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THE EXAMINATION OF COMPOUND II FORMATION IN CYTOCHROME P450S

LEON LIN
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Reviewed and approved* by the following:

Michael Green
Associate Professor of Chemistry
Thesis Supervisor

Pshemak Maslak
Associate Professor of Chemistry
Honors Adviser

Thomas E. Mallouk
Professor of Chemistry

* Signatures are on file in the Schreyer Honors College.

ABSTRACT

Cytochrome P450's are cysteine ligated heme enzymes found in a large variety of organisms and are able to oxidize C-H bonds, which are typically unreactive, and create functional substituents. The ability of P450 to catalyze oxidations on unreactive substrates is quite remarkable and is used in many processes such as metabolism of pharmaceuticals. The power of P450s to oxidize C-H bonds lies in their reactive intermediates compound I and compound II. The specific P450 being examined, CYP158A1 from *Streptomyces coelicolor*, yields significant amounts of compound II after site-directed mutagenesis. The electron reduction potential of compound I and the pKa of compound II plays a crucial role in the driving force for C-H hydroxylation. In order to examine compound II, CYP158A1 must be manipulated to increase the yield and stability of compound II. This is done through the changing of buffer environments and residues around the heme. Site-directed mutagenesis is used to replace tyrosine residues with phenylalanine residues which allow a high yield of compound II to be produced via peroxide shunt. The determination of the third P450 ferryl pKa of 12.615 is also done through the use of a pH jump experiment in conjunction with stopped flow spectroscopy.

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Chapter 1

Introduction

Enzymes

Enzymes run the world. The super-heroes of the chemistry world, enzymes assist in important chemical reactions, but do not appear in a standard written reaction. Enzymes catalyze reactions from production of DNA to photosynthetic phosphorylation. Enzymes themselves are as diverse as the reactions they catalyze. Enzyme cofactors are needed to assist the enzyme in chemical catalysis. These cofactors range from heme to iron-sulfur and dictate the identity and functionality of the enzyme. Metallo-enzymes, in particular, facilitate numerous cell functions such as protein transport, storage, and signal transduction to name a few[1, 2]. Metallo-enzymes differ in structure from a typical protein by incorporating metal ion(s) and an amino acid residue that is ligated to it. The difference in structure is what specializes each enzyme to do a specific reaction and what makes these enzymes so powerful. These ion cores, in addition to the differing ligation and protein scaffold that surround the core, can affect the way the active site accepts a molecule and how it is reacts. [3]

Heme

Heme proteins are a subset of metallo-enzymes where an iron is ligated in the middle of a porphyrin, which is a heterocyclic organic ring. In all cases, a heme contains an iron ion in the center with six coordination sites. Four of these sites are taken by the porphyrin nitrogens, located radially, which leaves two sites open for the binding of an amino acid residue and a divalent atom[1]. The iron core is coordinated by proximal and distal ligands which influences how the enzyme functions. The proximal ligand in this set up is an amino acid residue which is typically a cysteine, histidine, or a tyrosine residue. These different amino acid residues typically correspond to cytochrome P450s, peroxidases, and catalases respectively[1]. The differing amino acid ligation is a large factor in the type of reaction the enzyme can facilitate. The classes of heme proteins consist of cytochromes, heme-containing oxygenases, and globins. These classes of heme proteins function as sources of oxygen transport, electron transfer[4]. Additionally, heme irons can serve as an electron source and sink which can assist and catalyze redox chemistry and functionalize inert bonds in organic chemistry[5]. Heme proteins are able to perform powerful chemistry by activating bonds that are typically unreactive. Interestingly enough, the thiolate ligated P450, on paper, looks less reactive than the histidine ligated peroxidase due to its electron donating nature[6]. However, in application, P450s can functionalize C-H bonds while peroxidases are unable to perform such chemistry. Peroxidases, a histidine ligated system, typically perform oxidation of aromatic compounds, epoxidation, and the oxidation of hydrogen peroxide to oxygen[7]. An exception to this rule is seen in chloroperoxidases which is a type of peroxidase that is similar to the P450s in that is has a cysteine axial ligand[7]. This unexpected behavior between the powerful chemistry of the P450s ability to activate C-H bonds and the inability of histidine ligated peroxidases to the same is

hopefully explained by the amino acid ligation of cysteine and the protein scaffold that separates

these two proteins.
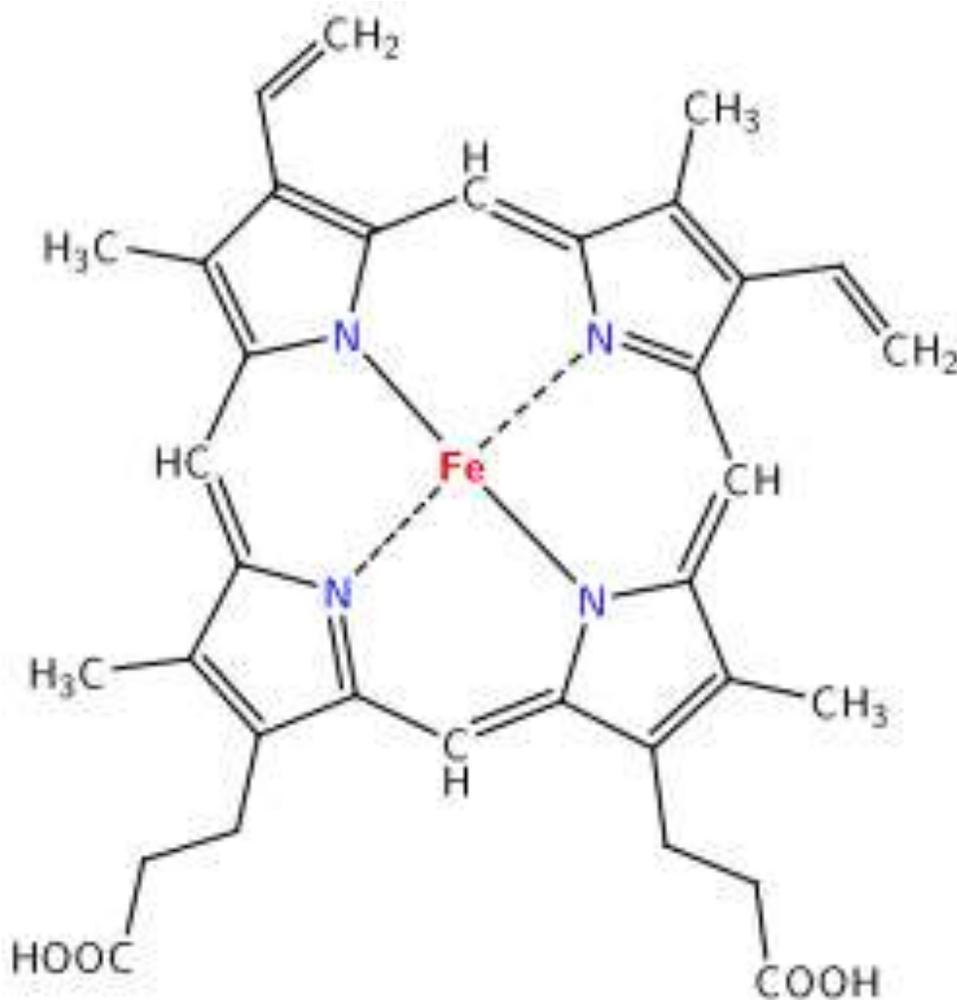
Figure 1 Ferric protoporphyrin IX. This protein scaffold is found in many heme enzymes such as P450s and peroxidases

Cytochrome P450s

Cytochrome P450s are cysteine ligated heme enzymes found in a large variety of

organisms and are able to oxidize C-H bonds, which are typically unreactive, and create

functional substituents[3, 8]. The ability of P450 to catalyze oxidations on un-reactive substrates is quite remarkable and is used in many processes. In mammals, P450s are used to metabolize drugs and convert unsaturated fatty acids to biologically active molecules. The chemical reaction of a P450 in the presence of substrate is typically seen as follows[9]:

$$R\text{-}H + O_2 + NAD(P)H \rightarrow R\text{-}OH + NAD(P)^+ + H_2O$$
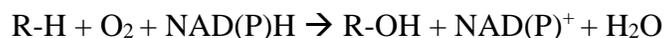
Cytochrome P450s and peroxidases have a ferric resting state, which differs from the ferrous resting state of globins which are known to bind dioxygen. The catalytic cycle of P450s is instigated by the binding of substrate which increases the reduction potential of the heme so it can be more easily reduced. Only after the P450 is in the ferrous resting state can dioxygen bind to the active site and proceed with the mechanism[3]. Peroxidases, on the other hand, will react with peroxides to generate oxidizing intermediates which can be further used in redox chemistry. The functionality difference of having a thiolate ligand in a P450 as opposed to a histidine ligated system of a peroxidase seems to be the key in distinguishing the functionality of these two types of enzymes. The mystery of heme proteins, however, lies in not only the character of the axial ligand but also in the residues that surround the heme which can heavily affect the enzyme's receptiveness and functionality[10].

Using Chloroperoxidases as a Model for P450s

Chloroperoxidase is a unique protein from the peroxidase family that exhibits a thiolate-ligated system which contrasts from the typically seen histidine-ligated peroxidase systems. Unlike the histidine-ligated peroxidases which are unable to perform P450-like chemistry, chloroperoxidases have been seen to perform reactions that are similar to P450s[11]. Although chloroperoxidases are comparable to P450s

in regards to the active site, it resembles a more peroxidase-like distal pocket which distinguishes

chloroperoxidases and allows the protein to accommodate larger substrates[12].

Chloroperoxidases are useful for modeling P450 chemistry because the high valent reactive

intermediates are more stable than its P450 counterpart[13]. Because of the similar thiolate-ligation of the

two systems, it is inferred that the intermediates (compound I and compound II) will resemble each other

in electronic structure[14]. By studying compound I and compound II in chloroperoxidases, a better

understanding of P450s and their ability to perform such powerful chemistry can be gleaned by

comparing the two similar proteins.

Mechanism of P450s

The beginning of the cycle starts with substrate binding to low-spin ferric

enzyme which allows the water to dissociate from the heme and converts it to high

spin[13]. The ferric enzyme can now be reduced as its reduction potential increases

with a higher spin and now receives an electron from NADPH or NADH via an electron

transport protein consisting of a reductase and a ferrodoxin protein or a single reductase

flavoprotein which produces a ferrous substrate bound enzyme[3]. The conversion to

high spin results in an increase in reduction potential. In the next step, dioxygen binds

to the heme iron forming a ferric superoxide complex. Another reduction of the iron

generates a ferric peroxo species which is then protonated at the distal oxygen to form a

ferric hydroperoxo complex. The distal oxygen is cleaved heterolytically when another

proton is added to the distal oxygen which yields Compound I, an oxo ferryl with a

delocalized radical. Compound I goes on to abstract a hydrogen from the substrate to

create compound II, a protonated ferryl species, and a substrate radical which

recombines to create hydroxylated product and ferric enzyme which re coordinates with

the heme to finish the cycle and produces resting low-spin ferric enzyme[15].

The Catalytic Cycle of Cytochrome P450
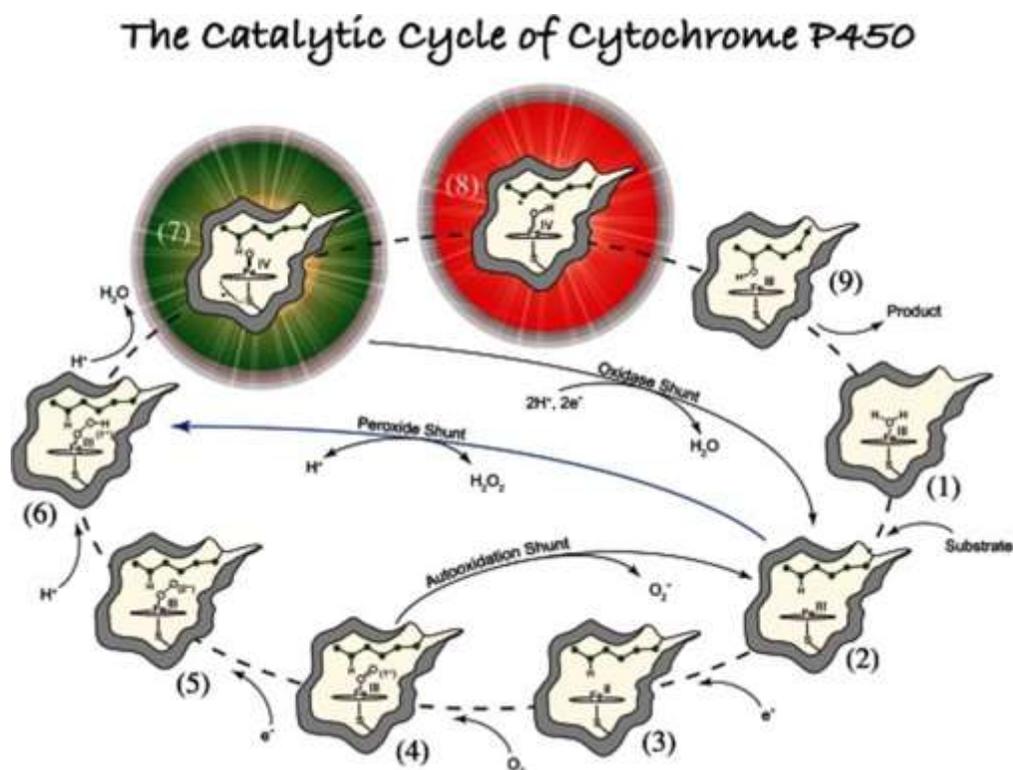
Figure 2. P450-Catalyzed Hydroxylation Mechanism

Starting from the resting low-spin ferric enzyme, the binding of substrate to the enzyme induces a structural change that involves the dissociation of the distally coordinated water and the conversion of the heme from low to high spin. Dioxygen then binds to the heme forming a superoxide complex. This species then reduces to form a ferric peroxo species which can be protonated at the distal oxygen to generate a ferric hydroperoxo complex. An additional proton induces the O-O bond to be cleaved, yielding compound I. Compound I then abstracts hydrogen from the substrate to yield compound II (8) which can then recombine with the substrate radical to yield the hydroxylated product and ferric enzyme.[15]

Peroxide Shunt

In an effort to avoid the need of a substrate to create the reactive intermediates of P450, a peroxide shunt method is used to generate the P450 reactive intermediates known as compound I and compound II[15]. The peroxide shunt pathway has low-spin ferric P450 react with peroxide to skip the necessity of dioxygen binding to the heme. The peroxide shunt pathway proceeds directly to the product of the first protonation in which, from there, the peroxo O-O bond is cleaved forming compound I[15]. Bypassing several mechanistic steps of the P450 catalytic cycle allows for a much higher yield of compound I and compound II.

Reactive Intermediates

With the reaction of ferric P450 with *meta*-chloroperbenzoic acid, the UV/Vis spectrum of compound I (P450-I) is produced. The UV/Vis spectrum of P450-I is derived using difference spectrum techniques[16]. Compound I is a highly reactive intermediate with its iron core in the IV oxidation state and a radical delocalized throughout the heme. This compound had been hypothesized for over 40 years but was only recently observed to exist with the help of a shunt pathway. When thiolate-ligated p450 systems are reacted with MCPBA, the reactive intermediate took on the spectral characteristics of a ferryl-oxo porphyrin cation radical which was identified by the absorption maxima at 370, 610, and 690 nm[17].

Compound II is characterized as a protonated ferryl species with a radical delocalized around the porphyrin. The UV/Vis spectrum for compound II is characterized by a shifting of the sorret from 416 nm to 426 nm[18]. These two intermediates have a distinct spectrum that can be distinguished using stopped flow UV-Vis spectroscopy among other things.
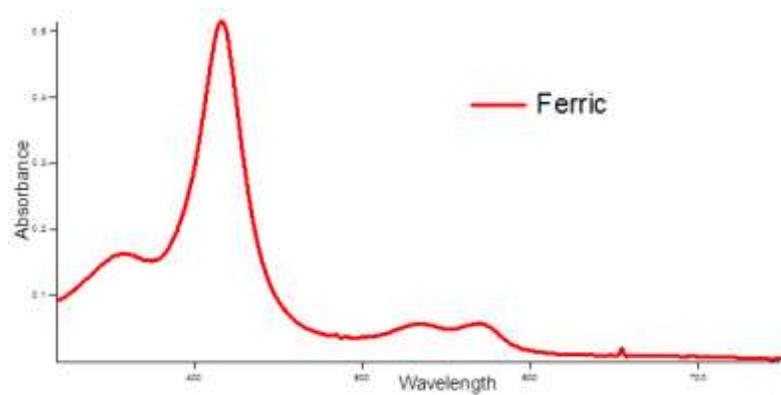
Figure 3 UV-Visible Spectrum of P450 Ferric CYP158A1
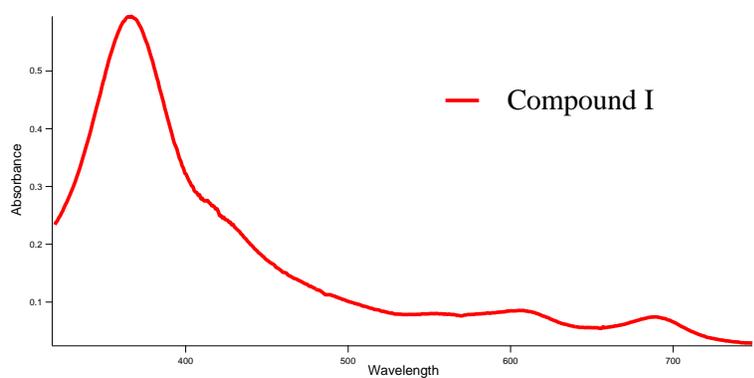
Figure 4 UV-Visible Spectrum of Compound I derived from difference spectrum techniques [16]
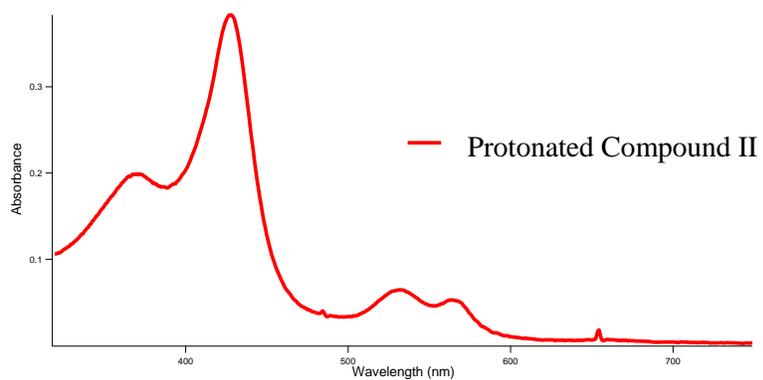
Figure 5 UV-Visible spectrum of protonated Fe-Hydroxy compound II [18]
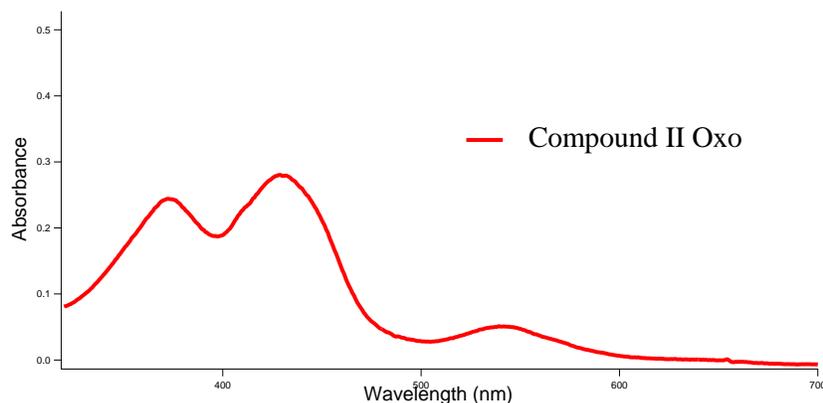
Figure 6 UV-Visible Spectrum of unprotonated Fe-Oxo compound II [18]

Importance of the Axial Ligand

The thiolate ligand plays an important role in ensuring the mechanistic cycle of C-H activation can occur efficiently and provide a driving force for heterolytic O-O cleavage. As discussed before, compound I is formed through a heterolytic cleavage of the O-O bond. The heterolytic O-O cleavage must be effective and without fault or the production of hydrogen peroxide may occur, which can be detrimental to the organism. The thiolate ligand in P450s serve as an electron donor which has been shown to push the heterolytic bond cleavage of O-O to completion[19, 20]. To demonstrate this effect, experiments done on myoglobin, in which the axial histidine is replaced with a negatively charged cysteine, have shown to increase the rate of heterolysis of the O-O bond and thus increasing its reactivity.

The ferryl form of histidine-ligated peroxidases have been seen to be Fe^{IV} oxo species due to examination of resonance raman and EXAFS data pointing to a bond length on the order of 1.65 Å[21]. Chloroperoxidases, on the other hand, has been reported to have a bond length of 1.82 Å which compliments the calculated value of a thiolate-ligated Fe^{IV} OH porphyrin bond

length of 1.81 Å. This supports the idea that CPO-II is indeed protonated. CPO has been used

as a model for P450 thiolate ligated systems due to its stable compound I and compound II

intermediates[22] and gives some insight into the importance of the thiolate ligand.

Additionally, experimental evidence from resonance Raman spectroscopy, EXAFS, and

Mossbauer spectroscopy from the reactive intermediates of catalase and myoglobin have

pointed towards the presence of an Fe^{IV} oxo species as opposed to a protonated species. From

the evidence, an upper limit of pKa ≤ 4 is placed on myoglobin and horseradish peroxidase

compound II and a pKa ≤ 7 for catalase compound II[21]. The difference of axial ligand plays

a large part in determining the identity and function of the enzyme. The thiolate-ligated system

allows the pKa of the reactive intermediate, compound II, to be much higher than that of a

histidine-ligated system due to its ability to stay protonated in pH of 7 and places a lower limit

pKa ≥ 8 for the thiolate-ligated chloroperoxidase. This is conducive to an increased ability to

functionalize C-H bonds.

Dependence on Ferryl pKa

As seen previously, the mechanism of P450s involves the abstraction of hydrogen, a

crucial process that is prevalent in many biochemical reactions. The driving force in the

hydroxylation of compound II from compound I is the difference in reductive potentials

between compound I and compound II. The strength of the O-H bond formed is related to the

strength of the C-H bond that can be broken for the reaction to be favorable[23].

$$[D(O\text{-}H) = 23.06 * E^0_{\text{compound I}} + 1.37 * pK_{a \text{ compound II}} + 57.6 \pm 2 \text{ kcal/mol}]$$

The pKa of compound II plays a large role in the reduction potential as it dictates the strength and stability of the O-H bond in the iron(IV)-hydroxide complex. Essentially, the ability of compound I to abstract a hydrogen is dependent on the one-electron redox potential of compound I and the pKa of the protonated species (compound II)[24].

It is hypothesized that the donating nature of the axial thiolate ligand is what allows the pKa of the reactive intermediate compound II to be so high. Contrast this to histidine-ligated systems such as peroxidases which show an Fe^{IV} oxo character with pKa ≤ 4.0[21]. This work can be used to explain how P450s are able to abstract hydrogen from C-H bonds at biological conditions. By determining the pKa of compound II and the redox potential of compound I, the potential ability of P450s to abstract a hydrogen from substrate can be deduced. This allows scientists to look at the feasibility of the enzyme in different environments and when it is exposed to different substrates.

Chapter 2

Materials and Methods

Purification and Overexpression

50µL of Rosetta Blue (DE3) pLysS E.*coli* competent cells were mixed with 2 µL of CYP158A1 (38ug/ul) plasmid. This mixture was incubated on ice for 30- 40 minutes, heat shocked at 42°C for 60 seconds, and then placed on ice again for 5 minutes. This mixture is put in 500uL NZY media and incubated at 37 °C for one hour. The transformed *E.coli* is plated onto LB agar plates with ampicillin and chloramphenicol antibiotics added.

One colony is placed into 250 mL TB media containing chloramphenicol and ampicillin. After the starters were put in the incubator and shaken for 10-15 hours at 37 °C and 225-250rpm and the OD is approximately 1.1. A 2L baffled flask containing 1L of modified M9 minimal media (6.8g Na_2HPO_4, 3g KH_2PO_4, 0.5g NaCl, 1.0g NH_4Cl) was inoculated with 15mL of starter culture. This was also supplemented with 100mg ampicillin, 34mg chloramphenicol, thiamine/proline (1.5M proline, 0.56M Thiamine), 15ml 40% glucose, 2.5ml of 1M $MgSO_4$, 125ul of 1M $CaCl_2$. This was shaken at a speed of 225-250 rpm at 37 °C. When the culture OD was approximately 0.6, the cultures were chilled on ice for 45 minutes and induced. For induction, the following solutions were added per liter, 1ml of 50mg/mL ampicillin, 1ml of 34mg/mL chloramphenicol, 1mL of trace elements, 0.5mM of IPTG, 0.5mM δ-aminolevulinic acid, and 1.5 mg of $FeCl_3$. Following induction, the shaking speed was reduced to 100rpm and the temperature lowered to 28 °C. After 25-30 hours, the cells were pelleted by centrifugation at 7,000xg. The cell paste was frozen in liquid nitrogen and stored at -70 ^{O}C.

The cell paste was resuspended in lysis buffer (20mm Tris, 4mM imidazole, 10% glycerol, 1mm DTT and 500mm NaCl). While the cells were resuspending into this solution, lysing enzymes were added. The cells were lysed using a micro-fluidizer. In this case, it was done using the micro-fluidizer. The lysate was loaded on a nickel agarose column and washed with 300 mL of lysis buffer. The protein, was eluted with 20mM Tris, 200mM imidazole pH 7.5 was used. Fractions that looked red were collected and concentrated using an amicon. Next, the protein was loaded onto a Q-sepharose column and washed with 400mL 10% glycerol, 1mm DTT, and 20mM Tris pH 7.5. A 40% gradient was set up to wash through the q-seph over 700 mL's to elute the protein with a 20mM Tris, 10% glycerol, 1mm DTT, 1M NaCl high salt solution. This particular protein eluted at 18% of the high salt, so approximately 200mM NaCl. The fractions with an Rz value of greater than or equal to 1.50 were kept. Rz is calculated by (415nm/280nm). The resulting protein was concentrated down to around 1-2mL. Lastly, this protein was loaded onto a size exclusion column and washed with low salt buffer. The fractions with Rz >1.51 are collected and concentrated further.

Site-Directed Mutagenesis

CYP158A1 Y349F

The objective of this mutation was to change the phenylalanine at residue 349 to a tyrosine. The forward primer was 5'-CGC ACC TGG CGT **TT**G GCA ACG GGC ACC -3' and the reverse primer used was 5'-GGT GCC CGT TGC C**AA** ACG CCA GGT GCG-3' The nucleotides bolded are the ones that are changed. The nucleotide mutation changed TAC to TTT. In order to do this transformation, a reaction and control are used to verify the success of the mutation. The recipes for the reaction and control reaction are as follows:

Table 1 Conditions for site-directed mutagenesis of the Y349F mutation

Recipe:	Rxn (µL)	Ctrl(µL)
75ng Template	0.77 of 98µg/µl	0.77 of 98µg/µl
225 ng Forward	6.81 of 4µM sol'n	0
225 ng Reverse	6.75 of 4µM sol'n	0
DNTP Mix	5	5
PFu Rxn Buffer	5	5
PFu Turbo AD	1	1
H2O	24.67	38.23

The control and reaction of 50ul each are put into the PCR for 5 hours. An annealing temperature set at 55 °C

CYP158A1 Y199F and CYP158A1 Y321F

Mutations of Y199F were done in a similar manner.

The Y321F forward primer used was 5'–CGG TGT ACG TCT CGT TTC TGG CCG CCA ACC G- 3'

The Y321F reverse primer used was 5'–CGG TTG GCG GCC AGA AAC GAG ACG TAC ACC G- 3'

The Y199F forward primer used was 5'–GGC AAA CGG GGC CTG **TT**T GGG TGG ATC ACC G- 3'

The Y199F reverse primer used was 5' –CGG TGA TCC ACC CA**A A**CA GGC CCC GTT TGG C- 3'

The YC405S forward primer used was 5' –CGC ACG CTG CCC AGC ACC TGG CAT C- 3'

The YC405S reverse primer used was 5' –GAT GCC AGG TGC TGG GCA GCG TGC G- 3'

CYP158A1 Y349F Y199F

The double variant produced in these experiments were made through the use of the single variant Y349F made earlier and then put through PCR again with the Y199F primers. The identity of the double variant was confirmed through sequencing.

CYP158A1 Y349F Y199F Y321F

The triple variant produced in these experiments were made through the use of the double variant Y349F Y199F made earlier and then put through PCR again with the Y321F primers. The identity of the Triple variant was confirmed through sequencing.

CYP158A1 Y349F Y199F C405S

The triple variant produced in these experiments were made through the use of the double variant Y349F Y199F made earlier and then put through PCR again with the C405S primers. The identity of the Triple variant was confirmed through sequencing.

Chapter 3

CYP158A1

Using CYP158A2 as a model for CYP158A1

Cytochrome P450 158A1 (CYP158A1) is found in *Streptomyces coelicolor* which protects soil bacterium from the harmful effects of UV radiation. CYP158A1, discussed in this paper, is particularly interesting because a close relative of the protein with 61% amino acid identity[25], wild type CYP158A2, has been shown to produce a large and stable amount of compound II when reacted with MCPBA[18]. Additionally, after the removal of nearby oxidizable tyrosine residues, CYP158A2 Y326F is shown to produce a buildup of compound I in the presence of MCPBA and no substrate via peroxide shunt pathway. A similar approach will be taken with CYP158A1 in attempts to procure a large and stable amount of compound I and compound II and further characterize these interesting reactive intermediates. Furthermore, a characterization of CYP158A1-II pKa can be done if the reactive intermediate is stable enough and produced in large yields.

As stated above, wild type CYP158A2 has revealed compound II spectroscopically when reacted with MCPBA via the peroxide shunt pathway[18]. Additionally, replacing a tyrosine residue with a phenylalanine residue, CYP158A2 (Y326F) shows a buildup of compound I, when reacted with M-CPBA in stopped flow experiments. Wild type CYP158A1, a close relative to CYP158A2, differs in the number of tyrosine residues located near the active

site. In the absence of substrate, compound I can oxidize nearby residues such as tyrosine and

receive a proton from solvent to rapidly form compound II, thus eluding spectroscopic capture.

Wild type CYP158A1 has three tyrosine residues close to the active site as opposed to only two

tyrosine residues in wild type CYP158A2. The extra tyrosine residue in CYP158A1 is believed

to be the difference in these two proteins and hopefully with the mutation of this residue, a

result similar to CYP158A2 is seen and a large amount of compound II is produced.

Phenylalanine is used as a place holder for the mutated tyrosine due to its similarity and size

and its inability to be oxidized. Using wild type CYP158A2 as a model, CYP158A1 will

hopefully exhibit similar behavior and provide spectroscopic evidence for compound I and

compound II after manipulating the residues near the active site. Hopefully, this work will also

deliver another compound II pKa and further insight into the factors that go into the

stabilization of compound I and compound II.

Wild Type CYP158A1

Wild type CYP58A1 did not yield any measurable amounts of compound I or II using

stopped flow experiments in the presence of MCPBA. The reaction of MCPBA with w-type

CYP158A1 in 100 mM Tris at pH 7, 8 and 9 and 100 mM Potassium Phosphate at pH 7, 8 and

9 resulted in an irreversible decrease in the Soret indicating degradation of the heme cofactor.

UV Vis evidence of compound I and compound II is hypothesized to be due to cross linkage

between tyrosine residue 349 or 199 near the active site and the porphyrin. In order to

eliminate the cross link effect, the tyrosine residue closest to the active site, tyrosine 349, of

wild type 158A1 is replaced with a phenylalanine. The productive pathway of compound I

decay, in P450s, is the abstraction of a hydrogen from substrate to form another intermediate, compound II. The non-productive decay of compound I may come from the oxidation of a tyrosine residue within the protein which leads to a deprotonated tyrosine residue and the loss of the delocalized radical on compound I.
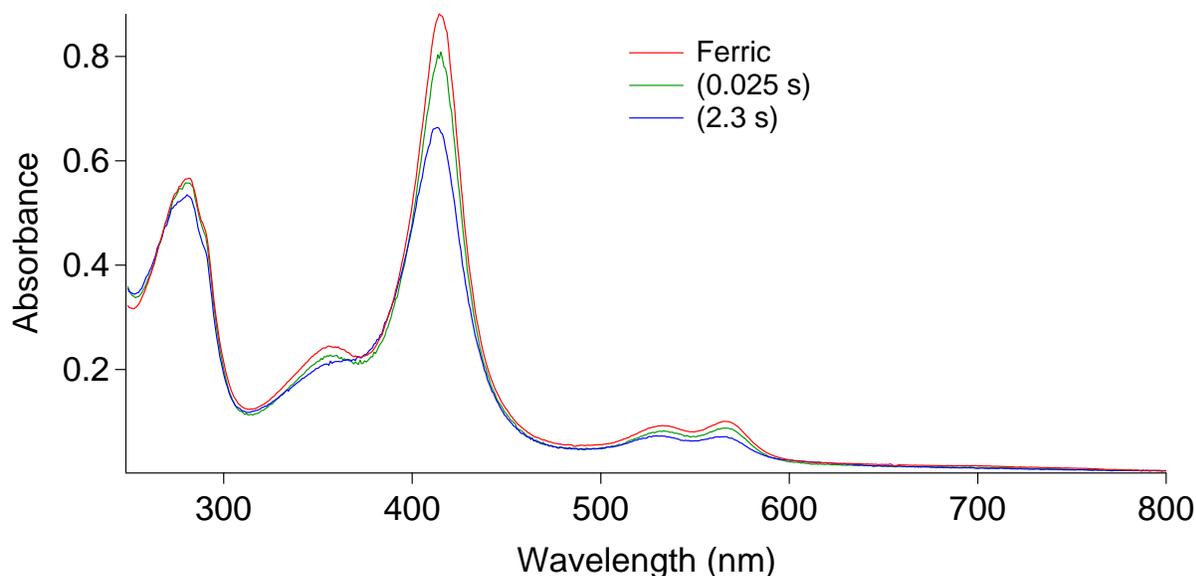
Figure 7 UV/visible spectra obtained from the stopped-flow mixing of 20 μM ferric CYP158A1 (100 mm Kphos pH 7.0) with 200 μM *m*-CPBA at 4⁰C.

Variant CYP158A1 Y349F

Wild type CYP158A1 appears to exclusively degrade in the presence of MCPBA (5 equivalents. However, with the replacement of the 349 tyrosine residue to a phenylalanine, a high yield of compound II is observed in 158A1 Y349F via UV/vis stopped flow spectroscopy. As can be seen in figure 9, a max yield of compound II was seen 1.725 seconds after reaction with MCPBA (60% yield). In figure 10, a max yield compound II was seen at 1.57 seconds. The UV-Vis spectrum is seen to shift from 416 nm to 424 nm with an absorption maxima at 371 and 422 nm, a clear indication of compound II presence see in figure 10. However, when

CYP158A1 Y349F variant is dissolved in tris buffer at a pH range from 7-9, the reaction with

MCPBA results in nothing but decay as seen by a decrease in Soret absorbance. However,

only when the protein is dissolved in Kphos of Borate with a pH range of 7-9, does the

evidence of compound II show itself. Target testing reveals small amounts of compound I is

also present in the reaction in 100 mM Kphos pH 7 and 8 and 100 mM borate pH 9.
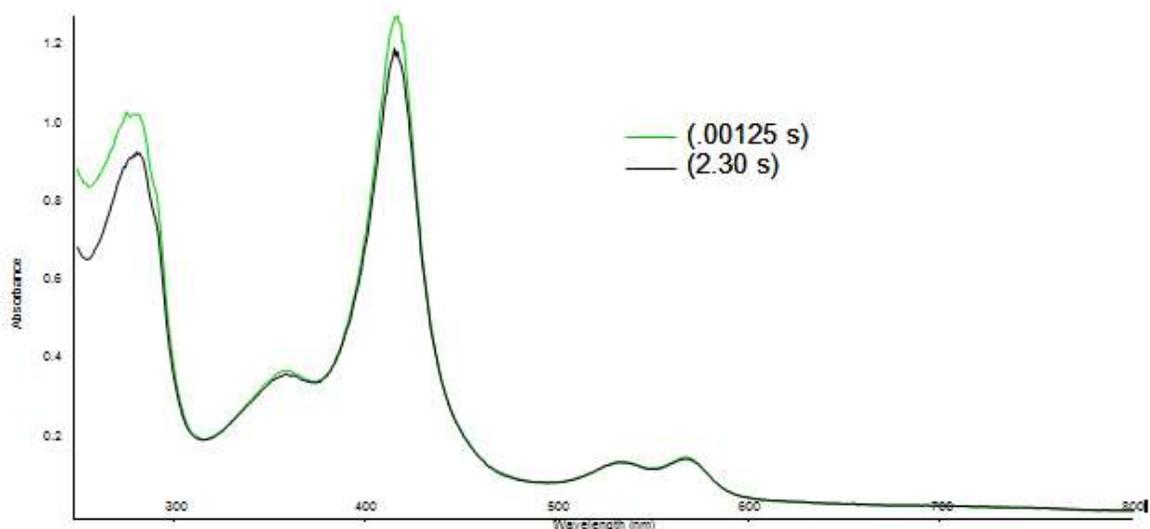
**Figure 8 20 μM CYP158A1-Y349F in 100 mm ph9 Tris buffer reacted with 100 μM M- CPBA. No major sorret shift is seen.**
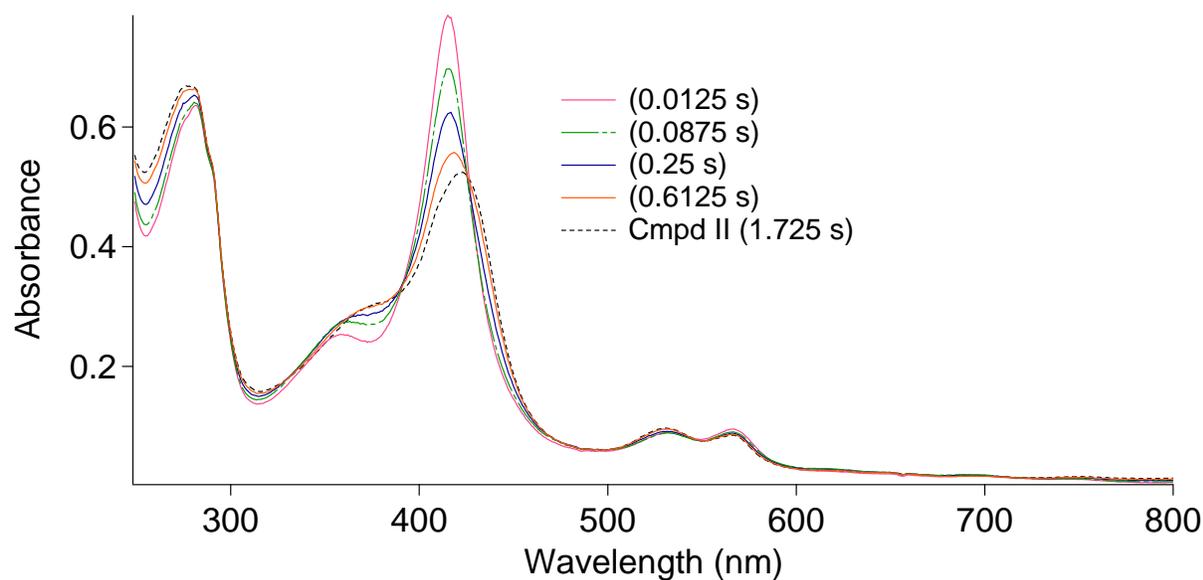
Figure 9 UV/visible spectra obtained from the stopped-flow mixing of 20 μM ferric CYP158A1-Y349F (100 mm Kphos pH 7.0) with 100 μM *m*-CPBA at 4⁰C.

Figure 10 UV/visible spectra obtained from the stopped-flow mixing of 20 μM ferric CYP158A1-Y349F (100 mm Borate pH 9.0) with 100 μM *m*-CPBA at 4⁰C.

Single Variant CYP158A1 Y199F

It is also interesting to note that with another single variant of Cyp158A1 of the 199 tyrosine residue changed to a phenylalanine, there was no compound I or II observed. Instead, there seemed to be slight, irreversible decay of the Soret (figure 11), but nothing comparable to the magnitude of the decay that wild type showed. The tyrosine residue 199   The reasons for this behavior are not completely known but may be linked to the fact that the vicinity of the 199 tyrosine residue is not as close to the active site as the 349 residue. As such, the behavior exhibited by this variant is very limited in what it shows spectroscopically. The slow decay signifies that it may be going through the P450 catalytic cycle at an extremely fast rate or is a product of non-productive decay due to a tyrosine residue near the active site (hypothesized to be the 349 tyrosine residue) still being oxidized by the radical of compound I.
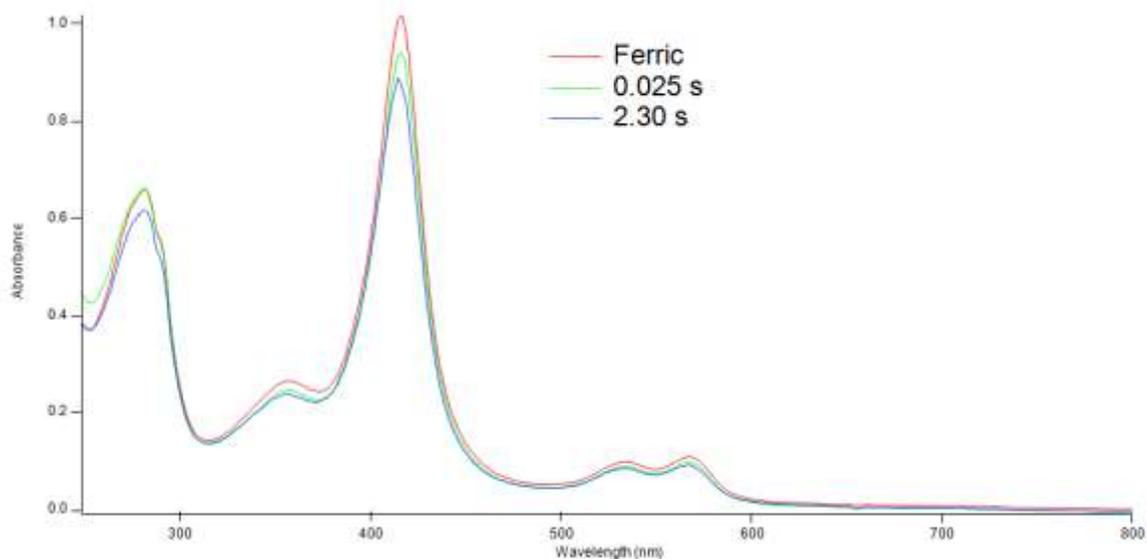
Figure 11 20 μM CYP158A1-Y199F in 100 mm ph7 Kphos buffer reacted with 40 μM M-CPBA. No capture of compound I or compound II, only decay is seen

Double Variant CYP158A1 Y349F Y199F

Further manipulation of Y349F yielded a double variant where Tyr199 and Tyr349 are substituted with phenylalanine residues. This variant shows even greater compound II yield than the single variant Y349f with a Soret shift from 416 nm to 426 nm (a more dramatic change compared to the single variant Y349F (seen in figure 12). In similar fashion, this double variant was tested in a pH range from 7 to 9 and in a variety of buffers including tris, Kphos, and borate buffer. Target testing CYP158A1 Y349F Y199F did not yield any measurable amounts of compound I. This immense compound II yield of 90% is encouraging because it shows that the replacement of tyrosine to phenylalanine is still lowering the oxidation potential of the residues near the active site but perhaps not to the point where a buildup of compound I can be observed. This points to the fact that there must still be another oxidizable residue near the active site that is allowing compound I to oxidize and form compound II. This large build-up of compound II is quite rare and has only been formed in high yield in two other P450 systems[18].

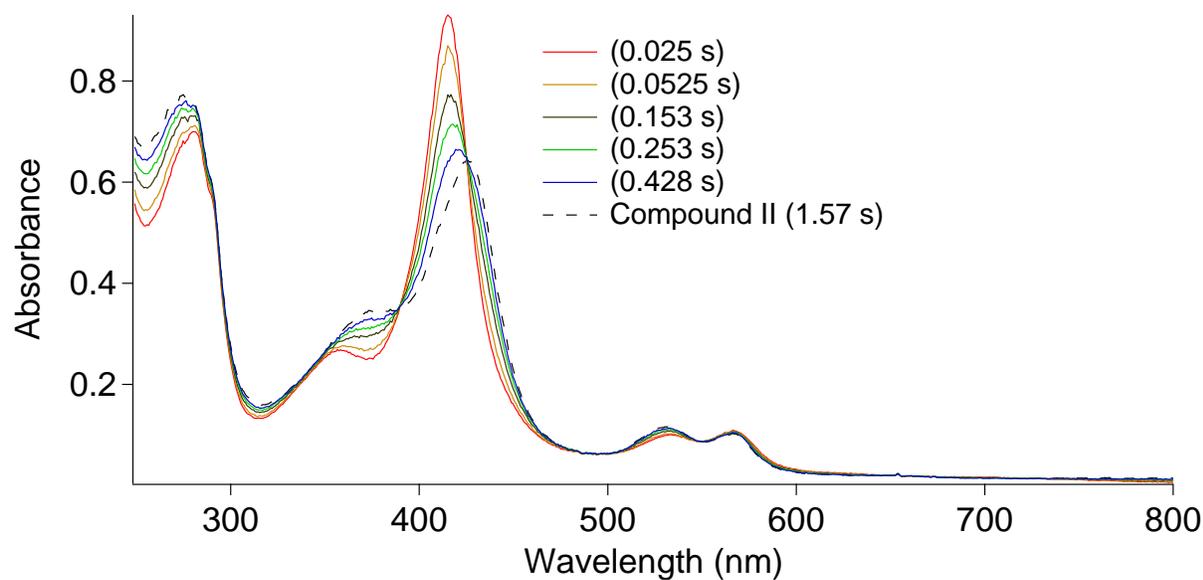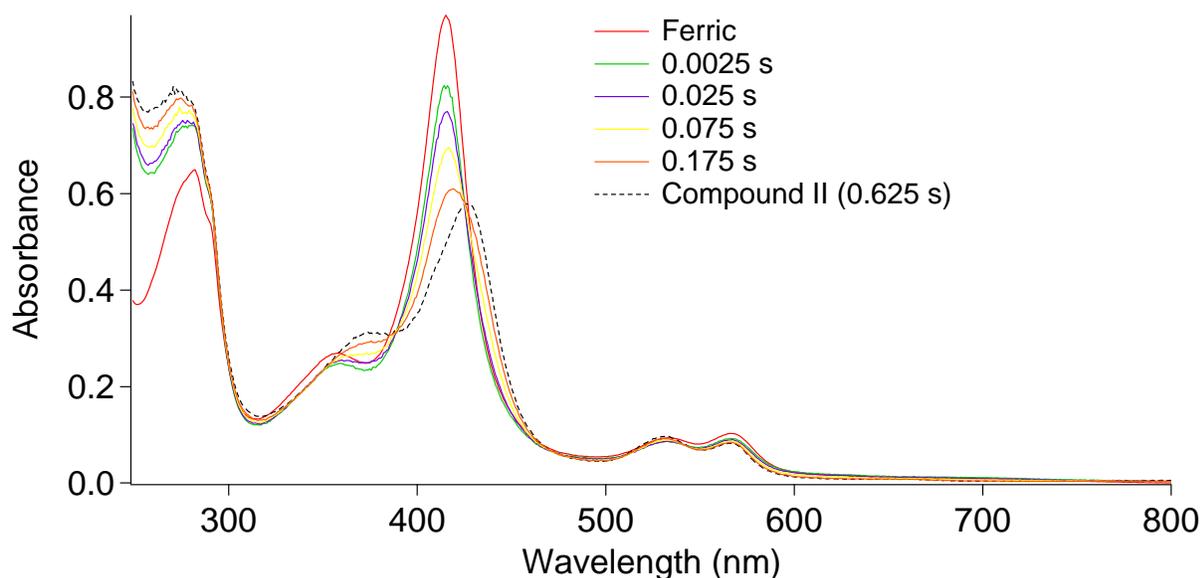**Figure 12 UV/visible spectra obtained from the stopped-flow mixing of 20 μM ferric CYP158A1-Y349F-Y199F (100 mm Borate pH 9.0) with 200 μM *m*-CPBA at 4⁰C. The soret is seen to shift from 416 to 426 nm.**

Triple Variant CYP158A1 Y349F Y199F C405S

The Triple Variant CYP158A1 Y349F Y199F C405S was made in hopes of combatting some of the protein stability issues that were occurring through these multiple variants. The issue of protein crashing out in solution and becoming unstable was a cause for concern and the replacement of the 405 cysteine residue on the exterior of the protein to a serine is done in hopes of providing a better folding structure and prevent disulfide bonds from forming between proteins. This variant was noticeably more stable in solution and provided a larger protein yield per batch than the previous variants. When this triple variant is reacted with MCPBA in 100 mM pH 9 borate, a similar production of compound II is formed as the previous double variant. However, the max formation time of only 0.473 seconds is quicker than the double variant and it has a Soret shift from 416 nm to 425 nm seen in figure 13. The compound II yield is slightly smaller than the double variant CYP158A1 Y349F Y199F for reasons that are unknown other than simply having a different protein scaffold.
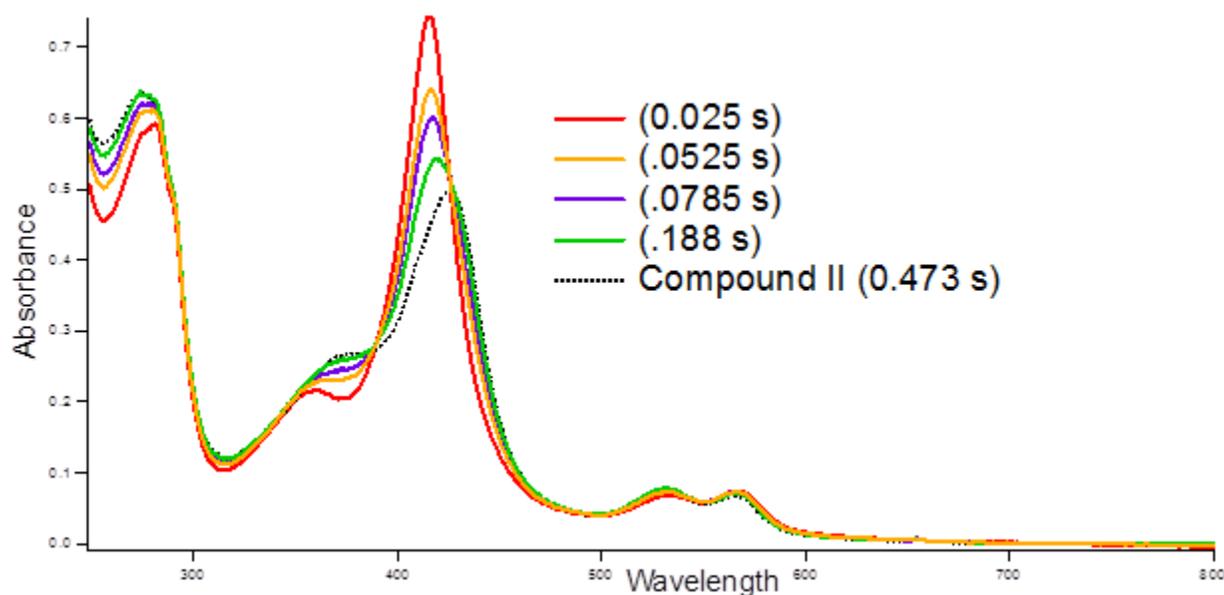
Figure 13 20uM CYP158A1-Y349F-Y199F-C405S in 100 mm ph9 Borate buffer reacted with 200μM MCPBA at 4°C. The soret is seen to shift from 416 nm to 425 nm

Triple Variant CYP158A1 Y349F Y199F Y321F

The second triple variant has removed all of the suspected tyrosines near the active site that may be responsible for being oxidized by compound I leading to non-productive decay of the heme. This variant, when reacted with MCPBA in the present of 100mm pH 9 borate buffer did not show any spectroscopic characteristics of compound I or compound II. The reasons for this are unknown but may be attributed to the ridiculous unstableness of the protein. The preparations of this protein required very careful watching and babysitting through the purification process as it would crash out very easily. Many of the purification steps were modified to increase its stableness in buffer by adding more ions in each buffer to hopefully assist its solvation. From figure 14 one can see an irreversible decrease of the Soret with very minimal shift.
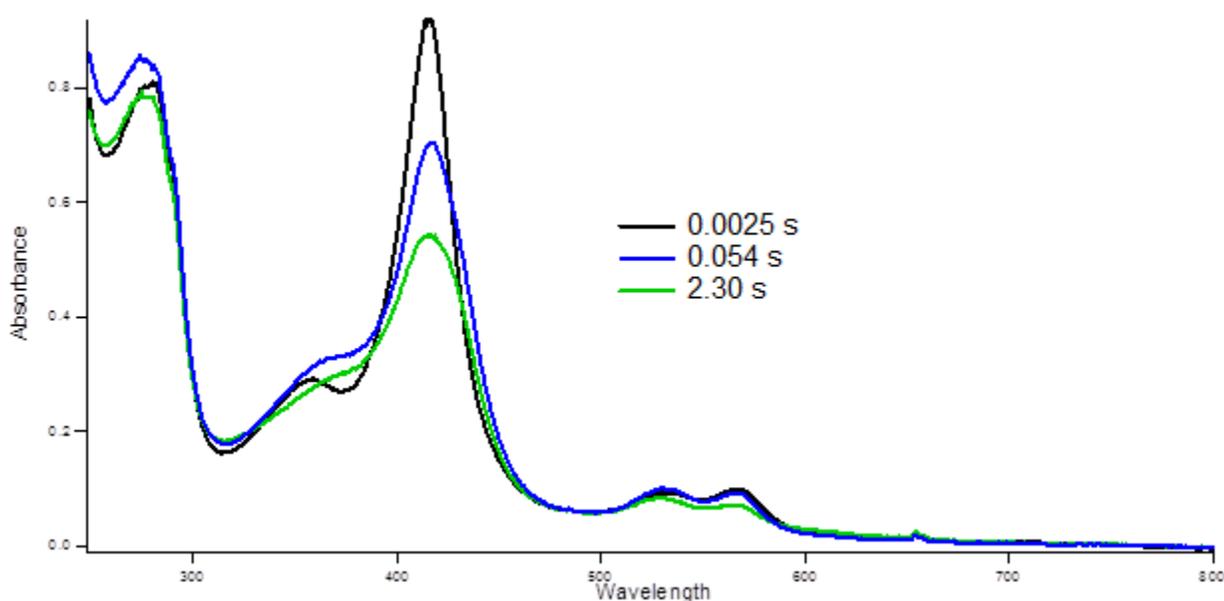
Figure 14 20 µM CYP158A1-Y349F-Y199F-Y321F in 100 mm ph9 Borate buffer reacted with 200 µM MCPBA at 4ºC.

Determining the pKa of CYP158A1

Examination of the double variant CYP158A1 Y349F Y199F in pH jump experiments was done in an attempt to find the ferryl pKa of the protein. It is already understood that the driving force for C-H bond activation is positively correlated with the difference in energy of the C-H bond broken and the O-H bond formed[23].

$$\Delta G_p = D(C\text{-}H) - D(O\text{-}H)$$

This results in a free energy relation that encompasses both the reduction potential and pKa. It has been proposed that the ferryl pKa and its difference in thiolate-ligated p450 systems compared to peroxidases is the key difference in understanding the reactivity for C-H bond activation[18]. Applying this concept, the energy of the bond being formed, D(O-H), is related to the one-electron reduction potential of compound I and the pKa of compound II. The pKa of compound II can then be seen as equivalent to a 59 mV drop in the one electron

potential of compound I per unit of pKa in compound II[18]. If the electron reduction potential of compound I is lowered, the system can become effective in hydrogen atom abstraction while also evading destruction of the protein through undesirable oxidations of the protein scaffold.

The pKa of a species can be determined using a pH jump experiment which is an experiment that incrementally increases the pH of a species and comparing the spectroscopic differences. Efforts to find the ferryl pKa of P450s have been mostly futile because in order to be able to do a pH jump experiment, high stability and yield of compound II are very important facets which could not be done in previous experiments. If compound II were to degrade too quickly, then spectroscopic characterization of the species could not be done. With the high yield and relative stability of compound II in the double variant CYP158A1 Y349 Y199F, a pH jump experiment was performed to determine its ferryl pKa. The double variant Y349F Y199F is first mixed with MCPBA and allowed to react at pH 9 until maximum yield of compound II is produced at 0.750 seconds. When max yield of compound II is reached after 0.750 seconds, a strong buffer is mixed with the compound II to increase the pH to a designated point.

Figure 15 depicts the spectrum of compound II at a variety of pH ranging from 9.71 to 14. As the pH increases, the spectra of compound II can be seen to transform to a different species different from that of the protonated ferryl species. This result is very similar to pervious work with CYP158A2-II [18] and displays all of the same characteristics of the compound II species transitioning from a protonated to a de-protonated system. The spectra goes from protonated (split Soret band at 370 and 428 nm with Q-bands at 532 and 565 nm) to a new spectrum representing the Fe-oxo species which is also seen to have a split soret (371

nm and 440 nm) and a presence of only a single Q-band located at 545 nm[18]. This new spectrum is characteristic of an Fe-oxo species based on similarities to CYP158A2 compound II oxo species.

The reported pKa value of 12.615 for CYP158A1 (figure 16) is much higher than the upper pKa ≤ 4 limits placed on the histidine ligated peroxidases and globins that were looked at earlier[21]. Using the D(O-H) equation and the pKa value of 3.5 for histidine ligated systems, a C-H bond difference of 12 kcals/mol is what separates the activation potential for a histidine ligated system from a thiolate ligated system.

The pKa reported for two other P450s, CYP119V-II and CYP158A2 are 12.1 and 11.9 respectively[18] which is in the ballpark of the value reported for CYP158A1. This confirms that at physiological pH, P450-II is a iron(IV) hydroxide species. The cysteine ligated system of P450s supports the formation of a protonated P450-II which is beneficial in increasing the ability of P450s to hydroxylate hydrocarbons.
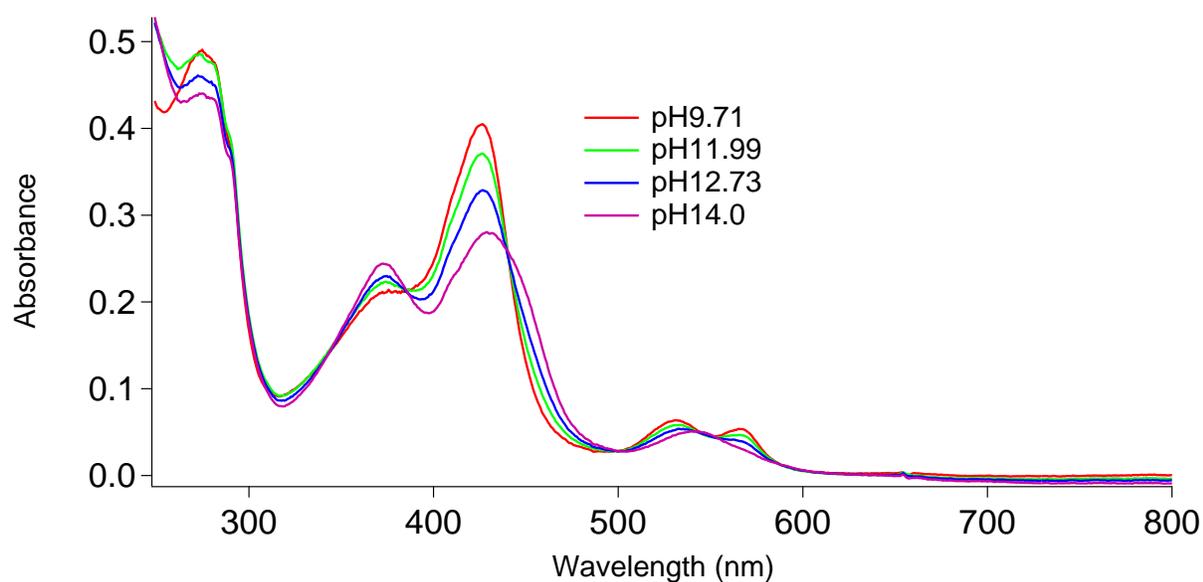
Figure 15 pH Jump experiment with CYP158A1-Y349F-Y199F using a 20µM protein solution in 100µM Borate pH9 buffer. Protein solution was mixed with 10 equivalents of MCPBA in a delay line for 750ms and then mixed with a strongly buffered solution of 200mM Borate, 200mM Phosphate, and 200mM Carbonate at a pH of that specified above.

pKa = 12.615
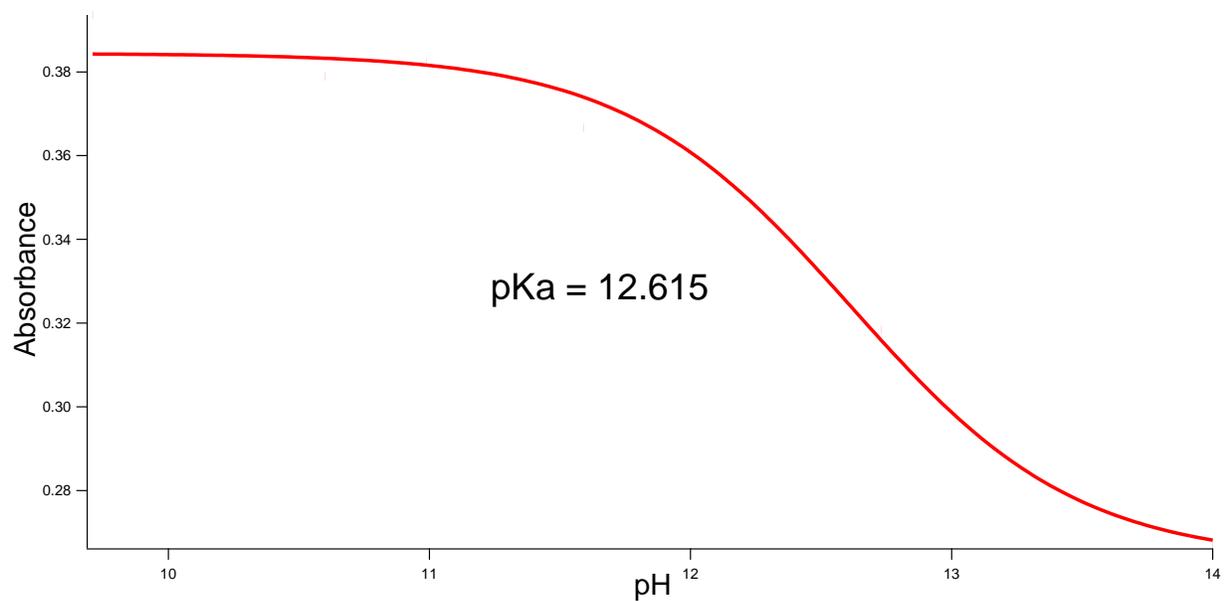
Figure 16 pH titration curve for CYP158A1-II obtained by plotting the absorbance at 422 nm against the pH of the protein solution.

Conclusion

The oxidation of inert molecules is an impressive feat. The inability for scientists to accomplish oxidation of C-H bonds in standard conditions is indicative of the importance of this enzyme. While the mechanism and characteristics of the intermediates of P450s is clearer than ever, the knowledge of how residues and solvent interact with the active site are still not completely known. The stability and interaction of protein with solvent is also an interesting point of contention. It seems that all efforts to react CYP158A1 with MCPBA while in tris buffer, regardless of pH, are futile. The pKa value of 12.615 reported in this paper is now among only two other reported values of ferryl pKa and continues to shed light on how the basicity of compound II relates to its reactivity. The journey of CYP158A1 and its numerous variants allowed a better view on how some residues affect the formation of compound I and compound II in the presence of MCPBA.

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ACADEMIC **VITA**

Leon Lin

103 Kaufman Run Blvd, Mars, PA 16046
Cell: 412-651-6932 : LYL5134@psu.edu

Education

Pennsylvania State University, Schreyers Honors College Class of 2015
Major: Chemistry

Activities and Positions Held

Delta Kappa Epsilon Fraternity (Officer) 2011-Present
- Fraternity Brother, initiated 11/21/11
- Executive Vice-President, Vice-President of Recruitment, Webmaster
- Participation and leadership of various philanthropies and community service activities. These activities include Cakes for Kids, THON Pan-Hellenic Dance Marathon, Kicks for Kids, and Walk for a Cure. Logged over 100 Hours of work since 2011.

Phi Eta Sigma National Honors Society (Officer) 2011-Present
- As scholarship chair, write the application for multiple $1,000+ scholarships and evaluate the applicants and reward them with a scholarship based on their scholastic and personal achievements.

Penn State Science Lion Ambassador 2012-Present
- Student and community outreach. Helping new science students and advising them on how to start their education on the right foot while answering any questions they might have. Creating a network of support and stability to students with science majors.

Work Experience

Research Assistant - Green Group located in Chemistry 306, Fall 2011-Present
Research assistant to Dr. Green and mentored by the graduate students of Green Lab. Proficient in FPLC, NMR, UV-Vis, and stop-flow. Current research delves into Cytochrome P450 in particular the variant cyp-158A1 and the creation of intermediates compound I and II through manipulations of residues using site-directed mutagenesis.

Chemistry Teaching Assistant – Analytical Chemistry
Lead work sessions outside of class once a week to help the students understand the material. Assist with the lectures and help with problems that students may have. Hold review sessions before midterm and final exams.

Clinical Shadowing, Volunteering and Research at University of Pittsburgh Medical Center - Pittsburgh, PA
Research at the radiology department of UPMC during the hours of 8am-11:30am under the supervision of Dr. He with a focus in breast cancer in-vivo nuclear magnetic radiation. During the afternoon hours of 1pm-5pm I was able to volunteer on the clinical side under the direction of registered nurses. My tasks included transporting patients, alerting staff if something is required by the patient, and stocking linens. I was also able to shadow Dr. Chen and Dr. Stefanovic at UPMC East and UPMC Presbyterian. Over 150 hours of shadowing and volunteering in a clinical setting.