are observed are consistent with one-electron reduction of LS-Fe(III)•BLM. It appears CR of LS-Fe(III)•BLM yields both HS-Fe(II) and LS-Fe(II) complexes. Of the four lines, the outer two are less intense (~9% each) than the middle two (~14% each), suggesting that they are paired in this fashion. A least-squares fit with two quadrupole doublets to the data results in the following parameters: $\delta_1 = 1.07$ mm/s and $\Delta E_Q,1 = 3.26$ mm/s (1.0% intensity, green line; Table 2-1 “CR LS-Fe(III) A”); $\delta_2 = 0.55$ mm/s and $\Delta E_Q,2 = 1.08$ mm/s (1.5% intensity, blue line; Table 2-1 “CR LS-Fe(III) B”). The parameters of the first species are typical of HS Fe(II), although they are clearly distinct from those of HS-Fe(II)•BLM...
generated by anaerobic addition of Fe(II) to BLM (see above). The second species is assigned to a LS-Fe(II)•BLM complex, because (i) this form is generated by cryoreduction of a Fe(III) complex and (ii) its parameters are in the range observed for LS-Fe(II) complexes, e.g. LS-Fe(II) heme proteins and models thereof with a similar N-rich ligand environment. (17)
HS-Fe(III)•BLM and its Cryoreduced Form

Figure 2-10: 4.2-K/53-mT Mössbauer spectra of HS-Fe(III)•BLM before (A) and after (B) cryoreduction. A) HS-Fe(III)•BLM reference; 10% LS-Fe(III)•BLM reference, spring green; B) CR HS-Fe(III)•BLM raw; 50% HS-Fe(III)•BLM reference (3A), black; C) Spectrum 3B – 50% 3A; 5% CR LS-Fe(III)•BLM reference, red; D) CR HS-Fe(III)•BLM reference; CR HS-Fe(III)•BLM reference fit, purple; HS-Fe(II)•BLM reference, blue.
The EPR spectrum of a sample of BLM in phosphate buffer, to which Fe(III)\textsubscript{aq} was added aerobically (Figure 2-9B) reveals a prominent signal at $g_{\text{eff}} = 4.3$, which arises from the middle Kramers doublet of a HS-Fe(III) ion with a near-rhombic zero-field splitting tensor ($E/D \approx 1/3$). As reported previously the addition of phosphate buffer results in a change of the spin state of the iron center. (7) The EPR spectrum also reveals the spectral features associated with LS-Fe(III)$\cdot$BLM (signals at $g = 2.45, 2.18, \text{and } 1.89$). Comparison of the intensity of the features of LS-Fe(III)$\cdot$BLM in this spectrum (Figure 2-9B) to those of the spectrum of LS-Fe(III)$\cdot$BLM (Figure 2-9A), which was recorded under identical conditions, suggests that the sample contains $\sim0.15$ mM LS-Fe(III)$\cdot$BLM (10\% of total Fe in the sample). The 4.2-K/53-mT Mössbauer spectrum of this sample (Figure 2-10A, vertical bars) is consistent with the EPR-spectra. The 10\%-contribution of LS-Fe(III)$\cdot$BLM is shown as a solid green line. The remainder of the spectrum displays multiple lines ranging from -8 mm/s to +9 mm/s. These features are typical of the magnetically split spectra of HS-Fe(III) centers. We have not attempted to simulate this component further, because of the large number of parameters required for simulation and, as seen above, it does not match any other HS-Fe(III) species generated in this study.

The 4.2-K/53-mT Mössbauer spectrum of this sample after CR (4 MRad radiation dose, Figure 2-10B vertical bars) is markedly different from that before CR. The intensity of the magnetically split features is diminished at the expense of two strong peaks in the central part of the spectrum, which result from the quadrupole-doublet features of the one-electron-reduced form. The reference spectrum of CR HS-Fe(III)$\cdot$BLM was generated by removing the features of the other components [non-CR HS-Fe(III)$\cdot$BLM, CR LS-Fe(III)$\cdot$BLM, and non-CR LS-Fe(III)$\cdot$BLM]. Shown as a solid black line in Figure 2-10B is the spectrum of this sample before CR (2-10A), scaled to 50\% of the total original intensity. This subspectrum corresponds to (presumably) 45\% HS-Fe(III)$\cdot$BLM and $\sim5$\% LS-Fe(III)$\cdot$BLM (see above). Because the original
sample of HS-Fe(III)•BLM contains ~10% LS-Fe(III)•BLM impurity and the CR yield of LS Fe(III)•BLM is ~50%, the CR sample now contains 5% CR LS-Fe(III)•BLM (see above, Figure 2-8D, shown as red line in Figure 2-10C). Removal of these features yields the reference spectrum of CR HS-Fe(III)•BLM (Figure 2-10D, vertical bars). This spectrum is virtually identical to that of HS-Fe(II)•BLM (solid blue line in Figure 2-10D) and its simulation is shown in the purple line in Figure 2-10D using parameters $\delta = 1.15$ mm/s and $\Delta E_Q = 2.83$ mm/s (Table 2-1 “CR HS Fe(III)”).

**Activated Bleomycin (ABLM) and its Cryoreduced Form**

For this study two sets of LS-HOO•Fe(III)•BLM (activated bleomycin, ABLM) samples have been generated. The main difference in the samples is one contains a higher concentration of BLM than the other. Further details regarding the preparation of ABLM samples can be found in the Materials and Methods appendix. Because of some variation in the preparation of these samples, there are expected to be some small variations in sample composition.

**High Concentration Sample**

As already discussed, the ABLM samples are not expected to be pure ABLM, and the EPR spectrum of the 3.5 mM sample before CR (Figure 2-9E) confirms the presence of HS-Fe(III)•BLM and ABLM (g= 2.26, 2.17, 1.94), but no LS-Fe(III)•BLM. The corresponding Mössbauer spectrum (Figure 2-11A) confirms the presence of HS-Fe(III)•BLM through the presence of peaks at -7.5 mm/s and +8.5 mm/s. Simulating these peaks with a theoretical spectrum (magenta, parameters in Table 2-1 “Theory HS Fe(III) B”) suggests the sample contains 30% HS-Fe(III)•BLM. Using previously published Mössbauer parameters for ABLM
(Table 2-1 “ABLM”) the spectrum 2-11A is estimated to also contain 60% ABLM (orange line) based on the peak at approximately 2 mm/s. (14) The additional 10% area of the spectrum not otherwise accounted for is again likely attributable to heterogeneity in the sample either in the simulation of HS-Fe(III)•BLM or ABLM, however the complexity of the sample prevents us from knowing exactly.

**Figure 2-11:** 4.2-K/53-mT Mössbauer spectra of 3.5 mM BLM sample containing ABLM before (A) and after (B) cryoreduction. A) 3.5 mM BLM containing ABLM; 60% ABLM theory, orange; 30% HS-Fe(III)•BLM theory, magenta; B) 3.5 mM CR ABLM containing CR ABLM; 50% 3.5 mM BLM containing ABLM (2-12A), black dashed; 12% ABLM theory, orange; C) Spectrum 5B minus 50% 5A and 12% ABLM theory; 15% CR HS-Fe(III)•BLM reference, purple; D) 3.5 mM CR BLM containing CR ABLM reference; Simulation of species generated from CR of ABLM, (Fe(IV)-BLM), black.
A secondary analysis utilizing the intensity of the peaks in Figure 2-9 corresponding to each species corroborates the above findings. Ideally the area under the doubly integrated curve is calculated to determine the amount of each species present in an EPR spectrum, however the spectra generated in Figure 2-9 have proven difficult to integrate. For this reason, the intensity of the g=2.45, g=2.26 and g=4.3 peaks is used to provide an estimation of the amount of LS-Fe(III)•BLM, ABLM and HS-Fe(III)•BLM respectively in the EPR spectra. The Mössbauer spectra of LS-Fe(III)•BLM (Figure 2-8) contains 78% LS-Fe(III)•BLM and the spectrum of HS-Fe(III)•BLM (Figure 2-10) contains 90% HS-Fe(III)•BLM. These values are used in conjunction with the spectra in Figure 2-9A and B to determine the amount of LS and HS components in the ABLM samples relative to the peak heights of the standards.

The intensity of the g=4.3 peak in spectrum 2-9E corresponds to this sample containing approximately 27% HS-Fe(III)•BLM which agrees well with the estimate of 30% HS-Fe(III)•BLM used for Mössbauer simulations. The intensity of the g=4.3 peak in spectrum 2-9F decreases by 46% relative to 2-9E corroborating the approximately 50% CR yield observed in the Mössbauer spectra of the HS-Fe(III)•BLM control sample (Figure 2-10).

CR of the higher concentration ABLM sample (Figure 2-11B) generates new spectral features at approximately -1, 0, and +1 mm/s. The spectra of CR ABLM are analyzed in the same manner as the CR controls. Non-CR spectral components are removed followed by any CR spectral components attributed to non-ABLM species. Having previously established that HS-Fe(III)•BLM is CR with 50% efficiency, and presumably ABLM may be CR to the same degree, 50% of spectrum 2-11A (black line) is removed from 2-11B. This matches and therefore completely removes the HS-Fe(III)•BLM contribution to the CR spectrum, however the broad feature from -5 to -2 mm/s that belongs to ABLM is underestimated by the 50% of 2-11A (30% ABLM). To completely remove this feature and therefore any non-CR ABLM contributions, an
additional 12% ABLM (orange line) must be subtracted from 2-12B to generate spectrum 2-11C. This indicates that the CR yield of ABLM is less than 50% and is actually approximately 30%. The intensity of the g=2.26 peak in 2-9F relative to 2-9E decreases by approximately 40% again suggesting that the CR yield of ABLM is significantly less than 50%.

Since HS-Fe(III)•BLM is present prior to CR, Mössbauer spectrum 2-11C still contains 15% CR HS-Fe(III)•BLM (50% of the 30% before CR in 2-11A). Subtraction of the 15% CR HS-Fe(III)•BLM reference (purple line) yields 2-11D, the reference spectrum for this CR ABLM sample. The peaks at 0 and +3 mm/s are likely due to the fact that the HS-Fe(III)•BLM reference spectrum does not match the HS-Fe(III) species in the other BLM samples so the CR HS-Fe(III)•BLM is likely not a perfect match for the CR HS-Fe(III) species in CR ABLM samples. As a result, spectrum 2-11D is fit to 18% of a single QD with \( \delta = 0.00 \) mm/s and \( \Delta E_Q = 1.57 \) mm/s which is consistent with an intermediate spin (\( s=1 \)) Fe(IV) containing species (Table 2-1 “CR ABLM”). Other \( s=1 \) Fe(IV) containing species that have been characterized include low pH catalase (Fe(IV)=OH, \( \delta = 0.07 \) mm/s and \( \Delta E_Q = 1.47 \) mm/s) and horseradish peroxidase Compound II (Fe(IV)-OH, \( \delta = 0.03 \) mm/s and \( \Delta E_Q = 1.61 \) mm/s).
Low Concentration Sample

Figure 2-12. 4.2-K/53-mT Mössbauer spectra of the 1.5 mM BLM sample containing ABLM before (A) and after (B) cryoreduction. A) 1.5 mM BLM containing ABLM; 60% ABLM theory (published), orange; 20% HS-Fe(III)•BLM theory, magenta; 10% LS-Fe(III)•BLM reference, spring green; B) 1.5 mM CR BLM containing CR ABLM; 50% 1.5 mM BLM containing ABLM (4A), black dashed; 12% ABLM theory, orange; C) Spectrum B minus 50% A and 12% ABLM theory; 10% CR HS-Fe(III)•BLM reference, purple; 5% CR LS-Fe(III)•BLM reference, red; D) 1.5 mM CR BLM containing CR ABLM reference; Simulation of species generated from CR, black; new species 1- LS-Fe(II)•BLM, green; new species 2- Fe(IV)•BLM, dark blue.
The spectra of the 1.5 mM samples can be treated similarly to those of the 3.5 mM samples. The EPR spectrum of the 1.5 mM sample before CR (Figure 2-9C) indicates the presence of HS-Fe(III)•BLM and LS-Fe(III)•BLM in addition to ABLM. The corresponding Mössbauer spectrum (Figure 2-12A) confirms the presence of HS-Fe(III)•BLM and simulating these peaks with the same theoretical HS-Fe(III) species used in the 3.5 mM spectra (Table 2-1 “Theory HS Fe(III) B”) suggests the sample contains 20% HS-Fe(III)•BLM (magenta line). Visual inspection of the EPR spectrum suggests there is a small amount of LS-Fe(III)•BLM relative to ABLM, however the actual amount cannot be determined outright from the Mössbauer spectrum since the LS-Fe(III)•BLM features are completely overlapped by the ABLM features. In this instance, the EPR spectrum is used to estimate the presence of 10% LS-Fe(III)•BLM (green line) based on the height of the g=2.45 peak relative to the same peak in the LS-Fe(III)•BLM control (2-9A) which corresponds to 78%. The remaining spectral area can then be simulated to 60% ABLM (orange line) using the previously published parameters. Again the unaccounted for 10% area of the spectrum is likely due to heterogeneity or slight variation in the shape of the overlapping species.

Spectrum 2-12B resulting from the CR of the 1.5 mM sample again contains noticeable new features at -1, 0 and +1 mm/s. The presence of both LS- and HS-Fe(III)•BLM before CR complicates this analysis slightly relative to the 3.5 mM sample as the CR spectral features of each needs to be accounted for. Subtracting 50% of 2-12A (black dashed line) from 2-12B again completely removes all non-CR HS-Fe(III)•BLM and should also completely remove any remaining non-CR LS-Fe(III)•BLM because the CR yield of LS-Fe(III)•BLM is 50%. As with the 3.5 mM sample, an additional 12% ABLM (orange line) needs to be removed from 2-12B to remove the -5 to -2 mm/s feature of ABLM and generate spectrum 2-12C. Reduction in g=2.26 intensity of 2-9D relative to 2-9C again indicates a CR yield of approximately 40%. In addition
to the 10% CR HS-Fe(III)•BLM (purple line) still present in spectrum 2-12C, there also is 5% CR LS-Fe(III)•BLM (red line) that must be removed to generate the reference spectrum for this sample of CR ABLM, 2-12D. The feature between +2 and +3 mm/s is likely another result of the close but imperfect HS-Fe(III)-BLM reference spectrum subtraction. The two sharp peaks at -1 and 0 mm/s and the wider peak at +1 mm/s suggest the presence of two, quadrupole doublet based species. Analysis of the higher concentration CR ABLM sample yielded a single species on CR with parameters consistent with an Fe(IV) species. Fitting 2-12D with the same Fe(IV) parameters and an additional set of floating parameters to account for the second species yields the black line fit comprised of the QD in blue and the QD in green. The green QD has parameters consistent with a LS-Fe(II) species ($\delta = 0.53$ mm/s and $\Delta E_Q = 1.03$ mm/s) that is very similar to the LS-Fe(II)•BLM species generated from CR of LS-Fe(III)•BLM. The LS-Fe(II)•BLM species accounts for approximately 10% of the species in this CR sample of ABLM, however the 10% LS-Fe(III)•BLM in the starting material (2-12A) indicates only 5% LS-Fe(II)•BLM should be generated via CR. This discrepancy may be due to the possibility that the LS-Fe(III)•BLM in spectrum A may actually be a decay product of ABLM and has slightly different parameters than that of the reference LS-Fe(III)•BLM species. This species may subsequently generate a different spectrum upon CR than the reference LS-Fe(III)•BLM spectrum and has resulted in an imperfect subtraction. In this sample, the Fe(IV) species comprises 11% of the CR ABLM sample as opposed to the 18% in the 3.5 mM sample. This again supports the hypothesis that the O-O bond in ABLM can be homolytically cleaved to generate an O=Fe(IV)-BLM species during the reaction mechanism.
Annealing of CR ABLM

Figure 2-13: 4.2-K/53-mT Mössbauer spectra of 3.5 mM BLM containing ABLM before and after annealing at 200-K for 20 minutes. A) Annealed 3.5 mM CR ABLM; B) Annealed minus cryoreduced 3.5 mM CR ABLM (2-11B); 33% 2-11C, grey-blue; C) Annealed ABLM reference; 30% HS-Fe(III)•BLM raw (2-10), purple, 30% HS-Fe(III)•BLM theory, magenta.

Annealing provides further insight into the species produced by CR based on new species that are generated by the annealing process. The spectra in Figure 2-13 show what happens when the high concentration sample of CR ABLM is annealed at 200-K for 20 minutes. It is useful to subtract the CR spectrum (2-11B) from the annealed spectrum (2-13A) to emphasize any spectral features that decay (upward pointing features) or form (downward pointing features) due to the...
annealing process (2-13B). In this case, 33% of a reference spectrum containing both CR ABLM (18%) and CR HS-Fe(III)•BLM (15%) (spectrum 2-11C, grey-blue in 2-13B) can be added back to smooth the “negative” features. Since the original CR yield of ABLM was 35%, adding back 33% indicates essentially all of the CR species were annealed. In this case the Fe(IV) and Fe(II) species appear to have reacted in a comproportion-like reaction to generate a HS-Fe(III)•BLM species shown in 2-13C. Spectrum 2-13C can also be fit with the “Theory HS Fe(III) B” spectrum (magenta) reasonably well. The HS-Fe(III)•BLM reference spectrum (purple) once again does not resemble the HS-Fe(III)•BLM present in this sample. Since this theoretical HS-Fe(III) simulation fits the spectra of both ABLM samples and the annealing sample reasonably well it is likely all three samples contain the same HS-Fe(III)•BLM species, likely one of the poorly defined BLM decay products. (7)
Parallel vs Perpendicular Spectra of ABLM

Figure 2-14: 4.2-K/53-mT Mössbauer spectra of 3.5 mM ABLM run in parallel (//) and perpendicular (-/) magnetic fields. A) 3.5 mM sample run in parallel field; B) 3.5 mM sample run in perpendicular field; C) Spectrum A minus spectrum B; Simulation of 60% ABLM theory in // field minus 60% ABLM theory in -/ field, peach.

Additional information can be gleaned from MB spectra run in a perpendicular (-/) magnetic field when compared to the spectra run in a parallel (//) magnetic field. This is typically most informative for the paramagnetic species. In Figure 2-14 the spectra of high concentration ABLM collected in // and -/ field are shown (2-14A and B respectively). Subtracting spectrum A from spectrum B to yield C showcases the difference between the spectra collected in the two field orientations. The fit for 2-14C (peach) is generated by taking two theoretical fits of 60%
published ABLM, one simulated with // field, the other with -/-, and subtracting the one (//=) from the other (-/-). The fact that the amplitude and shape of the fit are very similar to the experimental difference spectrum indicates both that the parameters used to simulate ABLM are good and that the estimation of 60% ABLM in the sample is correct. The minor differences that are present in this spectrum may be due to slightly different parameters for ABLM as well as field dependent contributions from the middle Kramers doublet of the HS-Fe(III)•BLM species. One may wonder why the outside lines of the HS species completely cancel each other on subtraction and this is because the ground Kramers doublet of the HS-Fe(III)•BLM that produces a 3:2:1:1:2:3 peak intensity ratio is actually field independent. As just mentioned, the middle Kramers doublet is not field independent so may be contributing to the difference spectrum.

**Bleomycin Summary**

This study shows that the cryoreduction of BLM in absence of DNA can generate an Fe(IV) containing species. The Mössbauer spectra containing a species with $\delta = 0.00$ mm/s and $\Delta E_Q = 1.57$ mm/s and the decreased intensity of EPR spectral features support the generation of the Fe(IV) species. The Mössbauer parameters ($\delta = 0.00$ mm/s and $\Delta E_Q = 1.57$) mm/s are reasonably similar to Fe(IV) containing spectra obtained from a sample of low pH catalase (Fe(IV)=OH, $\delta = 0.07$ mm/s and $\Delta E_Q = 1.47$ mm/s) and horseradish peroxidase Compound II (Fe(IV)-OH, $\delta = 0.03$ mm/s and $\Delta E_Q = 1.61$ mm/s). (17) The generation of an Fe(IV) containing species is further supported by the annealing of the cryoreduced sample containing Fe(II) and Fe(IV) to generate an Fe(III). It is also likely that the HS-Fe(III)•BLM species present in both ABLM samples and generated in the annealing sample is an ABLM decay product.
References


2. Cancer Research Funding - National Cancer Institute, National Cancer Institute.


Chapter 3

Taurine:α-Ketogluturate Dioxygenase

Taurine:α-Ketogluturate Dioxygenase Background

Taurine:α-ketogluturate dioxygenase (TauD) is a member of the Fe(II)- and α-ketoglutarate (αKG)- dependent oxygenase enzyme family. Members of this family are involved in a wide variety of functions including, but not limited to, collagen synthesis, degradation of xenobiotics, synthesis of antibiotics, repair of alkylated DNA, and oxygen and iron homeostasis.  

(1, 2) A number of diseases, including alcoholic liver cirrhosis, hawkinsinuria and Refsum’s disease, arise due to malfunction of members of the enzyme family. (1) TauD and the majority of Fe(II)- and αKG- dependent oxygenases maintain a conserved iron binding motif consisting of a “facial triad” of two histidine residues and one carboxylate (aspartate or glutamate) residue. These residues occupy three of the six coordination sites of the iron center. (1) The other three coordination sites are generally occupied by water molecules while the enzyme is at rest.

In 1982, Hanauske-Abel and Günzler proposed a mechanism for the collagen prolyl-4-hydroxylase (P4H) that has held up as a working hypothesis for all dioxygenases in the family (Figure 3-1). (2) The mechanism involves the activation of O₂ and formation of a high-valent iron-oxo intermediate that attacks and hydroxylates a substrate or carries out a similar two-electron oxidation. (2) The resting state of the enzyme is shown in the upper left of Figure 3-1 with three water molecules occupying the open coordination sites. Bidentate coordination of αKG via C-1 carboxylate and the ketone oxygen displaces two of the water ligands. Subsequent binding of substrate (taurine in the case of TauD) displaces the third water ligand. The taurine does not actually coordinate to the iron; it binds in the vicinity but leaves an open iron coordination site that allows a molecule of O₂ to bind to the iron. The uncoordinated oxygen
atom then attacks the carbonyl of αKG, leading to formation of an Fe(IV)=O containing species and the oxidative decarboxylation of αKG to succinate. The Fe(IV)=O species then abstracts a hydrogen from C1 of taurine, and the –OH moiety rebounds as •OH to the taurine C1 radical to complete hydroxylation.

**Figure 3-1:** Working hypothesis for the mechanism of the Fe(II)- and α-ketoglutarate (αKG)-dependent oxygenase enzyme family. (3)

Until the early 21st century, no direct spectroscopic evidence of the Fe(IV)=O species had been generated. J.C. Price, a former member of the Bollinger/Krebs research group, first observed evidence of two transient species in the TauD reaction via stopped-flow (SF) absorption spectroscopy. (1) The absorbance-versus-time (kinetic) traces at 320 nm and 520 nm proved to be diagnostic for two species in the TauD hydroxylation reaction. The 320 nm wavelength trace exhibited a maximum absorbance at 20 ms after mixing the TauD•Fe(II)•αKG•taurine quaternary
complex with an oxygenated buffer solution at 5 °C (Figure 3-2). The 520 nm wavelength trace had a minimum absorbance at 210 ms. The nature of the absorbance changes suggests a corresponding formation and decay (or decay and formation) of a transient species in the reaction. Absorbance at 520 nm had previously been attributed to αKG binding (increase in absorbance) and dissociation (decrease in absorbance) due to a metal to ligand charge transfer transition. (2) The faster 320 nm associated transient feature had not been investigated previously and became the focus of subsequent study.

![Figure 3-2: Comparison of SF absorbance versus time (kinetic) traces at 320 nm and 520 nm from TauD and P4H when mixing enzyme•Fe(II)•substrate with oxygenated buffer at 5 °C. Red and blue symbols are experimental data and black lines are simulations of the data. Modified from (2, 3)](image)

As previously discussed, to characterize in greater detail a potential reactive intermediate, samples containing maximal amounts of the intermediate are generated via the appropriate quenching process. A sample of the TauD reaction mixture frozen at 20 ms via freeze-quenching techniques (more information can be found in the Materials and Methods appendix of this document) and analyzed with a Mössbauer spectrometer at 4.2-K with a 53-mT, parallel (∥)
magnetic field exhibited a new feature with parameters $\delta = 0.31$ mm/s and $\Delta E_Q = 0.88$ mm/s (Figure 3-3). (1) Parameters from the 53-mT spectra combined with data from higher magnetic field Mössbauer spectra (up to 8 T) and the fact that cryoreduction of the sample yielded a high spin Fe(III)-containing species suggested the sample corresponding to the 320 nm feature contains an Fe(IV) species. (1)

Subsequent studies showed the presence of a kinetic isotope effect (KIE) on the decay of the Fe(IV)-containing species when substituting C-1 taurine hydrogens for deuteria. (4) As seen in Figure 3-2, the KIE manifests as a larger amplitude and slower decay in the presence of deuterated substrate as opposed to protiated substrate. The deuterium isotope effect was also observed via freeze-quench Mössbauer studies (Figure 3-3). The KIE manifests as the Mössbauer spectral feature at approximately 0.8 mm/s but occupies a larger percentage of the total area of the spectrum and persists for a longer time than identical samples generated with non-labeled taurine. (4) Quantum mechanical principals dictate that C-D bonds require more energy to break than C-H bonds and the less massive hydrogen is more likely to tunnel through its energy barrier which further reduces the energy necessary to cleave a C-H bond. (5) These quantum mechanical principals were invoked when the decay of the Fe(IV) was slowed in the presence of deuterated substrate to conclude that the Fe(IV) containing species was involved in the hydrogen abstraction step. The KIE ($k_H/k_D$) on the decay of the Fe(IV) species was initially determined to be approximately 37, but the observation of uncoupling in the reaction with deuterated substrate means that the measured KIE underestimates the true KIE of the reaction which is now reported as approximately 50. (4) Resonance Raman data observed a spectral feature in the 800 cm$^{-1}$ region that is downshifted by 35 cm$^{-1}$ in the presence of $^{18}$O which is a hallmark of Fe(IV)-oxo species. (2) EXAFS data were subsequently recorded that detected a short, 1.62 Å Fe-O interaction. (2) All of these observations further solidify the identity of the Fe(IV) intermediate as an Fe(IV)=O in agreement with the predictions of Hanauske-Abel and Günzler. (6)
Figure 3-3: Mössbauer spectra (4.2-K, 53-mT //) of samples containing the Fe(IV)=O intermediate of TauD reaction generated with A) TauD•Fe(II)•H$_4$-taurine•αKG mixed with oxygenated buffer at 5 °C and reacted for 0.084 s before freezing. B) Same sample as A except generated with D$_4$-taurine instead of H$_4$-taurine. C) Same reaction mixture as B but allowed to react 0.11 s before freezing. Solid lines are simulation of the spectrum of the Fe(IV)=O intermediate scaled to 22% (A), 46% (B) and 64% of the total area of the experimental spectrum. (4)

Kinetic studies utilizing TauD have helped elucidate a number of finer points of the reaction mechanism. (7) Any species that form after addition of O$_2$ but before the generation of the Fe(IV)=O species do not accumulate, likely due to unfavorable kinetics for accumulation. (7) At least one Fe(IV)=O precursor may have been observed through the work of Proshlyakov et al. which provides resonance Raman evidence of a species that has a spectral band shift from 583 cm$^{-1}$ to 555 cm$^{-1}$ when the reaction is carried out with $^{18}$O$_2$. (7, 8) This observation helps explain variations of experimental kinetic data from simulated data as the experimental data consistently presented with slightly greater absorbance in the very early times at 320 nm than the simulations predicted. When the simulations were adjusted to account for an additional absorbing species
prior to the Fe(IV)=O species, the simulations matched experimental data much more closely. (7) Additionally, this Fe(IV)=O precursor may build up to 0.3 equivalents of the enzyme but the formation maximizes at times shorter than quenching processes utilized thus far are able to access. (7) The recent development of “hyper-quenching” techniques may permit further characterization of this species in the future.

Another study investigating the decarboxylation of αKG determined that CO₂ formation occurs with a rate constant of $1.6 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ (second order with respect to $[\text{O}_2]$). (7) This value agrees well with the value obtained in the simulations ($1.5 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$) and suggests decarboxylation occurs during the formation of the Fe(IV)=O intermediate. (7) SF kinetic traces of O₂ free reactions varying the order of co-substrate addition suggest that when TauD•Fe(II)•taurine is mixed with αKG, there is a significant lag phase for binding of αKG compared to when taurine and αKG together are mixed with TauD•Fe(II). (7) The slowed binding of αKG to the TauD•Fe(II)•taurine complex combined with more rapid binding of αKG to TauD•Fe(II) and the rapid binding of taurine to TauD•Fe(II)•αKG suggests that there is a preferred, ordered binding of substrates (αKG then taurine) in TauD. (7) Knowing the preferred binding order of substrates also lends insight into another variation between experimental data and simulations. The 520 nm absorbance-versus-time trace of the TauD•Fe(II)•αKG•taurine complex mixed with limiting $[\text{O}_2]$ revealed a slower regeneration phase than expected. (1) Because αKG binds before taurine it is likely that 1-hydroxytaurine dissociates before succinate. It is also likely that, in the presence of excess taurine, taurine could rebind before succinate releases and slow dissociation of succinate, thereby delaying rebinding of αKG and accounting for the slow regeneration phase of the 520 nm feature. (7) Confirmation of this theory was provided by variation of the concentration of taurine in the reaction. Greater concentrations of taurine lead to inhibition of the reaction, likely due to the inability of complete product release to occur before another taurine enters the active site to bind rapidly. (7)
**Prolyl-4-Hydroxylase Background**

The Fe(II)- and αKG-dependent dioxygenases known collectively as prolyl-4-hydroxylases (P4H) are known to hydroxylate the C-4 carbon of proline residues in proteins. (3) P4H plays a pivotal role in the cross-linking of collagen, and lack of function by P4H due to vitamin C deficiency is the cause of scurvy. (3) As previously discussed, P4H was postulated to function via the mechanism in Figure 3-1. Like the other Fe(II)- and αKG-dependent dioxygenases, P4Hs contain the facial triad motif that binds the iron cofactor. (3) Due to the prior success of dissecting the TauD mechanism and TauD’s similarity to P4H, it was decided that the 27 kDa P4H from *Paramecium bursaria Chlorella* virus 1 (PBCV-1) would be a good candidate to generate another observable Fe(IV)=O containing species. For these studies, a short peptide, (Pro-Ala-Pro-Lys)₃ [(PAPK)₃], was used as substrate because this length of peptide had been shown to have the minimum $K_m$ of the chain lengths tested and any longer chains would become more prohibitive to handle. (3)

As with TauD, the affinity for the enzyme to bind αKG was investigated in the presence and absence of the peptide substrate via the absorbance changes at 520 nm. Again, this feature arises due to the metal-to-ligand charge transfer transition associated with chelation of the cofactor by αKG. (2, 3, 7) P4H exhibits a $K_D$ for αKG binding of about 27 μM. However, unlike TauD, the presence or absence of peptide substrate does not affect this value. (3) Also, P4H does exhibit substrate triggering like TauD, because, as discussed in the introductory chapter, the reaction with enzyme and $O_2$ proceeds very slowly when no substrate is present. (3)

SF studies confirmed the characteristic loss of absorption at 520 nm during the reaction of P4H•Fe(II)•αKG•(PAPK)₃ with $O_2$ followed by the return of the absorbance to the baseline level that was also observed in the TauD reaction (Figure 3-2). However, P4H does not exhibit the lag phase in the initial decay of the absorbance at 520 nm that TauD does (Figure 3-2 top
panels). This difference is attributed to the fact that the Fe(IV)=O species also absorbs slightly in the 500 nm region (not strictly at 320 nm) so the lag phase in the 520 nm trace of TauD is due to accumulation of the Fe(IV)=O species followed by the generation of a more transparent product complex. (3) The lack of the lag in the P4H reaction combined with lack of an absorbing feature at 320 nm suggested that the Fe(IV)=O species does not accumulate in the reaction with non-labeled substrate. (3) Again, using lessons learned from TauD, a substrate containing perdeuterated proline residues was generated for SF studies. This reaction exhibited significant accumulation at the 320 nm wavelength and simulation of the traces yielded a KIE of about 60 (Figure 3-2, lower left panel). (3)

Freeze quench Mössbauer spectroscopy samples again showed evidence of an Fe(IV) containing species with $\delta=0.30$ mm/s and $\Delta E_Q=0.82$ mm/s (4.2-K, 53-mT/ℓ), with both parameters being very similar to those of the TauD Fe(IV)=O species ($\delta=0.31$ mm/s and $\Delta E_Q=0.88$ mm/s) (Figure 3-4). (1, 3) The spectral splitting pattern generated by high field (8T) Mössbauer studies indicates it is again a high spin Fe(IV). (3) The kinetics of the 320 nm traces were also compared to the kinetics of the Fe(IV) species as determined by Mössbauer samples and both matched very well, providing further confirmation that the 320 nm feature corresponds to the Fe(IV)=O species. (3)
Figure 3-4: Mössbauer spectra (4.2-K, 53-mT/\( \text{mT} \)) monitoring the P4H Fe(IV)=O intermediate formation and decay generated by mixing P4H•Fe(II)•\( ^{3}\text{H}-[\text{PAPK}]\), with oxygenated buffer at 5 \(^\circ\text{C}\). The solid line in the top spectrum is the derived spectrum of the Fe(IV)=O species. (3)

Iron- and \( \alpha \)-Ketoglutarate-Dependent Aliphatic Halogenases

Another \( \alpha \)KG-dependent enzyme relevant to this work is the aliphatic halogenase SyrB2. This enzyme has some interesting differences from TauD and P4H, however. Most notably, rather than a facial triad of two histidines and one carboxylate ligand, SyrB2 contains two coordinating histidine residues, but the carboxylate residue is replaced with an alanine residue. (9) This change in the iron binding pocket allows coordination of a halogen anion so that, instead of rebounding a hydroxyl anion after hydrogen abstraction, as seen in Scheme 3-1, SyrB2 rebounds a halogen radical onto its substrate. (10) The substrate for SyrB2 is an L-threonine moiety attached to a carrier protein via a thioester bond and a phosphopantetheine arm as seen in Figure 3-5. (11)
Scheme 3-1: Possible rebound pathways and products of the reaction of SyrB2. Scheme kindly provided by Dr. M. Matthews.

Figure 3-5: Cartoon representation of the substrate required by SyrB2 that includes the carrier protein SyrB1, a phosphopantetheine arm, a thioester linkage and an amino acid moiety. The amino acid moiety (L-Threonine in this figure) may be varied.

Studies investigating substrate triggering of SyrB2 have generated several interesting observations. First of all, the enzyme requires a full SyrB1•phosphopantetheine arm•thioester•amino acid substrate (Figure 3-5) for triggering of the enzyme to occur, similar to TauD and P4H requiring all of their substrates/co-substrates to be present for the fastest possible reaction with O₂ to occur. (II) Neither SyrB1 missing any of the above constituents nor plain amino acid are able to trigger the enzyme. (II) It also appears that the carrier protein is the most important component to generate a triggered. (II) The amino acid side chain attached to the SyrB1 cofactor can be modified and still cause triggering of the reaction with O₂ and, remarkably, the amino acid’s identity even contributes to the stabilization of the Fe(IV)=O intermediate. In
the case of the SyrB1 substrate complex bound to cyclopropylglycine (Cpg), the Fe(IV)=O species persists with a remarkably long half life of about 1.8 h. (11) This is fascinating in that the enzyme not only stabilizes the Fe(IV)=O species for previously unheard of lengths of time but also prevents its decay through other, off pathway reactions as has been seen with TauD uncoupling. (1, 11)

Interestingly, in the presence of chloride and the natural L-Thr substrate, SyrB2 strictly halogenates the L-Thr moiety despite the fact that the Fe(IV)=O still abstracts a hydrogen from threonine. (10) Theoretically, if the Fe(IV)=O is close enough to abstract a hydrogen from the substrate, the hydroxyl moiety should be close enough to rebound onto the threonine. Studies with the A118E variant of SyrB2, which replaced the alanine with a glutamic acid in order to restore the facial triad, showed that SyrB2 can hydroxylate substrate but the resulting reaction is fairly inefficient and suggests that other adaptations besides removal of the carboxylate have made SyrB2 a halogenase. (10)

Studies varying the amino acid bound to the otherwise complete carrier protein substrate generated some very interesting results in regards to “tuning” the halogenase versus hydroxylase activity of SyrB2. (10) Relative to TauD and P4H, SyrB2 (and its cousin CytC3) abstract hydrogen from substrates very “sluggishly” at a rate of about $10^{-1}$ s$^{-1}$ (5 °C). (10) As already discussed, the activity of SyrB2 on L-Thr results in essentially all halogenation. However, removal of the β-hydroxyl of L-Thr to generate L-2-aminobutyryl-SyrB1 (Aba) accelerates the hydrogen abstraction portion of the reaction mechanism. (10) Increasing the rate of hydrogen abstraction correlates with more hydroxylation of the target amino acid. (10) As seen in Figure 3-6, it is believed the β-hydroxyl of L-Thr (blue) likely acts as an electrostatic anchor to keep the tail of the substrate positioned more closely to the halogen anion than to the oxygen of the ferryl. (10) When L-norvaline (Nva) (black), which has an additional methylene unit, is tethered to SyrB1, its hydrogen is abstracted 10 times faster than the hydrogen of Aba (red) (and 130 times
faster than L-Thr). (10) Nva is almost exclusively hydroxylated at the C-5 position, but will undergo primarily halogenation at the C-4 position when the C-5 position is deuterated. (10)

Figure 3-6: Cartoon of amino acid positioning around iron in the SyrB2 active site. A) L-Thr in blue and Aba in red. B) Aba in red, Nva in black. Cartoons kindly provided by M. Matthews.

Unpublished studies by Megan Matthews have utilized size exclusion chromatography to examine the stability of the enzyme•substrate complex before, during and after the reaction. When enzyme and substrate are, under anaerobic conditions, passed through a size exclusion chromatography column, SyrB1 and SyrB2 can be partially resolved from one another. Similarly, if the reaction of enzyme, substrate and O₂ is allowed to proceed to completion, the substrate and halogenase can be separated chromatographically. Interestingly, when utilizing the stable cyclopropylglycine substrate, initiating the hydroxylation reaction and passing the reacting components through the size exclusion column results in the elution of a stable complex of the enzyme and its substrate. Locking of the enzyme•substrate complex together during the reaction is not an unexpected result, as dissociation of the substrate during the reaction might allow for quenching of the ferryl complex and resultant uncoupling of αKG decarboxylation from substrate halogenation.
The similarities already established between the halogenases and hydroxylases have generated two more areas of interest in TauD and P4H. For one, because SyrB2 has been shown to have some hydroxylase activity upon substitution of one residue in the active site, it is of interest to know whether removing the corresponding carboxylate residue from TauD and P4H will generate halogenase activity. Also, we wondered if TauD could “lock” its small molecule substrate in place during its turnover reaction as SyrB2 has been established to do.

**TauD and P4H Results and Discussion**

**Tuning Reactivity of TauD and P4H**

Some preliminary data concerning the modification of reactivity in TauD and P4H has been obtained. Because the primary active site difference between the hydroxylases TauD and P4H and the halogenase SyrB2 is the absence of a carboxylate ligand in the halogenase, variants, mutants of TauD (D101A) and P4H (D154A) were generated to investigate if their reactivity could be “tuned” to result in halogenation in a manner similar to the SyrB2 reaction. Experimental details of generating plasmids for expression of the proteins as well as details of purification can be found in the Materials and Methods appendix.

An assay based on UV-Visible spectrophotometry has been developed to determine if a given anion can bind to the Fe(II) cofactor in SyrB2. Typically, anion binding is indicated by an increase in the intensity of the metal to ligand charge transfer band (in the range of 440 to 700 nm) upon addition of anion into an enzyme•Fe(II)•αKG solution. (12) The increase in absorbance is occasionally accompanied by a shift in the wavelength exhibiting maximum absorbance, and examples of titrations using SyrB2 and an assortment of anions can be seen in
Figure 3-7. (12) These effects have been tested for a large number of anions in both wild type and A118G SyrB2 and seem to be a very reliable indicator of anion binding.

The spectra in Figure 3-7 are generated by correcting for the dilution of the sample by addition of anion, setting the absorbance at 800 nm to zero and subtracting the spectrum of the enzyme•Fe(II)•αKG complex before the addition of any anion. All subsequent spectra of TauD and P4H have been generated in a similar fashion unless indicated otherwise. The difference spectra allow for determination of $K_D$ for each anion by plotting the absorbance values at a given wavelength against the concentration of anion added. Fitting the resulting data with a hyperbolic binding equation (when in a weak binding regime) or a quadratic binding equation (when in a tight binding regime) provides a numerical output that includes the $K_D$. 
Figure 3-7: UV-Visible difference spectra of the effects of anion binding on the metal to ligand charge transfer band of WT (A-F) and A118G (G-L) SyrB2. All reactions contain 0.75 mM Fe(II), 5 mM αKG, and 1 mM WT or A118G SyrB2. (A, G) Cl⁻ titration; (B, H) Br⁻; (C, I) N₃⁻; (D, J) OCN⁻; (E), HS⁻; (F, L) CN⁻; (K), SCN⁻. Spectra were generated as described in the text. From (12).
TauD Titrations

In order to ensure spectral changes that are due to anion binding to TauD or P4H and not an off pathway reaction of the enzyme or solution chemistry, several control titrations were performed. Figure 3-8 compares spectra for the addition of chloride to wild type (WT, left panel) and D101A TauD•Fe(II)•αKG complexes (right) at 5 °C in an anaerobic environment. Addition of chloride to WT TauD (left panel) yields no consistent spectroscopic changes. Addition of chloride to TauD D101A (right panel) generates a set of spectra that resemble the changes in the SyrB2 anion binding spectra, with increase in absorbance at 650 nm and a shift in the maximum absorbance from 610 nm to 650 nm.

**Figure 3-8:** UV-Visible spectrophotometry absorbance change-versus-wavelength difference spectra monitoring the metal to ligand charge transfer band regions of WT and D101A TauD to determine if D101A can bind Cl\(^-\). Each reaction contains 625 μM enzyme, 500 μM Fe(II) and 1.5 mM αKG, with the solution maintained at 5 °C in the spectrophotometer.

The difference spectra in Figure 3-8 are not simple spectra with single maxima and likely include multiple absorbing species in the sample. In order to accurately determine the absorbance of a single species at a given wavelength (e.g. 650 nm), a 3 point dropline analysis was used to remove absorbance of any overlapping species at that wavelength. Details of this analysis can be found in Materials and Methods. Plotting the corrected absorbance values at 650 nm versus NaCl...
concentration generates Figure 3-9 which is fit with the hyperbolic binding equation to yield a $K_D$ of approximately 2.5 mM for NaCl binding. Repetition of this titration yields a $K_D$ of $2.1 \pm 0.5$ mM ($n=5$) for binding of Cl$^-$ to TauD D101A. Since the binding of Cl$^-$ to TauD D101A generates similar changes to those in SyrB2 as seen in Figure 3-7, it is believed that TauD D101A is capable of binding chloride (albeit weakly) so it may be able to generate halogenated product as well.

![Figure 3-9:](image)

**Figure 3-9:** 3 point dropline corrected absorbance (650 nm)-versus-[NaCl] plot of the titration of NaCl into TauD D101A•Fe(II)•αKG complex (5 °C) for the determination of the $K_D$ for NaCl binding. The red line represents a fit of the equation for the hyperbolic regime to the data and yields $K_D = 2.5$ mM for this plot. The cuvette contained 625 μM D101A, 500 μM Fe(II) and 1.5 mM αKG at the start of the titration.

Other useful dissociation constants can be obtained from titrations of TauD D101A with Fe(II) or αKG. Figure 3-10 contains the difference spectra and absorbance-versus-[titrant] plots for the addition of iron to D101A•αKG and the addition of αKG to D101A•Fe(II). The difference spectra in the left panels display the typical metal-to-ligand charge-transfer bands at 520 nm that WT TauD (P4H and SyrB) exhibit upon generation of the enzyme•Fe(II)•αKG complex. For
these experiments, the 520 nm versus [iron] or [αKG] plots can be fit with a quadratic binding equation as the complex forms in the tight binding regime. The resulting $K_D$ values for these and subsequent titrations can be seen in Table 3-1 along with previously reported values for WT TauD. (13) Unless otherwise indicated, all reported $K_D$ values are the average from at least 3 trials, with the standard deviation of the range in each individual $K_D$.

The $K_D$ of Fe(II) binding to WT TauD in the presence of αKG is approximately 8 μM and the $K_D$ of αKG binding WT in the presence of Fe(II) is approximately 300 μM. (13) The $K_D$ of Fe(II) binding to D101A TauD in the presence of αKG is approximately 40 μM and the $K_D$ of αKG binding in the presence of Fe(II) is approximately 130 μM. These results suggest that TauD D101A binds Fe(II) less tightly than WT and αKG more tightly. Again, since this mutation affects the iron-binding facial triad it is not surprising that this variant does not bind Fe(II) as tightly as WT. Tighter binding of αKG in the variant may aid in titrations of taurine into D101A•Fe(II)•αKG(•Cl) solutions that will be discussed below.

The quadratic binding equation also contains an estimate for the receptor concentration, $[R]_0$, in the reaction in addition to reporting a $K_D$ for each data set. Each of the fits from Figure 3-10 yields a receptor concentration of approximately 330 μM. TauD WT typically is about 80% active. In these reactions, 625 μM TauD D101A would theoretically yield 500 μM receptor concentration. However a receptor concentration of 330 μM suggests D101A may only be about 50% active instead of 80%. 
In order for the reactivity of TauD D101A to be successfully altered to halogenation, the protein must bind taurine in addition to iron, αKG and chloride. Figure 3-11 contains the difference spectra and resulting absorbance-versus-[taurine] plot to determine a $K_D$ for taurine binding. This value has been difficult to obtain for WT TauD, as the αKG tends to complex with other components of the reaction and precipitate over time making reproducible results difficult to obtain. Interestingly, TauD D101A does not suffer from precipitation issues to the same extent.
that the WT protein does and the $K_D$ for taurine binding to TauD D101A is reported in Table 3-1 below. The loss of intensity of the metal-to-ligand charge transfer band may occur because the D101A•Fe(II)•αKG and D101A•Fe(II)•αKG•Cl complex should shift from a six-coordinate to a five-coordinate system upon introduction of taurine. The somewhat tighter binding of αKG to D101A than to WT TauD may be the reason this $K_D$ is obtainable.

Figure 3-11: UV-Visible difference spectra and Absorbance (520 nm)-versus-[taurine] plots to determine the $K_D$ of taurine binding to D101A•Fe(II)•αKG (top) and D101A•Fe(II)•αKG•Cl$^-$ (bottom) at 5 °C. The cuvette contained 625 μM D101A, 500 μM Fe(II), 1.5 mM αKG, and 150 mM Cl$^-$ (when present) before adding taurine.
The titration of taurine into D101A•Fe(II)•αKG yields essentially identical spectra as when taurine is titrated into D101A•Fe(II)•αKG•Cl. The fact that the $K_D$ for taurine binding is essentially the same in the presence and absence of Cl$^-$ suggests the presence of Cl$^-$, even in mM quantities, does not perturb taurine binding to TauD D101A. Future experiments should investigate the reaction of these complexes with $O_2$ and if the kinetic rates of ferryl formation are perturbed.

**Table 3-1:** $K_D$ values obtained from fitting absorbance versus [titrant] plots from titrations of varying combinations of TauD D101A, Fe(II), αKG, NaCl and taurine. WT values are published $K_D$ values from (13). (n) indicates number of repetitions from which the standard deviation was calculated.

<table>
<thead>
<tr>
<th>Reactants in Solution</th>
<th>Titrant</th>
<th>$K_D$ (mM) (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-Fe(II)</td>
<td>αKG</td>
<td>0.3</td>
</tr>
<tr>
<td>WT-αKG</td>
<td>Fe(II)</td>
<td>0.008</td>
</tr>
<tr>
<td>D101A-Fe(II)</td>
<td>αKG</td>
<td>0.13± 0.023 (3)</td>
</tr>
<tr>
<td>D101A-αKG</td>
<td>Fe(II)</td>
<td>0.041± 0.017 (3)</td>
</tr>
<tr>
<td>D101A-Fe(II)-αKG</td>
<td>Cl</td>
<td>2.1 ± 0.47 (5)</td>
</tr>
<tr>
<td>D101A-Fe(II)-αKG-Cl</td>
<td>Taurine</td>
<td>0.020± 0.0023 (2)</td>
</tr>
<tr>
<td>D101A-Fe(II)-αKG</td>
<td>Taurine</td>
<td>0.024± 0.0041 (3)</td>
</tr>
</tbody>
</table>

**P4H Titrations**

Based on alignment of the protein sequences, the cognate residue of TauD D101A is D154A in P4H. Unfortunately, there are no spectral changes on addition of chloride to P4H•Fe•αKG that suggest this variant binds chloride (data not shown). To establish if all other constituents still bind, titrations of αKG to P4H WT•Fe(II) (Figure 3-12 A) and D154A•Fe(II) (C) complexes were carried out. Addition of αKG to D154A•Fe(II) generates atypical absorbance changes in the region of the metal-to-ligand charge transfer band, indicating that αKG does not bind to this variant as it does to WT P4H. WT P4H exhibits a typical metal-to-ligand charge-transfer band at 520 nm upon addition of αKG to WT•Fe(II) (A), and the data from this titration
yield a $K_D$ of approximately 220 $\mu$M (D). Addition of the peptide substrate (PAPK)$_3$ to the P4H•Fe•αKG•Cl$^-$ solution also does not generate any changes to the absorption spectrum, further indicating that this variant does not properly bind substrates (data not shown). It is possible that this residue plays a more critical role in iron binding and subsequent substrate binding in P4H than in TauD and that modification leads to inactivation of the enzyme.

**Figure 3-12:** UV-Visible difference spectra from titrations of P4H WT and D154A at 5 °C. A) Difference spectra generated by titrating αKG into WT-P4H•Fe(II). B) The $A_{520 \text{ nm}}$-vs-[αKG] plot to determine $K_D$ for αKG binding to WT-P4H•Fe(II); C) Difference spectra generated by titrating αKG into D154A-P4H•Fe(II). Each titration contained 625 $\mu$M enzyme and 500 $\mu$M Fe(II).
Substrate Exchange in the Ferryl State of TauD

As previously discussed, SyrB2 locks its bulky substrate in place while in the Fe(IV) state and there is no evidence for substrate dissociation before reaction completion in P4H. However, an observation by Eric Barr, a former researcher in the Bollinger/Krebs group, suggested that TauD might be different. To investigate this possibility further, (SF) absorption experiments were conducted.

Prior to investigating whether substrate can exchange in the ferryl state, the first experiment carried out was to establish that the current enzyme preparation exhibits the same kinetics as the original preparations for which rate constants were determined. A simple single-mixing SF experiment was conducted, in which mixing TauD•Fe(II)•αKG•H₄-taurine quaternary complex was mixed with oxygenated buffer giving final concentrations of 250 μM TauD, 200 μM Fe(II), 1.25 mM αKG, 220 μM H₄-taurine, and 190 μM O₂. All previously reported data have been from reactions carried out at 5 °C. It was expected that increased temperature might lead to more rapid exchange of substrate, and so the temperature dependence of the reaction was investigated by repeating these experiments at 15 °C and 25 °C (Figure 3-13).
Figure 3-13: Absorbance (320 nm)-versus-time traces from a SF experiment in which TauD•Fe(II)•αKG•H₄-taurine quaternary complex was mixed with oxygenated buffer to confirm and determine formation and decay rates of the H₄-taurine containing turnover reaction of WT TauD at 5 °C (red), 15 °C (orange), and 25 °C (blue). Final concentrations after mixing were 250 μM TauD, 200 μM Fe(II), 1.25 mM αKG, 220 μM H₄-taurine and 190 μM O₂.

Figure 3-13 displays the changes of the absorbance at 320 nm with time which is associated with the formation (increasing absorbance) and decay (decreasing absorbance) of the ferryl species. All absorbance versus time traces reported in this document are generated by averaging at least 3 experimental traces to improve the signal-to-noise ratio. Fitting of each absorbance versus time trace yields the rates in Table 3-2. As expected, formation and decay rates increase with increasing temperature. The reported formation rate at 5 °C for the ferryl species of 145,000 M⁻¹s⁻¹ corresponds to a formation rate constant of 29 s⁻¹ in the presence of 200 μM quaternary complex which agrees well with the observed formation rate constant of 29 s⁻¹. Previous studies have also reported that decay of the ferryl species generated with H₄-taurine at 5 °C occurs with a rate constant of 13 s⁻¹. (7) Table 3-2 shows that the fit of the 5 °C trace yields a decay rate constant of about 9 s⁻¹. The discrepancy results from assuming pseudo first-order conditions are present, however this requirement is not met for this experiment. The
experimental rates obtained suggest that the current preparation of the protein behaves in the same manner as the protein from the original experiments.

**Table 3-2:** Observed formation and decay rate constants of the WT TauD ferryl intermediate at three temperatures (from the experiment in Figure 3-13).

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>k\text{formation} (s(^{-1}))</th>
<th>k\text{decay} (s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>5°C</td>
<td>29</td>
<td>9.4</td>
</tr>
<tr>
<td>15°C</td>
<td>53</td>
<td>15</td>
</tr>
<tr>
<td>25°C</td>
<td>90</td>
<td>21</td>
</tr>
</tbody>
</table>

Because the focus of this study is the possibility that the taurine substrate still has the ability to bind and detach from the enzyme active site in the Fe(IV)=O stage of the reaction, the 320 nm wavelength is again used as an indicator for monitoring the formation and decay of the ferryl species. Exchange of taurine in the active site should generate variation in the kinetics of ferryl decay in the course of the turnover reaction when one isotopically substituted taurine is exchanged for another. To properly evaluate whether taurine can exchange, the length of time required for all of the reactant TauD•Fe(II)•αKG•taurine complex to react with O\(_2\) must be established so no variation is attributable to free enzyme that had not yet reacted. Figure 3-14 shows the 320 nm kinetic traces following sequential mixing of the TauD•Fe(II)•αKG•D\(_4\)-taurine quaternary complex with oxygen to initiate the ferryl formation and the solution was then mixed 50 ms later with an anoxic buffer solution (final concentrations after mixing were 250 μM TauD, 200 μM Fe(II), 1.25 mM αKG, 220 μM D\(_4\)-taurine and 95 μM O\(_2\)). The sequential mixing setup was used as a control for subsequent experiments that would require this method and a 50 ms delay between the first and second mixes was used to minimize any mixing artifacts that might be visible with shorter delay times. D\(_4\)-taurine was used instead of H\(_4\)-taurine because it allows greater accumulation of the ferryl species.
Figure 3-14: Absorbance (320 nm)-versus-time traces following the mixing of TauD•Fe(II)•αKG•D_4-taurine with O_2 and then with anoxic buffer with a 50 ms delay between the first and second mixes. The experiment was carried out at 5 °C, 15 °C, and 25 °C and the final concentrations of reactants after mixing were 250 μM TauD, 200 μM Fe(II), 1.25 mM αKG, 220 μM D_4-taurine and 95 μM O_2.

The experiment in Figure 3-14 provides the proper length of time to allow the reaction to proceed to ensure that any quaternary complex capable of reacting with O_2 has done so before introducing additional taurine for exchange. Here it was decided to wait until approximately halfway down the decay phase of the reactions above before mixing with the unlabeled substrate. For subsequent experiments, the delay in mixing for 5 °C was set for 2.0 s, for 15 °C is set to 1.0 s, and for 25 °C to 0.5 s, as indicated by the vertical black lines in Figure 3-14.

The subsequent experiment tested for changes in the kinetics of the decay of the ferryl species when the second mix that was previously just buffer instead delivered a large excess of H_4- or D_4-taurine. The conditions of the experiment in Figure 3-15 were the same as for the previous experiment in Figure 3-14, except that, when substrate was added in the second mix, it was present at a final concentration of 5 mM.
Figure 3-15: Absorbance (320 nm)-versus-time traces of reactions at multiple temperatures exhibiting differences in decay kinetics when the ferryl state of TauD is exposed to substrate isotopologs. In all panels, the red trace is the control experiment with a second mix with buffer; the orange trace is the reaction containing D_4-taurine mixed with a large excess of D_4-taurine; the blue trace is the reaction containing D_4-taurine mixed with an excess of H_4-taurine. Panel A shows traces from the set of reactions carried out at 5 °C with delay of 2.0 s between the first and second mix. Panel B shows traces from the reactions run at 15 °C with a delay of 1.0 s. Panel C shows traces from reactions run at 25 °C with a delay of 0.5 s. Final concentrations of the reactants after mixing were 250 μM TauD, 200 μM Fe(II), 1.25 mM αKG, 220 μM D_4-taurine, 95 μM O_2, and 5 mM D_4- or H_4-taurine (when added in second mixing step).

Figure 3-15 shows that there is variation in the kinetics of ferryl decay when the ferryl state generated with D_4-taurine is exposed to excess H_4-taurine. If there were no effect on the kinetics of decay, all three traces of each panel should be identical. The separation of the blue
trace from the orange and red traces shows that addition of H₄-taurine to the ferryl complex generated with D₄-taurine accelerates the decay of the ferryl species. To quantify this acceleration, the decay phases were fit by the equation for exponential decay to yield the rate constants in Table 3-3.

**Table 3-3**: Table of decay rate constants for the TauD ferryl species from the experiment in Figure 3-15 at three temperatures.

<table>
<thead>
<tr>
<th>2nd Mix</th>
<th>$k_{\text{decay}}$ 5°C (s⁻¹)</th>
<th>$k_{\text{decay}}$ 15°C (s⁻¹)</th>
<th>$k_{\text{decay}}$ 25°C (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vs Buffer</td>
<td>0.33±0.03</td>
<td>0.56±0.05</td>
<td>1.8±0.3</td>
</tr>
<tr>
<td>Vs D₄</td>
<td>0.35±0.02</td>
<td>0.63±0.07</td>
<td>2.0±0.2</td>
</tr>
<tr>
<td>Vs H₄</td>
<td>0.50±0.05</td>
<td>0.99±0.05</td>
<td>3.5±0.3</td>
</tr>
</tbody>
</table>

As Table 3-3 shows, the mixes with buffer and D₄-taurine produce very similar decay rate constants within each temperature. When the second mix contains the H₄-taurine substrate, there is a significant acceleration of the decay phase, indicating that there can indeed be exchange of the taurine substrate, even when the enzyme is in the ferryl state. Crystal structures of TauD have shown that conformational changes occur when both αKG and taurine are bound. These changes have been likened to a lid closing over the active site. (14) This fact would seem to suggest that, once substrates are bound, they should be locked in place as in SyrB2, thus, the clear variation of decay when an alternate taurine is added in the midst of the ferryl state is unexpected and very interesting.

**Substrate Exchange in the Ferrous State of TauD**

Given that TauD can exchange taurine in the ferryl state, it should be able to exchange rapidly in the ferrous state as well. Subsequent experiments were aimed at determining the kinetics of taurine exchange in the ferrous state of the enzyme. The on-rate of taurine has
previously been reported to be greater than $140 \text{s}^{-1}$, but, again, the $K_D$ for taurine in WT TauD has not been determined. (13)

The first experiment of this series yielded insight into the binding order of substrates. In this single mixing SF experiment a TauD•Fe(II)•taurine complex was mixed with a solution containing $O_2$, $\alpha$KG, and taurine at 5 °C and the ferryl state was monitored at 320 nm. Figure 3-16 contains the resulting kinetic traces from the reactions under different conditions. The figure legend indicates which taurine ($H_4$ or $D_4$) is present in which solution, so the “$H_4$ vs $D_4 + O_2$” condition indicates the ternary complex contained $H_4$-taurine and was mixed with the oxygenated solution containing $\alpha$KG and $D_4$-taurine. Final concentrations after mixing were 250 μM TauD, 200 μM Fe(II), 2 mM taurine (from 1st syringe), 130 μM $O_2$, 2mM $\alpha$KG, and 40 mM taurine (2nd syringe).

Figure 3-16: Investigation of the substrate binding order of WT TauD. Absorbance (320 nm)-versus-time traces of a SF experiment (5 °C) mixing TauD•Fe(II)•Taurine with a solution of $O_2$, $\alpha$KG, and Taurine. $H_4$- and $D_4$-taurine were added in varying order as indicated by the figure legend. Final concentrations of the reactants after mixing were 250 μM TauD, 200 μM Fe(II), 2 mM taurine (from the 1st syringe), 130 μM $O_2$, 2mM $\alpha$KG, and 40 mM taurine (2nd syringe).
Figure 3-16 shows the absorbance (320 nm)-versus-time traces from mixing a TauD•Fe(II) and taurine solution with a solution of O₂, αKG, and taurine. H₄- and D₄-taurine were added in different combinations as indicated in the figure legend. Reactions in which only one form of taurine was present generated kinetic traces that were essentially identical to each other. For example when the TauD•Fe(II) solution with H₄-taurine was mixed with O₂ and αKG (red trace) it produced an absorbance-versus-time trace that was nearly identical to the trace generated by mixing the TauD•Fe(II) and H₄-taurine solution with a solution of O₂, αKG and additional H₄-taurine (orange trace). Interestingly, when the opposite isotopolog of taurine was added with O₂ and αKG (purple and black dashed traces), the 320 nm absorbance-versus-time trace generated was nearly identical to the control traces that correspond to the excess taurine. In other words, when the TauD•Fe(II) and H₄-taurine was mixed with a solution of O₂, αKG and D₄-taurine, the resulting 320 nm trace was nearly identical to the traces containing only D₄-taurine. This observation suggested that when αKG was added after taurine, any taurine in the active site must leave so that αKG could bind. The excess taurine then out competed the lower concentration taurine to initiate the turnover reaction. If the taurine in solution with the TauD•Fe(II) complex was not required to exit the active site for αKG to be able to bind, the resulting kinetic traces after mixing with O₂, αKG and the opposite taurine isotopolog should appear one of two ways: either the trace would look like the control trace of the initial taurine, or the trace would have an amplitude and decay phase partway between the single taurine control experiments. This experiment confirmed previously published results that αKG binds TauD before taurine. (1)

One can see that the traces from each experiment with the same excess taurine look fairly similar (green solid, dark green solid and purple dashed and red solid, orange solid and black dashed), but it is useful to fit these traces for quantitation purposes. Figure 3-17 shows each set of traces with their respective fits. Again, it is likely that the fits are not perfect due to the lack of
pseudo first order conditions in the experiment. The extracted formation and decay rates can be found in Table 3-4.

![Figure 3-17: Fitting of the kinetic traces from the experiment in Figure 3-16. Solid circles are experimental data and solid lines are generated from the “B in A to B to C” equation.](image)

**Table 3-4:** Formation and decay rate constants of the ferryl species generated from mixing ternary TauD•Fe(II)•taurine with a solution of O₂, αKG, and taurine.

<table>
<thead>
<tr>
<th>Condition</th>
<th>k\text{formation} (s^{-1})</th>
<th>k\text{decay} (s^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₄ vs O₂</td>
<td>23 ± 0.4</td>
<td>1.7 ± 0.04</td>
</tr>
<tr>
<td>H₄ vs H₄ + O₂</td>
<td>22 ± 0.5</td>
<td>2.4 ± 0.05</td>
</tr>
<tr>
<td>D₄ vs H₄ + O₂</td>
<td>23 ± 0.5</td>
<td>1.6 ± 0.03</td>
</tr>
<tr>
<td>D₄ vs O₂</td>
<td>4.9 ± 0.04</td>
<td>0.15 ± 0.001</td>
</tr>
<tr>
<td>D₄ vs D₄ + O₂</td>
<td>5.8 ± 0.05</td>
<td>0.19 ± 0.002</td>
</tr>
<tr>
<td>H₄ vs D₄ + O₂</td>
<td>6.2 ± 0.05</td>
<td>0.21 ± 0.002</td>
</tr>
</tbody>
</table>

Again, the formation rate for the ferryl species of 145,000 M^{-1}s^{-1} corresponds to 29 s^{-1} in the presence of 200 μM TauD•Fe(II). (1) When formed with H₄-taurine, the ferryl species should decay at 13 s^{-1} and when formed with D₄-taurine it should decay at 0.35 s^{-1} at 5 ℃. (4) From Table 3-4, the rate constants comparing each excess taurine reagent are all very similar to each other (e.g. H₄ + O₂, H₄ vs H₄ + O₂ and D₄ vs H₄ + O₂). All formation rates should be the same irrespective of the form of taurine present and only the decay rates should differ. It is likely the
variation in generated rate constants from reported constants was again in part due to the lack of pseudo first-order conditions. The variation likely also arose in part from the fact that the “B in A to B to C” equation fits the kinetic traces as though they were two step processes with one absorbing species. In reality, these traces were generated by a four step reaction where the initial taurine must dissociate, αKG was bound followed rapidly by O₂ to generate the ferryl species, and the ferryl decayed to release the products. The fidelity of fit appears better for the reactions with D₄-taurine in excess, so those rate constants are likely more similar to the actual values. Even though the fitting was not perfect it did provide enough information to make one important set of conclusions. Both of the “vs H₄ + O₂” traces could be fit with essentially identical formation and decay rate constants and both “vs D₄ + O₂” traces could be fit with identical rate constants. This again affirmed that the initial taurine needed to leave the active site for αKG to bind which biased the reaction to whichever isotopolog of taurine was in excess. It was not surprising that the decay rate constant of “D₄ vs H₄ + O₂” was slightly slower than “H₄ vs H₄ + O₂” because there would likely be some small fraction of enzyme that contained D₄-taurine during turnover. Similarly, it was not surprising that “H₄ vs D₄ + O₂” decay was slightly faster than “D₄ vs D₄+ O₂”.

In order to tease out more kinetic parameters related to the exchange of substrate in the ferrous state, another set of sequential mixing SF experiments was performed. In this case, quaternary complex containing TauD•Fe(II)•αKG•taurine isotopolog A was mixed with a deoxygenated solution containing taurine isotopolog B. This solution was then aged for varying delay times before being mixed with oxygenated buffer. The absorbance at 320 nm was monitored to determine the similarity of the kinetics of the reaction to either a strictly H₄- or D₄-taurine reaction (Figure 3-18). The final concentrations of reactants after mixing in the experiment in Figure 3-18 were 250 μM TauD, 200 μM Fe, 1.25 mM αKG, 500 μM taurine (A), 10 mM taurine (B) and 130 μM O₂ with the temperature maintained at 5 °C.
Figure 3-18: Absorbance (320 nm)-versus-time traces generated by mixing TauD quaternary complex containing taurine isotopolog A with taurine isotopolog B and aging for various lengths of time before mixing with oxygenated buffer. The times in the figure legend are the delay between the first and second mixes, i.e. how long each form of taurine is allowed to exchange before \( \text{O}_2 \) is introduced. The final concentrations of reactants after mixing were 250 μM TauD, 200 μM Fe, 1.25 mM αKG, 500 μM taurine A, 10 mM taurine B, and 130 μM \( \text{O}_2 \) with the temperature maintained at 5 °C.

Figure 3-18 shows the 320 nm kinetic traces that resulted from the above described reaction varying the time that the two taurine isotopologs were allowed to exchange before the complex was reacted with \( \text{O}_2 \). The times in the figure legends are the delay times between the first and second mixes. In the panel on the left, the quaternary complex was generated with \( \text{H}_4 \)-taurine and then mixed with an excess of \( \text{D}_4 \)-taurine and, in the right panel, the quaternary complex was generated with \( \text{D}_4 \)-taurine and then mixed with \( \text{H}_4 \)-taurine before \( \text{O}_2 \) was added. It is apparent that, within 20 ms of introducing the second taurine, significant exchange occurs. These traces are significantly different from the control traces from experiments involving a second mix with more of the original taurine. Within 10 s, the excess second taurine has completely exchanged for the original taurine, as is evidenced by the similarity of the control traces containing only the second taurine to the 5 and 10 s exchange traces.

These traces can be analyzed as a summation of two so-called “B in A to B to C” equations (Equation 3-1). The equation utilizes a common formation rate (\( k_{\text{form}} \)), because the
kinetics of ferryl formation are the same, regardless of which taurine isotopolog is bound. Equation 3-1 accounts also for the amplitude of absorbance by both H₄- and D₄-taurine (Ampₐ-initial and Ampₐ-excess taurine) as well as the decay rate constant of each species (k_decayA-initial and k_decayB-excess) in addition to an offset value to account for any non-zero initial absorbance (c). The decay of the process associated with H₄-taurine should be approximately 13 s⁻¹ and the decay of the process associated with D₄-taurine should be about 0.35 s⁻¹.

\[
\left(\text{Amp}_A \times \frac{k_{\text{form}}}{(k_{\text{decay}A} - k_{\text{form}})} \times \left(e^{-k_{\text{form}}x} - e^{-k_{\text{decay}A}x}\right) + \text{Amp}_B \times \frac{k_{\text{form}}}{(k_{\text{decay}B} - k_{\text{form}})} \times \left(e^{-k_{\text{form}}x} - e^{-k_{\text{decay}B}x}\right) + c\right)
\]

Equation 3-1: Equation for fitting the absorbance (320 nm)-versus-time traces from Figures 3-18 and 3-19.

![Figure 3-19](image)

**Figure 3-19**: Fitting of the traces in Figure 3-18 using Equation 3-1. Panel A is the experiment monitoring the absorbance at 320 nm with time when the quaternary complex generated with H₄-taurine is mixed with excess D₄-taurine (H₄ vs D₄ vs O₂). Panel B shows the absorbance changes at 320 nm with quaternary complex generated with D₄-taurine and mixed with excess H₄-taurine (D₄ vs H₄ vs O₂).
Figure 3-19 contains the kinetic traces from Figure 3-18 (dashed lines) and the fits generated by Equation 3-1 (thin solid lines). The resulting amplitudes and rate constants can be found in Table 3-5.

Table 3-5: Values of amplitudes and rate constants obtained from Equation 3-1 for both sets of experiments observing the exchange of substrate in the ferrous state of TauD.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Delay (s)</th>
<th>AmpA</th>
<th>k_{form} (s^{-1})</th>
<th>k_{decayA} (s^{-1})</th>
<th>AmpB</th>
<th>k_{decayB} (s^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₄ vs D₄ vs O₂</td>
<td>0.02</td>
<td>0.081</td>
<td>30</td>
<td>0.38</td>
<td>-0.026</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.093</td>
<td>28</td>
<td>0.38</td>
<td>-0.035</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>0.11</td>
<td>25</td>
<td>0.39</td>
<td>-0.040</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.12</td>
<td>24</td>
<td>0.38</td>
<td>-0.043</td>
<td>3.0</td>
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Theoretically, all formation rates and each decay rate should be the same value within each condition, with only the amplitudes changing. Exchange from one taurine to the other before addition of O₂ caused less of the 320 nm absorbance change to be due to the initial taurine and more due to the excess taurine. If the amplitudes of each process are plotted versus the delay time and fit with an exponential function, one of the values obtained is actually the off rate of the first taurine species. The D₄ vs H₄ vs O₂ traces showed a higher fidelity of fit and are likely more
accurate than the H\textsubscript{4} vs D\textsubscript{4} vs O\textsubscript{2} traces. The amplitudes from this set of data are used for plotting in Figure 3-20.

![Figure 3-20](image)

**Figure 3-20**: Reported amplitudes from the fits of D\textsubscript{4} vs H\textsubscript{4} vs O\textsubscript{2} traces from Figure 3-19 plotted versus delay time. The data were fit with an exponential function to obtain the $k_{off}$ for taurine binding.

The fitting of the two sets of data in Figure 3-20 provides one value of $1.2 \pm 0.2$ s\textsuperscript{-1} and a second value of $0.88 \pm 0.09$ s\textsuperscript{-1} for the off rate of the first taurine. These values are in good agreement with each other.
As Table 3-5 and Figure 3-19 A show, the $H_4$ vs $D_4$ vs $O_2$ condition was not well fit with equation 3-1. A simple analysis, in which the amplitude of the kinetic trace at 0.45 s, which was associated with the $D_4$-taurine process, was plotted against the delay time in Figure 3-21. The fit of this data set yields a $k_{\text{off}}$ of 0.98 s$^{-1}$ which agrees very well with the $k_{\text{off}}$ values generated through fitting the data sets in Figure 3-20. The on rate of taurine has been reported to be greater than $3 \times 10^4$ M$^{-1}$s$^{-1}$. (2) The $k_{\text{off}}$ and $k_{\text{on}}$ are related through the equation $K_D = k_{\text{off}}/k_{\text{on}}$, which means that $K_D$ for taurine binding to WT TauD is less than 1.0 s$^{-1}$/30 mM$^{-1}$s$^{-1} = 0.033$ mM. Incidentally, this is very similar to the value of 0.02 mM that was determined for the $K_D$ of taurine binding to D101A TauD.

**Conclusions**

The TauD D101A variant appears to be able to coordinate Cl$^-$ at its Fe(II) cofactor, and further experimentation should be done with this enzyme to determine if it can halogenate taurine. This variant appears to bind Fe(II) more weakly and $\alpha$KG more tightly ($K_D = 0.04$ mM.
and 0.13 mM respectively) than TauD WT. The $K_D$ for taurine binding to D101A TauD is 0.02 mM whereas the $K_D$ for WT TauD is not able to be determined through similar titrations.

The cognate mutation of D101A in P4H is D154A. Asp154 may play a more critical role in binding Fe(II) in P4H than Asp101 does in TauD. Substitution of this residue seems to inactivate the enzyme, as UV-Visible spectra indicate the enzyme is unable to bind αKG and Fe(II).

WT TauD can exchange taurine substrate even while in the ferryl state, as demonstrated by an effect on the rate constant for decay of the ferryl species upon addition of an alternately labeled taurine. It has been reconfirmed that αKG binds before taurine in an ordered sequential reaction for the turnover reaction to commence. Exchange of taurine in the ferrous state occurs with an off rate of about $1 \text{s}^{-1}$ that when combined with the on rate estimate of $30,000 \text{M}^{-1}\text{s}^{-1}$ yields a $K_D$ for taurine binding to WT TauD of less than 0.033 mM. This value is very similar to the $K_D$ of 0.02 mM obtained for D101A TauD.
References


The initial goal of this work was to characterize a P450 Compound I utilizing a novel peroxygenase P450. After the successful characterization of a P450 Compound I by the Green research group, this work was not pursued with as much rigor. Published information suggested the novel peroxygenase P450 fatty acid hydroxylating enzyme from *Bacillus subtilis* should be a good candidate for employing the strategies discussed above to trap Compound I. During experimentation to attempt to trap Compound I, several additional observations that had not previously been reported yielded additional insight into this enzyme and are reported in this unedited appendix.

**Appendix A**

**P450<sub>BSβ</sub>**

Heme containing enzymes have been studied for more than 50 years with a seminal study on the family including the characterization of a P450 enzyme from liver microsomes in the early 1960s by Omura and Sato. (1, 2) The subfamily of P450 enzymes received its name due to the UV-Visible feature that develops at 450 nm when the enzyme is exposed to carbon monoxide. (1) Heme proteins perform a number of biologically critical roles including oxygen transport via hemoglobin and storage via myoglobin and reactive oxygen species detoxification via catalase. (3, 4)

**P450 Consensus Mechanism**

The P450 family of enzymes is involved in such wide ranging functions as vitamin D regulation, quorum sensing, and degradation of xenobiotics. (5-7) P450s operate via the consensus mechanism seen in Figure **A-1** where the resting low spin (LS)-ferric center (species 1)
is coordinated by the four nitrogens of the heme macrocycle, as well as a protein ligand that is either a histidine or a cysteine, and a water. (8) The binding of substrate (RH) in the active site displaces the water ligand causing the iron to leave the plane of the heme and shift into a high spin (HS) state (2). This shift increases the redox potential of the heme so that an electron can be shuttled into the active site from an NAD(P)H or similar electron donating molecule via a separate reductase domain. (8) The electron reduces the HS-ferric center to a HS-ferrous center (3) which allows oxygen to bind (4) and subsequently rearrange electronically to generate a ferric superoxide species (5). Introduction of a second electron from the reductase domain, potentially the rate limiting step of the reaction, yields a ferric peroxide species (6) which will readily bind a proton to form a ferric hydroperoxide complex (7). (8) A second protonation on the ferric hydroperoxide causes heterolytic cleavage of the O-O bond to release water and generate the ferryl (Fe(IV)=O) and porphyrin radical containing species referred to as Compound I (8). This highly reactive species has the potency to abstract a hydrogen atom from the substrate leading to rebound of a hydroxyl moiety back onto the substrate radical resulting in hydroxylation of the substrate and returning the enzyme to its resting state.
Figure A-1: Consensus mechanism for the reaction of the P450 enzyme family. Adapted from (8).

Peroxidase Enzymes

A closely related family of heme containing enzymes to that of the P450s is the peroxidase family. These enzymes are named for the fact that they do not require molecular oxygen for their reactions but can utilize hydrogen peroxide effectively to carry out a variety of reactions. The chloroperoxidase from *Caldariomyces fumago* has multiple reactivities that can decompose H$_2$O$_2$ to molecular oxygen and water in a catalase type mechanism, as well as generate a substrate-halogen bond at low pH and also carry out peroxidative oxidation at higher pH. (9, 10) The chlorination reaction utilizes a mechanism that, for P450s, is termed a peroxide...
shunt (Shunt in Figure A-1). The hydrogen peroxide provides an oxygen source as well as electrons, without the need of a bulky reductase domain, to rapidly generate the Compound I species and carry out a hydrogen abstraction and subsequent halogenation. (10)

**Compound I Characterization**

Compound I has been observed in a variety of peroxidase enzymes including chloroperoxidase and horseradish peroxidase. (11) The EPR spectrum of Compound I (Figure A-2) shows a radical feature at approximately g=2 associated with the porphyrin radical, as well as a broad feature at approximately g=1.73. (11) The Mössbauer spectrum of Compound I (Figure A-3) can be simulated with parameters of δ=0.15 mm/s and ΔE_Q=1.02 mm/s which are typical of a ferryl species. (11) The Mössbauer spectrum also exhibits magnetic hyperfine structure that is highly field dependent which advocates the presence of a magnetic species with an effective spin (S^eff) of ½. (11) The effective spin results from coupling of the Fe(IV)=O with S=1 and the porphyrin π-cation radical with S=½. (11)
Figure A-2: EPR spectrum of Chloroperoxidase Compound I (g= 2, 1.73) and native chloroperoxidase (g=2.62, 2.26, 1.82). From (11).

Figure A-3: Mössbauer spectrum of chloroperoxidase Compound I run at 4.2-K in a 34-mT parallel magnetic field (a), a 34-mT perpendicular field (b), and the difference spectrum of b-a. From (11).
While Compound I has been observed in multiple members of the peroxidase family, until very recently Compound I had not been characterized in a member of the P450 family. (12, 13) Green et al utilized the thermophilic CYP119 from *Sulfolobus acidocaldarius* and the oxygen donor m-chloroperbenzoic acid (mCPBA) to generate Compound I in high yields for spectroscopic characterization. (12) This discovery will be discussed in greater detail later.

**P450\_\text{BSβ} Background**

The initiation of this research occurred prior to the characterization of the CYP119 Compound I and one of the initial goals of this study was to attempt to observe Compound I in a P450 enzyme. In order to do this, a unique P450 enzyme from *Bacillus subtilis* was isolated and purified (details in Materials and Methods appendix). This P450 has been dubbed P450\_\text{BSβ} (systematic name, CYP152A1) and is not an oxygenase but has been characterized as a peroxynoenase. (14) This classification as well as several other aspects of the enzyme discussed below suggested this enzyme would be a good candidate to characterize Compound I.

The peroxygenase designation indicates that the enzyme utilizes H\(_2\)O\(_2\) instead of O\(_2\) as an oxygen atom donor to hydroxylate the α and β positions of fatty acid substrates. (14) As previously discussed, H\(_2\)O\(_2\) not only provides the oxygen for hydroxylation but also provides the electrons necessary to generate Compound I without the need for a reductase domain to shuttle electrons. Since the electron shuttling and accompanying conformational changes in the P450s can be rate limiting, the “shortcut” of consuming H\(_2\)O\(_2\) may theoretically shift the rate limiting step to the chemical hydrogen abstraction. This should facilitate observation of Compound I utilizing deuterium kinetic isotope effects and other strategies discussed in the introduction for trapping reactive intermediates.
Another advantage of an enzyme that efficiently utilizes H$_2$O$_2$ instead of O$_2$ (as opposed to an enzyme that will survive one or two turnovers before inactivating due to H$_2$O$_2$) is that concentrations of H$_2$O$_2$ in the molar range can be obtained at standard temperatures and pressures whereas O$_2$ concentrations in the millimolar region are the maximum obtainable concentrations at atmospheric pressure. (15) This eases some experimental constraints as one can have millimolar concentrations of protein and still be able to saturate the enzyme in excess oxygen donor to allow the enzyme to operate at maximal turnover rate (5 s$^{-1}$ for myristic acid hydroxylation). (16)

Another potential indicator to be able to characterize a reactive intermediate with P450$_{BS\beta}$ that it contains a heme ligated by the thiolate moiety of a cysteine residue. (17) Green et al. have discussed the role of thiolate ligation as opposed to ligation via a histidine residue. (18) It is believed the thiolate ligand mainly aids in the activation of dioxygen by acting as an internal electron donor to facilitate O-O cleavage and the generation of the ferryl species. (18) Additional experimental evidence provided by Green et al. has suggested also that the majority, if not all, thiolate ligated hemes will generate basic ferryl species to aid in hydrogen abstraction. (18)

Despite utilizing nearly every strategy available to us to trap Compound I, it was never observed in P450$_{BS\beta}$. However, the experiments attempting to trap Compound I yielded several insights into the reactivity of P450$_{BS\beta}$ that suggest why Compound I was not observed. The hydroxylation reaction seems to uncouple considerably upon the addition of perdeuterated substrate and O$_2$ is evolved from the reaction to hinder the accumulation of any reactive species. It is believed that instead of Compound I, Compound II may actually be observed but this species does not appear to accumulate significantly or persist very long. The specific strategies and interesting observations from the investigation of P450$_{BS\beta}$ are discussed below.
P450_{BSβ} Results

Reaction Uncoupling

The first avenue of study to investigate the reaction of P450_{BSβ} is to investigate the activity of the enzyme. Matsunaga et al have developed HPLC and mass spectrometry assays that were adapted for use in this study (see Materials and Methods appendix). (16) Optimal conditions for the HPLC column prevented a combination HPLC-MS assay, but the two assays in conjunction provide initial insight into the reaction of P450_{BSβ}. Additionally, a horseradish peroxidase assay (discussed in Materials and Methods) was employed to determine the amount of H₂O₂ that has been consumed at the end of each reaction.
**Figure A-4:** HPLC generated chromatograms of standards of myristic acid, D_{27}-myristic acid, α-hydroxymyristic acid and β-hydroxymyristic acid. Experimental details are discussed in Materials and Methods.

Figure A-4 contains the HPLC generated chromatograms from the standards of myristic acid, D_{27}-myristic acid, α-hydroxymyristic acid (α-OH) and β-hydroxymyristic acid (β-OH). The hydroxylated standards (i.e. product standards) are well resolved from the non hydroxylated standards. The α-OH myristic acid elutes approximately 30 s after the β-OH standard under the conditions discussed in Materials and Methods. The D_{27}-myristic acid standard is also resolvable from the non-labeled standard as it elutes approximately 45 s before the unlabeled standard. The area under the curve of each peak is calculated by the HPLC software and used to determine the concentration of substrates and products remaining in the acid quenched samples that will be discussed below.

**Figure A-5:** [Fatty Acid] and [H_2O_2] remaining-versus-[H_2O_2] plot of an HPLC based activity assay containing 1 μM P450_{hsp} and 100 μM Myristic acid initiated with varying [H_2O_2] and allowed to react for 5 minutes at 22 °C before quenching with acid.
Figure A-5 displays the results of an activity assay containing 1 μM P450<sub>hsβ</sub> and 100 μM Myristic (tetradecanoic) acid initiated with varying [H<sub>2</sub>O<sub>2</sub>] and reacted for 5 minutes before quenching with acid. Substrate remaining minimizes and products formed maximize when 100 μM H<sub>2</sub>O<sub>2</sub> (stoichiometric to substrate) initiates the reaction. H<sub>2</sub>O<sub>2</sub> in excess over substrate concentrations produces less than the maximum yield of product. These observations suggest a large excess of H<sub>2</sub>O<sub>2</sub> over enzyme causes at least partial inactivation of the enzyme. To minimize this, no more than 20 fold excess H<sub>2</sub>O<sub>2</sub> to enzyme concentrations was used in subsequent experiments.

![Graph showing fatty acid vs. H<sub>2</sub>O<sub>2</sub>]  

**Figure A-6:** [Fatty Acid]-versus-[H<sub>2</sub>O<sub>2</sub>] added plot from the results of an HPLC based activity assay containing 100 μM P450<sub>hsβ</sub> and 500 μM myristic acid initiated with varying [H<sub>2</sub>O<sub>2</sub>], reacted for 5 minutes at 22 °C.

With complete H<sub>2</sub>O<sub>2</sub> consumption, the coupling of H<sub>2</sub>O<sub>2</sub> and myristic acid consumption to product formation can be investigated. Fitting the initial linear decay phase for substrate consumption in Figure A-6 with a straight line (red line) results in a slope of -0.86. This indicates that for every 100 μM H<sub>2</sub>O<sub>2</sub> consumed, 86 μM myristic acid is consumed for a coupling factor of
86%. The turnover reaction involving naturally occurring (not modified) myristic acid is therefore highly coupled. As previously mentioned, a positive indicator for being able to observe a reactive intermediate is if a modified substrate also produces a highly coupled reaction. An assay identical to that shown in Figure A-6 can be seen in Figure A-7 with the only variation being that the enzyme was provided per-deuterated substrate (D$_{27}$-myristic acid).

![Figure A-7](image)

**Figure A-7:** [Fatty Acid]-versus- [H$_2$O$_2$] added plot of an HPLC based activity assay containing 100 μM P450$_{2BS}$ and 500 μM D$_{27}$-myristic acid initiated with various [H$_2$O$_2$] and reacted at 22 °C for 5 minutes.

Analyzing the turnover reaction that utilizes D$_{27}$-myristic acid instead of H$_{27}$-myristic acid in Figure A-7 produces a coupling ratio of 58%. This suggests the reaction with per-deuterated substrate is less coupled than that of protonated substrate. These findings were also repeated via mass spectrometry based assays not shown.

An attempt was made to develop a combined gas chromatography-mass spectrometry assay but the detection abilities were not as sensitive as hoped. Further refinement of this assay may provide more confirmation of these results however the trends observed from the liquid
chromatography and mass spectrometry have been observed on numerous occasions, particularly the larger uncoupling that occurs with per-deuterated substrate over protonated substrate.

**Search for Compound I of P450$_{\text{BS\beta}}$**

As mentioned above, the initial goal of this study was to attempt to observe and characterize a Compound I species using P450$_{\text{BS\beta}}$ and its unique peroxygenase properties. Since stopped-flow spectroscopy has proved invaluable in the observation of transient species, studies utilizing this instrument were the primary focus of the following experiments.

Initial studies of the turnover reaction yielded spectra seen in Figure A-8. Unfortunately all studies of this enzyme have yielded similar looking spectra in which very small changes are observed. Wavelengths of interest in these experiments are 390 nm, 418 nm, 430 nm, 500 nm, and 690 nm. The set of spectra in Figure A-8 was collected using a 2 mm light path length in the spectrophotometer to try to maximize the amplitude of any observable changes while minimizing experimental noise. As a result, absorbance in the spectrophotometer maximized in the linear range, less than 1 at the Soret band maximum 418 nm, but Figure A-8 was generated by multiplying the absorbance features by 5 to produce a spectrum normalized to what would have been observed if the 10 mm light path was utilized. Due to the minor spectral changes that occur with time, any further spectra shown here will be presented as difference spectra. All difference spectra are generated by subtracting a very early timepoint spectrum from subsequent spectra to highlight any changes that occur in the UV-Visible spectrum.
Figure A-8: UV-Visible spectra collected with a stopped-flow spectrophotometer to show the small amplitude of change that occurs over 30 s of the P450$_{BSβ}$ hydroxylation reaction. The reaction contained 150 μM enzyme and 1 mM myristic acid, and was initiated with the addition of H$_2$O$_2$.

Reactivity of P450$_{BSβ}$ with Varying [H$_2$O$_2$]

As previously discussed, H$_2$O$_2$ grants an advantage over dissolved O$_2$ since it can be administered in much higher concentrations. It is wise to investigate the reactivity of the enzyme when exposed to varying amounts of H$_2$O$_2$. This can be seen in Figure A-9 for reactions involving 30 μM P450$_{BSβ}$ and 100 μM myristic acid and varying amounts of H$_2$O$_2$ as indicated in the figure.
Figure A-9: UV-Visible difference spectra generated from the 22 °C P450<sub>BSβ</sub> reactions containing 30 μM P450<sub>BSβ</sub> and 100 μM myristic acid initiated with varying amounts of H<sub>2</sub>O<sub>2</sub> as indicated in each panel.

The changes that occur with time upon initiating the turnover reaction with varying amounts of H<sub>2</sub>O<sub>2</sub> are seen above. There are several trends that appear through the panels. These spectra are generated by subtraction of the spectrum recorded at 10 ms so it is not surprising that the spectra do not return to a flat baseline at longer reaction times since there is likely some reaction occurring already at the 10 ms time point. The 10 ms spectrum is used instead of a much later time point spectrum because it is likely that some of the later spectra are perturbed by photobleaching of the reaction mixture by the intense light of the spectrophotometer. An
example of the perturbation of absorbance values due to light intensity in provided in Figure A-10 and will be discussed in greater detail below.

In Figure A-7 in the 7.5 μM H₂O₂ (the limiting reagent) panel, from 20 ms to 1 s the absorbance at 380 nm decreases while the absorbance at 418 nm increases. This is a typical indicator of consumption of substrate as the enzyme shifts from a five coordinate to a six coordinate system and back again. (19) This trend also occurs in the presence of 30 μM H₂O₂ (stoichiometric to [enzyme]) except that the feature maximizes for 10 s instead of 1 s before decaying. The reaction initiated with 100 μM H₂O₂ starts with increased absorbance at 380 nm and decreased absorbance at 418 nm that decays and increases respectively until 10 s and then returns to nearly the same position as the 20 ms initial spectrum. Similar changes occur with the reaction initiated with 500 μM H₂O₂ however the loss of absorbance at 380 nm as well as at 418 nm starting at 30 s suggests that in addition to changes generated by photobleaching of the sample, the H₂O₂ concentration may be detrimental to the enzyme and cause its inactivation as was seen in Figure A-4 where not all H₂O₂ was consumed in reaction mixtures where [H₂O₂] was in large excess over [enzyme].
Figure A-10: $A_{419\ nm}$-versus-time traces showing variations in data collected with the stopped-flow spectrophotometer’s two collection modes—photodiode array (red circles) and photomultiplier tube (blue squares).

Figure A-10 shows the loss of absorbance (photobleaching) that occurs when conducting experiments using the photodiode array (PDA) detector as opposed to the photomultiplier tube (PMT) detector of the stopped-flow spectrophotometer. While the PDA provides data on all wavelengths simultaneously, it uses an essentially unfiltered and very intense light source that is known to cause photobleaching/decay. This appears to be happening to some extent here as the overall absorbance is decreased after 100 ms. As a result, the transient feature observed at 418 nm does not build up to as large an extent and decays continuously for as long as exposed to the light source in a PDA experiment. However, no kinetics are significantly impacted as the feature still maximizes between 1 and 2 s. The lack of noise in the PMT data is also evident in the orange trace of Figure A-10 and is another advantage of PMT data collection. The reason PMT detection was not used for more experiments is that only one wavelength may be observed at a time, so in the spectroscopically rich spectra produced by the P450s, this would require a very large volume of reagents to monitor each changing wavelength individually.
The activity assays of Figures A-4 to A-7 have shown that turnover is occurring when enzyme, substrate and H$_2$O$_2$ are present. Figure A-9 indicates that some different reactivity may occur with increasing [H$_2$O$_2$]. These assays also indicate that the reaction uncouples more when per-deuterated substrate is added, which is a small “red flag” in the ability to be able to observe a transient intermediate.

Substrate Triggering

Another hallmark for being able to potentially observe a transient intermediate is whether the reaction is substrate triggered or not. The results of the triggering experiments are shown in Figures A-11 to A-13. To more easily observe changes with time, these experiments monitor absorbance changes with time of two or three wavelengths (390 nm, 418 nm and 431 nm) to generate the kinetic traces in these figures. These two wavelengths exhibit the largest absorbance changes in the reaction. Any other wavelengths that exhibit absorbance changes with time vary with the same kinetics as 390 nm and will not be shown for simplicity.
Figure A-11: Absorbance (391 nm)-versus-time traces from SF experiments comparing with and without substrate reactions of P450<sub>BSβ</sub>. The reactions contained 30 μM P450<sub>BSβ</sub> and 200 μM myristic/ D<sub>27</sub>-myristic acid (when present) and 7.5 μM H<sub>2</sub>O<sub>2</sub>.

As seen in Figure A-11, the P450<sub>BSβ</sub> turnover reaction is indeed a triggered reaction. Again, triggering is indicated when the reaction proceeds more quickly in the presence of all (co)substrates as opposed to when one of the substrates is absent. When no H<sub>2</sub>O<sub>2</sub> is present (red and purple traces), no reaction occurs regardless of whether a fatty acid substrate is present or not. When both H<sub>2</sub>O<sub>2</sub> and a fatty acid substrate are present (blue and green traces), the absorbance at 391 nm changes with a formation rate of approximately 5.5 s<sup>-1</sup> and a decay rate of approximately 0.1 s<sup>-1</sup> as reported in Table A-1. Formation in this case is the initial loss of absorbance followed by the decay phase that presents as a gain of absorbance at 391 nm. When H<sub>2</sub>O<sub>2</sub> is present but no fatty acid substrate is available (orange trace), the absorbance at 391 nm is perturbed but at a rate approximately 100 times more slowly than when fatty acid is present confirming the substrate triggers this enzyme.
Table A-1: Formation and decay rates obtained from fitting traces in Figure A-11.

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<td>Myristic</td>
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<tr>
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<td>0.11±0.002</td>
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Varying Fatty Acid Concentration

Figure A-12: Absorbance-versus-time traces from three wavelengths monitoring the P450$_{BS\beta}$ reaction containing 35 μM P450$_{BS\beta}$ and 25 μM H$_2$O$_2$ with varying amounts of myristic acid.
Figure A-12 is further confirmation of the triggered reaction. In this figure, individual kinetic traces from different conditions were arbitrarily adjusted along the y-axis to better visualize trends that develop with increasing concentrations of myristic acid. Again, the kinetics of the reaction at 390 nm shifts from a slow reaction to a faster reaction in the presence of increasing concentrations of myristic acid (red to violet). Comparison of the 391 nm blue and purple traces containing 100 μM myristic acid and 200 μM myristic acid shows they are nearly identical and therefore the substrate has saturated the enzyme under these conditions. The 418 nm traces mirror the 391 nm traces for the reason indicated previously although photochemistry may be suppressing the lower myristic acid concentration feature that should occur around 100 s. The 433 nm kinetic traces exhibit a loss of absorbance from the very early time points until approximately 1 s in the presence of substrate. It is believed that the initial absorbance is not a baseline but is actually due to formation of an absorbing species in the dead time of the instrument and subsequent decay of this species is what is observed. This is believed to be a transient species and more support for this will be presented below. Also notable is the fact that any absorbance change at all occurs when no fatty acid is present. This again may indicate a different reactivity of some sort is occurring with the enzyme. At this time, however, it is not clear what changes to the enzyme may be occurring when no fatty acid is present to generate UV-Visible changes.

**Varying H₂O₂ Concentration**

Since triggering has been repeatedly established and substrate saturation conditions can be obtained, investigation of the reactions varying the concentration of H₂O₂ has been carried out. Figure A-13 exhibits the changes in kinetic traces when varying the amount of H₂O₂ are used to initiate the turnover reaction. The reactions in Figure A-13 all contain 30 μM P450_{Bsa} and 100
μM myristic acid with the reaction initiated with the concentration of H₂O₂ listed in the figure legend.

Figure A-13: A₃₉₀nm-versus-time kinetic traces from reactions containing 30 μM P450ₐₜ and 100 μM myristic acid initiated with varying [H₂O₂] indicated in each legend.

Figure A-13 shows what appears to be different regimes or reactivity of the P₄₅₀ₚₜ enzyme by following the kinetic traces of reactions at 390 nm. At the lowest [H₂O₂] (panel A, orange trace) the reaction appears to proceed via a single turnover pathway based on the simple decay and rise in absorbance in the trace. Concentrations of H₂O₂ up to 30 μM (stoichiometric with [enzyme]) initially were believed to show multiple turnover, but on further consideration it was realized this is more likely a new reactivity. Multiple turnover would present as either a
plateau as in the 15 μM H₂O₂ trace or as a shoulder with a smaller change in amplitude from the baseline, not greater as appears in the 30 μM H₂O₂ trace.

Something more interesting may occur in the lower left panel. These concentrations of H₂O₂ appear to show the suppression of the initial faster regime and a transition into a slower reactivity. The reactions initiated with even higher concentrations of H₂O₂ presented in the lower right panel initially suggest that the enzyme reacts more quickly with more H₂O₂, however these changes are more likely bleaching of the heme absorbance and possibly destruction of the heme and/or enzyme at the highest [H₂O₂] as seen in the activity assays above.

Possible Reactive Intermediate Observation in the P450<sub>BSβ</sub> Reaction

Subsequent experiments have been designed to maximize the amount of the faster reactivity while preventing the possibility of multiple turnover by using limiting H₂O₂. This is because it is more likely that a highly reactive intermediate like Compound I would form faster rather than slower. Figure A-14 shows the difference spectra generated on initiation of the turnover reaction with limiting H₂O₂.
Figure A-14: UV-Visible difference spectra generated by hydroxylations reactions with conditions identified in each panel. These spectra are used to illustrate the conditions under which the species that absorbs at 430 nm is present.

The series of spectra in panel A of Figure A-14 is generated using limiting H$_2$O$_2$ similar to the reaction of Figure A-9 in the upper left panel. Figure A-9 shows evidence of a small shoulder at approximately 440 nm. Panels A, C and D of Figure A-14 confirm this shoulder is a real and reproducible feature of this reaction. In fact this feature appears as an actual peak that maximizes at approximately 30 ms and decays to generate absorbance at 418 nm as the 390 nm absorbance decays. Panel B contains a reaction with excess H$_2$O$_2$ (similar to the reactions in the bottom panels of Figure A-13) and shows that under this regime the 418 nm feature has already formed in the first 30 ms of the reaction and continues to grow until it maximizes at about 1 s.
Again, it appears this excess H$_2$O$_2$ has suppressed the formation of the faster transient feature at 435 nm.

The only difference between the reactions in panels C and D of Figure A-14 is that panel C uses unmodified myristic acid as a substrate and panel D uses per-deuterated substrate. If the species at 440 nm is involved in C-H/D cleavage the use of the per-deuterated substrate likely would cause this species to either form to a larger extent or persist for a longer amount of time. It appears the peak at 440 nm in panel D may persist slightly longer than the peak in panel C. However, these difference spectra are not simple peak or trough spectra but complex multifeatured traces, making it necessary to perform a 3 point dropline analysis to accurately assess whether a species has different kinetics between different reactions. This analysis of several wavelengths was carried out to determine the true kinetics of several features and is seen in Figure A-15.
Figure A-15: 3 point dropline analysis generated absorbance-versus-time spectra of 380 nm, 418 nm and 435 nm wavelengths from reactions containing 150 μM P450BSβ, 37.5 μM H₂O₂ and 1 mM myristic (red) or D₂₇-myristic acid (blue).

Figure A-15 shows that the 3 point dropline analyzed 418 nm traces are essentially identical between the myristic (red) and D₂₇-myristic acid (blue) traces. It appears that in the 380 nm traces more of the species generated via D₂₇-myristic is present early in the reaction however this wavelength is not an indicator for any previously characterized Compound I species, so it is likely not relevant here. The 435 nm analysis shows there is some difference between the myristic and D₂₇-myristic acid traces, however these occur on a timescale much too slow to indicate variation in the early transient species. This also suggests there is no kinetic isotope effect on the transient species presenting as the peak at 435 nm. The lack of a kinetic isotope
effect is another “strike” against being able to build up a transient species for further characterization.

The reactions observed in Figures A-9 and A-14 were carried out at room temperature (22 ± 3 °C). The 3 point dropline analysis indicates the transient species associated with the 435 nm feature is maximizing between 10 to 30 ms. It is possible to obtain quenched samples in this time scale, however it would be advantageous to attempt to slow the reaction further to capture the rise phase and potentially a greater percentage of the transient species to aid in its characterization. In order attempt to slow the reaction, subsequent experiments were carried out at 5 °C, the results of which can be seen in Figure A-16.

![Figure A-16](image)

**Figure A-16:** UV-Visible difference spectra generated from 5 °C reactions containing 150 μM P450\textsubscript{BSII} and 1 mM myristic (left) or D\textsubscript{27}-myristic acid (right) initiated with 37.5 μM H\textsubscript{2}O\textsubscript{2}. These spectra were used to aid in the determination for whether or not the peak at 435 nm forms and decays with different kinetic rates than the 23 °C reactions.

Figure A-16 shows that the results of the turnover reaction run at 5 °C are very similar to the reaction run at 23 °C. Three point dropline analyses of the individual wavelengths of the reactions of Figure A-16 produce results that are essentially invariant from those in Figure A-15. Again, by 20 ms the 435 nm species has maximized and does not persist considerably longer at 5
°C than 23 °C. Also, there is still no kinetic isotope effect on the decay of this 435 nm species either.

While it is impossible to definitively identify a species based solely on a UV-Visible feature or spectrum, it is possible to make conjectures of the potential identity of a transient species. Figure A-17 contains a set of features typical of the heme containing transient intermediates Compound I and Compound II. Chloroperoxidase has become a model enzyme for how the transient species of a number of members of the P450 family will manifest in the UV-Visible spectrum since it is also a thiolate-ligated heme containing enzyme.

![Figure A-17: Absorbance-versus-wavelength spectra showing the typical UV-Visible spectral features of chloroperoxidase resting enzyme (CPO), Compound I (I) and Compound II (II) species. From (20).](image)

Figure A-17 shows that typical resting state chloroperoxidase enzyme has a large Soret band peak around 400 nm. This is similar for all heme containing proteins as the Soret peak lies anywhere from 400 to 430 nm with P450_{HSP} containing a peak at 418 nm. Observation of Compound I across multiple heme containing families has shown a decrease in absorbance (possibly due to a smaller molar absorptivity) at the Soret maximum as well as a slight shift of the
maximum to lower wavelengths. Most interesting for this document is the typical spectrum of a Compound II species. This species presents with increased absorbance in the form of a peak around 440 nm for chloroperoxidase. This peak is shifted to larger wavelengths than that of the Soret feature and is accompanied by decreases in absorbance at the Soret as well. Based on these trends of UV-Visible changes it appears that the transient feature presenting with a peak at 435 nm may be a Compound II species. In addition to the increased absorbance at 435 nm, this feature also includes a decrease in absorbance from 390 nm through 418 nm relative to the even earlier spectrum used for generation of the difference spectra, agreeing with the changes seen in Figure A-17.

Figure A-18 provides further evidence that the transient species in the stopped-flow reactions is not Compound I and contains the UV-Visible spectrum of Compound I of chloroperoxidase and CYP119 Compound I. (13)
Figure A-18: UV-Visible spectra from chloroperoxidase (A) and CYP119 (B) showing the spectra of Compound I (red) and ferric enzyme (Black) with combinations of the two in dotted and dashed spectra. From (13).

Figure A-18 shows that on generation of a Compound I species the absorbance at approximately 360 nm should increase while the Soret band decreases. Also typical of many Compound I species is the generation of a peak between 650 and 700 nm as is seen in the red spectra of panels A and B in Figure A-18. No features between 600 and 700 nm are generated in the experiments of Figures A-14 and A-16 again suggesting this transient feature is not Compound I.

The structure of Compound I has been identified as a formally Fe(V)=O species that has been observed spectroscopically as an Fe(IV)=O species coupled with a porphyrin π-cation radical (Figure A-1 species 8). Compound II is the one electron reduced form of Compound I and
has been characterized as either an Fe(IV)=O or Fe(IV)=OH, neither of which contains the porphyrin π-cation radical. (21) Compound II is most likely generated from the hydrogen abstraction from the substrate and is an intermediate between Compound I and the resting ferric enzyme as seen in Figure A-19.

Figure A-19: Scheme for the conversion of P450 Compound I to ferric enzyme via hydrogen abstraction from C-H (substrate) to generate Compound II. Modified from (22).

The original published data for P450BSβ suggested it may be a good candidate for characterizing the first P450 Compound I species. Again, the data published by Matsunaga et al. indicated this is a unique peroxygenase enzyme capable of efficiently utilizing H₂O₂ in its reaction mechanism to hydroxylate the α-OH and β-OH positions of fatty acids. (14) Subsequent studies even suggested the presence of a moderate kinetic isotope effect on the hydroxylation of myristic acid. (16) However the uncoupling of the reaction observed here in conjunction with the lack of an observable kinetic (deuterium) isotope effect seems to be enough to prevent buildup of the only transient species observed.

In order to better characterize the species associated with the 435 nm kinetic changes, samples for EPR spectroscopy were prepared. Typically EPR spectroscopy is less time and material intensive than Mössbauer spectroscopy so it is often performed first when paramagnetic species are expected to be present. Figure A-20 shows the results of two freeze-quench EPR
experiments, one with excess H$_2$O$_2$ and no substrate and one with limiting H$_2$O$_2$ and excess D$_{27}$-myristic acid substrate.

**Figure A-20:** Freeze-quench EPR spectra from samples containing the reactions of either excess H$_2$O$_2$ and no substrate (A) or limiting H$_2$O$_2$ and excess substrate (B). The samples in A were generated by reacting 500 μM P450$_{BSβ}$ with 8.5 mM H$_2$O$_2$ at 22 °C for the length of time indicated in the figure before freezing. The samples in B were generated by reacting 150 μM P450$_{BSβ}$ and 1 mM D$_{27}$-myristic acid with 37.5 μM H$_2$O$_2$ at 5 °C for the length of time indicated in the figure. The samples were run at 10-K, 20 μW power, 10 G modulation amplitude with a 9.51 GHz microwave frequency.

The EPR experiment in Figure A-20 A was performed early in research when it was first observed that more H$_2$O$_2$ led to more dramatic changes in the UV-Visible spectra of the stopped-flow spectrophotometer. It is likely that the large excess of H$_2$O$_2$ in panel A inactivated the enzyme as several features (indicated with vertical black lines) in the no H$_2$O$_2$ starting material go away within 400 ms and do not return. There appears to be a radical feature that maximizes at 10s, possibly a buildup of reactive oxygen species or a radical in the active site.
Panel B in Figure A-20 contains the EPR spectra of an experiment performed under similar conditions to those that generated the Compound II like species in Figure A-16. Interestingly, features with the same field parameters as those that disappear and never return in panel A (2550 G, 3025 G, 3680 G) form and decay in panel B. In the 20 and 80 ms samples nearly 50% of the starting material intensity disappears and is not accounted for in new features. This indicates there may be an EPR silent species generated at this point, which would agree with the hypothesis of a Compound II like species that would be an Fe(IV). The features at 2720 G, 3000 G, and 3600 G likely are a low spin ferric heme center with substrate bound as these features decay and reform through the course of the H\textsubscript{2}O\textsubscript{2} limiting, excess substrate reaction. A new feature at ~3050 G forms from 0.02 to 5 s and a radical like feature also maximizes at 3375 G at 80 ms.

**P450\textsc{bsβ} Alternate Reactivity**

Despite not observing Compound I, several other avenues of study for this enzyme were investigated to further characterize the reactivity of the enzyme. Uncoupling measurements revealed that H\textsubscript{2}O\textsubscript{2} was being consumed in greater amounts than substrate. The main focus of this section is on the alternate reactivity of P450\textsc{bsβ} and what reaction is occurring when the enzyme consumes all available H\textsubscript{2}O\textsubscript{2} but does not produce a corresponding amount of hydroxylated product (i.e. when the enzyme uncouples). Effluent from stopped-flow reactions containing large amounts of H\textsubscript{2}O\textsubscript{2} contained bubbles, which suggested that gas was being evolved from the reaction. To test this, an O\textsubscript{2} sensor (details available in the Materials and Methods appendix) was used to determine if O\textsubscript{2} was the gas evolving from the system, the results of which can be seen in Figure A-21.
Figure A-21: \( \text{O}_2 \) evolution-versus-time plots from \( \text{O}_2 \) sensor based experiments to determine if \( \text{P}450_{\text{BSβ}} \) can liberate \( \text{O}_2 \). The left panel contains reactions of 50 \( \mu \text{M} \) \( \text{P}450_{\text{BSβ}} \) and 500 \( \mu \text{M} \) myristic acid with 500 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) (Blue) or 12.5 \( \mu \text{M} \) catalase with 500 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) (Red). The right panel contains reactions involving 200 \( \mu \text{M} \) \( \text{P}450_{\text{BSβ}} \), 1 mM myristic acid and 75 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) (Blue); 200 \( \mu \text{M} \) \( \text{P}450_{\text{BSβ}} \), and 75 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) (Green); or 50 \( \mu \text{M} \) catalase and 75 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) (Red). \( \text{H}_2\text{O}_2 \) was added at 33 ± 2 s.

In Figure A-21, catalase is used as a control for oxygen liberation since its reaction has been well documented to consume two equivalents of \( \text{H}_2\text{O}_2 \) for every \( \text{O}_2 \) liberated. This provides a maximum for the amount of \( \text{O}_2 \) that can be generated in any reaction. Catalase at 1/4 the concentration of \( \text{P}450_{\text{BSβ}} \) is used since catalase is a tetramer with each subunit containing a heme center for reaction. Therefore 1/4 the amount of catalase provides the same number of heme centers able to react with \( \text{H}_2\text{O}_2 \). The left panel shows that in the \( \text{P}450_{\text{BSβ}} \) reactions there is \( \text{O}_2 \) generated in the absence of substrate (green). Also, there is more \( \text{O}_2 \) generated when no substrate is present than in the presence of substrate. Here the reaction containing protonated fatty acid evolves ~30% of the oxygen generated by the catalase reaction. This corresponds to the HPLC and MS data that suggests under similar conditions the enzyme is ~ 80% coupled (Figure A-6). In the right panel, ~15 \( \mu \text{M} \) \( \text{O}_2 \) is generated from 75 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) again indicating ~20% uncoupling. This also agrees with the activity data discussed previously. In addition, in data shown below in Figure A-25, larger quantities of \( \text{O}_2 \) are evolved from reactions containing \( \text{D}_{27}\)-myristic acid than
reactions containing H$_{27}$-myristic acid. These experiments suggest P450$_{BSβ}$ can also carry out a catalase like reaction to generate O$_2$ from H$_2$O$_2$ in addition to hydroxylating fatty acid substrates.

**Alternative Fatty Acid Substrates**

Myristic acid or D$_{27}$-myristic acid turnover reactions using H$_2$O$_2$ have not generated significant amounts of transient species. To ensure myristic acid is the optimal length fatty acid for turnover, a stopped-flow experiment comparing an assortment of saturated fatty acids with P450$_{BSβ}$ and limiting H$_2$O$_2$. The 390 nm absorbance versus time trace has been established as a reliable indicator of substrate triggering so is used here to determine if any other lengths of fatty acids cause comparable or more hydroxylating activity. The results of this experiment can be seen in Figure A-22.

![Figure A-22: Absorbance (391 nm)-versus-time traces from reactions containing 35 μM P450$_{BSβ}$ and 200 μM of fatty acid substrate with the reaction initiated by 25 μM H$_2$O$_2$. The legend indicates the length of the unsaturated fatty acid chain present for each reaction.](image)

The legend in Figure A-22 indicates the number of carbons in the unsaturated fatty acid chain (e.g. C6 stands for hexanoic acid). For this experiment triggering efficiency is judged
relative to the C14 (myristic acid, dark blue) trace with a completely untriggered reaction judged relative to the no substrate reaction (red). The only fatty acid in this experiment that provides any sort of triggering is lauric acid (C12, cyan), however it does not generate the same magnitude of change as myristic acid so likely is not as efficient. It is possible the variation in the longer chain fatty acid traces (C15, C16, C18) is due to decreased solubility of the fatty acid. Also, the later time points generate the feature typical of the untriggered reaction indicating these molecules do not react like myristic acid and may be too long to fit (well) into the active site of the enzyme. Similarly, anything shorter than 12 carbons also generates features typical of the untriggered reaction. Tridecanoic acid has also been tested as well as per-deuterated tridecanoic acid in the event it performs similarly to myristic acid. Both substrates actually produce traces similar to lauric acid and do not appear to trigger the enzyme as much as myristic acid (data not shown).

The turnover reaction of P450_{HSP} when provided H$_2$O$_2$ and myristic acid generates two products: $\alpha$-OH and $\beta$-OH myristic acid. It is possible that the enzyme may hydroxylate each position through a different mechanism, so providing the enzyme with $\alpha$-OH myristic acid would require the enzyme to pass through a single mechanism to maximize accumulation of transient species. This theory is investigated below by providing mixtures of hydroxylated and myristic acid for reaction with H$_2$O$_2$ and the results are reported in Figure A-23.
Figure A-23: Absorbance-versus-time traces monitoring 373, 418 and 433 nm wavelengths from 5 °C reactions containing 35 μM P450<sub>BSβ</sub> and 150 μM of each substrate with the reaction initiated with 25 μM H<sub>2</sub>O<sub>2</sub>.

Again, the untriggered reaction (orange) in Figure A-23 has been observed and repeated over multiple experiments as has the triggered turnover reaction (yellow). These are the controls for comparison to other kinetic traces in this experiment in which various mixtures of hydroxylated fatty acids and myristic acid.

Unfortunately none of the product as substrate reactions produced UV-Visible changes similar to myristic acid (green, cyan, blue traces). Only when myristic acid was added with products was the trace similar (purple) to the triggered reaction (yellow). The decay phase of the purple trace is perturbed, possibly due to binding and dissociation of unreactive products before
the enzyme finds an unmodified myristic acid molecule. When only hydroxylated fatty acids are provided as substrates, the traces most closely resemble the untriggered reaction. Interestingly, when just α-OH myristic acid is present the magnitude of change is greater than when β-OH is included. At this time though, it is unclear what this may be due to.

Difference spectra of these reactions can be seen in Figure A-24 and present several trends. The difference spectra in figure A-24 panel A show that minimal spectroscopic changes occur when no substrate is present for the reaction and the changes that do occur are on a slow timescale. The control myristic acid difference spectra show a rapid shift from 380 nm to 415 nm that maximizes at 1 s and then decays again. As with the kinetic traces, none of the other difference spectra resemble the myristic acid control. The β-OH only and the α-OH + β-OH myristic acid spectra show similar kinetics and difference spectra (panels D and E). With these spectra, the feature at approximately 390 nm decays for about 50 s and then increases for the rest of the measured time. The α-OH difference spectra (panel C) somewhat resemble the α-OH + β-OH + myristic acid spectra (panel F). This likely only indicates the early spectra used for subtractions were similar as the changes in α-OH occur on a much slower timescale. Mass spectroscopy assays (not shown) do not show any evidence of multiply hydroxylated substrates so the enzyme likely is able to recognize a single hydroxylation and will not doubly hydroxylate a fatty acid.
Figure A-24: UV-Visible difference spectra from the experiment in Figure A-23. The combination of substrates is indicated in each panel. The 5 °C reactions contained 35 μM P450_{hsβ} and 150 μM of each substrate initiated with 25 μM H₂O₂.
The evolution of O$_2$ from the hydroxylated fatty acid as substrate reactions should yield more insight into what reactivity is occurring in the reactions and can be seen in Figure A-25.

**Figure A-25:** O$_2$ evolution-versus-time plot from the O$_2$ sensor based experiment to monitor O$_2$ evolution when hydroxylated myristic acids are provided to P450$_{BSeta}$ as a substrate. Reactions include 210 μM P450$_{BSeta}$ and 1 mM fatty acid initiated with 150 μM H$_2$O$_2$ (or 52.5 μM Catalase and 150 μM H$_2$O$_2$). H$_2$O$_2$ was added at 33 ± 2 s.

The O$_2$ evolution by P450$_{BSeta}$ when provided with various “products” as “substrates” (Figure A-25) yields some interesting results. As observed previously, the no substrate control (red) produces significant amounts of O$_2$ but only produces approximately 75% of the O$_2$ of the catalase control. Also as seen before, more O$_2$ is generated when D$_{27}$-myristic acid is provided, again indicating more uncoupling of the reaction when compared to the non-labeled substrate. Here, 43% more O$_2$ is liberated when the enzyme is presented with α-OH myristic acid (20 μM) as when β-OH myristic acid is present (14 μM). This may indicate it is more difficult to
hydroxylate the β position and the enzyme instead utilizes its catalase reactivity. However the amounts of O₂ liberated are more similar to the established turnover reaction (myristic) levels (14 μM) than the substrate free levels (48 μM). This is somewhat contrary to the stopped-flow assays that indicate a more uncoupled reaction occurs with limiting H₂O₂ presented with hydroxylated fatty acid so one would expect O₂ liberation on levels more similar to the no substrate reaction. Since this avenue of study did not appear to encourage transient intermediate accumulation no mass spectrometry based assays have been carried out to try and identify if any novel products have developed but this may be a good experiment for future investigation. These results would likely reveal if P450hape can actually doubly hydroxylate a fatty acid or if another reaction altogether occurs.

**m-Chloroperbenzoic Acid**

Since P450hape is able to uncouple its hydroxylation reaction to carry out catalase type reactivity, another strategy to try and accumulate more transient species is to try to suppress the catalase activity. m-Chloroperbenzoic acid (mCPBA) has been utilized as an alternative O atom donor and may reduce or slow the catalase activity since it has a bulky benzyl ring attached to one oxygen. This is also the O atom donor that was successfully employed by J. Rittle to detect the P450 Compound I species of CYP119. (12) To determine any optimal ratio of P450hape to mCPBA that may exist, a stopped-flow titration of increasing amounts of mCPBA to enzyme was carried out, the results of which can be seen in Figure A-26.
Figure A-26: Absorbance-versus-time traces from reactions containing 35 μM P450_{BSβ} and 150 μM myristic acid initiated with either 25 μM H₂O₂ or varying [mCPBA] identified in the legend.

CYP119 Compound I was successfully observed using only 2 equivalents of mCPBA per enzyme (see Figure A-18). Figures A-26 and A-27 show that the reaction of P450_{BSβ}, myristic acid with mCPBA does not accumulate more transient intermediate than H₂O₂ does. The kinetic traces of 391 nm and 418 nm indicate that as the amount of mCPBA increases the changes occurring in the Soret region continuously enlarge, which is likely a bleaching of the Soret band. Figure A-27 shows there is a peak around 485 nm that seems to decrease with increasing mCPBA. This peak occurs at such long times however it is likely irrelevant to the formation of a transient Compound I or Compound II like species. Amounts of mCPBA greater than seven equivalents lead to formation of a peak from 0.1 to 1 s at 485 nm. Since similar features appear in the 690 nm kinetic trace it is likely a systematic shift in all spectra of the reaction that only is noticeable in the kinetic traces that have small amplitudes of change.
Figure A-27 A shows that some of the changes in these reactions are more visible than those with small amounts of $H_2O_2$ but difference spectra still provide the best method of viewing the changes in the reacting spectra. As mentioned above, Figure A-27 shows the 390 nm and 418 nm wavelengths exhibit changes proportional to the amount of mCPBA in the solution, specifically loss of absorbance through these wavelengths. Since this is essentially just a decaying feature and there is no concomitant absorbance rise anywhere, this is likely bleaching of the heme chromophore. Again it is unclear what the feature at 485 nm is due to, however it forms at such a slow rate it is highly unlikely it is of any interest as a transient species.
Figure A-27: Absorbance-versus-wavelength spectra (A) and difference spectra (B-F) from reactions containing 35 μM P450<sub>BSβ</sub> and 150 μM myristic acid initiated with varying [mCPBA] to determine if mCPBA can generate any larger amounts of the transient species that absorbs at 430 nm.
In order to determine if any O$_2$ can be evolved by P450$_{BSβ}$ reacted with mCPBA, an oxygen sensor experiment was performed, the results of which can be seen in Figure A-28.

![Graph showing O$_2$ generation versus time](image)

**Figure A-28:** O$_2$ generation-versus-time plot of O$_2$ sensor based experiments monitoring O$_2$ generation from reactions containing 210 μM P450$_{BSβ}$ and 1 mM substrate (when present) initiated with 150 μM H$_2$O$_2$ or 1.5 mM mCPBA. Catalase reactions included 52.5 μM catalase and 150 μM H$_2$O$_2$ or 1.5 mM mCPBA. H$_2$O$_2$ or mCPBA were added at 33 ± 2 s.

Figure A-28 contains the results of experiments performed to see if more (or less) O$_2$ is evolved when P450$_{BSβ}$ is provided with the O atom donor mCPBA instead of H$_2$O$_2$. Previous consistent trends have been discussed above as relevant to the H$_2$O$_2$ traces and continue to hold true. Again D$_{27}$-myristic acid generates more O$_2$ than unmodified myristic acid. All reactions
except the red “myristic H$_2$O$_2$ Tris” trace were carried out with phosphate buffer since mCPBA is able to react with the primary amine present in the buffer structure. 1.5 mM mCPBA was used for these experiments since excess mCPBA in previous experiments exhibited greater effects on the UV-Visible absorption and less mCPBA did not appear to produce any evidence of a transient feature.

Catalase (black trace) produces approximately 3 times more O$_2$ with 150 μM H$_2$O$_2$ than with 1.5 mM mCPBA or 1 O$_2$ for every 2 H$_2$O$_2$ molecules as opposed to 1 O$_2$ for every 25 mCPBA molecules. Catalase should have access to 1.5 mM O-OH bonds to cleave which should in turn generate 750 μM O$_2$ from mCPBA. Since it generates so much less O$_2$ with 10x more O-OH bonds this indicates the mCPBA molecule is likely too large of a substrate and very few molecules are cleaved to generate O$_2$.

For P450$_{BSβ}$, the stopped-flow data above indicates that mCPBA either is not a good co-substrate or just does not provide moderate UV-Visible changes in quantities that should be relevant to generating interesting intermediates (based on what was observed with the CYP119). Moderate quantities of mCPBA did generate some UV-Visible changes which is why 1.5 mM mCPBA is used here to determine if the changes may coincide with O$_2$ generation. As seen in Figure A-28, P450$_{BSβ}$ can utilize mCPBA to a small extent. As with H$_2$O$_2$, mCPBA results in more O$_2$ evolution when P450$_{BSβ}$ is provided with perdeuterated substrate than with protonated substrate. It seems unlikely based on the lack of UV-Visible perturbation, but it is possible that the lack of O$_2$ generation means the substrate is more strictly hydroxylating substrate. This could be confirmed or denied by mass spectrometry based assays but again, since this is not contributing to transient intermediate accumulation further assays have not been done.
P450<sub>BSβ</sub> As An Oxygenase

Matsunaga et al tested a variety of reductase domains to determine if P450<sub>BSβ</sub> will interact with any of them; however this was not the case. One may wonder whether one could reduce the heme center artificially and therefore allow the iron to react with O<sub>2</sub> as in a traditional P450 oxygenase reaction. This was investigated here as one potential hypothesis for the catalase activity is that perhaps the enzyme generates its own O<sub>2</sub> to be used in a subsequent reaction. One artificial reductant that is sufficient to reduce the majority of metal centers in proteins is that of dithionite (sodium hydrosulfite). This reductant readily reacts with O<sub>2</sub> so the first experiment to determine if P450<sub>BSβ</sub> can behave as an oxygenase must be a titration to determine the proper amount of dithionite to fully reduce the iron center without having large quantities of dithionite available to compete for O<sub>2</sub> consumption. Figure A-29 contains the results of the titration of P450<sub>BSβ</sub> by dithionite.

![Figure A-29](image)

**Figure A-29:** Absorbance-versus-[dithionite] plot from the titration of 17.5 μM P450<sub>BSβ</sub> with dithionite (500 μM stock). Red circles are 320 nm data and green squares are 390 nm data.
The 320 nm and 390 nm wavelengths were chosen to determine the optimal amount of dithionite to reduce P450<sub>BSβ</sub> for the following reasons: 320 nm is where unreaceted dithionite absorbs, so significant increase in absorbance at this wavelength indicates excess dithionite. Based on the difference spectra, it appears that 390 nm (as well as 460 nm and several other wavelengths) absorbance increases to a certain point and then remains static. This indicates the maximum change due to dithionite reduction has occurred. As seen in Figure A-29, the 320 nm data exhibits absorbances that remain essentially static until 20 μM dithionite is added to the sample containing 17.5 μM P450<sub>BSβ</sub>. Similarly, the 390 nm absorbance increases through the addition of about 15 μM dithionite and then plateaus. This indicates that at approximately stoichiometric amounts of dithionite to P450<sub>BSβ</sub> the enzyme is fully reduced.

Dithionite is actually a two electron reductant so in theory only half the concentration of dithionite to enzyme should be necessary for full reduction. There are two likely explanations for this discrepancy. Despite efforts to make the protein solution 100% anoxic, there is always a small amount of contaminating oxygen and the fact that the difference spectra remain fairly static through the addition of 5 μM dithionite is likely due to the amount of contaminating oxygen in the solution. The second likely reason for discrepancy in the amount of dithionite necessary for enzyme reduction is that the powder stock of dithionite is only about 75% active. Taken together, these explanations account for the requirement of the extra dithionite. To ensure complete reduction of the heme center for subsequent stopped-flow experiments a slight excess of dithionite over [P450<sub>BSβ</sub>] was employed.

The stopped-flow experiments to determine if P450<sub>BSβ</sub> may behave as an oxygenase are fairly straightforward to set up. P450<sub>BSβ</sub> reduced with a slight excess of dithionite is mixed with an oxygenated buffer solution and the absorbance across the UV-Visible spectrum is monitored. Even though the slight excess of dithionite will react with O<sub>2</sub>, it is of a sufficiently small quantity so as to not significantly affect the amount of O<sub>2</sub> dissolved in the buffer. The experiment in
Figure A-30 was carried out with multiple dissolved O$_2$ concentrations, however all resulting spectra and kinetics are very similar so only the highest concentration of dissolved O$_2$ spectra are reported here.

**Figure A-30:** Absorbance change-versus-wavelength difference spectra from the reaction containing 35 μM P450$_{BSβ}$, 150 μM myristic acid, 40 μM dithionite and 260 μM O$_2$ to determine if P450$_{BSβ}$ can act as an oxygenase.

The spectra resulting from the reaction of reduced P450$_{BSβ}$ with dissolved O$_2$ is seen above in Figure A-30. Very minimal spectral changes occur, and the predominant changes occur at very slow times indicating there is no fast (hydroxylation) reaction occurring and the late changes again are likely photochemistry. In the absence of substrate (Figure A-31), there are some changes occurring on a faster timescale than that of the with substrate reaction.
Figure A-31: Absorbance change-versus-wavelength difference spectra from the reaction containing 35 μM P450<sub>BSβ</sub>, no myristic acid, 40 μM dithionite and 260 μM O<sub>2</sub> to determine if P450<sub>BSβ</sub> can act as an oxygenase enzyme.

In Figure A-31 the 435 nm feature returns in the early spectra and decays within 10 s to form a peak at 420 nm. Surprisingly, this is very similar to what has been observed in the presence of substrate and limiting H<sub>2</sub>O<sub>2</sub> seen in Figure A-32 (from panel C in Figure A-14). The decay of this feature occurs much more quickly with H<sub>2</sub>O<sub>2</sub> and substrate than with no substrate and O<sub>2</sub>. 
Figure A-32: Absorbance change-versus-wavelength difference spectra from the 20 °C P450_{BSβ} reaction containing 150 μM P450_{BSβ}, 1 mM myristic acid and initiated with 37.5 μM H_{2}O_{2}.

It is unclear whether the similarities are anything more than a coincidence of the subtraction process or if O_{2} can react with the enzyme active site when no fatty acid is present to generate these spectra. Regardless, some sort of change is occurring in the substrate free reaction that does not occur when substrate is present.

The 435 nm feature in the no substrate reaction above is the only potential alternative intermediate that forms and on the same timescale as the catalase like reaction. It is possible this may be involved in the alternate reactivity of P450_{BSβ} that occurs in the absence of substrate.

Conclusions

Several aspects of the P450_{BSβ} reaction have proven detrimental to the capture and characterization of a Compound I like species. Assays monitoring the consumption of H_{2}O_{2} and myristic acid along with the production of hydroxylated fatty acid indicate that the hydroxylation reaction uncouples more with larger amounts of H_{2}O_{2} and when D_{27}-myristic acid is used in place of H_{27}-myristic acid. Assays to detect the generation of O_{2} in solution have shown that some
oxygen is liberated even under the most coupled conditions with limiting H$_2$O$_2$ when myristic acid substrate is present. Large quantities of O$_2$ are generated when no substrate is present and reactions containing D$_{27}$-myristic acid produce more O$_2$ than reactions with unmodified myristic acid. The uncoupling of the hydroxylation reaction, presumably to consume H$_2$O$_2$ in a catalase type reaction, prevents accumulation of Compound I, but may allow observation of another species at very early times with limiting H$_2$O$_2$ as seen from stopped-flow based experiments. The reproducible species with an absorbance peak at 435 nm produced in stopped-flow experiments may be Compound II. EPR data suggests that by 20 ms into the reaction of P450$_{hsb}$ with D$_{27}$-myristic acid and limiting H$_2$O$_2$, an EPR silent species has formed and then rapidly converts to a low spin ferric species.

Even though the enzyme appears to readily uncouple, it is still substrate triggered, therefore becoming much more reactive (a rate increase of approximately 100 fold) to H$_2$O$_2$ in the presence of substrate. Further stopped-flow studies have indicated that there may be two reaction phases depending on the amount of H$_2$O$_2$ presented to the enzyme. At very low (sub stoichiometric) amounts of H$_2$O$_2$ to enzyme, a fast phase of the reaction generates absorbance versus time kinetic traces that appear to be single turnover reactions. H$_2$O$_2$ concentrations similar to enzyme concentration generate a slower reaction phase that appears to suppress the faster phase as H$_2$O$_2$ concentration continues to increase. Any further increase of H$_2$O$_2$ beyond approximately 10 equivalents of H$_2$O$_2$ to enzyme appear to cause the destruction of the heme chromophore.

Despite a reported kinetic isotope effect on $V_{\text{max}}$ of the hydroxylation reaction, no evidence of a kinetic isotope effect has been observed throughout these experiments. (16) This is likely due in large part to the uncoupling of the reaction. The apparent lack of a kinetic (deuterium) isotope effect and uncoupling of the hydroxylation mechanism are the likely reasons for not being able to build up a ferryl intermediate in the reaction of P450$_{hsb}$ with H$_2$O$_2$. 
Further efforts to re-couple or optimize the reaction have not proven effective. Myristic acid appears to be the optimal length fatty acid for the hydroxylation reaction. Matsunaga reported that providing selectively deuterated myristic acid (on the α position) generated a significant deuterium isotope effect on the formation of the α-OH myristic acid product while not perturbing the rate of formation for the β-OH product. (16) Providing the enzyme with singly hydroxylated product does not appear to generate any multiply hydroxylated products however. This may suggest the enzyme active site generates an electrostatic repulsion to hydroxylated fatty acid that prevents multiple hydroxylations or simply cannot accommodate the extra bulk of another hydroxyl group addition to the substrate.

Efforts to “re-couple” the mechanism by suppressing the catalase reactivity have shown that less O₂ is evolved from reactions with m-chloroperbenzoic acid as compared to equivalent amounts of H₂O₂. It seems likely, however, that mCPBA is simply too bulky to act as an efficient O atom donor for P450₉₉β as no stopped-flow data from these experiments resembles data from confirmed hydroxylation reactions.

Finally, P450₉₉β does not appear to react as an oxygenase. Artificially reducing the enzyme in the presence of substrate and then mixing with oxygenated buffer simply generates spectra that appear to show photochemistry of the heme with the strong light source. Adding more reducing equivalents would likely only result in further reaction of the dithionite with O₂ rather than more reduction of the heme center. The reason for changes in the UV-Visible spectrum upon addition of O₂ to substrate free P450₉₉β is still unclear at this time but may be interesting to investigate in the future if it is indeed a Compound II species being generated.
References


3. Antonini, E. (1965) Interrelationship between structure and function in hemoglobin and myoglobin, *Physiological Reviews* 45, 123-&.


for catalytic chloride binding-site on Compound-I *Journal of Biological Chemistry* 264, 15284-15292.


Appendix B

Materials and Methods

B-1: Bleomycin

Preparation of BLM samples for CR

A 10 mM stock of bleomycin sulfate (Blenoxane, a gift of Bristol Laboratories) was generated by adding 550 μL distilled deionized water to the vial containing lyophilized solid. An anaerobic $^{57}$Fe(II) stock was generated by dissolving $^{57}$Fe(0) in 2 N H$_2$SO$_4$ in an anaerobic glove box to a final concentration of 518 mM. An $^{57}$Fe(III) stock was generated by dissolving $^{57}$Fe metal in 3 M HCl in air to a final concentration of 575 mM. All control samples contained 2.0 mM bleomycin (BLM), 1.5 mM $^{57}$Fe, 20 mM sodium HEPES (pH 7.8), and 50 % ethylene glycol.

The anaerobic HS-Fe(II)•BLM control sample was made in an anaerobic glove box with the $^{57}$Fe(II) solution that was neutralized in HEPES buffer before adding to the BLM solution. The solution was placed in an EPR tube or Mössbauer sample holder, sealed and quickly frozen outside of the glove box in liquid nitrogen. All other control samples were made with the $^{57}$Fe(III) stock. The HS-Fe(III)•BLM control was made by adding 200 mM sodium phosphate buffer (pH 7.8) to a final concentration of 20 mM in the sample.

Two sets of ABLM samples were generated for this study, one containing 1.5 mM LS-Fe(III)•BLM and one containing 3.5 mM LS-Fe(III)•BLM. Both sets of samples of ABLM were generated in a 5 °C cold room by adding H$_2$O$_2$ to 60 mM. The 1.5 mM samples were frozen in liquid nitrogen within 60 s of H$_2$O$_2$ addition and the 3.5 mM samples were frozen 45 s after addition of H$_2$O$_2$. 
Cryoreduction (CR) of BLM samples

Frozen samples for CR were irradiated in the γ-irradiation facility of the Breazeale nuclear reactor at the Pennsylvania State University using a $^{60}$Co-source. A total dose of 4 Mrad was delivered to samples maintained at 77-K in liquid nitrogen.

Annealing of CR BLM samples

Samples of CR ABLM were annealed by submerging the frozen sample for 20 minutes in a 2-methylbutane (isopentane) bath maintained at 200-K by addition of liquid nitrogen.

Mössbauer spectroscopy

The spectrometers have been described previously. (1) Specific conditions are given in the appropriate figure legend. Simulation of Mössbauer spectra was carried out using WMOSS (WEB Research, Edina, MN).

EPR spectroscopy

EPR spectra were recorded on an ESP300 spectrometer from Bruker (Billerica, MA) equipped with an ER 041 MR Microwave Bridge and a 4102ST X-band Resonator from Bruker. The temperature was maintained at 10 K by immersion of the sample in a cryostat cooled by a continuous flow of liquid helium. (1) Samples were run at 20 μW power, 10 G modulation amplitude with a 9.51 GHz microwave frequency.
B-2: TauD

Stopped Flow

All SF reactions were carried out on an Applied Photophysics SX20 Stopped-Flow instrument equipped with a Xenon lamp and pph Spectrakinetic monochromator. In addition, the actual sample mixing portion of the apparatus was housed in an anoxic chamber and connected to either a photodiode array detector or photomultiplier tube detector depending on the experiment. Any relevant conditions are discussed in the text.

Anaerobic protein solutions were generated by placing a desired amount of apo-protein into a vacuum flask and gently evacuating and backfilling the headspace of the sealed flask with argon ~40 times in an hour on a vacuum line. Anaerobic buffer solutions were generated in a similar fashion although typically the flask(s) would be evacuated for ~20 minutes and then back filled with argon for ~5 minutes with this cycle repeated ~10 times over 2 or 3 hours. Oxygenated solutions were generated by pulling hard vacuum on a flask in an ice bath for about 5 minutes then back filling with pure oxygen for 5 minutes and repeating ~ 10 times. The flask would then be allowed to sit under oxygen for ~30 minutes to encourage full saturation of the solution and equilibration with the headspace.

D101A Purification

TauD D101A cell pellet was purified in the same manner as WT TauD. (J) Modifications of the procedure in reference 1 include the following: 2 passes through the pressure cell of a microfluidizer instead of a French press; an AKTA purification system controlled the passage of
soluble protein solution through the DEAE-Sepharose FF column; pooled fractions were concentrated in YM-30 Centriprep (Millipore) concentrators.

**P4H D154A Plasmid Generation**

Previous work has identified D154 of the prolyl-4-hydroxylase (P4H) from *Paramecium bursaria Chlorella virus-1* (PBCV-1) to be the cognate residue of D101 in TauD. (2) This residue is the carboxylate member of the 2 histidine, 1 carboxylate facial triad that binds iron to the protein. PCR primers

5’-CTATTATCATCATTACGCCTGGAGATGACTGCACG-3’ and

5’-CGTCGCAGTCATCTCCAGCGTAATGATGATAATAG-3’ were synthesized by Integrated DNA Technologies and were used to generate a D to A point mutation in the P4H PBCV-1 WT gene previously cloned into a pET-15b vector from Novagen. The underlined portions are the site of the desired mutation. The WT vector is ampicillin resistant and produces a protein with a His tag for easy purification. Successful PCR reactions contained 50 ng WT template, 125 ng of each primer, 250 μM each (1mM total) dNTPs, and 1x reaction buffer in a 50 μL solution. 1 μL PfuUltra HF AD polymerase (Agilent) initiated the PCR reaction which was carried out with 1 cycle of 95 °C for 30 s followed by 16 cycles of 95 °C for 30 s, 55 °C for 60 s and 68 °C for 7 minutes. PCR product was cooled to 37 °C and 1 μL DPN1 was added and incubated at 37 °C for 1 hour to digest methylated WT DNA.

The resulting PCR product was transformed into supercompetent cells and plated on 100 μg/mL ampicillin containing LB-agar plates. Resulting colonies were amplified via a miniprep (Qiagen) protocol. Samples were sent to the Pennsylvania State University Genomics Core Facility for sequencing to confirm the presence of the D to A sequence mutation.
Plasmid containing D154A P4H PBCV-1 was transformed into BL21 cells for expression of protein. Cells were grown in 20 L of Luria-Bertani broth supplemented with 100 μg/mL ampicillin at 37 °C to OD<sub>600 nm</sub> of 0.8 with constant agitation in a 30 L fermentor. The temperature of the reaction was then reduced to 15 °C and overexpression was induced by the addition of 0.8 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were harvested by centrifugation 4 hours after induction and were subsequently frozen in liquid nitrogen before storing at -80 °C.

**P4H D154A Purification**

P4H purification was carried out at 5 °C. The frozen cell pellet was resuspended in 2x the volume of the cell pellet of lysis buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris pH 7.9) and the cells were lysed by passage through a microfluidizer. The lysate was passed through the pressure cell at least two times to ensure thorough lysing. The lysate was centrifuged at 38,000 xg for 30 minutes and the soluble fraction was applied to a Ni-NTA affinity column (Qiagen). Extraneous protein was removed by washing with at least 5 column volumes of P4H wash buffer (60 mM imidazole, 0.5 M NaCl, and 20 mM Tris, pH 7.9). Bound P4H D154A was eluted by washing with several column volumes of P4H elution buffer (500 mM imidazole, 0.5 M NaCl, 20 mM Tris, pH 7.9). Fractions were analyzed by SDS-PAGE to determine which fractions contained protein. These fractions were pooled and dialyzed with 0.5 M NaCl and 20 mM Tris, pH 7.9, 10% glycerol buffer to remove imidazole before storing at -80 °C for use in assays.
**B-3: P450<sub>bsβ</sub>**

*Bacillus subtilis* genetic material was a kind gift from Dr. Paul Babitzke, Professor of Biochemistry and Molecular Biology, Pennsylvania State University.

**Cloning P450<sub>bsβ</sub> into pET28a**

The P450<sub>bsβ</sub> gene was amplified via PCR using the forward primer 5′-GCG GCG TCC ATA TGA ATG AGC AGA TTC CAC ATG ACA AAA GTC TCG-3′ and reverse primer 5′-GCG GCG TCC CAA GCT TTT AAC TTT TTC GTC TGA TTC CGC-3′ on the *Bacillus subtilis* genome. Successful PCR conditions utilized the protocol for Vent DNA polymerase that suggests 1 cycle of 5 minutes at 95 °C and 35 cycles of 1 minute at 95 °C, 1 minute at 70 °C, and 3.5 minutes at 72 °C followed by one 10 minute cycle at 72 °C. Each 50 μL reaction tube contained 1X Thermal polymerase buffer, 1 mM of each forward and reverse primer, 1 to 2 ng/μL of *Bacillus subtilis* genomic DNA, 300 μM dNTPs, and 1 μL of stock Vent DNA polymerase.

To determine the success or failure of the PCR procedure a 1% (w/v) agarose gel was made and samples were run at 90 V for 1 hour. Visualization showed clear amplification of a 1.4 kb fragment which is the expected size of the P450<sub>bsβ</sub> gene. The successfully amplified products were then extracted from the gel using a well established Qiagen brand gel extraction kit and protocol.

The PCR amplified product was then restriction digested with NdeI and HinDIII restriction enzymes according to their recommended protocols. A parallel sample of pET28a vector from Novagen was also digested with NdeI and HinDIII at the same time. These samples were again run on a 1% agarose gel and bands believed to belong to the digested products were gel extracted as above.
Ligation reactions were done with either a 3:1 or a 1:3 ratio of pET28a:P450 using 50 ng of whichever species is the limiting reagent. Also included in the 15 μL reaction mixture were 1 μL of T4 ligase and 1.5 μL of commercial ligation buffer. The reaction mixtures were then allowed to sit at room temperature for 2 hours before being transformed into GC5α competent cells.

Transformation of either 1 or 5 μL of ligation product into 50 μL GC5α was accomplished by incubating the ligation product with competent cells on ice for 30 minutes and then heat shocking at 42 °C for 45 seconds. At this point 250 μL SOC broth was added and gently mixed via pipetting. This mixture was then incubated for 1 hour at 37 °C with shaking at ~200 rpm and the entire reaction mixture was plated onto 50 μg/mL kanamycin LB plates. The plates were then allowed to incubate at 37 °C overnight for approximately 16 hours.

All ligation reaction mixtures generated colonies and several colonies were chosen to undergo established MINI and MIDI procedures using the Qiagen protocols. Samples were sent to the Penn State University Nucleic Acid Facility for sequencing and determined to be the desired P450_{BSβ} gene inserted into the pET28a vector that provides a hexa-histidine tag for purification simplification.

For expression and purification purposes, P450_{BSβ} was then transformed into BL21 competent cells following the same protocol as transforming into GC5α competent cells.

**Expression of P450_{BSβ}**

Cells were grown in M9 minimal media. One 100 mL starter culture and six flasks containing 1 L media each were prepared. The starter culture and flasks containing water and M9 salts, were autoclaved. Once ready for inoculation, 2 mM MgSO₄, 100 μM CaCl₂, 30 μg/mL kanamycin, and 0.4% dextrose were added. All non-autoclaved solutions were filter-sterilized.
A P450_{BSβ} colony from a fresh transformation was inoculated into the 100 mL starter culture and allowed to incubate at 37 °C with shaking for approximately 18 hours. This starter culture was then equally distributed into the 6 1 L M9 minimal media. The cells were grown at 37 °C with shaking until reaching a optical density (OD)_{600nm} of 0.8. The flasks were removed from the shaker and placed in an ice bath to cool. At this point 500 µM of heme precursor, δ-aminolevulinic acid, and 1mL of a trace elements solution was added to each 1L media. The trace elements solution is generated by making a 1 L solution containing 10% HCl, 1 g of ZnCl$_2$-4H$_2$O, 0.2 g CoCl$_2$-6H$_2$O, 1 g of Na$_2$MoO$_4$-2H$_2$O, 0.5 g CaCl$_2$-2H$_2$O, 1 g CuCl$_2$ and 0.2 g H$_3$BO$_3$. Once the cells and the shaker were cooled to about 16 °C, 500 µM IPTG was added to induce expression of P450_{BSβ}.

The cells were harvested by centrifugation at 6000 rpm for 20 minutes in JLA 8.1000 rotor in Beckman centrifuge. Upon completion, the supernatant was discarded, and the cell pellet was frozen in liquid nitrogen and stored at -80 °C in preparation for purification.

**Purification of P450_{BSβ}**

The cell pellet was thawed with stirring in 2 times the volume of Lysis buffer (50 mM Tris pH 7.5, 300 mM NaCl, 10 mM imidazole) and 10 µL/mL of 10 mM PMSF. The cells were lysed by two passes through a french pressure cell at 10000 psi. The lysate was then centrifuged at 50,000 xg for 45 minutes. The supernatant was reserved and loaded onto a 50 mL bed volume Ni-NTA column equilibrated with 2-3 bed volumes of Lysis Buffer. Once the supernatant was loaded, the column was washed with several bed volumes of Wash buffer (50 mM Tris pH 7.5, 300 mM NaCl, 30 mM imidazole). The protein was eluted with Elution buffer (50 mM Tris pH 7.5, 300 mM NaCl, 200 mM imidazole). Fractions containing red color were collected and analyzed by UV-Visible spectrophotometry to determine which fractions had the best heme to
protein ratio (421 nm/280 nm). Any fractions with 421/280 ratio greater than 0.6 were pooled for dialysis to remove the imidazole. Dialysis was carried out over night in 2 L of dialysis buffer (50 mM Tris pH 7.5, 300 mM NaCl) with two subsequent buffer exchanges. On completion of dialysis, 10-20% glycerol was added to the protein for cryoprotection on freezing. In a YM-30 centrifprep concentrator, centrifuged at 1500 xg, the protein was concentrated to 2-3 mM. Once sufficiently concentrated, the protein was aliquoted into 1.5 mL microcentrifuge tubes and stored at -80 °C.

**Activity Assays**

Assays for activity were carried out in two ways, one involving HPLC/MS (high performance liquid chromatography/ mass spectrometry) and the other involving a colorimetric assay and horseradish peroxidase. MS samples were prepared in a total volume of 600 µL with 200 mM NaCl, 200 mM Tris pH 7.5. Protein, substrate and H₂O₂ concentrations varied with experiments, but in general, excess substrate over protein was used. H₂O₂ was always added to tubes last and after 5 minutes the reaction was quenched by adding 6 N HCl to pH ~1. At this point, an aliquot of the reaction mixture was frozen and stored at -80 °C in preparation for a colorimetric horseradish peroxidase assay. To the remaining reaction mixture, 100 µM D₂₇-myristic acid purchased from CDN Isotopes was added as an internal standard. The fatty acids were then extracted three times with 0.5 mL of ethyl acetate and then washed with 1 mL of ddH₂O. Half of the extracted mixture was added to a new set of tubes for 9-anthryldiazomethane (ADAM) derivatization and the other half of the mixture was added to a set of tubes for mass spectrometry analysis. When possible, the ethyl acetate was evaporated from both sets in a Savant SC110 Speedvac with Refrigerated Vapor Trap RVT4104. If not dried in the Speedvac, samples were allowed to slowly evaporate in a fume hood. 50 µL of 1 mg/1 mL ADAM in ethyl acetate
was added to each tube in one set and allowed to sit over night in the dark. The set of ADAM derivatized samples was re-dissolved in 50 µL mobile phase and the set of non-derivatized samples was reconstituted in 20 µL mobile phase. A Waters Micromass ZQ LCMS, with a 1525µ Binary HPLC Pump and a 2996 Photodiode Array Detector was used for these experiments. For HPLC analysis, a Waters Free Fatty Acid HPLC column (Part no WAT011690) with a mobile phase consisting of 45% acetonitrile : 20% tetrahydrofuran : 35% ddH₂O : 0.025% triethylamine and flow rate of 0.8 ml/min was used. The mobile phase for MS analysis contained 45% acetonitrile : 55% ddH₂O : 0.05% triethylamine and was run at 0.05 mL/min as a direct injection through the probe with the following settings: Source temperature = 80 °C, Desolvation Temperature = 150 °C, Capillary voltage = 2.0 kV, Cone = 25 V, Extractor = 5 V, RF lens = 3.0 V.

The horseradish peroxidase colorimetric assay quantifies the amount of H₂O₂ in a solution. Frozen samples for horseradish peroxidase measurements were prepared by adding the thawed aliquots to a solution containing 4 U/mL horseradish peroxidase, 5 mM 4-aminoantipyrine, and 10 mM vanillic acid in a 0.2 M NaCl, 0.2 M MOPS (pH 7.5) buffer. The solutions were mixed to a final volume of at least 500 µL and to produce an absorbance between 0.1 and 0.9 when measured at 498 nm in a UV-Visible spectrophotometer.

**Stopped Flow of P450<sub>BSβ</sub>**

All stopped flow spectrophotometer based experiments were carried out on the same instrument described above for TauD. Any anoxic solutions necessary were generated and handled in the same manner as above.
FQ EPR

The apparatus and procedure for generating FQ samples have been described elsewhere. (4) In one experiment, a solution of 1 mM P450BSβ (in 50 mM Tris, pH 7.6, 300 mM NaCl, and 10% glycerol) and a solution of 17 mM H₂O₂ (in 50 mM Tris, pH 7.6, 300 mM NaCl and 10% glycerol) were loaded into syringes for mixing in a 1:1 ratio at 22 °C. In a second experiment, a solution of 300 μM P450BSβ and 2 mM D27-myristic acid (in 50 mM Tris, pH 7.5, 300 mM NaCl, and 10% glycerol) and a solution of 75 μM H₂O₂ were loaded into separate syringes for mixing in a 1:1 ratio at 5 °C. In both experiments, the reacting solution was passed through an aging hose of appropriate length to produce the desired reaction time before spraying into a -150 °C 2-methylbutate solution for quenching. Quenched samples were run on the Bruker EPR described above with 20 μW power, 10 G modulation amplitude and a 9.51 GHz microwave frequency.

O₂ Sensor

A fiber optic oxygen sensing FOXY probe connected to a single channel multifrequency phase fluorometer (MFPF) from Ocean Optics was used to measure the partial pressure of O₂ in solution. The probe is a chemical sensor that receives blue LED excitation light from the fluorometer on the thin-film coating of its tip. O₂ in solution diffuses into the coating and quenches the fluorescence. The loss of fluorescence is relayed back through the bifurcated optical fiber assembly to be measured by the fluorometer. Data was recorded with the provided OOISensors and TauTheta software.
Solutions were kept sealed by a rubber septum in small test tubes with minimal headspace to prevent any evolved O\textsubscript{2} from diffusing into the head space. The solutions were also stirred vigorously to prevent any localized depletion of O\textsubscript{2} by the probe.
References


