Heme Iron Catalysis: Contrast to Non-Heme Iron Enzymes

Kednerlin Dornevil
Georgia State University

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ABSTRACT

Metalloenzymes catalyze a wide range of chemical transformations. Their remarkable versatility is imparted to them by their metal centers that are redox active or function as Lewis acids. They utilize transition metals such as iron that has accessibility to a variety of redox states, allowing them to efficiently activate and insert molecular oxygen into a wide range of unactivated organic substrates. The work described in this dissertation is the structural and mechanistic characterization of both heme and non-heme iron-dependent metalloenzymes. Precisely, the focus is placed on three enzymes, a cytochrome P450, CYP121, a tyrosine hydroxylase, LmbB2, and an extradiol dioxygenase, 3-hydroxyanthranilate 3,4-dioxygenase (HAO). The first two enzymes expand the repertoire of activities performed by hemoproteins. CYP121 catalyzes an unusual C-C crosslinking reaction that is distinct from traditional oxygenase chemistry performed by this
family. LmbB2 is one of the first enzymes that mediate aromatic amino acid hydroxylation without using a pterin cofactor. Finally, HAO serves as the prototype for type III extradiol dioxygenase chemistry. A broad spectrum of biochemical and spectroscopic techniques was used to investigate the oxygen activation techniques by both LmbB2 and CYP121 and the role of second sphere ligands in the HAO active site. Specifically, rapid-kinetic methods, EPR spectroscopy, and X-ray crystallography allowed the elucidation of enzyme structure and function properties. In CYP121, using peracetic acid as an oxidant, similarities with canonical P450 enzymes through the use of a catalytic shunt pathway were demonstrated. We also identify the first intermediate in this pathway, an alkyperoxo species with an unusual 5/2 spin signal observed by EPR spectroscopy. In the LmbB2 study, a compound ES-like species was observed by UV-Vis and EPR spectroscopy. This intermediate was shown to be catalytically competent and able to react specifically to the substrate L-tyrosine. Finally, the role of a second sphere Pro97 residue in the active site of HAO was investigated using enzymatic assays. Long-range remote structural influences were observed using protein crystallography techniques between the active site and the conformation of the surface residues.

INDEX WORDS: Oxygenase, Hydroxylase, Heme, Non-heme Iron, P450, Oxygen Activation, Crystallography, Extradiol Dioxygenase,
HEME IRON CATALYSIS: CONTRAST TO NON-HEME IRON ENZYMES

by

KEDNERLIN DORNEVIL

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HEME IRON CATALYSIS: CONTRAST TO NON-HEME IRON ENZYMES

by

KEDNERLIN DORNEVIL

Committee Chair: Aimin Liu

Committee: Donald Hamelberg

Jenny Yang

Electronic Version Approved:

Office of Graduate Studies
College of Arts and Sciences
Georgia State University
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DEDICATION

I dedicate this dissertation to my family. Specifically, to my father, Kedner Dornevil, my mother Nesline Paul Chalumeau and finally my sister, Chrissie Dornevil. Without your motivation and support, I could not have made it this far. With your motivation and support, I can go as far as I want.
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I graduated with my B.S. eight years ago in 2009 with a major in chemistry. At the time, I could not imagine that eight years later I would have still been in school and successfully defended a dissertation to obtain a Ph.D. in biochemistry. These past eight years represent a third of my life and nearly all of my adult years thus far. There have been times when I was excited about what’s to come, and life could not have been better. There have also been times where I had serious doubts and questioned whether this was the right path for me; and times where I could not imagine I would ever reach the end of this journey and bring my dreams to reality. Through all of this, I have been fortunate to have met many individuals, some in passing and others who have become lifelong friends. I have been fortunate that many of these people have taken particular interest in seeing me succeed and have provided the means to do so. Whether it has been some words of wisdom, emotional or financial support, or just an ear to let me vent my frustrations, everyone in their way has helped to keep me upright, make sure I didn't fall and allowed me to reach my destination.

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1 INTRODUCTION

1.1 Metalloenzymes in biology

Metalloproteins make up approximately 50% of the total proteins found in biology (1) and nearly one-third to one-fourth of all proteins require a metal for activity (2). They perform numerous physiologically relevant functions from storage transport (3), signal transduction (4) and redox sensing (5), DNA and RNA repair (6), antibiotic biosynthesis (7) and cofactor biosynthesis (8,9) as well as catalyzing a wide range of chemical transformations. Additionally, metal ions play a significant role in the infectious disease since bacteria often sequester trace elements from the host as nutrients to promote their own cell growth (10). The most common coordinating environments for the metal ions are nitrogen, oxygen, and sulfur (11). These are often provided by key conserved protein residues such as histidine, cysteine, and aspartic/glutamic acid. Exogenous chelators are also used to bind the metal, the most famous and common one being a macrocycle, protoporphyrin IX.

Two major categories of metalloenzymes include the heme and non-heme iron-dependent enzymes (12). These two together catalyze a wide range of chemical transformations (13-15). Some of the chemistries carried out by metalloenzymes include amino acid hydroxylation (16-18), monooxygenation (19), dioxygenation (20-23), hydrolysis (24) and C-C bond formation (11,25) among many others. These two categories of metalloenzymes catalyze a wide range of chemical transformations which in some cases overlap with each other. While the two systems recruit metal ions to activate molecular oxygen, their overall mechanisms diverge from each other owing to the different cofactors available to each. The mechanisms employed by both heme and non-heme iron-dependent enzymes are a direct consequence of the cofactors used by each.
1.2 Contrasting cofactors for heme/non-heme enzymes

The heme cofactor is composed of a tetrapyrrole macrocycle with an iron atom at its core. There are currently seven identified hemes differing amongst each other by their type of substitution and binding mode to the enzyme (26-32). The most common heme group is heme b (protoporphyrin IX) and is found in many proteins including cytochrome P450s, peroxidases, catalases, hemoglobin and myoglobin (Figure 1.1). Additionally, heme a and heme c are also two common hemes that are found in nature. The hemes differ from each other according to the functional groups present at positions C3, C8 and C18. The hemes can either be attached to the protein matrix either by a covalent linkage (heme c) or through coordination of a protein ligand.

Figure 1.1 Chemical structure of common heme cofactors.
The heme cofactor provides a coordinating environment of four nitrogen atoms to the metal ion. An additional protein derived ligand; usually, a cysteine or histidine occupies the fifth position. A sixth coordination site, called the distal site, is often occupied by a water molecule which can be displaced for binding of O₂ or other exogenous ligands.

The mononuclear non-heme iron enzymes are classified broadly into two major categories. The first category activates substrate to allow for direct reaction with O₂ and comprises the intradiol dioxygenases (22). These enzymes contain ferric catalytic centers. The second category is ferrous enzymes that activate O₂ directly (33). The main binding mode for many of the non-heme iron-dependent enzymes is the canonical 2-His-1-carboxylate binding motif, also known as the facial triad (Figure 1.2) (33). Enzymes using this metallocenter motif include, but not limited to, the extradiol catechol dioxygenases, Rieske dioxygenase, pterin-dependent enzymes and α-ketoglutarate-dependent enzymes (33,34). In addition to the protein-derived ligands, an additional one, two, or three water molecules also coordinate the metal ion. The metal coordination for non-heme iron enzymes provides additional exchangeable sites for direct binding of substrate and/or cofactor to the metal center. Other metal binding motifs are also found in nature but are less prevalent, these include the 1-His-2-carboxylate (35), 2-His-2-Tyr (36), 3-His-1-carboxylate (37), 3-His (37) and 4-His (38) binding motifs.

Figure 1.2 Chemical structure of the facial triad.
The non-heme systems contain metal centers possessing open coordination sites that can allow for simultaneous binding of both the organic substrate and oxygen. This allows more flexibility for potential mechanistic routes towards substrate oxidation. Conversely, heme enzymes in general only possess a single open site for binding of the main oxidant and the organic substrate is not able to directly coordinate to the metal center. Thus, these two classes of enzymes diverge in the strategies employed for oxygen activation and substrate oxidation.

1.3 Structure and spectroscopic properties of iron in heme and non-heme iron enzymes

The flexibility of transition metals in biocatalysis is largely due in part to their ability to access multiple redox states and undergo redox cycling during catalysis. Iron has two readily accessible oxidation states used for catalysis in the ferric and ferrous forms (Figure 1.3). Additionally, the iron center is able to facilitate multiple coordinating environments to modify its spin state. The coordination of ligands to the metal allows for the electronic properties of the iron center to be more precisely tuned towards its catalytic activities. The redox potential of iron is also highly modulated depending on the ligand environment and geometry. The range can be from -600 mV for a ferredoxin site that is four-coordinate (4C) to 390 mV and higher for a 6C heme system (39).
Figure 1.3 Electronic configuration of ferric and ferrous oxidation states of iron in an octahedral environment.

Coordination of the iron by the porphyrin forms a square-planar complex geometry for the metal. Coordination of the two axial positions of the metal ligands results in an octahedral geometry. Properties of the metal ion such as size, oxidation state, spin-state and identity of the axial ligand can affect whether it is bound either in plane or out of plane of the porphyrin ring system. Larger metal ions typically adopt an out of plane coordination mode, however, changes in their oxidation state or spin state can affect the binding mode due to changes in the size of the metal ion. Iron typically adopts an out of plane binding mode, however, upon binding of either molecular oxygen or water in the sixth position a spin-shift to low-spin is observed and the geometry adopts an in-plane configuration. While the porphyrin molecule is typically planar, the heme cofactor can be ruffled when bound in the protein. In some situations, extreme ruffling is observed as is the case for the heme-degrading enzymes, IsdG and IsdI (40). The ruffling of the heme provides an additional method to fine tune its redox properties or catalysis (41).
Porphyrrins are highly conjugated systems whose aromaticity gives rise to its intense color \( (\varepsilon_{\text{Soret}} = 10^5 \text{ M}^{-1} \text{ cm}^{-1}) \). Their electronic structure is composed of 22p electrons of which 18p are directly involved in the conjugated system. Porphyrrins are easily oxidized and the resulting cationic radical is a key feature in the catalytic cycle of many intermediates in heme chemistry (42). Because of their conjugated pi system, heme possesses an intense characteristic Soret band and have historically provided a benefit for the spectroscopic characterization. The spectroscopic signature arises from \( \pi \rightarrow \pi^* \) transitions in the heme cofactor. This transition gives rise to the Soret band centered near 400 nm. The type of axial ligand provided by the protein can have significant effects on the absorption spectra of the Soret peak. Reduced heme systems exhibit a ca. 20 nm red shift from \( \sim 400 \) to 420 nm when complexed with CO if the protein ligand is a histidine. Conversely, P450 enzymes contain thiolate ligated heme which has a distinct absorption at 450 nm when a similar Fe\( ^{II} \)-CO complex is formed.

Non-heme enzymes have more options when coordinating the iron ion compared to heme enzymes. Because of the lack of an exogenous cofactor, non-heme enzymes can donate up to 6 potential protein ligands. Typically, the iron center contains 3-4 protein ligands with additional waters to saturate the coordination sites. The ligands donated are traditionally aspartates/glutamates and histidines; cysteine residues are used for iron-sulfur proteins. Multiple ligand combinations are used to tune the electronic properties of the iron center with the 2-His-1-carboxylate motif being the most prevalent. Unlike heme enzymes, the protein ligands are not oxidized during catalysis and thus do not participate directly in the chemistry. The flexibility of these enzymes, however, does allow for coordination of the primary substrate and co-substrate like \( \alpha \)-ketoglutarate (\( \alpha \)KG) to the iron. This allows the iron center to serve as a bridge to mediate the transfer of electrons from the primary substrate to the molecular oxygen substrate.
The non-heme ferrous systems do not possess the same signature transitions in the UV-Vis region, however, they do possess $d \rightarrow d$ transitions that occur in the near-IR region. These transitions have very low intensity in absorption spectroscopy, they do have intense magnetic circular dichroism (MCD) spectra at low temperatures and several spectroscopic methodologies have been derived to assist in the investigation of these enzymes (43-45).

A wide range of spectroscopic techniques has been developed to characterize both heme and non-heme enzymes. Several techniques such as electron paramagnetic resonance (EPR), MCD, resonance Raman, and Mössbauer spectroscopy provide information on the active site environment and spin state. They have been used successfully to characterize many intermediates in the reaction cycle of metalloenzymes. Other commonly applied techniques include resonance EXAFS and XANES have been critical in understanding the coordination of the metallocenters. Structural techniques like X-ray crystallography have been highly informational in characterizing active site geometry, ligand identification, and visualization of key reaction intermediates of metalloenzymes. Finally, computational methods such as QM/MM allow probing of the reaction landscape and to model reaction mechanisms \textit{in silico}. Predictions made from these studies often guide in the design and execution of experiments \textit{in vitro}. Many of these techniques are complementary to each other and offer a synergistic effect on the information obtained in studying metalloenzyme chemistry and have allowed for a remarkable advancement in the understanding of basic principles in the enzymology field.
1.4 Principles of electron paramagnetic resonance (EPR)

Electron paramagnetic resonance is a powerful tool for studying paramagnetic species. Many of the transition metals contain numerous unpaired electrons in their d-orbitals, thus, EPR has been routinely employed to study the electronic structure of metalloproteins. The spin states of unpaired electrons are degenerate, however, the degeneracy is lost when a strong magnetic field is applied to the electrons. This results in two spin states being created, $m_s = \pm \frac{1}{2}$. The two spin states signify electrons aligned either with or against the applied field and correspondingly have two different energy levels. The energy difference between the states is directly proportional to the strength of the applied magnetic field (Figure 1.4).

![Figure 1.4 Energy splitting of unpaired electrons in a magnetic field.](image-url)
An EPR spectrum is obtained by maintaining a fixed microwave frequency and sweeping the magnetic field. At specific magnetic field the energy difference between the two energy states will match the energy of the microwave frequency. When these conditions for EPR resonance are met, the free electrons will orient themselves antiparallel to the applied magnetic field and an absorption spectrum of the microwave energy is recorded by the spectrophotometer. A key principle component obtained by EPR experiments is the unitless g-factor which measures the intrinsic magnetic moment for an electron. In the most straightforward system, a free electron, the g-factor has a value of 2.0023. Unpaired electrons can be differentiated from free radicals due to the interactions with their local environment, the features of the EPR spectra will be dependent on the nature of these interactions.

1.5 Oxygen activation in nature

Molecular oxygen is a very stable molecule with a triplet ground state from two unpaired electrons (Figure 1.5). This makes reactions with organic molecules in the singlet ground state a spin forbidden process (46). However, most oxygenase enzymes are able to catalyze reactions with \( \text{O}_2 \) by using transition metals such as iron, copper or manganese. Transition metals can react with one electron processes allowing for the activation and subsequent insertion of \( \text{O}_2 \) into the organic substrate. Oxygen is activated by converting the triplet ground state to a more reactive species such as a singlet, denoted as \( ^1\Delta_g \) with no unpaired electrons or a doublet with a single unpaired electron. Both heme and non-heme iron-dependent enzymes employ their own distinct mechanisms to activate molecular oxygen for insertion into organic molecules.
There are two possible methods for O$_2$ activation (Figure 1.6). The first method is a spin inversion of one of the radicals to generate a singlet. This is a highly endothermic process making it very difficult and unlikely to occur. The second method proceeds by reductive activation. In this process, an external electron source is required to supply electrons to the oxidant to convert the oxygen to the doublet form. Some sources of electrons are flavin molecules which can interact with oxygen directly or a reductase system that couples the oxidation of NADPH to the reduction of molecular oxygen. Many biological systems have been primarily observed to activate molecular oxygen via the reductive activation method (47).

Figure 1.5 Molecular orbital diagram of triplet oxygen.
1.5.1 \( \text{O}_2 \) activation in heme enzymes

Because the heme cofactor possesses only a single open site for binding of exogenous ligands, there is less mechanistic flexibility in activating \( \text{O}_2 \) and many of the heme enzymes share a very similar paradigm regarding this process. One key difference between heme and non-heme systems is that the binding of \( \text{O}_2 \) to the heme center rarely occurs in the side-on binding mode as observed in the non-heme counterpart (48-50). Heme enzymes can activate either molecular oxygen or hydrogen peroxide for use in oxidation chemistry. The oxidant binds to the open axial position of the heme center. The type of oxidant able to be used is dependent on the oxidation state of the metal center, molecular oxygen is activated by ferrous centers while hydrogen peroxide is activated by ferric centers. After binding of molecular oxygen to generate the peroxoanion species, a supplemental source for an electron and proton are required by the system to generate the next ferric hydroperoxo intermediate. For many heme enzymes, typically the final activated form of oxygen is expressed as a ferryl(oxo) species. The ferryl(oxo) species comes in two forms that differ from each other by one oxidizing equivalent. The first form is named compound II and is an
iron(IV) species, the second, termed compound I, has an additional oxidizing equivalent provided by the porphyrin or a nearby aromatic amino acid in the form of a cation radical. The porphyrin (as well as the cysteine ligand of P450s) is a readily oxidizable cofactor and is able to donate an electron to facilitate heterolytic scission of the O-O bond to generate the terminal oxidant after formation of the ferric hydroperoxo intermediate (51). The ferryl(oxo) intermediate is not the only oxidant of choice for heme enzymes, in heme oxygenase (HO) the species responsible for hydroxylating the heme is the ferric hydroperoxo species (52). This reaction proceeds by an electrophilic attack by the terminal oxygen atom on the \( \alpha \)-meso carbon to generate \( \alpha \)-hydroxyheme.

1.5.2 \( O_2 \) activation in non-heme enzymes

Rieske cis-diol forming dioxygenases

This group of enzymes is responsible for activating aromatic ring systems, functionalizing them by the addition of two oxygen atoms at positions adjacent to each other. Their products are further processed downstream by ring cleaving enzymes. Rieske enzymes require an external supply of electrons during activity to account for the breaking of a single O-O bond and the formation of two new C-O bonds. The Rieske center is a [2Fe-2S] cluster that stores and supplies an electron to the active site iron during catalysis. Regeneration of the center is achieved by coupling the oxidation of NADH to the reduction of the Rieske cluster. The identity of the key oxidizing species in this group of enzymes has not been fully elucidated, though current proposals favor one of two possible intermediate species (47,53). The first species is an Fe(III)-OOH with the peroxide bound in an end-on manner. The second species, an Fe(V)-oxo-hydroxo can be formed by heterolytic cleavage of the O-O peroxy group. While the Fe(V) species has yet to be
directly observed and characterized, several pieces of data (EPR spectroscopy and MS) have supported its presence in the mechanism (54,55). The ferryloxo would be a more potently oxidized form of O₂ compared to extradiol dioxygenases, this is required because the substrate is a very stable and unactivated aromatic ring system. However, both species are likely capable of inserting both atoms of oxygen into the substrate.

**Extradiol Catechol Dioxygenase**

Compared to Rieske dioxygenases, no external reductants are needed and all the electrons required are supplied by the organic (primary) substrate. During the activation process, the organic substrate binds and chelates the Fe(II) in a bidentate monoanionic mode which allows preservation of the neutral iron center compared to the resting enzyme. This process causes a release of the water ligands. The Fe(II) center which is now five coordinated, has an open position for molecular oxygen to enter and bind the iron in an end on manner. Next, a single electron is transferred from the primary substrate to oxygen allowing for both to be activated simultaneously. After activation of both substrates, the two substrate-based radicals combine allowing the reaction to continue through the resulting iron-bound alkylperoxo intermediate.

**Intradiol Dioxygenase**

Intradiol dioxygenase enzymes possess a ferric iron center coordinated by two histidine and two tyrosine ligands. The electrons needed for dioxygen reduction, like extradiol dioxygenases, are all provided by the catechol substrate and thus, no additional electron donor is required. Before activation of oxygen can occur, the primary substrate molecule is required to first bind to the active site and coordinate the iron center. A single electron is transferred from the
substrate to the iron ion generating the ferrous center and an accompanying substrate radical. The reduced metal center is able to bind molecular oxygen in an end-on manner. The ferrous center subsequently reduces the oxygen to superoxide. During the activation process, the iron center serves as a conduit to funnel electrons from the substrate to oxygen. In the doublet form of molecular oxygen can react directly with the substrate radical to form an alkylperoxo intermediate which will collapse and result in the insertion of the first oxygen atom to the substrate. The reaction proceeds with the second oxygen atom being inserted by the resulting ferric-hydroxide species formed after the collapse of the alkylperoxo.

**Alpha-ketoglutarate (αKG) Dependent dioxygenases**

Compared to the previously described Rieske and extradiol catechol dioxygenase enzymes, the αKG-dependent enzymes utilize a much more distinctly different method for the activation of molecular oxygen. The first unique aspect is the usage of a co-substrate αKG which bidentately chelates the Fe(II) center and is positioned adjacent to the O₂ binding site. Both primary organic substrate and αKG are required to properly bind and activate O₂. Binding of O₂ to the iron center in the active site generates a ferric superoxide intermediate which then performs a nucleophilic attack on the oxo acid of the second substrate, αKG. This process results in lengthening the O-O bond, cleavage, and one of the oxygen atom going into αKG to yield CO₂ and succinate as reaction byproducts. The second oxygen remains bound to the Fe ion and becomes a highly oxidatively powerful Fe(IV)=O species capable of transferring an oxygen atom to the primary substrate.
Tetrahydropterin (pterin)-dependent dioxygenases

In contrast to the previously described mechanisms, neither the substrate nor the pterin co-substrate chelates the iron center during oxygen activation for this group of enzymes. Binding of the pterin co-substrate in the active site is followed by the substrate in a sequential and ordered manner. This causes changes in the active site that promotes binding of one oxygen atom. Of the non-heme iron-dependent enzymes, the mechanism of oxygen activation is the least understood in the pterin-dependent subfamily. However, it has been proposed that oxygen must react directly with the pterin co-substrate due to the formation of a 4a-hydroxypterin as a byproduct of the reaction. Currently, two mechanisms have been proposed for oxygen activation. The first mechanism involves a slow and non-enzymatic one electron transfer from the pterin to oxygen, generating a pterin radical and superoxide. The pterin radical can combine with superoxide to generate a 4a-peroxypterin. The 4a-peroxypterin can then react with the active site ferrous ion to generate a Fe(II)-peroxypterin intermediate. In the second pathway, oxygen reacts directly with the ferrous ion to generate a ferric superoxide complex. This species can react with the pterin to generate an Fe(II)-peroxypterin intermediate. In both pathways, the Fe(II)-peroxypterin intermediate can undergo heterolytic cleavage of the O-O bond to directly generate both the 4a-hydroxypterin side-product and the key Fe(IV)=O species (56).

1.6 Catalytic mechanisms of representative metalloenzymes

1.6.1 Catalytic mechanism of cytochrome P450

P450 enzymes are well known for their ability to perform a variety of chemical transformations while working on diverse substrates (13,14,57-59). P450s are also highly relevant in human medicine due to their presence in human bodies (57 distinct P450 enzymes) and ability
to oxidize a broad array of pharmaceutical drugs (60). The P450 mechanism is one of the most studied in enzymology (61-63) (Figure 1.7). Over the past 50 years, a broad mechanistic understanding of P450 chemistry has developed, with this information now at the level of textbook material (64).

Figure 1.7 Consensus catalytic mechanism of CYP450. Both electrons and protons are supplied by NAD(P)H and NAD(P)H reductase protein.
The P450 catalytic cycle contains at least 6 steps and the iron undergoes multiple redox changes. The reaction cycle begins with the primary organic substrate binding to the active site but not coordinated to the heme. However, this binding triggers structural changes in the enzyme that promotes the release of the water ligand of the heme. This changes the spin state of the heme from low-spin \((S = \frac{1}{2})\) to high-spin \((S = 5/2)\) and the redox potential of the heme moiety. At the same time, it also opens up a position on the iron center for oxygen to bind once the iron ion is at the reduced state. The five-coordinate high-spin ferric heme is subsequently reduced by a single e\(^-\) transfer step from NAD(P)H that is mediated by an associated reductase protein (65,66). This changes the redox state of the iron from ferric to ferrous and allows for the binding of molecular oxygen to generate the first oxy ferrous or ferric superoxide complex intermediate (67,68). The proceeding steps consist of a second electron transferred to the iron center followed by a single proton to form a ferric hydroperoxo species (68-70). It is currently not known whether the proton is added to the system simultaneously with the second electron or proceeds it in a synchronous manner going through a ferric peroxo intermediate. While much is known about the oxygen activation steps of the P450 mechanism, there are still remaining points of contention that need to be addressed to fully detail out each of the chemical steps. The mechanism continues with the addition of a second proton to the distal oxygen of the peroxide intermediate which then decomposes by splitting the O-O bond and releasing a water molecule. The O-O bond cleavage results in the formation of the intermediate in the P450 mechanism shown in Figure 1.7, compound I, responsible for the insertion of oxygen into a wide variety of unactivated C-H bonds (71). The compound I intermediate is composed of an iron center oxidized to the ferryl state and has an accompanying cation radical localized on the heme cofactor. Both the ferryl iron and radical are strongly coupled to each other, together this system is equivalent to an Fe(V) in oxidizing potential.
Compound I is a highly oxidizing species and is capable of abstracting the electron and proton directly from a C-H bond. This results in its reduction and becoming a protonated compound II species while the organic substrate being oxidized to a radical speices. A rapid recombination of the OH and the substrate radical, known as “radical rebound”, results in the formation of the hydroxylated product which disassociates from the active site (72). The resting ferric enzyme is regenerated with a new water molecule binding to the ferric center.

The compound I intermediate has been the most elusive species in the P450 mechanism to trap at high yields for spectroscopic characterization. A strategy that has been used to study P450 mechanism has been the “shunt pathway.” The use of H$_2$O$_2$, peroxyacetic acid (PAA), and meta-chloroperbenzoic acid (mCPBA) have helped to facilitate the characterization of ferryl species in P450 and other heme enzymes (73,74). In fact, this final piece of the mechanism was obtained when Rittle and Green demonstrated the ability to obtain high yields of P450 compound I (~70% yield) allowing for the first robust kinetic and spectroscopic characterization of the intermediate (71).

1.6.2 Catalytic cycle of tryptophan 2,3-dioxygenase

Tryptophan 2,3-dioxygenase (TDO) forms the first step in the main metabolic pathway for L-tryptophan metabolism. It is responsible for breaking open the indole ring and inserting two oxygen atoms from molecular oxygen and forming N-formyl-L-kynurenine (NFK). This step commits L-tryptophan to be further metabolized and leads to the production of NAD$^+$. An alternate enzyme known as indoleamine 2,3-dioxygenase (IDO) catalyzes the same reaction as TDO with the main difference that IDO has a broader substrate-specificity than TDO. In contrast to the well-
studied monooxygenase chemistry of the P450 enzymes, the dioxygenase chemistry of TDO/IDO is less established.

The mechanism for oxygen activation and insertion into L-tryptophan is currently in contention with several models being proposed (20,75-83). The first proposal for dioxygenase activity by TDO was an active site base mechanism. This mechanism requires an active site base to deprotonate the indole ring during the formation of a 3-indolenylperoxo intermediate. After formation of the peroxo intermediate one of two branching steps are proposed in which either each oxygen atom is incorporated sequentially followed by a Criegee rearrangement, or added simultaneously in the dioxetane pathway. Previously an active site L-histidine was suspected to play the role of the key base. A second mechanism was proposed that proceeds through a high-valent ferryl species and formation of an epoxide substrate intermediate. This proposal bypasses the need for an active site base, the radical character of a ferric superoxide species formed upon binding of O2 allows for the direct addition of oxygen to the C2 position of the substrate.

Research on TDO has yielded several pieces of data that favor the ferryl/epoxide pathway. The first piece of evidence comes from Geng et al. who were able to clarify the role of the L-histidine and demonstrate that an active site base was not required to observe the TDO dioxygenase activity (84). This was achieved through a combination of site-directed mutagenesis experiments and the use of a substrate analogue. Mutants of L-histidine revealed its role was primarily the stabilization of a high-valent ferrylloxo species. When an N-methyl-L-tryptophan substrate analogue was assayed, a significant amount of activity and formation of the final NFK product was observed. The next piece of data provided by Basran et al, is the detection of a small amount of the proposed epoxide species by MS (85). This data also supports the sequential addition of oxygen into the substrate.
While TDO is active as a ferrous enzyme, work has also been performed on the ferric oxidation state and a unique reactivation mechanism has also been described which enables the enzyme to mitigate oxidative damage and maintain activity \textit{in vivo} (86). Additionally, the biomedical relevance for TDO is of significant interest as a potential target in certain cancers and in the clinical evaluation of patients (87). A general catalytic strategy has emerged for TDO/IDO enzymes that is consistent with the established experimental results (Figure 1.8), however, more data is needed before a mechanism as detailed as that of P450 enzymes can be realized.
Figure 1.8 Working mechanism of TDO dioxygenase activity.
1.6.3 Catalytic cycle of homoprotocatechuate 2,3-dioxygenase

The extradiol dioxygenase enzymes are currently one of the best well-characterized and most studied of the dioxygenases. Many of the key intermediates and mechanistic insights for this family have been obtained from studies performed on HPCD. This has resulted in a very clear and concise proposal for the oxygen activation and insertion steps consistent with the observed biochemical, spectroscopic and kinetic data observed (Figure 1.9). Herein the mechanism for extradiol dioxygenase enzymes will be discussed in the context of HPCD chemistry and the studies supporting the catalytic cycle.

The reaction mechanism begins with a ferrous ion center that is coordinated by two histidine and one glutamate residues as well as 3 water molecules. The kinetics of substrate binding were teased out by using a slower substrate, 4-nitrocatechol (4NC) (88). Binding of the substrate is expected to proceed in three steps (89,90). After initial binding to the active site, ionization of the substrate through deprotonation allows it to coordinate to the iron center and release two water ligands. A final reorganization then occurs to open a site for molecular oxygen to bind in a side on manner (91). In the next step, binding of oxygen to the iron center generates the ternary complex. The iron center serves as a conduit allowing for one electron transfer from the substrate to the oxygen giving both species radical character and generating a superoxide intermediate. The two radicals can combine to generate a peroxy bridge between the Fe and the substrate. A nearby His200 plays the role as an active site base and protonates the iron bound oxygen to promote O-O bond cleavage. Breaking of the O-O bond results in the incorporation of one atom of oxygen into the substrate to form a seven-membered lactone ring intermediate. Finally, hydrolysis of the lactone promotes ring opening and insertion of the second oxygen atom generates the final ring-opened product.
Figure 1.9 Extradiol dioxygenase reaction mechanism of HPCD.

The mechanism for the extradiol dioxygenase reaction is supported by extensive kinetic, spectroscopic and computational data. Many of the intermediates have been successfully trapped and characterized either in crystallo or in solution (91,92). The reaction of the wild-type enzyme is fast making detection of reactive intermediates difficult in the native system. However, several methodologies were used in combination to afford a dramatic reduction in the catalytic turnover and several catalytic steps were slowed down allowing for their isolation and subsequent
characterization. The first method was the use of the substrate analogue 4NC which remained active with the WT enzyme however the activity was only 5% of the native substrate, homoprotocatechuate (88). The second key method was the identification of the key active site base in the catalytic cycle. Mutation of the His200 slowed down the reaction, and in one case helped change HPCD activity from extradiol to intradiol (93,94).

When HPCD was crystallized, the crystals could be soaked with 4NC and exposed to low concentrations of O₂. The crystals were determined to be catalytically competent (91). Experiments on this system allowed for the key substrate-alkylperoxo-Fe(II) species to be trapped and its first structural characterization in HPCD. The ternary ES(O₂) complex and the final ring cleaved product were also trapped in protein crystals. In the solution state, several intermediates have also been characterized. These include the HPCA⁺-Fe(II)-(hydro)peroxo, HPCA⁺-Fe(II)-O₂, Fe(II)-alkylperoxo and lactone intermediates (92). Nearly all of the important intermediates in the reaction cycle have been observed and characterized.

1.6.4 Catalytic mechanism of tyrosine hydroxylase

The common amino acid hydroxylase enzymes for L-phenylalanine, L-tyrosine, and L-tryptophan are all pterin-dependent enzymes. They require an additional cofactor, either pterin or BH₄ to provide two additional electrons during the reaction cycle to fully reduce O₂. The catalytic cycle of tyrosine hydroxylase (Figure 1.10) begins with a ferrous center coordinate by the 2-His-1-carboxylate facial triad. After initial binding of the pterin co-substrate to the active site, the tyrosine ligand changes its binding to a bidentate mode and the ferrous ion converts from a 6C to 5C center through the loss of two water molecules. L-tyrosine binds to the active site, followed by oxygen to the open position of the metal center to generate the productive catalytic complex. There
are currently two proposed mechanisms for activation of molecule oxygen by the enzyme. Tetrahydropterins are known to react non-enzymatically with molecular oxygen (95,96). Therefore, the first mechanism involves a one-electron transfer from pterin to oxygen from direct interactions with each other; this generates a superoxide and a pterin radical in the active site. The pterin radical and superoxide combine to form a 4a-peroxypterin intermediate species. The 4a-peroxypterin reacts with the iron center to generate a ferrous-peroxypterin intermediate. Neither of the peroxypterin intermediates have been observed directly. However, the non-productive decay products of the intermediate, hydrogen peroxide and quinonoid dihydropterin have been observed (97). Heterolytic cleavage of the peroxypterin intermediate will yield the key ferryloxo species in the cycle (56).

After activation of the oxygen molecule, hydroxylation of the L-tyrosine substrate proceeds through a cationic substrate intermediate. An electrophilic attack on the ring system forms a new C-O bond between the substrate and the oxo group causing the substrate L-tyrosine to lose its aromaticity (98). In the next step, a 1,2-hydride shift results in the carbon adjacent to the C-O bond to possess two hydrogen atoms (99). The ring system of the substrate is re-aromatized when either one of the hydrogen atoms is lost. The final re-aromatization step is not stereospecific suggesting an active site base is not required for this process to occur.
Figure 1.10 Proposed mechanism of L-tyrosine hydroxylase.
2 CROSSSLINKING OF DICYCLOTYROSINE BY P450 ENZYME CYP121 THROUGH SHORT CIRCUIT

(The main text in this chapter is reproduced from a previously published research article by KD and coauthors: Crosslinking of dicyclotyrosine by the cytochrome P450 enzyme CYP121 from *Mycobacterium tuberculosis* proceeds through a catalytic shunt pathway. Dornevil K., Davis I, Fielding A.J., Terrell J.R., Ma Li, and Liu A. J. Biol Chem., 2017, 292(33), 13645-13657)

2.1 Abstract

CYP121, the cytochrome P450 enzyme in *Mycobacterium tuberculosis* that catalyzes a single intramolecular C-C crosslinking reaction in the biosynthesis of mycocyclosin, is crucial for the viability of this pathogen. This C-C coupling reaction represents an expansion of the activities carried out by P450 enzymes distinct from oxygen insertion. Although the traditional mechanism for P450 enzymes has been well-studied, it is unclear whether CYP121 follows the general P450 mechanism or uses a different catalytic strategy for generating an iron-bound oxidant. To gain mechanistic insight into the CYP121-catalyzed reaction, we tested the peroxide shunt pathway by using rapid kinetic techniques to monitor the enzyme activity with its substrate dicyclotyrosine (cYY) and observed the formation of the crosslinked product mycocyclosin by LC-MS. In stopped-flow experiments, we observed that cYY binding to CYP121 proceeds in a two-step process, and EPR spectroscopy indicates that the binding induces active site reorganization and uniformity. Using rapid freeze-quenching EPR, we observed formation of a high-spin intermediate upon addition of peracetic acid to the enzyme-substrate complex. This intermediate exhibits a high-spin (S = 5/2) signal with g values of 2.00, 5.77, and 6.87. Likewise, iodosylbenzene could
also produce mycocyclosin, implicating compound I as the initial oxidizing species. Moreover, we also demonstrated that CYP121 performs a standard peroxidase-type of reaction by observing substrate-based radicals. On the basis of these results, we propose plausible free radical-based mechanisms for the C-C bond coupling reaction.

2.2 Introduction

*Mycobacterium tuberculosis* causes more deaths annually worldwide than any other known pathogen. As the causative agent of tuberculosis in humans, it is one of the most dangerous and difficult to combat bacterial infection. Approximately 10.4 million people suffered from tuberculosis in 2015 with 1.5 million deaths (100). A primary reason for the effectiveness of the pathogen is the recent development of drug and multi-drug resistant *M. tuberculosis* strains. Nearly 10% of new infection cases developed are multi-drug resistant tuberculosis. Resistance to common antibiotics makes treatment very difficult. As the number of strains resistant to frontline drugs grows, pressure is increasing for the identification of potential new targets to combat *M. tuberculosis* infections and the development of new types of drugs and drug classes (101).

A significant milestone in the molecular biology of *M. tuberculosis* was the sequencing of the full genome in 1998. The results revealed a large number of genes encoding for cytochrome P450 enzymes (102). A total of 20 different P450 encoding genes were found in *M. tuberculosis*, far more than any previously sequenced bacterial genome at the time. Two P450 enzymes were quickly identified as potential new drug targets, CYP51B1 and CYP121. CYP51B1 was found to contain sterol demethylase activity (103-105). Initial studies found that both CYP51B1 and CYP121 had low binding constant values toazole- and triazole-based drugs with CYP121 having a higher affinity (105,106). A knock-out study of *rv2276*, the gene encoding for CYP121 in *M.
*tuberculosis*, highlighted the physiological importance of CYP121 (107). These findings set the stage for CYP121 as a possible novel target to combat *M. tuberculosis* infections.

The first breakthrough in the biochemical characterization of CYP121 was the initial determination of the X-ray crystal structure solved at atomic resolution by Munro and coworkers (108). The structure has allowed for the characterization of various small molecules binding to the enzyme active site. The CYP121 structure has complemented the research approach for finding new azole-based inhibitors and characterizing its interactions with current azole-based drugs (109-115). Binding constants for several azole molecules were determined, and these values correspond very closely to minimum inhibition constants against *M. tuberculosis*, further validating CYP121 as a viable drug target (107).

The next breakthrough in the study of CYP121 was the report identifying the native substrate. The gene encoding CYP121 in *M. tuberculosis* was found in an operon-like structure with the gene *rv2275* (102,116). Characterization of Rv2275 in *Escherichia coli* revealed that the products were mainly tyrosine-containing cyclo dipeptides, the majority of which were cyclo-(L-Tyr-L-Tyr) (cYY) (117). At the same time, the crystal structure for CYP121 in complex with cYY was also solved by Belin *et al.* (118). Assays conducted on CYP121 utilizing a ferredoxin and ferredoxin reductase system demonstrated that CYP121 catalyzes multiple turnovers of cYY to form mycocyclosin as the single major product in the presence of NADPH (118). The product exhibits a cross-link between the respective carbons in the *ortho* position of cYY tyrosine moieties. P450 enzymes are normally known to promote a wide range of catalytic activities of aliphatic and aromatic hydroxylation, dealkylation, desaturation, epoxidation, deamination, dehalogenation, dehydration, and isomerization. The C-C bond formation represents an unusual activity of the P450 enzyme superfamily (11,119).
However, important chemical and biological questions remain unanswered regarding CYP121. The mechanism for cross-link formation and the identity of the oxidizing species or the physiological relevance of mycocyclosin are still unclear. A quantum mechanics/molecular mechanics study supports the catalytic mechanism via formation of a diradical intermediate species with the cross-link being formed non-enzymatically in solution (120). The current work expands on the previous studies by investigating the reaction pathway of the CYP121 system. CYP121 is uniquely attractive because of the non-canonical P450 chemistry it catalyzes, and whether or not it follows the classical mechanism of P450s. The mechanistic question under investigation includes the “short circuit,” also known as the catalytic “peroxide shunt” pathway, for the formation of ferric hydroperoxide adduct complex and the subsequent oxo-ferryl species (Figure 2.1) (75,121). Towards this aim, we have carried out rapid kinetics, spectroscopy, and LC-MS analysis to investigate the CYP121 reaction mechanism.

Figure 2.1 Proposed peroxide shunt pathway of CYP121 reaction within the general P450 mechanism. The shunt pathway directly arrives at the hydroperoxo intermediate without the need for receiving electrons and a proton from an external donor (i.e. NAD(P)H) via a reductase system.
2.3 Materials and methods

2.3.1 Expression and purification

The CYP121 gene \textit{rv2276} was synthesized using an outsourced gene synthesis company, GenScript. The gene sequence encoding CYP121 was cloned into vector pET28a-TEV and expressed in \textit{E. coli} strain BL21 (DE3). Conditions used for expression of a CYP121 protein with heme incorporation were incubation at 37°C under kanamycin selection with constant agitation at 220 rpm until a value of 0.6 \( A_{600} \) is reached. Immediately before induction, the cells were inoculated with 5-aminolevulinic acid and ammonium iron(II) sulfate to a final concentration of 300 and 35 \( \mu \text{M} \) respectively. The cells were then induced with a final concentration of 400 \( \mu \text{M} \) IPTG and left to continue shaking over night at a reduced temperature, 28°C. Cells were harvested the next morning by centrifugation and stored in -80°C until further use.

Before purification cultured cell pellets were resuspended in lysis buffer (50 mM potassium phosphate, pH 8.0, 300 mM NaCl) and passed through an LS-20 cell disruptor (Microfluidics) to lyse cells. The resulting suspension was centrifuged, and the supernatant was collected. Clarified cell extract was applied to a Ni-NTA affinity chromatography column, and bound protein was eluted using elution buffer (50 mM potassium phosphate, pH 8.0, 300 mM NaCl, 500 mM imidazole). Fractions were collected during purification and pooled together; the sample was then buffer exchanged into 50 mM Tris-HCl pH 7.4, 5% glycerol buffer. The purified protein was flash frozen in liquid nitrogen and stored in -80°C until required.

2.3.2 Pre-steady state kinetics

Stopped-flow studies were carried out in single kinetic traces and multi-wavelength data sets on an Applied Photophysics SX20 stopped-flow system. The assay conditions were 5 – 25
μM CYP121 and varied concentrations of each oxidant, from 0.1 – 20.0 mM. Assays with cYY used a constant 400 μM concentration of substrate. The stock solution of cYY was made in dimethyl sulfoxide and a desired amount of the solution was incubated with CYP121 prior the kinetics experiments (113). The experiments were carried out at room temperature in 50 mM Tris-HCl, pH 7.4 buffer, and dimethyl sulfoxide was less than 2% (v/v).

2.3.3 Rapid freeze-quench EPR spectroscopy

Rapid freeze-quench experiments were carried out using a System 1000 Chemical/Freeze-Quench Apparatus made by Update Instruments, Inc. A typical freeze-quench set-up consisted of a liquid ethane bath at -130 °C. Reactions were carried out by mixing 500 μM CYP121 or the enzyme-substrate complex in 1:1 ratio with 2 – 10 mM peracetic acid (PAA). The reaction was carried out at room temperature in 50 mM Tris-HCl pH 7.4 buffer. The reaction mixture was rapidly frozen in liquid ethane after reacting for different time intervals. The frozen samples were transferred into EPR tubes, and excess liquid ethane was vacuumed off. Quenched samples were stored in a liquid nitrogen dewar before analysis.

EPR spectra were recording at 9.6-GHz microwave frequency with a Bruker E560 EPR spectrometer with a dual mode resonator at 100 kHz modulation frequency. The temperature was maintained at 10 K by a cryogen-free 4 K temperature system. Frozen sample solutions were analyzed in 4-mm quartz EPR tubes.

2.3.4 LC-MS analyses

A typical assay experiment was performed using 20 – 30 μM CYP121, 40 μM of cYY and 10 eq of oxidant relative to the enzyme. The assay was carried out in 5 mM Tris-HCl buffer, pH
7.4. Oxidant was titrated over 10 additions on ice with 1-min intervals between each addition to avoid pronounced peroxide damage to the enzyme. After the 10-min reaction, the final sample was centrifuged for 15-min using an Amicon Ultra centrifugal filter with 10-kDa cut-off. Chromatographic separations and mass spectrometry analyses were performed on an Acquity UPLC system directly coupled to a triple quadrupole detector (Waters Corp.). For chromatography, solvent A was an aqueous solution containing H$_2$O plus 0.1% formic acid, while solvent B was acetonitrile plus 0.1% formic acid. Separation was achieved at a flow rate of 0.2 mL/min using a Synergy 4u Fusion-RP 80A column (50 x 2.00 mm, C18, 4-μm particle size) from Phenomenex. For each analysis, 5 μL of sample was injected onto the column. Mass spectra for full scans and selected-ion monitoring were acquired in negative ionization mode in the range $100 \leq m/z \leq 1000$. Capillary, cone and extraction potentials were maintained at 3.5, 35, and 3 V, respectively. The source block temperature was 120 °C, and the desolvation temperature was 250 °C. The cone gas flow rate was 50 liters/h. Product detection was performed by selected-ion monitoring in which the quadrupole was used to filter for the substrate (325.1 m/z) and the product (323.1 m/z).

### 2.3.5 Peroxidase activity assay

Using ABTS as a substrate, we tested the ability of CYP121 to generate substrate-based radicals. A representative assay shown in Fig. 2.9A was conducted using an Agilent 8453 UV-visible spectrometer. The steady-state kinetic analyses were performed with single-wavelength traces monitored at 420 nm for formation of ABTS$^+$ using an Applied Photophysics SX20 stopped-flow spectrometer (Leatherhead, UK). The assay conditions for the determination of kinetic parameters were 100 nM CYP121, 2 mM ABTS, and the concentration of PAA was
varied between 0.02 and 5 mM. The concentration of ABTS radical formed during the reaction was determined using $\varepsilon_{420}$ 36,000 M$^{-1}$ cm$^{-1}$ (122). The experiments were performed at room temperature in 50 mM Tris-HCl, pH 7.4 buffer.

2.4 Results

2.4.1 Spectral properties of cYY binding to CYP121

The as-isolated CYP121 exhibits a Soret peak centered at 416 nm. Two absorbance features at 538 and 565 nm in the $\alpha/\beta$ region and an additional minor band at 648 nm are also present. CYP121 displays type I characteristic spectral changes in the UV-visible heme Soret spectrum upon binding of cYY (Figure 2.2A), as is frequently observed in P450 enzymes during binding of endogenous substrates and xenobiotics (63). After substrate binding, the Soret peak blue-shifts to 395 nm. However, a significant shoulder peak remains even when saturating concentrations of cYY (400 µM, 80:1 ratio of the substrate over enzyme, 20 times the cYY $K_d$ value of 21.3 µM) (118) were used. This phenomenon has previously been observed among the P450 family as well as in CYP121 (118). In the $\alpha/\beta$ region additional changes occurred with the loss of the two peaks to generate a new broad feature at 516 nm with shoulders at 541 and 571 nm, while the charge transfer peak at 651 nm increased in intensity (118). The spectroscopic changes observed for substrate binding are summarized in Table 2.1. The spin transition was less pronounced when monitored by low-temperature EPR spectroscopy for samples slowly frozen by liquid ethane. The incomplete spin-state conversion most likely originates from the low temperature used in the EPR studies. A slight decrease in the $g$-anisotropy of the low-spin species and a new resonance at $g = 8$ is observed (Figure 2.2B).
Figure 2.2 Spectroscopic characterization of substrate cYY binding to CYP121. All substrate binding experiments were monitored using nearly saturated concentrations of cYY, 400 µM. A, UV-visible spectra of CYP121 (10 µM) incubated with cYY; B, EPR characterization of CYP121 (250 µM; black spectrum) binding to CYY (red spectrum); C, stopped-flow UV-visible spectroscopic monitoring of the kinetics of cYY binding to CYP121 shows complete conversion to the ES complex form within the first 250 ms.
Table 2.1 Summary of spectra profile changes for cYY binding and ES-complex reaction

<table>
<thead>
<tr>
<th>Transition</th>
<th>positive peak (nm)</th>
<th>negative peak (nm)</th>
<th>isosbestic point (nm)</th>
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<tbody>
<tr>
<td></td>
<td>#1</td>
<td>#2</td>
<td>#3</td>
</tr>
<tr>
<td>cYY Binding</td>
<td>393</td>
<td>508</td>
<td>651</td>
</tr>
<tr>
<td>ES + PA</td>
<td>433</td>
<td>573</td>
<td></td>
</tr>
</tbody>
</table>

2.4.2 Stopped-flow kinetic studies of cYY binding

Stopped-flow experiments to determine the microscopic rate constants \((k_{on} \text{ and } k_{off})\) for cYY binding to Fe(III)-CYP121 were performed by monitoring the formation of the ES complex at 388 nm using a stopped-flow spectrometer. This wavelength was chosen because in the difference spectra, the largest amplitude change is at 388 nm (Figure 2.2C). Figure 2.3A shows stopped-flow time-traces varying the [cYY] from 20 to 700 µM. Fitting the kinetic traces to single exponential equations resulted in a very poor fit with significant residual amplitudes that show systematic dependence with [cYY]. In contrast, a very good fit was obtained by using a two-exponential equation (Figure 2.3A).
Figure 2.3 Kinetic characterization of substrate binding to CYP121. A, single-wavelength stopped-flow data collected at 388 nm monitoring formation of enzyme-substrate complex. Reaction conditions were pH 7.5, 21°C, 5.05 µM heme after mixing. Residuals from fitting stopped-flow data with single- and double-exponential curves are shown. B, plot of reciprocal relaxation times from double-exponential fitting of single-wavelength stopped-flow data collected at pH 7.5 (5 µM heme after mixing). The slower phase shows saturation behavior at high cYY concentrations. Experiments were limited by low solubility of cYY in water. Error bars originate from fitting S.D. values from fitting multiple experimental data sets. C, replots of cYY concentration dependence data showing the sum and product of two observed reciprocal relaxation times from double-exponential fitting of data.

The plots of the observed rates (1/\(\tau_1\) and 1/\(\tau_2\)), from double exponential fittings of the [cYY] dependence are shown in Figure 2.3B. While 1/\(\tau_1\) shows linear [cYY] dependence, 1/\(\tau_2\) shows parabolic concentration dependence, suggesting a stepwise mechanism for cYY binding (Reaction 1) to form the binary ES complex. Fitting both 1/\(\tau_1\) to a linear equation and 1/\(\tau_2\), to a hyperbolic equation yielded non-zero y-intercepts, suggesting that both steps are reversible. From the reploting of the [cYY] dependence data (Figure 2.3C), taking both the sum (1/\(\tau_1\) + 1/\(\tau_2\)) and the product (1/\(\tau_1\) x 1/\(\tau_2\)) of the observed reciprocal relaxation times, the microscopic rate constants
(\(k_1, k_{-1}, k_2,\) and \(k_{-2}\)) can be calculated from the slope and the y-intercept from the two graphs (Reaction 1) (123). The second-order rate constant \((k_1)\) of 0.065 ± 0.002 \(\mu\text{M}^{-1}\ \text{s}^{-1}\) is fairly low where the initial binding step is slower than both \(k_1\) and \(k_2\) \((k_1 < k_{-1}\) and \(k_2\) when \([cYY] < 62 \ \mu\text{M})\).

The reverse rates \((k_{-1}\) and \(k_{-2}\)) are also on the same order of magnitude and have similar values to \(k_2\). These suggest a highly reversible system where \(\text{ES}^*\) and \(\text{ES}\) are in rapid equilibrium.

\[
\begin{align*}
k_1 &= 0.065 \pm 0.002 \ \mu\text{M}^{-1}\ \text{s}^{-1} \\
k_2 &= 4.0 \pm 1.2 \ \text{s}^{-1} \\
k_{-1} &= 4.2 \pm 0.6 \ \text{s}^{-1} \\
k_{-2} &= 3.2 \pm 0.9 \ \text{s}^{-1}
\end{align*}
\]

Reaction 1 shows the proposed multistep mechanism for cYY binding to Fe(III)CYP121, with rate constants obtained from double exponential fitting of stopped-flow data from [cYY] dependence data (Figure 2.3).

2.4.3 Transient kinetic studies of the reaction of CYP121 with peracetic acid in the presence and absence of substrate cYY

If CYP121 follows the general cytochrome P450 mechanism, it would be able to generate the enzyme-based key oxidant, compound I, through the well-established peroxide shunt pathway. The peroxide shunt pathway bypasses the need for NAD(P)H and a redox mediator system to supply electrons and protons to the heme-bound \(\text{O}_2\) (124-126). Among \(\text{H}_2\text{O}_2\), cumene peroxide, \(t\)-butyl peroxide, \(meta\)-chloroperoxybenzoic acid, and peracetic acid (PAA) tried at pH 7.4, only \(\text{H}_2\text{O}_2\) and PAA gave significant reaction. Because PAA has the most apparent reaction at lower concentrations during our initial tests, the following work described in this study was mostly
focused on PAA. The reaction was initiated by incubating the peroxide oxidant with 5 μM of CYP121 pre-mixed with cYY to determine if the shunt pathway would be a viable route to generate the C-C cross-linked product. A parallel experiment was performed in the absence of substrate as a control to assist identification of the intermediate species.

The enzyme was first pre-incubated with the substrate (600 μM) and subsequently mixed with increasing concentrations of PAA, and the reaction was monitored for 30 s by stopped-flow UV-Vis spectroscopy (Figure 2.4A). Over the course of the reaction, the difference spectra show several transitions. During the first 300 ms, the ES complex absorbance at 395 nm decreases concomitant with an increase at 427 nm. As the reaction continues, the 395-nm Soret peak of the ES complex continues to decrease while the 427 nm peak increases and red-shifts to 433 nm. Finally, when the reaction proceeds for longer than 10 s, the isosbestic points become less clear, suggesting that heme bleaching becomes a contributing factor in the reaction. Monitoring the reaction rate as a function of PAA concentration allows for the determination of the apparent pseudo first order rate constant $k_{obs}$ of $(7.2 \pm 0.1) \times 10^{-4} \text{ s}^{-1}$. 
Figure 2.4 Transient kinetic analysis of the ES complex (10 µM CYP121 and 400 µM cYY) reacting with peracetic acid (2 mM). A, full spectra of the first 10 s of reaction showing decay of the ES complex back to resting. B, difference spectra showing return back to the resting enzyme within 10 s with multiple clear isosbestic points signify a clear transition back to the resting state during this time period and showing the development of a 427-nm intermediate between 5 and 449 ms. After this time, the intermediate decayed and shifted to 433 nm. C, single-wavelength kinetic traces monitoring the regeneration of the resting enzyme at the expense of the ES complex. D, plots of substrate binding (described in the previous figure) showing the formation of the ES complex.
For comparison, when the enzyme is reacted with PAA in the absence of substrate, the first 100 ms generate a new intermediate species (Figure 2.5A). The formation of the intermediate species is described by a decrease in the intensity of the Soret peak with an increase in the shoulder peak near 379 nm. Near the α/β region of the spectra, a decrease at 543 and 574 nm are observed with formation of two minor peaks at 616 and 704 nm. The difference spectra clearly show the formation of a new species. Five isosbestic points at 300, 392, 447, 517, and 584 nm are also observed in the difference spectra (Figure 2.5A, inset). The clear isosbestic points suggest a direct transition from resting state heme directly to the first intermediate species. From 100 ms to ~ 2 s, a second transition occurs forming a second intermediate species (Figure 2.5B). This second intermediate is distinct with isosbestic points at 296, 340, 427 and 580 nm. The second intermediate is highlighted by two noticeable absorption changes at 421 and 440 nm, decreasing and increasing respectively. The final transition occurs from 2 to 20 s of CYP121 reacting with PAA (Figure 2.5C). The difference spectra contain two prominent peaks centered at 412 (increasing) and 440 (decreasing) nm (Figure 2.5C, inset). A complete profile of the transitions observed is compiled in Table 2.2. The 433-nm species found in the reaction of ES complex with PAA is not present in the reaction when cYY is absent.
Figure 2.5 Stopped-flow UV-visible kinetic characterization of CYP121 (5 µM) reacting with peracetic acid (2 mM). A, 0 - 100 ms; B, 100 ms – 2 s; C, 2 – 20 s. Insets, difference spectra showing three intermediates observed for each transition.
Table 2.2 Summary of intermediate spectra profiles.

<table>
<thead>
<tr>
<th>Transition</th>
<th>positive peak (nm)</th>
<th>negative peak (nm)</th>
<th>isosbestic point (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1  #2  #3  #4  #5 #1  #2  #3  #4 #1  #2  #3  #4</td>
<td>#1  #2  #3  #4</td>
<td>#1  #2  #3  #4</td>
<td></td>
</tr>
<tr>
<td>100 ms     379  469  616  704</td>
<td>421  543  574</td>
<td>313  392  601</td>
<td></td>
</tr>
<tr>
<td>2 s         318  382  440  535  571</td>
<td>359  421  587</td>
<td>296  340  427  580</td>
<td></td>
</tr>
<tr>
<td>20 s        412  495  595  624  681</td>
<td>374  440  538  572</td>
<td>393  425  583</td>
<td></td>
</tr>
</tbody>
</table>

2.4.4 Detection of mycocyclosin from the peroxide shunt pathway

Because the P450 enzyme-mediated C-C bond coupling mechanism has not yet been elucidated, one cannot assume that the shunt pathway in CYP121 will lead to the generation of the reported product. To demonstrate the relevance of PAA as an oxidative source for mechanistic studies, the reaction mixture was characterized by LC-MS. When the substrate was analyzed alone or pairwise in the presence of either PAA or CYP121 only, a single peak elutes with a retention time of ~ 8 min (Figure 2.6A). This peak possesses an m/z of 325, which is the expected value for the cYY substrate (Figure 2.6B). When all three components, CYP121, cYY and PAA are combined and allowed to react (see “Experimental Procedures”), the reaction mixture contains new peaks and the peak with retention time close to 5 min shows an m/z of 323, which is consistent with the cross-linked mycocyclosin product (Figure 2.6 C and D). These data demonstrate that CYP121 can utilize the shunt pathway to carry out the C-C crosslinking reaction on cYY and generate mycocyclosin.
Figure 2.6 Mass spectrometry analysis of ferric CYP121-mediated cYY crosslinking using peracetic acid as the oxidant. A, total ion count of enzyme assay mixture; B, mass-spectral detection of cYY (325 m/z); C, selected ion monitoring of both cYY and mycocyclosin shows a new peak at 4.8 min after incubation of reaction mixture for 10 min; D, molecular mass detection of the cross-linked mycocyclosin (323 m/z) after incubation of the enzyme, cYY, and peracetic acid.

2.4.5 Characterization of the shunt reaction by EPR spectroscopy

To gain more insight into the CYP121 cross-linking reaction, rapid freeze-quench EPR samples were made in which CYP121 was rapidly mixed with PAA in either the presence or absence of cYY before quenching in liquid ethane at various time points. The quenching times chosen were guided by our stopped-flow studies described above. When the reaction of the ES complex with PAA is quenched at 5 ms, EPR data reveals a near complete disappearance of the ES
complex EPR signal (≥75%). Instead, the EPR spectra from samples trapped at different time points in the millisecond time windows show a new high-spin ($S = 5/2$) ferric heme species with $g$ values of 6.87, 5.77, and 2.00 (Figure 2.7). When allowed to react for longer times, the high-spin species decreases in intensity (Table 2.3). However, the decay of the high-spin species was not accompanied by regeneration of the low-spin ferric signal. Noticeably, a new EPR-silent heme species was formed during this time. The remaining minor low-spin heme signal also continuously decays during this period. During the same time window, the 427-nm species was developed and shifted to 433 nm in the stopped-flow experiments. The final sample quenched at 10 s after mixing gives a spectrum with no sign of the low-spin heme. The high-spin ferric signal intensity is significantly reduced compared to the 5 ms sample. A new significant portion of adventitious iron, presumably from heme degradation, and a free radical species are observed. Minor EPR species at $g = 2$ are observed in the samples, but unlike any characterized tyrosyl radicals. Thus, the radical species are unlikely to be associated with the cYY reaction but are more likely to be intermediates of side reactions towards heme degradation. When the rapid freeze-quench EPR study was performed with 0.4 mM PAA with more time points in the first 180 s, similar results were obtained, *i.e.*, the $g = 6$ species maximizes and then starts to decay. The only difference is that the $g = 6$ species is lower in intensity to compared to the 10 mM PAA, and the heme bleaching $g = 4.3$ signal does not occur at the end of the reaction.
Figure 2.7 EPR analysis of the enzyme-substrate complex (250 µM) reacting with peracetic acid (10 mM). The black trace shows the ES complex of cYY and CYP121. A new high-spin ferric heme intermediate is formed in 5 ms with concomitant decay of the low-spin ES complex (red trace). The EPR spectra also include the new high-spin intermediate at 160 (blue) and 300 ms (green). The final decayed reaction complex after 10 s of reaction is shown by a dashed line, which does not contain the intermediate. EPR spectra were obtained at 5 K, 9.6-GHz microwave frequency, and 1-milliwatt microwave power. mT, millitesla.

Table 2.3 Summary of the resonance components in the rapid freeze-quench EPR spectra of ES complex reacting with PAA*

<table>
<thead>
<tr>
<th>g value</th>
<th>ES complex</th>
<th>5 ms</th>
<th>160 ms</th>
<th>300</th>
<th>10 s</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.77</td>
<td>0</td>
<td>100</td>
<td>98.9</td>
<td>80</td>
<td>36</td>
</tr>
<tr>
<td>6.87</td>
<td>0</td>
<td>100</td>
<td>77</td>
<td>80</td>
<td>0</td>
</tr>
<tr>
<td>8.11</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

*Numbers are given in percentage of the amplitude of the resonance using the 5-ms intermediate sample as a reference.
For the reaction of CYP121 alone with PAA, a series of freeze-quench samples were prepared and analyzed by EPR (Figure 2.8). After quenching at 100 ms, a significant decrease in the low-spin heme signal was observed with the formation of a minor new high-spin species with \( g \) values of 6.67, 5.77, and 2.0. This species is distinctly different from the high-spin heme intermediate observed in the reaction of the ES complex with PAA. After reacting for longer times, the 2-s sample resulted in a further reduction of the low-spin heme and a continued increase of the high-spin species. The high-spin heme was present as a heterogeneous species with three peaks featured on the spectral profile. Allowing the reaction to proceed further and quenching at 20 s causes a greater formation of the high-spin species, with the \( g = 6.67 \) feature becoming more prominent and distinguished.
Figure 2.8 EPR analysis of the resting enzyme (250 µM) reacting with peracetic acid (2 mM). The resting enzyme of CYP121 is present as a heterogeneous low-spin ferric heme species (black trace). CYP121 was mixed in 1:1 ratio and quenched in liquid ethane after reaction times of 100 ms (navy trace), 2 s (blue), and 20 s (cyan). The reaction generated at least one high-spin species at \(g = 6.67, 5.77, \text{ and } 2\) that increased in intensity after longer reaction times. EPR spectra was obtained at 5 K, 9.6-GHz microwave frequency, and 1-milliwatt microwave power. \(mT\), milliteslas.

### 2.4.6 The shunt reaction using iodosylbenzene

The oxidant of cYY could be a ferric peroxide intermediate or a compound I species. In fact, the fatty acid peroxide-metabolizing P450s, CYP5, CYP8A, and CYP74, are believed to directly utilize a ferric peroxide to initiate substrate oxidation reactions to generate a compound II and a substrate radical (127-130). In C-C bond cleavage reactions exhibited by 11A1, 17A1, 19A1,
and 51A1 the active oxidant has been controversial (131,132). Our EPR detection of a ferric intermediate prompted us to consider if the intermediate is an active species or it is the precursor of a compound I intermediate as illustrated in Figure 2.1. Following the strategy of the 17A1 study using iodosylbenzene by Guengerich and coworkers (131), we performed a comparative study with PAA under the exact same reaction conditions. Figure 2.9 shows that the anticipated product was formed using iodosylbenzene as the oxidant, although additional side products were also produced. This result sheds light on the catalytic mechanism and is in favor of the compound I intermediate as the catalytic oxidant, because iodosylbenzene cannot possibly form a peroxo intermediate. However, such evidence does not preclude the possibility of the ferric peroxide intermediate detected in the PAA reaction from being an active species.
Figure 2.9 HPLC chromatogram (monitored at 280 nm) showing formation of mycocyclosin from reactions of 5 µM, 400 µM cYY and iodosylbenzene (PhIO, red trace), or 400 µM PAA. The product mycocyclosin elutes at 4.3 minutes. The PhIO reaction generates more side products than the PAA reaction. For the PhIO reaction, the background at 358 nm was subtracted to correct the baseline. mAU, milliabsorbance units.

2.4.7 The P450 enzyme CYP121 catalyzes a peroxidase reaction

If the cross-linking of cYY is a radical mechanism, then the CYP121-mediated enzymatic action is a peroxidase-like reaction, where two substrate-based cation radicals would be expected to form by compound I and then compound II in succession. We found that CYP121 is able to perform catalytic turnover of the peroxidase substrate, 2,29-azino-bis(3 ethylbenzothiazoline-6-sulfonate) (ABTS), to generate ABTS cation radical (Figure 2.10). When the [PAA] is varied the
kinetic parameters, $k_{\text{cat}}$ and $K_m$, are $4.1 \pm 0.1 \text{ s}^{-1}$ and $1.5 \pm 0.1 \text{ mM}$, respectively. Because of the relatively high $K_m$ for PAA, the $k_{\text{cat}}$ and $K_m$ cannot be accurately determined as a function of [ABTS] due to complications of enzyme damage and nonenzymatic reaction between ABTS and PAA at high oxidant concentrations. Nonetheless, the results shown in Figure 2.10 show that CYP121 is able to function as a peroxidase and generate substrate-based radicals.

![Figure 2.10](image_url)

Figure 2.10 Steady-state kinetic analysis of the peroxidase activity of CYP121 (100 nM) with ABTS (2 mM) and PAA (0.02-5 mM). A, representative spectra of CYP121 peroxidase activity as monitored by UV-visible spectroscopy for 1.5 min. B, EPR spectroscopic detection of ABTS$^+$. C, single-wavelength stopped-flow data monitoring formation of ABTS$^+$ at 420 nm. D, Michaelis-Menten fit to the kinetic data as a function of PAA concentration. AU, absorbance units.
2.5 Discussion

In addition to CYP121, several other P450 enzymes have been identified to perform C-C coupling reactions. For instance, the biosynthesis of staurosporine requires an intramolecular C-C bond between two indole rings at the C2 position and is catalyzed by P450 StaP (133). In mammals, several P450s are reported to form C-C bond in the synthesis of salutaridine, a morphine precursor (25,134). In plant secondary metabolism P450 enzymes CYP80G2 and CYP719B1 catalyze intramolecular C-C phenol coupling reactions (11). The C-C bond formation represents a novel and small, but growing activity of the P450 enzyme superfamily (119). The work described here represents an initial effort aimed at a better understanding of the C-C bond coupling mechanisms mediated by the P450 enzymes.

2.5.1 Structural reorganization and active site uniformity introduced by substrate binding

The binding of cYY to CYP121 appears to be a complicated multistep process similar to binding studies of other P450 systems (63). There are several possible models to explain these results. In the first model, the high-spin and two different low-spin states of the heme in the active site have different affinities for the substrate. The second model is that the initial binding of cYY is followed by reorientation of the substrate within the active site. The first model is believed to be an unlikely scenario; whereas the heme is present in two conformations, the overall configuration within the active site is highly conserved before and after binding of substrate. The ligand-free and the substrate-bound active site structures of the enzyme are nearly identical (108,118). The second model is more likely due to the bulky nature of the substrate and the conformational freedom at α-carbons of both tyrosine ends in cYY. The as-isolated CYP121 contains two EPR resonance components in the low-spin region: an outer component, species A,
and an inner component, species B with smaller $g$-anisotropy (Figure 2.5B). Species B increases in intensity upon binding of the substrate at the expense of species A. While the UV-visible spectra of CYP121 demonstrate typical type I binding of cYY with a low-spin to high-spin transition, this conversion does not appear to be complete even under saturating cYY concentrations. In EPR experiments, the ratio of the low-spin to high-spin transition of the enzyme-substrate complex appears to be dependent on freezing speed, with faster freezing producing more high-spin heme signal (data not shown), suggesting a dynamic equilibrium exists between the two spin states.

### 2.5.2 Mechanistic implications

The ability of CYP121 to react with a hydrogen peroxide analogue (i.e. peracetic acid) was studied to understand the heme-based oxidant behind the unusual intramolecular C-C coupling reaction chemistry carried out by this enzyme. As an established strategy, peracids have been utilized in place of hydrogen peroxide in the catalytic shunt pathway to generate and characterize high-valent ferryl species to probe P450 reaction mechanisms (135,136). The advantage of using a peracid is that it has an active leaving group, which helps to promote heterolytic cleavage of the O-O bond to generate an oxoferryl porphyrin cation radical (known as compound I) in P450 systems (137). Here, we show that peracetic acid could react with the heme center of CYP121 to produce the C-C bond coupling product mycocyclosin. Based on the shunt pathway illustrated in Figure 2.1, either a ferric peroxo, a compound I intermediate, or both, should be part of the catalytic cycle. Compound I is a strong spin-coupled system carrying two oxidizing equivalents on a single heme. The P450 compound I intermediate is unfortunately not a spectroscopically long-lived species that previously eluded EPR and Mössbauer spectroscopic detection for several decades until recent characterization of the thermophilic P450 CYP119 at 4 °C through the catalytic shunt reaction using an organic peroxide (71). The understanding of C-C coupling of cYY has stagnated
for lack of a detectable intermediate in the typical catalytic reaction with O\textsubscript{2} and the ferredoxin system (110,112-114,118,138,139). The slow formation of an active intermediate from the alkylperoxo and its rapid reaction with cYY is the most likely reason for its lack of spectroscopic detection.

The investigation of the peroxide shunt pathway carries several mechanistic implications toward understanding the CYP121-mediated C-C bond formation. We established the shunt pathway in the CYP121 reaction, which often suggests the involvement of a high-valent ferryl species in the catalytic cycle. A key mechanistic difference between CYP121 and other P450 enzymes is that CYP121 does not perform a hydroxylation reaction on cYY (118), and thus the quintessential radical rebound mechanism found in traditional P450 mechanisms does not apply in the C-C bond formation reaction (140).

Our LC-MS analysis confirmed the cYY-to-mycocyclosin conversion. However, this reaction is limited to only a few turnovers, presumably due to complications of enzyme damage from PAA. When monitored by EPR spectroscopy, the reaction of the ES complex with PAA shows the low-spin heme signal nearly disappears, and a new high-spin intermediate was formed with subsequent slow conversion to an EPR-silent heme species and an organic radical. This high-spin intermediate is different from traditional P450 high-spin systems that are characterized with g-values at 8, 4, and 1.7 (141).

The most possible candidate for the rapid and nearly full production of the high-spin ferric intermediate from the ES complex reaction with PAA is an alkylperoxo (\textit{i.e.} a side-on Ac-O-O-Fe(III) intermediate). Typically, ferric hydroperoxo species in heme-based systems are present at the low-spin state and ferric alkylperoxo is less studied. High-spin, peroxo-bound species have been reported in the study of ferric peroxide complex (47), binuclear cytochrome c oxidase mimics
iron complexes with tetra- and pentadentate ligands, and a superoxide reductase mutant with four equatorial histidines and an axial cysteine (144-146). The high-spin state is likely due to the side-on geometry of the peroxo moiety to the iron ion (hepta-coordinate) and dianionic (strong π-base) O_2^2- ligand causing weakening of the other axial ligand. It has been known through model complex studies that the spin state of the ferric-alkylperoxo complexes is important for the reactivity. The high-spin state in the nonheme complex presents a barrier for homolytic cleavage of the O-O bond relative to the low-spin complex (147). How the spin state effects the heterolytic cleavage of the O-O bond remains unexplored. The electronic structure of the high-spin ferric intermediate found in CYP121 and corresponding model complexes, once available, deserve further characterizations in subsequent studies.

The peroxide shunt study and our finding of the peroxidase activity of CYP121 provide insights into the mechanism of action for this unusual P450 enzyme. Taken together, we were able to put forth plausible catalytic mechanisms for the C-C bond coupling by the P450 enzyme. Figure 2.11 (A and B) illustrates that the compound I intermediate is the oxidizing species. It can perform hydrogen atom abstraction on the hydroxyl group of the cYY proximal tyrosine, generating a protonated compound II and a cYY radical. Likewise, two subsequent pathways could take place. The reaction proceeds via an electron tunneling or hopping step to generate a cation radical and phenolate species (Figure 2.11A). A second hydrogen atom abstraction at the ortho position by the protonated Compound II will generate the second radical on cYY. The two radicals of opposite charge on cYY would be expected to combine rapidly to give rise to a new C-C bond. Finally, deprotonation of the distal tyrosine by the phenolate will re-aromatize the final product. In Figure 2.10B, we propose an initial intramolecular proton-coupled electron transfer step to migrate the single radical to the distal tyrosine moiety. The protonated compound II then abstracts a hydrogen
atom from the hydroxyl moiety of the proximal tyrosine. The two phenoxy radicals are delocalized on the aromatic ring system allowing for the diradical to combine and form the C-C bond. The resulting diketone group can tautomerize to generate mycocyclosin. Because no substrate-based radical intermediate was observed, we therefore assume that the catalytic chemistry is fast and not rate-limiting.
Figure 2.11 Proposed mechanistic models for the CYP121-mediate cYY cross-linking reaction using peracetic acid as the oxidant. The top two mechanisms are proposed using compound I as the oxidant (A and B), and the bottom two using the peroxo intermediate as the active oxidant (C and D). In each case, two pathways are proposed for the formation of a second radical and subsequent C-C bond formation.
Our current understanding of the PAA based CYP121 shunt mechanisms shown in Figure 2.11 (A and B) are consistent with all the observations obtained so far. In the PAA reaction, heterolytic cleavage of the alkylperoxo will be expected to yield a compound I oxidant for the subsequent oxidation reactions. The production of compound I is expected to be rate-limiting based on our stopped-flow and RFQ-EPR data. Additionally, the observation of a species consistent with protonated compound II also supports mechanisms A and B in Figure 2.10.

Although compound I is probably the oxidant, our data cannot exclusively eliminate the possibility that the Ac-O-O-Fe(III) intermediate oxidizes the cYY directly. If so, an oxoferryl species and a substrate-based radical species that is electronically equivalent to compound I will be generated. After this initial phase, two possible diverging mechanisms for the formation of a second substrate radical and subsequent C-C bond formation are proposed (Figure 2.11, C and D). The first is a direct electron tunneling or electron/hole hopping via a protein residue to share the radical character with the remote tyrosine moiety (148-150). Subsequent oxidation of the proximal tyrosine generates a second radical for radical-radical coupling reactions (Figure 2.11C). An alternative proposal is proton-coupled electron transfer (PCET) (151-154). In contrast to the above model, the high-valent Fe intermediate will oxidize the hydroxyl group of the closer tyrosine again, yielding a diradical species on the cYY for a rapid C-C bond coupling (Figure 2.10D). In this model, an intranuclear tautomerization reaction must take place to produce the final product, mycocyclosin.
2.5.3 Concluding remarks

In summary, we have studied substrate binding kinetics and characterized the reactivity of CYP121 towards cYY and peracetic acid, an exogenous oxidant that bypasses the need to provide electrons through an exogenous reductase system. Iodosylbenzene was also employed as an oxidant of the reaction to support the catalytic competence of a putative compound I species in CYP121. Furthermore, CYP121 was shown to be able to perform peroxidase chemistry, generating substrate-based radicals. Together, we demonstrate that CYP121 can utilize the well-described shunt pathway of other P450 enzymes to generate the cross-linked product mycocyclosin from the cYY substrate. The formation of the postulated compound I is rate-limiting, while all the subsequent chemical steps are rapid. With these advances, we propose four detailed catalytic mechanisms for future investigations.
3 CRYSTAL STRUCTURE OF CYP121 IN COMPLEX WITH A DIATOMIC LIGAND

(The main text in this chapter is reproduced and modified in part from a previously published research article by KD and coauthors: Probing ligand exchange in the P450 enzyme in CYP121 from Mycobacterium tuberculosis: Dynamic equilibrium of the distal heme ligand as a function of pH and temperature. Fielding A.J., Dornevil K, Ma Li, Davis I, and Liu A. J. Am. Chem. Soc., In press DOI: 10.1021/jacs.7b08911)

3.1 Abstract

CYP121 is a cytochrome P450 enzyme from Mycobacterium tuberculosis that catalyzes the formation of a C-C bond between the aromatic groups of its cyclodityrosine substrate (cYY). The crystal structure of CYP121 in complex with cYY reveals that the solvent-derived ligand remains bound to the ferric ion in the enzyme-substrate complex. Whereas in the generally accepted P450 mechanism, binding of the primary substrate in the active-site triggers the release of the solvent ligand, priming the metal center for reduction and subsequent O₂ binding. Here we employed NaCN, a diatomic O₂ analogue to probe the metal-ligand exchange of the enzyme and the enzyme-substrate complex. CYP121 enzyme was co-crystallized with the native substrate, cYY. Soaking experiments with CN allowed for the determination of the structure for the ternary ESCN complex. The crystal structure of the ternary complex shows a rearrangement of the substrate in the active-site, when compared to the structure of the binary complex, upon cyanide binding. The presence of CN in the active site induces several active site changes as observed in the crystal structure. A water network that interacts with the hydroxy group of the tyrosine moiety near the heme center
is disrupted when CN is bound. The disruption of this network brings the hydroxyl group close to the heme center presumable allowing for easier deprotonation and oxidation of the cYY substrate. Additional changes are also observed in two active site residues, Arg386 and Ser237. Our data reveal the chemical and physical properties of the solvent-derived ligand of the enzyme, which will help to understand the initial steps of the catalytic mechanism.

3.2 Introduction

CYP121 has been crystallized both in the ferric resting state and in complex with its native substrate cYY at 1.06 Å and 1.4 Å respectively (108,118). The enzyme only structure contains a large active-site pocket for binding organic substrate hydrated with numerous water molecules. The heme is 6-coordinated with a cysteine as the axial ligand and a water molecule as the sixth ligand. The binary ES-complex structure shows that binding of cYY displaces many of the active site waters. Additionally, one of the tyrosine moieties is pointed towards the heme center and oriented appropriately for oxidation. Surprisingly, it was found that the ES-complex also possesses a single water molecule bound to the heme iron in the sixth position. This is uncharacteristic of typical P450 enzymes, which traditionally lose the coordinating water molecule upon binding of the organic substrate (155). The departure of the axial ligand converts the heme from a low-spin to high-spin electronic configuration allowing for reduction of the iron and creating an open site for binding of molecular oxygen (62,155,156). However, no significant changes are observed in the active site upon binding of cYY suggesting that binding of the organic substrate alone is not enough to prime CYP121 for reaction. In order to further investigate the structural properties that govern the reactivity of CYP121, NaCN was used as a molecular probe to mimic oxygen or hydrogen peroxide binding.
3.3 Materials and methods

3.3.1 Crystallization of the CYP121 ternary complex ESCN

Crystals of CYP121 were obtained using the hanging drop method. The 6xHis-tagged CYP121 was cleaved using the Thrombin Cleavage Kit (Sigma Aldrich) prior to crystallization and the enzyme was buffer-exchanged into 50 mM Tris-HCl pH 7.4. The crystallization conditions consisted of 100 mM MES buffer pH 5 – 6.5, 1.75 – 2.5 M ammonium sulfate and 400 µM cYY at 4ºC in a vibration-free crystallization refrigerator (Molecular Dimensions). The protein was mixed in a 1:1 ratio with the reservoir solution for a total drop volume of 3 µL. Crystals appeared within one week. To obtain the cyanide complex the crystals were transferred to a new buffer solution containing 100 mM Tris-HCl pH 8, 2 mM ammonium sulfate and 50 mM sodium cyanide and allowed to soak for two hours. The crystals were then mounted onto a loop and stored in liquid nitrogen prior to data collection.

3.3.2 X-Ray data collection and data processing

X-ray diffraction data were collected at the Stanford Synchrotron Radiation Lightsource beamline 9-2. Diffraction data were collected under liquid nitrogen, at 100 K and processed using the software HKL-2000 (157). The ternary [Fe(III)CYP121(cYY)CN] structure was solved by molecular replacement using coordinates from PDB 1N40 (108) as the search template and the Phaser-MR (158) software in the PHENIX program package. The software Coot (159) and Phenix (160) were used for model building and refinement.
3.4 Results

The crystal structure of the ternary ESCN complex (Figure 3.1) were obtained by soaking crystals of the ES complex in 50 mM NaCN at pH 8 for 2 hr. The ESCN crystals were found to belong to space group $P6_522$ (Table 3.1), as previously observed for crystals of the enzyme and enzyme-substrate complexes (118). The structure was solved by molecular replacement and refined to 1.4 Å. The $F_o-F_c$ map of the ESCN complex (Figure 3.1A) clearly shows the density for both the cYY substrate bound in the active site and the cyanide coordinating in a linear end-on binding mode to the six-coordinate iron. A 2.0 Å Fe-C distance was observed with a nearly linear Fe-C-N geometry of 175 degrees. Two active site residues, Arg386 and Ser237 were also perturbed due to the presence of the bound CN ligand. The distance between the proximal tyrosine OH of cYY and Arg386 is reduced by 0.5 Å from 3.5 to 3.0 Å. Moreover, the Ser237 is rotated ~20 degrees away from the CN binding site presumably due to steric clashing from the nitrogen atom.
Figure 3.1 Crystal structure of CYP121 ternary complex with cYY and CN. A, stereo view of the electron density map for ESCN crystal structure at 1.40 Å, cYY highlighted in yellow and heme is shown in dark raspberry. The 2Fo-Fc density (mesh) is contoured at the 3σ level. B, comparison of the ESCN complex (colored) with the ES complex (PDB 3G5H, 1.4 Å; gray). C, the H-bonded water network of the ES complex. D, overlay of the enzyme-cYF complex (PDB 4IQ9, 1.8 Å; gray) with the active site of the ESCN was overlaid with the.
3.5 Discussion

The overall position of the residues lining the substrate binding pocket was unaffected by CN binding when compared to the previously published binary ES complex (Figure 3.1B) (118). While the position of the distal tyrosine of the substrate was unaffected by cyanide binding, the binding geometry of the proximal tyrosine was significantly affected, which was observed to move by 1.0 Å toward the cyanide ligand. The binding of cyanide to the metal reorganizes the active site structure, and in turn, positions the phenol group of the proximal tyrosine, which would position the substrate closer to the heme for subsequent oxidation during catalysis.
### Table 3.1 X-ray crystallography data collection and refinement statistics

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*a*The values in parentheses are for the highest resolution shell.

*b*R_{merge} = \sum |I_{hkl}| - \langle |I_{hkl}| \rangle / \sum \sum I_{hkl}, where I_{hkl} is the observed intensity, and \langle |I_{hkl}| \rangle is the average intensity of multiple measurements.

*c*R_{work} = \sum |F_o| - |F_c| / \sum |F_o|, where |F_o| is the observed structure factor amplitude, and |F_c| is the calculated structure factor amplitude.

*d*R_free is the R factor based on 10% of the data excluded from refinement.
The cause of the above observed changes are likely due to the loss of a short hydrogen bonding network from the proximal tyrosine to the iron-bound water. This network involves two ordered water molecules, w1 and w2, which are observed in several published CYP121 substrate-complex crystal structures (3G5H, 1N4G, 4IQ9, 4IPW and 5IBI) (108,113,114,118). Except for 1N4G and 5IBI, the other 3 crystals all contain a substrate with an OH group positioned near the heme similar to the cYY-ES structure. The structure for 1N4G contains the small molecule 4-iodopyrazole which is bound far from the heme center, likely maintaining the integrity of the active site to resemble that of the enzyme only structure (108). Conversely, a similar case can be made for the 5IBI structure, the phenol groups are all pointed away from the heme cofactor. Thus, the binding site near the heme is less crowded allowing for more water molecules to be present which can maintain a hydrogen bond network with w1 and w2.

The other ES structures of CYP121 in the PDB contain small molecules that bind either distinctly compared to cYY or occupy the same space as one of the water molecules. The w1 molecule is bound directly to the Fe center and possess a hydrogen bond to w2, 2.4 Å away. The w2 molecule, in turn, forms a hydrogen bond to the phenol group of the proximal tyrosine moiety which is 2.6 Å away, Figure 3.1C. In the binary ES complex structure, this H-bond network is likely able to help position the hydroxyl group of the proximal cYY near the heme (118). Such a water-based H-bond network is found in both the enzyme only structure and the binary complex but is missing in the ternary ESCN complex. The binding of CN, and possibly O2, to the ES complex apparently displaces the two water molecules from the active site allowing for the reorientation of the proximal tyrosine, bringing it closer to the oxidizing species for reaction. Interestingly, it should be noted that in the previously published structure of CYP121 in complex with cyclo-(l-tyrosine-l-phenylalanine) (E-cYF, PDB entry: 4IQ9), the phenylalanine moiety was
observed to be positioned toward the heme and the second water involved in the H-bond network is not present in this complex (Figure 3.1D) (113). It is likely the more hydrophobic nature of the benzene ring compared to the phenol and the loss of a hydrogen bond to w2 is responsible for disrupting the network. In the structure of E-cYF the phenylalanine group is also observed to be positioned closer to the heme center than the ES with cYY. In the previous study, it was also found that the cYF was firstly hydroxylated to cYY and then reacted further to form the crosslink and mycocyclosin (113).

3.6 Summary

The crystal structure of CYP121 in complex with cYY and CN provide an opportunity to investigate the active site structure as it would mimic the catalytically competent structure with O2 or hydrogen peroxide. The use of the oxygen mimic has revealed several active site changes that prepare the enzyme for catalysis. The disruption of a nearby water network and conformational changes in nearby residues highlight the precise chemical control required for CYP121 to perform such a unique reaction.
4 EVIDENCE FOR INVOLVEMENT OF A HIGH VALENT FERRYL SPECIES IN LMBB2, A NOVEL L-TYROSINE HYDROXYLASE

4.1 Abstract

LmbB2 is a heme enzyme involved in the biosynthesis of lincomycin A as the first step. In contrast to the nonheme tyrosine hydroxylase that uses O$_2$ and tetrahydrobiopterin, LmbB2 is a peroxxygenase that utilizes H$_2$O$_2$ to oxidize L-tyrosine at the ortho position to generate L-DOPA. Here, we employed rapid kinetic methods stopped-flow UV-Vis and rapid freeze-quench EPR spectroscopy to characterize the LmbB2 reaction. We observed an intermediate of LmbB2 with H$_2$O$_2$ or peracetic acid as the peroxide oxidant in the absence of L-tyrosine substrate. The formation of this intermediate is rate-limiting in the presence of the primary substrate L-tyrosine. We identified the intermediate as a ferryl compound ES species, i.e., a ferryl heme and a protein-based cation radical. Double mixing experiments demonstrate that the compound ES species is catalytically competent and it reacts rapidly when mixed with the L-tyrosine substrate. From our results, we propose a possible mechanism for the ortho-hydroxylation of L-tyrosine by LmbB2.

4.2 Introduction

Actinomycetes are a group of gram positive bacteria well known for their abilities to produce a wide range of small molecules with many biomedical properties. These molecules include anthramycin, a pyrrolobenzodiazepine antimicrobial with antitumor properties and lincomycin, a lincosamide antibiotic among many others. Their structural similarities suggest that they are synthesized by a similar set of reactions, together they share a common hydroxyproline precursor that is derived from L-tyrosine (161-163). Previously, the biosynthetic pathway for these
small molecules as well as the hydropyrrole moiety have not been fully characterized. However, due to the need of developing new antibiotics, recent progress has been made to characterize the biosynthetic pathway for several of these molecules. Studies carried out on cell free extracts from the lincomycin A biosynthetic pathway has resulted in the assignment of L-tyrosine hydroxylase and L-DOPA dioxygenase activities for the first steps two steps (164).

The antibiotic lincomycin A is produced by Streptomyces lincolnensis. The gene cluster contains 26 ORFs (lmb genes). The first enzyme whose function was identified was encoded by the gene lmbB1, and catalyzes an extradiol cleaving reaction on L-DOPA. Both the lmbB1 and lmbB2 genes are translationally coupled to each other suggesting they may share a functional relationship between their product and substrates. After the initial identification the lmbB2 gene, its subsequent expression and isolation allowed for in vivo characterization of the expressed protein, LmbB2, and determination of its biological function (18,165). LmbB2 utilizes a His-ligated heme cofactor in the enzyme active site and the oxidant H₂O₂ to carry out the hydroxylation of L-tyrosine to form L-DOPA as the first step of the pathway (Figure 4.1).

The hydroxylation of aromatic molecules has been extensively characterized in biochemistry and is performed by a variety of enzymes each utilizing various cofactors (166). These enzymes comprise six groups known as monophenol monooxygenases and include the thiol-ligated heme-dependent P450s (75), mononuclear non-heme iron monooxygenases utilizing either pterin or alpha-ketoglutarate as an additional cofactor (167), di-iron hydroxylases (168), copper dependent monooxygenases (169), flavin-dependent monooxygenases (170), and the heme-dependent peroxygenases (166,171). LmbB2 is different from any of the aforementioned groups in that it catalyzes aromatic amino acid hydroxylation using a histidine-ligated heme belonging to peroxidase/peroxygenase group. The only other group able to carry out the same chemical
transformation is the non-heme pterin-dependent enzymes (16,166,172). Additionally, LmbB2 utilizes a heme cofactor and H₂O₂ as the oxidant for the same reaction making it a distinct new member of the peroxidase/peroxigenase group of enzymes.

Figure 4.1 Enzymatic pathway for hydropyrrole biosynthesis

4.3 Materials and methods

4.3.1 Expression and purification of ferric LmbB2

The LmbB2 construct was made by cloning the gene sequence into pET16 plasmid with ampicillin resistance. The plasmid was transformed into E. coli BL21 overexpression system. Additionally the construct contains 6xHis-tag at the N-terminus with a Factor Xa cleavage site. Cell cultures of LmbB2 were started by streaking frozen glycerol stock cells onto an ampicillin agar plate and incubated at 37°C overnight. A single cell colony from the agar plate was used to inoculate 10 mL of Luria-Bertani (LB) medium supplemented with 100 μg/mL ampicillin and incubated at 37°C and 220 rpm agitation until the OD₆₀₀ reached 0.6. The starter culture was used to inoculate into 50 mL of LB media containing 100 μg/mL ampicillin to a starting OD₆₀₀ of 0.002 followed by incubation at 37°C and 220 rpm agitation. At OD₆₀₀ 0.6, 500 mL of LB medium was
inoculated similar to the 50 mL media. At OD₆₀₀ value of 0.3, δ-aminolevulinic acid was added to a final concentration of 10 mg/500 mL culture. The cells were induced at OD₆₀₀ of 0.6 using a final concentration of 0.5 mM Isopropyl-β-D-thigalactopyranoside (IPTG) with continued incubation overnight at 28°C and 220 rpm agitation. The cells were harvested the next morning by centrifugation at 8000 x g for 20 minutes at 4°C and stored at -80°C until further use. Cells were re-suspended in lysis buffer (50 mM sodium phosphate buffer pH 8, 300 mM NaCl, 20 mM imidazole) supplemented with 1 mM PMSF protease inhibitor and lysed using a homogenizer (MicroFluidics M-110-P). Cell debris was removed by centrifugation at 27,000 g for 30 min at 4°C and the clarified supernatant was applied to Ni-NTA affinity chromatography resin pre-equilibrated with 50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl, 20 mM imidazole and 5% glycerol. LmbB2 was eluted out using 50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl, 500 mM imidazole and 5% glycerol in a gradient profile. The eluted protein fractions were collected and concentrated using an Amicon stirred-cell concentrator. The protein was then desalted into 50 mM Tris-HCl, 5% glycerol, 10 mM imidazole, pH 8.0 and stored at -80°C.

4.3.2 Pre-steady state kinetics

Rapid kinetic studies were performed on a SX20 Stopped-Flow System (Applied Photophysics). Each reaction assay consisted of 5-10 µM LmbB2 and the concentrations of either oxidant, H₂O₂ or peracetic acid was varied between 0.1 mM to 5 mM. The substrate L-tyrosine was prepared in 0.1 M HCl solution at a stock 20 mM concentrations. The final concentration for L-tyrosine was maintained at 1.5 mM for assay it was used. LmbB2 was pre-incubated with the substrate for several minutes before initiating with oxidant. All experiments were carried out in 100 mM sodium phosphate buffer at pH 8.


4.3.3 Rapid-freeze quench EPR

Rapid-freeze quench experiments were carried out using a System 1000 Chemical/Freeze Quench Apparatus (Update Instruments, Inc.). Experimental set up consisted of an isopentane bath for handling freeze-quenched samples and a secondary liquid ethane both at -130 °C to freeze quench the reactions.

Single mix reactions were carried out by mixing 500 µM LmbB2 in 1:1 ratio with various concentrations of oxidant, either H₂O₂ or peracetic acid. The reaction mixture was passed through an aging hose of various lengths and shot into liquid ethane to obtain samples quenched at various time intervals. Double mixing experiments used a similar setup with the addition of a third ram for the second mix. The reactions were carried out in 100 mM sodium phosphate buffer pH 8.0. The frozen samples were transferred into EPR tubes and excess liquid ethane was vacuumed off. The final frozen samples were stored in a liquid nitrogen containing dewar prior to analysis. EPR spectra were recording at 9 GHz microwave frequency with a Bruker EMX spectrometer sing a 4119HS resonator, perpendicular mode, at 100 kHz modulation frequency. The temperature was maintained at 10 K in an ESR910 liquid helium cryostat by an ITC503S temperature controller (Oxford Instruments). Frozen sample solutions as prepared above was analyzed in 4 mm quartz EPR tubes.

4.4 Results

4.4.1 UV-Vis characterization of enzyme and substrate complex of LmbB2

The EPR spectra of LmbB2 is consistent with that of a high-spin ferric heme. The resting enzyme contains a large signal at $g = 6$ and is typical for the resting state for histidine ligated heme systems (Figure 4.2A). The UV-Vis spectra for the as-isolated enzyme contains a ferric heme with
a Soret maxima at 404 nm (Figure 4.2B). The α/β region contains additional features with peaks found at 499, 543 and 625 nm (Figure 4.2B inset). When LmbB2 is mixed with its substrate, 2 mM L-tyrosine, a 1 nm red-shift is observed concomitant with a decrease in the Soret intensity. In the α/β region an overall slight increase in intensity was observed, however, no other changes were observed after substrate binding. The substrate analogue L-phenylalanine, was tested as well for binding to the enzyme. Similar changes were observed for binding of L-phenylalanine, however, the Soret intensity decreased to a lesser extent compared to the binding of L-tyrosine. These findings are consistent with the same enzyme from a different bacterium, currently named as Orf13, which exhibited very minor changes when incubated with tyrosine.
Figure 4.2 Spectroscopic characterization of LmbB2. A, UV-visible spectra of LmbB2 (5 μM) with native substrate L-tyrosine and substrate analogue L-phenylanine; B, EPR spectra of as-isolated LmbB2 (500 μM) is consistent with high-spin ferric heme at $g = 5.8$. Spectra was collected at 10 K, 9.6 GHz microwave frequency and 1 mW power.
4.4.2 HPLC detection of L-DOPA from LmbB2 assay

When an L-tyrosine/L-DOPA control mixture was injected onto the HPLC column, separation was achieved with L-DOPA eluting out first in 1.5 minutes and L-tyrosine second at 2 mins (Figure 4.3A). Full separation between the two molecules were obtained. HPLC activity assays were performed to determine whether the purified LmbB2 was able to catalyze the hydroxylation of L-tyrosine. LmbB2 was premixed with the substrate for 5 minutes at room temperature. To initiate the reaction, either H$_2$O$_2$ or peracetic acid (300 µM) was added to the reaction system and allowed to react for 10 minutes. The assay was quenched with 0.2 M HCl and centrifuged to pellet the precipitated protein. When the reaction mixture was injected into the HPLC column, a new peak was observed with retention time at 1.5 minutes. The retention time of the new peak matched exactly that of the L-DOPA control experiment. This data highlights that the purified protein is indeed LmbB2 and able to perform its native function in vitro.
Figure 4.3 HPLC analysis of LmbB2 hydroxylase activity with L-tyrosine and substrate analogue Me-O-L-tyrosine. LmbB2 (1 µM) was incubated with either L-tyrosine (1 mM) or Me-O-L-tyrosine (1 mM) and reacted for 10 minutes in the presence of H$_2$O$_2$ (300 µM). A, a new peak at 1.5 minutes is observed corresponding to formation of L-DOPA for the L-tyrosine assay; B, no additional peaks are observed when the analogue Me-O-L-tyrosine is used as the substrate.
4.4.3 Stopped-flow investigation of the reactivity of LmbB2 with peroxide analogues and substrates

To characterize the formation of any catalytic intermediates during the hydroxylation reaction, stopped-flow experiments in which LmbB2 (5 µM) was premixed with either 2.5 mM L-tyrosine, or L-phenylalanine prior to reacting with oxidant peracetic acid (PAA) (250 µM) was performed. When LmbB2, in complex with L-tyrosine was mixed with PAA, the spectra for the Soret peak underwent a small ~2 nm red shift from 405 to 407 nm (Figure 4.4A). During the time course, there was no significant amount of heme bleaching observed as is typical for hemoproteins reacting with peroxides. A slight increase in the broad α/β region was also observed. The substrate specificity for the homologue protein, Orf13 from the anthramycin biosynthetic pathway was tested suggesting this group of enzymes are highly specific to and require substrates that contain a phenol group at the para position to generate the corresponding catechol product (17). The spectral changes for when the substrate analogue, L-phenylalanine is bound to the enzyme was also tested as a control. Upon mixing the complex with PAA the Soret max red shifted 6 nm. During the reaction, a considerable amount of heme bleaching was observed as indicated by the decreased intensity of the Soret peak and a broad increase in the α/β region whose profile became less distinct. As a control, the substrate analogue L-phenylalanine was also tested. In the assay, a larger 6 nm red shift was observed suggesting an increased formation of the intermediate (Figure 4.4B). This data suggests that in the presence of substrate, the accumulation of the enzyme based intermediate is hampered. The data suggest that an observable intermediate species may form during the reaction cycle. The different changes observed in the Soret max for the L-tyrosine and L-phenylalanine suggest that the two different species may be formed.
Figure 4.4 Stopped-flow UV-Vis characterization of intermediate formation in the presence of substrates and substrate analogues. LmbB2 (5 µM) is premixed with: A, L-tyrosine (1.5 mM); B, L-phenylalanine (1.5 mM) and reacted with peracetic acid (250 µM); C, a double-mixing experiment for which LmbB2 (5 µM) was reacted with peracetic acid (250 µM) for the initial mix and followed by a second mix with L-tyrosine (1.5 mM).
As a control to the previous set of experiments, the reactivity of LmbB2 with different oxidants in the absence of substrates were also performed. The enzyme was reacted with either H₂O₂ or PAA and monitored by stopped-flow UV-Vis spectroscopy. When reacting LmbB2 with oxidant, H₂O₂ or an organic peroxide peracetic acid (PAA), the heme spectra undergoes several changes throughout the time course of the reaction (Figure 4.5). Upon reaction with 5 mM H₂O₂ a rapid decay and ~5 nm red shifting (405 nm to 410 nm) is observed for the Soret peak (Figure 4.5A). Concurrent with the changes in the Soret peak, the α/β region changed as well during the reaction cycle. Specifically, during the time course two new peaks centered at 540 nm and 574 nm both rise in intensity within 0.3 seconds after which these features decay at longer times (Figure 4.5A inset). A reaction of LmbB2 with peracetic acid produces similar spectral changes as with H₂O₂ with some differences described as followed. Peracetic acid produces a larger 8-nm shift to a new Soret max of 413 nm opposed to 410 nm and in the α/β region a new broad peak at with features at 525, 560 and 600 nm is generated (Figure 4.5B, inset). The maximum change for the intermediate formations were observed at 574 nm and 600 nm when reacting H₂O₂ and PAA respectively (Figure 4.5B, D). Fitting the single wavelength data yielded a formation rate for the H₂O₂ and PAA reactions formed with a \( k_{obs} \) of 3.22 ± 0.12 s⁻¹ and 16 ± 1 s⁻¹ respectively. The data were fit using a single exponential function for the first 300 ms for the H₂O₂ reaction and 10 s for the PAA reaction. While the changes observed in the Soret peak of LmbB2 are very similar when reacting with H₂O₂ or PAA, the α/β region regions behave differently depending on the source of oxidant. For both of the oxidants, the heme spectra does not return to the starting form, instead, continuous decay is observed at longer time periods. This is consistent with the known behavior of heme blenching during reactions with excess peroxides. The UV-Vis profile at longer times suggests that under these conditions LmbB2 undergoes considerable heme bleaching and loss. The
milder conditions required for reaction with peracetic acid and the larger Soret shift allowed for easier characterization of the intermediate species. Herein the following results described will focus on the reaction with PAA.

Figure 4.5 Stopped-Flow UV-Vis kinetic characterization of LmbB2 (5 µM) reacting with H2O2 (5 mM) and peracetic acid (250 µM). A, full spectra of the reaction with H2O2 as the oxidant monitored for 5 s; B, single wavelength trace of intermediate formation monitored at 574 nm; C, full spectra of the reaction with PAA as the oxidant and monitored for 10 s; D, single wavelength trace of the intermediate formation monitored at 600 nm. The insets show the α/β region of the starting spectra and intermediates.
4.4.4 Reactivity of the LmbB2 intermediate with L-tyrosine in sequential mixing experiments

To test the catalytic competency of the LmbB2-based species generated with PAA, a double mixing experiment was performed using the stopped-flow set up. The initial mix was used to generate the enzyme based intermediate followed by a second mix with L-tyrosine. Within the mixing time of the instrument, introduction of L-tyrosine resulted in almost complete decay of the enzyme intermediate. The initial spectra after mixing with L-tyrosine contained a Soret maximum at 407 nm which is closer to that observed for the starting enzyme as opposed to 413 nm which is observed when LmbB2 reacts with peracetic acid alone (Figure 4.4C). When the enzyme was allowed to react for longer times a new species is formed with the Soret max shifted to 410 nm.

4.4.5 Identification of the enzyme based intermediate using RFQ-EPR

In order to determine the nature of the enzyme based species, rapid freeze-quench (RFQ) experiments were performed and samples were analyzed using continuous-wave EPR spectroscopy. RFQ samples were prepared by mixing LmbB2 enzyme with peracetic acid and allowed to react for varying times before quenching in liquid ethane. The resulting samples were then analyzed by EPR at 10 K.

When LmbB2 is mixed with PAA and subsequently quenched in liquid ethane, a new radical species is observed at g = 2.0087 (Figure 4.6B). The new radical signal contains a peak-to-peak line width signal of 21 gauss, this high value is indicative of a radical localized on an aromatic amino acid such as L-tyrosine or L-tryptophan. Additionally, a decrease in the ferric signal is also observed, presumably due to the formation of an EPR silent species. A time course of the reaction shows the intensity of the radical signal is inversely related to the intensity of the ferric signal and are closely linked to each other (Figure 4.7). Together these changes indicate the formation of an
enzyme based intermediate which is assigned as a compound ES. This compound ES is an EPR silent ferryl iron associated with a nearby radical that is likely located on an aromatic amino acid.

Figure 4.6 EPR analysis of LmbB2 (250 µM) reacting with peracetic acid (250 µM). The reaction was quenched at multiple time points. A, EPR spectra of the ferric high-spin heme signal monitored during the reaction shows a decay and return of the signal; B, a new radical is observed during the time course of the reaction with a signal at $g = 2$. EPR spectra was obtained at 10 K, 9.6 GHz microwave frequency and 1 mW power.
Figure 4.7 Integration of the high-spin ferric signal and the g = 2 radical signal. Reaction of LmbB2 with peracetic acid shows the decay of the high-spin heme signal follows closely to the formation of the radical signal. Formation of an EPR silent species with a radical is consistent with the assignment of a ferryl intermediate and a protein based radical localized on an aromatic amino acid.

In order to test whether the observed compound ES species was reactive towards L-tyrosine to support LmbB2’s hydroxylase a double mixing EPR experiment was performed similar to the stopped-flow experiments. The first mix with oxidant was used to generate the enzyme based intermediate, a second mix with either L-tyrosine or L-phenylalanine were performed and the reaction mixture was immediately quenched in liquid ethane (Figure 4.8). When the LmbB2 intermediate species was mixed with L-tyrosine, a rapid decay of the radical was observed within the mixing time of the RFQ. In contrast, when L-phenylalanine was used for the second mix, the intermediate radical signal was present in the spectra and did not decrease. This data corroborates
the observations by stopped-flow and taken together suggests that the compound ES intermediate is catalytically competent and active towards the native substrate L-tyrosine.

Figure 4.8 EPR analysis of intermediate reactivity with substrates after double mixing. LmbB2 (250 μM) enzyme was first reacted with peracetic acid (250 uM) for the initial mix, a secondary mix was followed with either L-tyrosine or L-phenylalanine. The samples were quenched immediately after the second mixing. A compound ES species is formed after reacting with peracetic acid (black trace). Second mix with L-tyrosine shows decreased radical signal (red trace). A second mix with L-phenylalanine shows the compound ES unreactive towards the substrate analogue (blue trace). EPR spectra was obtained at 10 K, 9.6 GHz microwave frequency and 1 mW power.
4.5 Discussions

In this study, we identify an enzyme based intermediate from the reaction of LmbB2 with either H\textsubscript{2}O\textsubscript{2} or peracetic acid as the main oxidant source. The UV-Vis and EPR spectroscopic properties of this intermediate were characterized and support its identification and assignment as an ferryloxo species, compound ES. The catalytic competency of the compound ES was demonstrated through double mixing experiments by both EPR and stopped-flow UV-Vis.

Oxoferryl species have been studied and characterized in many different enzymes including dehaloperoxidase (173), eosinophil peroxidase (174), and ascorbate peroxidases (175), among others. The observed UV-Vis profile changes are similar with spectra profiles for ferryl species identified in other peroxidase enzymes (173). The profile is consistent with formation of either a compound II or compound ES species. While these two ferryl intermediates possess spectra signatures distinct from compound I, however, they cannot be distinguished from each other by UV-Vis spectroscopy alone. Compared to the enzyme only reactions, the intermediate did not accumulate when LmbB2 was premixed with L-tyrosine whereas the L-phenylalanine complex did present formation of the species. These findings highlight that the formation of the intermediate ferryl species is dependent on the absence or presence of a reactive substrate.

In order to support the assignment of a ferryloxo iron intermediate in the reaction cycle of LmbB2 with peroxide and to distinguish between compounds II and compound ES, EPR experiments were performed to further investigate the intermediate species. The EPR data provided strong evidence in support for the formation of a compound ES-type species specifically, instead of either a compound II or other non ferryl type intermediate. Upon mixing LmbB2 with oxidant, the high-spin ferric species signal of the resting state decays slightly without formation of any low-spin heme. The oxidant states of iron that are EPR silent are the ferrous and ferryl
oxidation states. This reaction system does not contain any reducing agents and peroxides are not able to donate electrons to the ferric ion. This provides evidence that the observed decay of the ferric signal is due to the formation of an EPR silent ferryl species. Coupled to the changes in the high-spin region of the EPR spectra, a new radical species was also observed during the reaction at the same time. Its formation was closely aligned with the decay of the ferric heme (Figure 4.7). The radical portion of this intermediate species as observed by EPR contains a large peak-to-peak linewidth at ca. 22 gauss. This is indicative of a protein-based radical that would be associated with the compound ES. The RFQ quench experiments for LmbB2 reacting with peracetic acid has allowed us to confidently assign the identity of the intermediate species observed by the stopped-flow experiments. The combined changes in the low and high g-value region of the EPR spectra suggest that the new intermediate is likely a high-valent, ferryloxo species. While only a compound ES-type intermediate is observed, we cannot preclude the possibility for the formation of a compound I-type of intermediate that does not accumulate, rather, would rapidly decay to compound ES.

After initial identification of the intermediate in LmbB2 as a compound ES-type species, we further demonstrate it is a catalytically competent species. Our previous experiments showed that the compound ES did not form when the known substrate was present in the active site. There are two possible hypotheses to explain the observations made for reactions in the presence and absence of L-tyrosine. The first hypothesis postulates that the intermediate rapidly reacts with the native substrate L-tyrosine when present in the active site. In this scenario the intermediate would not be allowed to accumulate to sufficient levels needed for detection. The second hypothesis is that the intermediate is off pathway, and only formed in the absence of substrate and not catalytically relevant. Upon binding of L-tyrosine, the addition of oxidant may initiate the natural
reactivity of LmbB2 which does not contain a compound ES but an alternate species to oxidize the substrate.

In order to test and differentiate between the competing hypotheses, double mixing experiments were carried out under multiple conditions. Using double mixing experiments, we tested if the decay of the compound ES species would be accelerated in the presence of substrate indicating that this intermediate is able to react with L-tyrosine. The results of the experiment showed a rapid decay of the compound ES intermediate back to the resting state when L-tyrosine was utilized for the second mix. The reaction was quenched using the shortest possible reaction time (15-20 ms), after which EPR analysis revealed a near complete depletion of the original radical signal. In contrast, when the substrate analogue L-phenylalanine was used, no reactivity towards the compound ES intermediate was seen. This is consistent with a previous finding that this family of enzymes possess structural selectivity to substrates that are substitute at the para position with a hydroxyl group and L-phenylalanine was not an active substrate. Double mixing experiments with L-tyrosine demonstrates that within the reaction cycle to generate L-DOPA, compound ES is likely an on-pathway intermediate, participating as an oxidizing species. Additionally, we demonstrate that the LmbB2 compound-ES species is also highly reactive towards the native substrate. This is evident by the near complete consumption of the compound ES radical in the enzyme within the mixing time of the experiment. The results from the double mixing experiments monitoring the reactivity of the compound ES species with substrate were observed in two independent experiments, stopped-flow UV-Vis and RFQ-EPR spectroscopy. The combined data is strong evidence that the most likely candidate for the intermediate that performs the ortho-hydroxylation on L-tyrosine is the compound ES species.
We propose a mechanism (Figure 4.9) for the hydroxylation of L-tyrosine by LmbB2 that proceeds via a high-spin iron(oxo) species. The reaction proceeds first with binding of the substrate and H₂O₂ to generate an iron(oxo) with a nearby cation radical located on a protein derived amino acid. Abstraction of the hydroxyl hydrogen atom by the intermediate will generate a substrate radical and protonated ferryl species. Migration of the radical to an ortho carbon allows a rebound-like mechanism for the addition of the OH group to L-tyrosine and finally yielding the product L-DOPA. To further test the proposed mechanism the substrate analogue Me-O-L-tyrosine was utilized which would inhibit the initial proton abstraction step by the compound ES species (Figure 4.3B). When incubating LmbB2 with the substrate analogue, no peak corresponding to DOPA or any other products were observed. This result strongly favors the proposal that the first step in the hydroxylation of L-tyrosine is deprotonation of the hydroxyl group.

Figure 4.9 Proposed mechanism for ortho hydroxylation of L-tyrosine by LmbB2.
4.6 Conclusion

LmbB2 and homologues represent a new class of heme peroxygenases that are able to catalyze the hydroxylation of aromatic amino acid residues. After the initial isolation and characterization of both LmbB2, we present the first investigation in the enzyme mechanism and reaction pathway. Our study of LmbB2’s heme reactivity with its native substrates L-tyrosine and H$_2$O$_2$ as well as with the oxidant analogue peracetic acid has allowed us to identify the formation of a ferryl species within the catalytic pathway. Our initial characterization of the nature of the intermediate suggests it is most similar to a ferryl(oxo) compound ES intermediate species. While ferryl species are very elusive and often difficult to capture due to their highly reactive nature with its native substrate, significant progress has been made into their characterization. Within LmbB2, the compound ES is found to be highly reactive and is presumably unstable. The formation of the compound ES does not accumulate enough to allow for more robust characterization. However, we were successfully in observing its high reactivity with the substrate L-tyrosine, suggesting it participates as in part of the mechanistic cycle.

LmbB2 utilizes a different mechanistic pathway for the hydroxylation of aromatic amino acids in nature, a reaction previously only performed by pterin-dependent enzymes. The non-heme enzymes utilized a pterin/BH$_4$ cofactor to provide additional electrons allowing for the activation and reduction of oxygen. In contrast, the heme enzymes utilize hydrogen peroxide as the oxidant which is already two electrons reduced compared to O$_2$. Both enzymes utilize a strongly activated oxygen in the form of a ferryl(oxo) signifying the difficulty in oxidizing very stable aromatic ring systems. The different cofactors used by these two enzymes may be related to the biological purpose of their reaction. In mammals, this reaction is used to synthesize several important neurotransmitters including dopamine, norepinephrine, and epinephrine. These neurotransmitters
a crucial for the proper functioning of many biological organisms. Conversely, this reaction is used to synthesize antibiotics in certain bacteria. This is not a critical reaction in bacteria, thus it could be assumed that nature would use a different strategy that uses less resources. The use of heme and hydrogen peroxide forgoes the need to use the more valuable pterin cosubstrate and makes this reaction more cost effective to produce the smaller quantities of antibiotics that may be needed.
5 INVESTIGATING THE ROLE OF A SECOND-SPHERE PROLINE RESIDUE OF AN EXTRADIOL DIOXYGENASE IN THE KYNURENINE PATHWAY

5.1 Abstract

3-Hydroxyanthranilic acid 3,4-dioxygenase (HAO) is a non-heme iron-dependent enzyme characterized as a type III extradiol dioxygenase belonging to the cupin superfamily. Comparison of the active site of HAO with well-characterized type I dioxygenases reveals similarities in regard to the metal binding ligands and their relative geometry despite distinct differences in their overall structural scaffolds. The second sphere ligands, however, are not as well conserved. In particular, an active-site histidine acid-base catalyst conserved in type I dioxygenases is not found in HAO, but a Pro97 residue is present in the equivalent position. In the present work, three proline mutants were generated with either an alanine, valine to probe the catalytic contributions of the residue, or with a histidine substitution to mimic the second sphere ligand found in type I dioxygenases. X-ray crystal structures were obtained for the P97A, P97V, and P97H mutants at 2.7, 2.35 and 1.79 Å, respectively. The crystal structures reveal that proline substitution induces significant conformational changes to the protein backbone distant from the active site. The conformation of several key residues within the active site are disrupted in P97H. Moreover, activity assays showed that while both alanine and valine mutants remained as active enzymes, P97H was nearly inactive. Structural studies on the mutants revealed conformation changes to two key arginine residues responsible for binding and properly orienting both the organic substrate and oxygen. This effect is most pronounced in the histidine mutant. These results suggest that Pro97 in HAO is important for the active site geometry.
5.2 Introduction

In nature, there exists a large group of non-heme, iron-dependent ring-cleaving dioxygenases capable of catalyzing the activation and insertion of molecular oxygen into a wide variety of substituted aromatic molecules \((22,47,53,176)\). This group of enzymes have been subdivided into two sets differentiated from each other by their mechanism and position to which \(O_2\) is inserted into the substrate to cleave the ring. The first set is termed extradiol dioxygenases, the second type being intradiol dioxygenases. The extradiol dioxygenases cleave the aromatic ring between a hydroxylated carbon and an adjacent non-hydroxylated carbon, whereas intradiol dioxygenases cleave the bond between two hydroxylated carbons \((22,53,89,176,177)\). Extradiol ring-cleaving dioxygenase genes are more commonly found in nature \((53)\).

Within the large family of extradiol dioxygenases, three distinct subfamilies designated as type I, II and III enzymes have been identified based on their primary sequence and overall tertiary structure fold \((178,179)\). Type I enzymes were the first to be characterized and share partial sequence similarity with the type II subfamily. Type II enzymes contain two domains with a similar folding pattern that are believed to have evolved through gene duplication of the type I family of enzymes \((178,179)\). Extensive biochemical work has been carried out on both the type I and type II enzymes which has yielded much information regarding their mechanism of action in that many of the key intermediates in the catalytic cycle have been captured and characterized \((22,23,47,53,89,176-180)\).

Studies on extradiol dioxygenases have yielded atomic level detailed structures of substrate- and product-bound complexes, as well as Fe-bound superoxo, alkylperoxo, and oxygen-inserted intermediates such as a gem diol species \((91,181)\). All these intermediates have been captured and characterized in the type I enzyme, homoprotocatechuate 2,3-dioxygenase \((2,3-\)
HPCD). Thus, the enzyme 2,3-HPCD is well-characterized and serves as the prototype for catalytic mechanism of the type I extradiol dioxygenases. The proposed catalytic cycle for 2,3-HPCD involves an active site acid-base catalyst, His200. This His200 is responsible for protonation of the Fe(II)-bound alkyperoxo intermediate during the reaction and thus serves as an active site acid assisting in O-O bond cleavage (94,182). While a significant amount of work has been carried out on type I enzymes, the details regarding oxygen activation and insertion of the type III enzymes remain dubious. Much less work has been carried out for the type III enzymes and therefore little is known about their overall mechanism.

The most well-studied member of the type III subfamily is 3-hydroxyanthranilic acid 3,4-dioxygenase (HAO) (183-202). HAO catalyzes the extradiol ring-cleaving addition of molecular oxygen to its substrate, 3-hydroxyanthranilic acid (3-HAA) (Figure 5.1) (183,203-205) producing an unstable product 2-amino-3-carboxymuconate-6-semialdehyde that spontaneously converts to quinolinic acid via autocyclization (202,206-209). HAO is found in the tryptophan catabolic pathway through kynurenine and in 2-nitrobenzene degradation pathway (200,210,211). While several studies have revealed insights regarding the details of how substrate and molecular oxygen, as well as several inhibitors, bind within the active site of HAO (192,193), there is yet not enough information to allow for the formulation of a consensus chemical model for either HAO or type III enzymes.

![Figure 5.1 Extradiol ring-cleaving reaction catalyzed by HAO](image-url)
Several crystal structures have been solved from various sources which show HAO is a member of the cupin superfamily (189,192,212,213). The active site iron of HAO contains the canonical 2-his-1-carboxylate coordination motif employed by many non-heme iron enzymes. While both 2,3-HPCD and HAO share the same set of metal ligands, as well as their relative positioning, no comparable histidine in the secondary ligand sphere functioning as an active site acid-base catalyst is found in HAO in the equivalent position (Figure 5.2). Additional investigation of the HAO active site did not reveal any other histidine residues nearby which can fulfill such a catalytic role. This may be explained that the substrate itself can play an acid-base function needed for catalysis. It has come to our attention that in the position equivalent to His200 in 2,3-HPCD, a Pro97 is present in the active site of HAO. This proline residue is located 3.5 Å away from the distal end of the Fe(II)-bound oxygen and is near to the substrate binding pocket. Also, a multiple amino acid sequence alignment after a blast search revealed that the Pro97 residue was strictly conserved among HAO sequences from various sources. Here, we intended to employ site-directed mutagenesis to transform the HAO type III active site into a type I-like catalytic center by altering the proline to histidine at the Pro97 position, and to simulate the removal of Pro97 to probe the role of the proline residue either by an alanine or valine. The alanine in P97A was chosen to decrease the hydrophobicity provided by the Pro97 residue within the local environment while also allowing for more flexibility due to a less rigid backbone conformation. In the second mutant, the valine served to maintain the same hydrophobicity as Pro97 since they both share a three-carbon backbone while simultaneously also allowing for more flexibility within the active site. These mutants were then characterized using kinetic assays and X-ray crystallography to elucidate the role, if any, played by Pro97 in the HAO mechanism.
Figure 5.2 Active site comparison of extradiol dioxygenases. A, type III dioxygenase HAO; B, type I dioxygenase HPCD.

5.2.1 Materials

3-HAA, ascorbate, EDTA, Tris-HCl, glycerol, and dithiothreitol were purchased from Sigma-Aldrich at the highest grades unless specified and used without further purification. MgCl$_2$ and PEG 8000 were purchased from Hampton Research.

5.2.2 Site-directed mutagenesis of HAO

The expression system of HAO from *Cupriavidus metallidurans* was previously described (193). P97A, P97V, and P97H mutants were constructed using a QuickChange Lightning site-directed mutagenesis kit (Agilent Technologies). The following complementary primer pairs were used for each mutant:

P97A-F: 5’-GTT CGC CAT TCC GCA CAG CGT CCC G-3’

P97A-R: 5’-CGG GAC GCT GTG CGG AAT GGC GAA C-3’
5.2.3 Protein overexpression and purification

HAO protein and the mutants were overexpressed in E. coli (DE3) cells (Novagen) and purified by nickel affinity chromatography as previously described (209). The protein was then treated with excess EDTA (20 mM) on ice overnight to generate apo-HAO. EDTA was removed by gel filtration chromatography using a G-25 desalting column on FPLC and buffer changed into 50 mM Tris-HCl pH 7.6, 5% glycerol. Purified apo-HAO was stored in -80 °C following flash freezing in liquid nitrogen. Because the holo-HAO binds two equivalents of metal ions in each polypeptide chain (192,209). The metal-free apo-HAO was reconstituted with 4 of freshly prepared Mohr’s salt solution anaerobically under argon protection immediately prior to assay.

5.2.4 Steady-state kinetics

In a typical kinetic experiment, the assay was carried out in 50 mM Tris-HCl buffer pH 7.6 at room temperature. Reactions were initiated by the addition of enzyme at a final concentration 0.05 – 1 µM to a solution of varied 3-HAA substrate concentration typically 1 - 200 µM. Enzyme activity was measured spectrophotometrically by following the formation of product 2-amino-3-carboxymuconic-6-semialdehyde at 360 nm ($\varepsilon_{360} = 47,500 \text{ M}^{-1} \text{ cm}^{-1}$) (193) using an Agilent 8453
UV-vis spectrophotometer. The enzyme activity was monitored for up to 30 s, and the resulting data were fit to the Michaelis-Menten equation shown below.

\[
\frac{v}{[E]} = \frac{k_{\text{cat}}[S]}{(K_m + [S])}
\]  

(1)

5.2.5 Crystallization of the HAO proline mutants

Prior to crystallizing HAO, an additional size exclusion column purification step was carried out to buffer exchange the protein into 20 mM Tris pH 7.6, 2 mM DTT and remove any trace contaminants. All HAO mutants crystallized in the same conditions as the WT enzyme with crystals the same shape as the WT enzyme appearing overnight. Crystals of HAO proline mutants were obtained using the hanging drop method as described previously (209). Briefly, holo-HAO protein (1.5 µL) was mixed in a 1:1 ratio with reservoir solution composed of 100 mM Tris-HCl, 200 mM MgCl\textsubscript{2}, 20% PEG 8000, and 1 mM DTT, pH 8.0 – 8.5. Crystals appeared overnight when incubated at 16°C and were allowed to continue to grow for one week at which point they were mounted onto a loop and stored in liquid nitrogen.

5.2.6 X-ray data collection and data processing

X-ray diffraction data were collected at the 22-ID beamline at the Advanced Photon Source (APS), Argonne National Laboratory, Argonne, IL. Data collection was performed at 100 K and processed using the software HKL-2000. All crystal structures were solved by molecular replacement using the Phaser-MR module found in the program package Phenix using HAO (PDB: 1YFU) as the search model. Electron densities were fit and refined using Coot and Phenix.
5.3 Results

5.3.1 Kinetic characterization of P97H, P97A and P97V mutants

To determine whether Pro97 residue is important for the activity of native HAO enzyme, the reconstituted mutant proteins were analyzed by steady-state kinetics with 3-HAA and O₂. The kinetic parameters of the mutants were compared to wild-type (WT)-HAO. A summary of all kinetic parameters acquired in this study can be found in Table 5.1. The results show that both P97A and P97V mutants contained less but modest levels of activity with $k_{cat}$ values of 1.49 ± 0.07 and 1.20 ± 0.06 s⁻¹, respectively, compared to the WT enzyme at 25 s⁻¹ (192). The $K_m$ values for both P97A and P97V mutants were determined to be 22 ± 3 and 40 ± 5 µM, respectively. The wild-type HAO has a $K_m$ value of 22.4 µM. The P97A mutant showed an identical $K_m$ value to wild-type HAO while the value for the valine was nearly doubled. The activity for the P97H mutant was undetectable at a comparable range of enzyme concentration to the other two mutant enzymes. Only when the P97H enzyme concentration was increased by 200-fold, a very small yet measurable activity was observed when the reaction was followed closely over several minutes (data not shown). However, this markedly decreased activity of the P97H mutant did not allow for determination of the kinetic parameters.

Table 5.1 Summary of kinetic parameters of CmHAO proline mutants.

<table>
<thead>
<tr>
<th></th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}/K_m$ (M⁻¹s⁻¹)</th>
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<tr>
<td>wt-HAO</td>
<td>25</td>
<td>22.4 ± 2.7</td>
<td>1.1 x 10⁶</td>
<td>(192)</td>
</tr>
<tr>
<td>P97V</td>
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<td>22 ± 3</td>
<td>6.72 x 10⁴</td>
<td>This work</td>
</tr>
<tr>
<td>P97A</td>
<td>1.20 ± 0.06</td>
<td>40 ± 5</td>
<td>2.98 x 10⁴</td>
<td>This work</td>
</tr>
<tr>
<td>P97H</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

ND, not detectable
5.3.2 Overall structure of Pro97 mutants

In order to develop a better understanding of the significance of the Pro97 residue and the kinetic differences that were observed after substitution, P97A, P97V, and P97H were each crystallized and their crystal structures determined at 2.7, 2.35 and 1.79 Å respectively (Figure 5.3). The cupin fold and conserved barrel domain previously described for HAO which is characteristic of the cupin superfamily of proteins are conserved in the core structure of all the mutants. Additionally, the crystal packing for both P97A and P97V mutants was not affected, both mutants crystallized with a single monomer in each asymmetric unit and in close contact with a neighboring monomer in the adjacent group. These structures were solved in hexagonal space group $P_{6_5}2_2$. Surprisingly, the crystal packing observed for the P97H is altered compared to the native enzyme and both mutants. P97H crystallized with two protein subunits packed into each asymmetric unit and was solved in a different space group, monoclinic $P12_11$ (Figure 5.4).
Figure 5.3 Subunit-subunit structural alignments of HAO. The wild-type HAO (Grey) with mutants A, P97H (Orange); B, P97A (Blue); C, P97V (Magenta).
Structural alignments of the mutants to WT-HAO revealed local changes along the backbone of the mutant enzymes for all three mutants. Both the P97A and P97V mutants present structural changes that are very similar with respect to each other with some minor differences between the two (Figure 5.3B, C). These structural changes are localized at the N-terminal of helix α3 and sheet β11, helix α2 is also slightly shortened. For P97A, sheets β5, 6 and 7 around the cupin barrel fold are both shortened whereas for P97V only minor changes are observed in the loop region between sheets β5 and 6. The jellyroll β-barrel remains mostly conserved among all the mutants.

Figure 5.4 Crystal packing of WT-HAO and P97H mutant. Structures are solved as a homodimer and asymmetric unit is color coded for clarity. A, WT-HAO contains a single protomer per asymmetric unit with each unit cell containing 12 asymmetric units with 6 pairs of dimers. B, the P97H mutant contains two protomers per asymmetric unit with each unit cell containing 3 asymmetric units.
In contrast to the previously described mutants, P97H exhibits a much greater extent of backbone conformational changes that are localized at both the N and C-terminals compared to the other two mutants (Figure 5.3A). Extensive changes covering the C-terminal are also observed. The most significant of these changes primarily affected the helix α2 and sheet β11, specifically, helix α2 moves ca ~2 Å away from the β-barrel and active site. The changes at the N-terminal primarily affect the loop between residues 21 - 26. This loop is angled closer to the active site. On the β-barrel, a second loop between residues 41 – 48 is also affected and the length of sheet β7 is extended.

In both P97A and P97V the position of both Arg99 and Arg47 residues are undisturbed and overlap with the original position of wild-type HAO (Figure 5.5A, B). In sharp contrast to the P97A and P97V mutants, the P97H mutation induced significant alterations in the conformation of the two arginine residues (Figure 5.5C). The newly introduced histidine side chain bends at an angle away from the metal center towards Arg99 to occupy its original position. This initiates several conformational changes which propagate throughout the enzyme active site. Steric clash with the histidine force the Arg99 side chain to rotate 115° out of its original position to move ca. 5 Å closer towards the Arg47 residue, this in turn causes the Arg47 to move from its original position. The Arg47 residue in P97H mutant is situated more than 5 Å from where the terminal oxygen would be in the HAO ternary complex while the Arg99 residue is 3 Å from the substrate carboxylate as well as facing the wrong direction. Since these residues have been identified to be important for the binding of both oxygen and substrate, this sequence of interactions effectively disrupt the stabilizing forces of both residues. The guanidine head of Arg99 is no longer positioned to properly interact and form a salt bridge with the carboxylate group of 3-HAA for optimum positioning, whereas disruption of Arg7 orientation may lower affinity for binding of molecular
oxygen to the ferrous enzyme. Attempts were made to obtain the binary complex structure for P97H mutant and 3-HAA, however no crystals diffracted with extra density near the active site that would result from substrate binding. It is interesting to note that while Pro97 is located on the cupin jelly roll fold, the effects of its mutation are not restricted to the local environment and instead major backbone changes are located primarily at the N-terminal.

Within the active site of HAO there are two arginine residues, Arg99 and Arg47. They are near to and responsible for proper binding and orientation of both substrates, 3-HAA and molecular oxygen, respectively. In the previously solved structure of HAO in complex with its substrate, 3-HAA, the substrate chelates the metal in a bidentate mode, and the carboxylate end is positioned 3 Å away from Arg99 to form a stabilizing salt bridge interaction. Crystal structures of HAO in complex with inhibitor 4-chloro-3-hydroxyanthranilate in the presence of either O2 or NO suggest a role for Arg47. Upon binding of O2 or NO the overall Arg47 sidechain shifts its position 0.8 Å closer to the terminal oxygen atom. Thus, the Arg47 mutant is expected to participate in the binding and stabilization of O2 in the active site.
Figure 5.5 Overlay of mutant HAO active site environments. Wild-type HAO (Grey) with mutants; A, P97A; B, P97V; C, P97H.
Table 5.2 X-ray crystallography data collection and refinement statistics

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<td>P 6 5 2 2</td>
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<td>1.23e&lt;sup&gt;-17&lt;/sup&gt; (1.65e&lt;sup&gt;-18&lt;/sup&gt;)</td>
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<sup>a</sup>The values in parentheses are for the highest resolution shell.

<sup>b</sup>\(R_{merge} = \frac{\sum |I_{hkl}| - \langle I_{hkl} \rangle}{\sum |I_{hkl}|} \), where \(I_{hkl}\) is the observed intensity, and \(\langle I_{hkl} \rangle\) is the average intensity of multiple measurements.

<sup>c</sup>\(R_{work} = \frac{\sum |F_o| - |F_c|}{\sum |F_o|}\), where \(|F_o|\) is the observed structure factor amplitude, and \(|F_c|\) is the calculated structure factor amplitude.

<sup>d</sup>\(R_{free}\) is the \(R\) factor based on 5% of the data excluded from refinement.
5.4 Discussion

Decreasing the hydrophobicity at the Pro97 position by introduction of an alanine or increasing flexibility while maintaining the same hydrophobic contribution via a valine substitution yielded mutants with comparable catalysis rates to each other, however the $k_{cat}$ values for each mutant were one order of magnitude less than that of the WT enzyme. Mutations within the active site of an enzyme can often have deleterious effects on the rate of catalysis even if the residue does not participate directly in the chemistry. One possibility for the observed activity of the Pro97V and Pro97A mutants can be ascribed to the disruption the dynamic properties within the active site. Pro97 is a very rigid residue and its close proximity to the catalytic center may suggest it plays a more structural or dynamic role to maintain the integrity of the active site during catalysis. The removal of a rigid proline ligand from within the HAO active site may allow more conformational dynamics of nearby residues. Another possibility for the observed effect may be the affinity for oxygen has been lowered in the two mutant enzymes due to affecting the hydrophobicity of the binding pocket. However, this scenario is less likely, while the alanine has decreased hydrophobicity relative to proline, the valine substitution should maintain very similar levels of hydrophobicity compared to the WT enzyme. Additionally, the observation that substitution of the Pro97 residue resulted in significant conformational changes in the protein backbone suggests that a conformational/dynamic role would best explain the observed findings.

Prior to this work, no study had been done on a type III extradiol dioxygenase to investigate the functional differences of second sphere ligands with type I extradiol dioxygenase. This work specifically focuses on the second sphere Pro97 ligand in HAO, a type III extradiol dioxygenase, that is spatially equivalent within the active site to a His200 found in the type I enzyme, 2,3-HPCD. The similarly positioned residue in the well-studied type I dioxygenase enzymes, His200 in 2,3-
HPCD, was determined to be responsible for protonating the bound oxygen atom of the alkylperoxo intermediate during catalysis. Less work has been done in type III extradiol dioxygenases with no information in the literature to implicate the Pro97 of HAO in the catalytic cycle. Mutant variants of the Pro97 residue were generated while enzymatic and structural characterizations were carried out on each mutant. The results show that the removal of Pro97 in the P97A and P97V mutants had little impact on the active site structure. Conversely the addition of a histidine, whose side chain has a much higher degree of freedom than proline, induced a alternative conformations of several key residues within the HAO active site, nearly abolishing its catalytic activity. It was also found that in the three mutant proteins the removal of the active site Pro97 prompted conformation changes throughout the protein backbone and were most pronounced in the P97H mutant. Additionally, the three loops previously found to move closer to the active site after substrate binding in the ES complex were also affected. These structural changes led to decreased enzymatic activity to near undetectable levels. While the Pro97 residue does not participate in the dioxygenase mechanism, it may function to preserve the integrity of the overall enzyme structure which can be important for maintaining high levels of activity. The most intriguing finding in the study were the long-range remote influences of a second-sphere residue on distant backbone conformations. These changes were centered on helix α2 and the rubredoxin-like site in P97H (209). In both P97A and P97V mutants the changes were spread throughout the protein backbone partially affecting helices α1 – α3, β-sheets β6 and β7 of the cupin fold, and β11. While type I dioxygenase enzymes contain a conserved histidine required to function as an active site base, this study demonstrates that this requirement is not held for.

The type I extradiol dioxygenase enzyme HPCD has been very well studied in the literature. Many of its mechanistic steps have been well studied and characterized, and the function
of key residues participating in the chemistry is known. Two such residues are Tyr257 and His200. The Tyr257 residue is responsible for deprotonating one of the substrate oxygens, while His200 plays key role as an active site acid catalyst and is responsible for facilitating the cleavage of the O-O bond in the alkylperoxo intermediate. The crystal structure of HAO suggests that the Glu110 residue is a likely candidate to replicate the function of Ty257. However, there is no equivalent histidine residue present in HAO to help cleave the O-O bond during the catalytic cycle. One possible proton source is the substrate itself. 3-HAA contains an amino nitrogen group that contains a proton that could be used to protonate the alkylperoxo intermediate, thus absolving itself of the need for a histidine residue. These points highlight the difference in the two types of extradiol dioxygenases enzymes and demonstrate that there is still much to be discovered within this enzyme family.
6 CONCLUSION

The idea that enzymes could incorporate molecular oxygen into small molecules was first proposed and demonstrated in 1955 (214,215). This gave rise to a new field of enzymology that allowed chemist to elucidate how nature is able to use the highly reactive oxygen molecule and insert it into a broadly diverse group of compounds. Since this initial landmark reporting, more than six decades have passed and the maturation of this oxygenase field has been tremendous and is now one of the most well studied enzymology fields. The work in this dissertation focuses on the oxygenase chemistry of three enzymes, CYP121, LmbB2 and HAO. While HAO has been previously well studied, both CYP121 and LmbB2 did not have a robust mechanistic understanding of the chemistries they catalyze. This dissertation attempts to fill in some of the gaps in knowledge for these enzymes.

CYP121, a P450 enzyme, performs a unique C-C crosslinking between two tyrosine moieties that are present on its substrate, cYY. CYP121 potentially represents a new and growing subclass of P450 enzymes not performing the oxygen insertion chemistry. We provide an initial understanding regarding the oxygen activation mechanism for this P450 enzyme. We trapped the first intermediate in the reaction cycle, an unusual alkylperoxo species. Initial EPR characterization of this intermediate revealed it to be a high-spin. Probing the nature of the substrate binding revealed a two-step process with each step being reversible. These two findings represent the first significant advancement made in the understanding of the initial reaction steps involving both substrates of CYP121.

The second enzyme presented in this dissertation, LmbB2, is a heme enzyme which catalyzes the ortho-hydroxylation of L-tyrosine. This reaction has previously been described in non-heme enzymes, however, LmbB2 and its analogues are the first examples of this reaction
being performed by heme enzymes. We generate and identify the first enzyme based intermediate in LmbB2, a high-valent ferryl species spectra properties similar to that of compound-ES that has been observed in other peroxidase enzymes. The compound-ES species is probed further by testing its reactivity towards tyrosine and several substrate analogues. By two complimentary rapid-kinetic methods we determine the intermediate is catalytically competent as well as being specific towards the native substrate of LmbB2.

The final enzyme presented in this dissertation is the extradiol dioxygenase, HAO, and is a non-heme iron-dependent enzyme. In contrast to CYP121 and LmbB2, HAO has been the most researched system. This dissertation takes a novel approach to obtaining a greater mechanistic understanding of HAO. We identify a strictly conserved Pro97 residue within the active site which is positioned similarly to a mechanistically critical His200 in another analogous system, HPCD. Structural characterization of a P97H mutant, mimicking the HPCD active site, reveals a surprising role of maintaining overall protein fold. Significant backbone changes are observed in the P97H mutant as well as a considerable reorganization of important ligands in the active site environment.

The work outlined in this dissertation focuses on developing a model for how metalloenzymes are able to carry out unique chemical transformations that are both physiologically and pharmaceutically important. The systems chosen for this study are unique and potentially represent a new subfamily of enzymes, two of the enzymes presented perform non-canonical chemistries for their cofactors. CYP121 performs an uncharacteristic C-C cross-coupling reaction as a P450 and LmbB2 possibly expands the many functions of heme-enzymes to include that of aromatic amino acid hydroxylation chemistry. The work presented in this dissertation also has significant relevance to those in the biomedical field, an example of which can be found in the work of CYP121. CYP121 contains potential as a promising drug target to
combat TB infection cases in patients. The work performed here regarding substrate binding is uniquely critical for further research into developing inhibitors that can bind tightly to the active site and limit activity \textit{in vivo}.
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