THOMPSON, JONATHAN IRA, M.S. The Reaction of Alternate Oxidants with Cytochrome P450BM3 and Mutants Generates Spectrally Detectable, High Valent Iron Intermediates. (2006)
Directed by Dr. Gregory Raner. 51 pgs.

The Cytochrome P450 family serves as the forefront for xenobiotic metabolism in the human body. An understanding of the mechanism behind this enzyme family is a necessity for later research in the areas of pharmaceuticals and biochemistry. It has been observed by previous researchers that the transient species compound I, a highly reactive π-cation radical intermediate, is able to be viewed with P450cam and other heme containing proteins with subjection to various oxidant compounds such as m-CPBA or peracetic acid. In this study Cytochrome P450BM3 and its' heme domain, P450BMH, has been reacted with various oxidant compounds to show evidence of this compound I intermediate by diode array spectrophotometry and electron paramagnetic resonance spectroscopy.

THE REACTION OF ALTERNATE OXIDANTS WITH CYTOCHROME P450BM3 AND MUTANTS GENERATES SPECTRALLY DETECTABLE, HIGH VALVENT IRON INTERMEDIATES

By

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A Thesis Submitted to
the Faculty of the Graduate School at
The University of North Carolina at Greensboro
in Partial Fufillment
of the Requirements for the Degree
Master of Science

Greensboro
2006

Approved By

_____
Committee Chair

To those who have helped me physically, mentally and emotionally through my journey and growth as a person and as a student. I am proud to have had the opportunity to know you and to be as much a part of your life as you have been in mine.

" The chief function of your body is to carry your brain around. "

-Thomas Edison

APPROVAL PAGE

This thesis has been approved by the following committee of the Faculty of the Graduate School at the University of North Carolina at Greensboro.

Committee Chair_____

Committee Members_____

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___11/16/2006_____
Date of Acceptance by Committee

_____11/16/2006_____
Date of Final Oral Examination

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CHAPTER I

INTRODUCTION

General Description of Cytochrome P450

Considered by most to be the "first line of defense" for xenobiotic metabolism, the Cytochrome P450 family uses its ability to oxidize reactants to form products that are more water soluble and thereby more easily released by the human body. A desire to understand the mechanism behind Cytochrome P450 enzymes has grown exponentially over the last three decades as a result. Originally thought to only be housed in the liver and small intestines of mammals, research has discovered P450 enzymes in the brain[1], kidneys[2] and skin[3] of mammals and in some plants[4], bacteria[5] and fungi[6] as well. As of today more than 50 genes have been mapped that code for P450 enzymes in humans. There are two general classes of P450 enzymes with which one can perform research to further the understanding of this family of enzymes. These classes may be described as bacterial P450's and microsomal P450's, both of which have unique characteristics that aid in the understanding of P450

enzymes as a whole. Bacterial P450's tend to be cytosolic in nature and their study has aided in the understanding of reaction mechanisms due to their greater ease of purification. Consequently, applications in the field of biotechnology involving P450 enzymes tend to make use of this class in general. Microsomal P450 research aims at understaning human xenobiotic metabolism, which is a major component of the field of pharmacology. Other areas impacted by P450 research include toxicology and general health and nutrition. Any science that in some way addresses the impact of foreign chemicals on human health must include some element of Cytochrome P450 biochemistry in their research efforts.

P450 nomenclature

In 1986 there were close to 30 genes reported which coded for P450 enzymes in humans and close to 40 genes in rats. This made the need for a systematic way of naming and categorizing different P450s essential. The nomenclature of a particular P450 enzyme is easy to break down and is done as follows.[7] Most often Cytochrome P450 is simply denoted as *CYP* for simplicity. The first number that comes after this notation corresponds to the P450

family to which the enzyme belongs. A letter following this number represents the enzymes' subfamily, which is used when two or more subfamilies are known to exist within the main family. A final number denotes the individual gene that codes for that particular P450.[7] As with all rules made in the scientific world there are exceptions. This holds true in P450 nomenclature as well. The nomenclature scheme described holds true for a majority of human P450 genes, however, if there appears to be no subfamily or second functional gene in a particular P450 family there is no need to assign a subfamily or individual gene when naming the enzyme. An example of this would be CYP21, which is the only functional gene in the CYP21 family, so the gene and enzyme can be simply referred to as CYP21. In addition to the systematic naming of genes, many P450 enzymes have common names based on their discovery in a new organism, or function. A few examples of this would include CYPBM3(CYP102) and CYPCam(CYP101) which are found in *Bacillus Megaterium* and *Pseudamonas Putida* respectively. Finally, when attempting to determine to which main family a P450 gene belongs the "40% rule" is implemented. The gene that codes for a newly discovered P450 enzyme is aligned with several genes that code for different P450

families to test for similarities between the two. If there is a 40% similarity or more between the two genes then the new P450 gene is a member of that family, however, if there is less than a 40% similarity between the new P450 gene and any other previously discovered P450 family then that gene is the first in a new P450 family.

Structure of Cytochrome P450

1. Overall Structure

What we know as of now about the overall structure of Cytochrome P450 can be mainly attributed to the crystal structures of P450cam and P450BM3, which were published in 1987.[8,9,10] Since then studies have shown that the overall fold of the P450 enzyme is extremely conserved, where no non-P450 enzyme shares a similar tertiary structure.[11,12] Most Cytochrome P450 enzymes contain a heme domain only, which is responsible for the biochemistry that takes place in the enzyme. However, several members of the P450 family have been identified that are functional fusion proteins in which the heme domain is connected to an auxiliary reductase enzyme that feeds needed electrons to the heme domain which are contributed by the biological electron

donor, NADPH or NADH. Within the P450 family the general rule is the further within the structure, or closer to the heme, the more conserved the enzyme structure between isoforms become. These secondary structures of the P450 enzyme include the I, L and G helices. The L and G helices are mainly responsible for the formation of the unique fold in the P450 tertiary structure. Also the L helix, along with the I helix, is in direct contact with the heme active site of P450. Just before the L helix lies the β bulge section, which is responsible for holding the cystiene residue in place that directly bonds to the iron center of the heme. One characteristic, or fingerprint, of each P450 enzyme is the B' helix whose orientation and component residues directly contribute to what type of substrates are allowed into the active site.

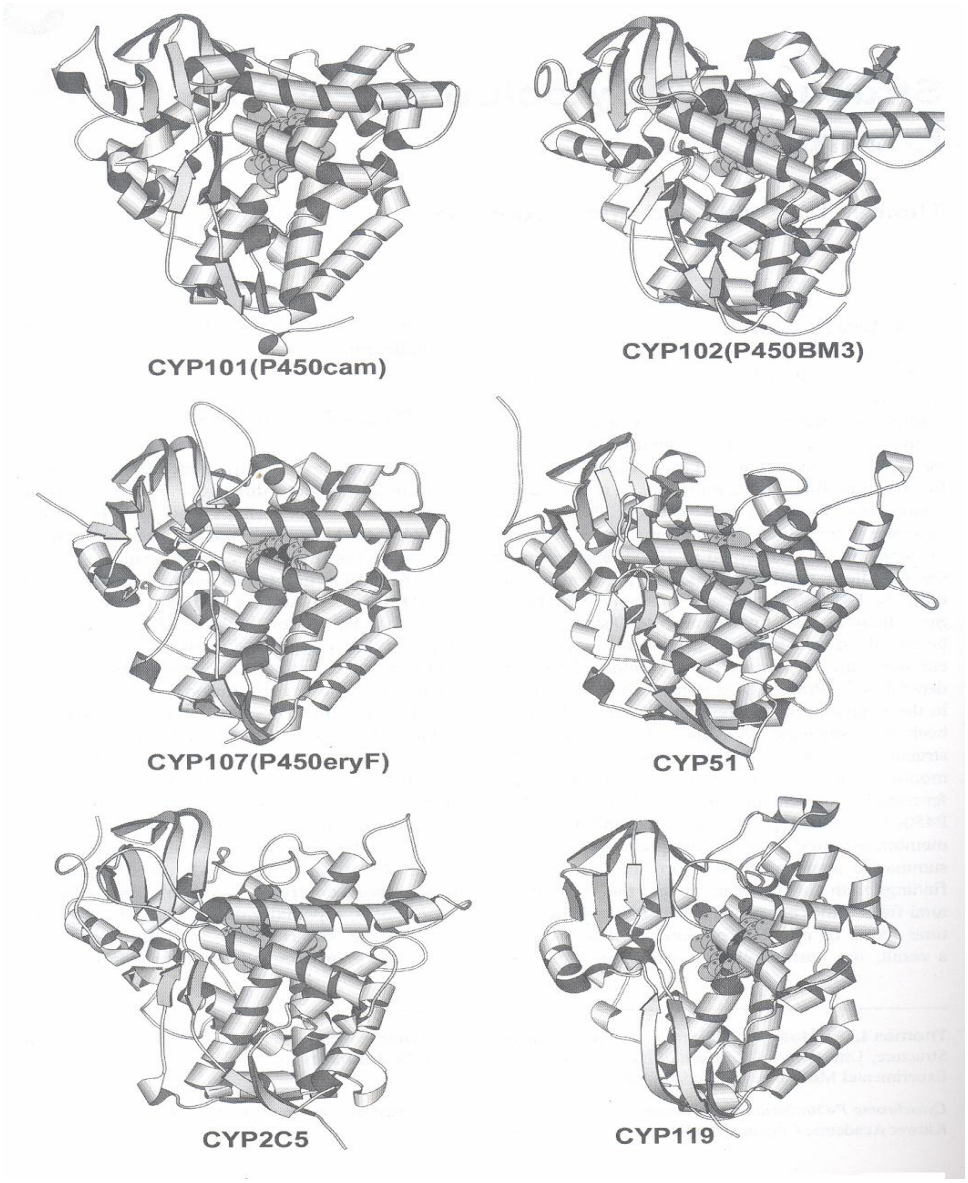


Figure 1: Overall tertiary structure of P450 enzymes.[13]

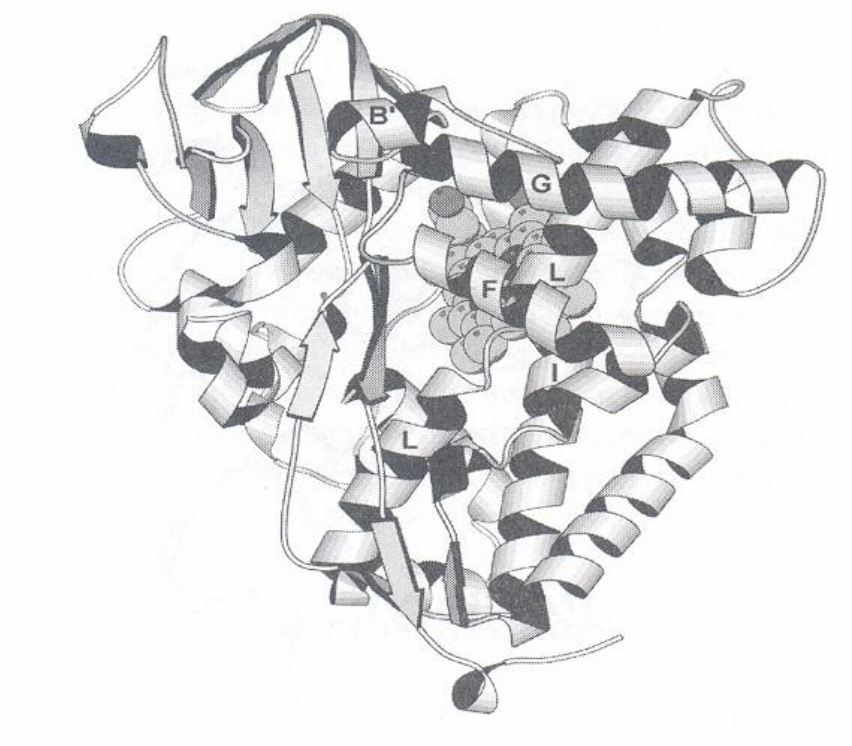


Figure 2: P450cam illustrating key conserved helical structures.[13]

2. Active Site Structure

As stated earlier, a cysteine residue housed by the β bulge, is responsible for holding the iron center of the heme active site in place. This heme active site is a porphyrin ring system that contains a redox active iron ion and is identical to the cofactor found in hemoglobin, myoglobin and many other biologically important redox enzymes. Several angstroms from the porphyrin ring system lie key residues that determine what particular substrate

will be allowed entry to the active site of the enzyme. Also these amino acid residues mediate electron transfer within the enzyme; several of these residues are shown in figure 3.

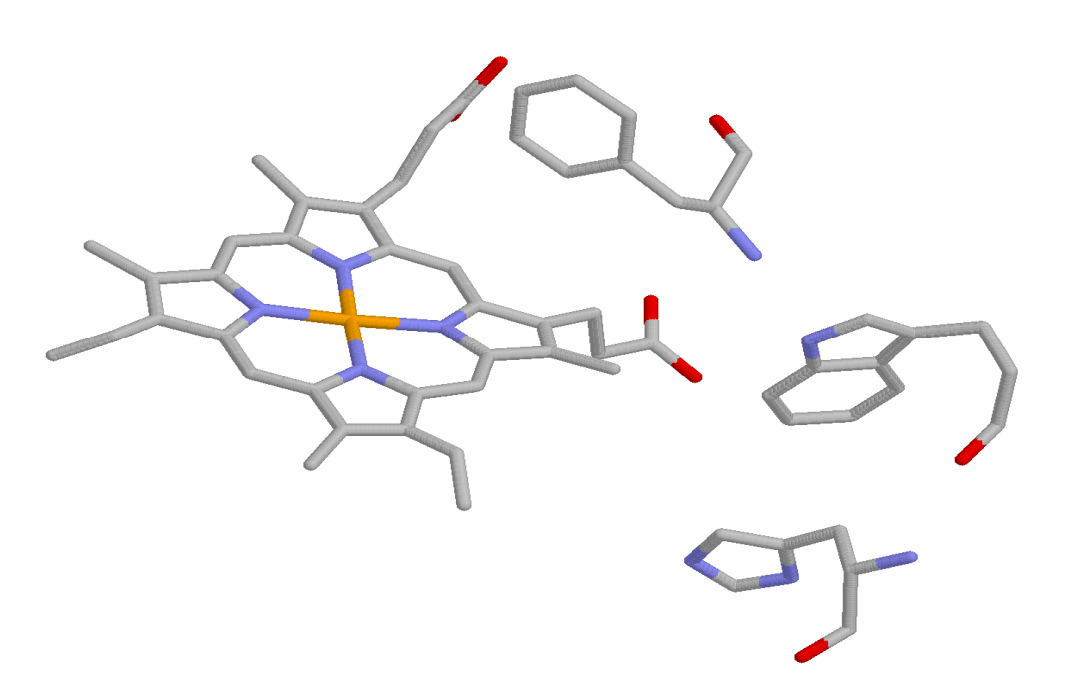


Figure 3: Active site structure of Cytochrome P450-BM3.[14]

Reaction Mechanisms of P450

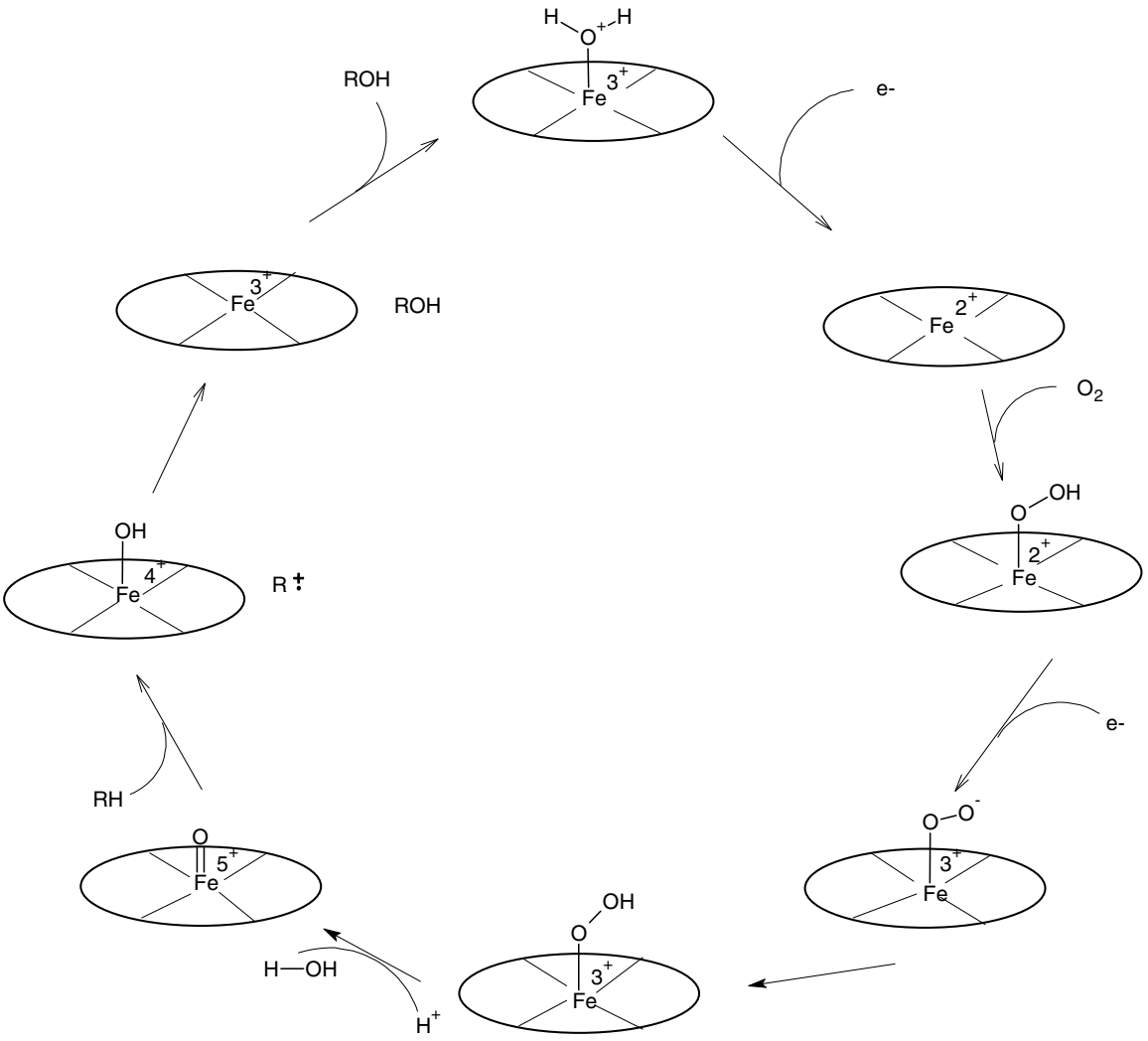


Figure 4: Catalytic cycle of Cytochrome P450.

The metabolic process of Cytochrome P450 takes place at its heme iron active site where a cyclic reduction-oxidation occurs converting substrate into an oxidized

product, often via the addition of a hydroxyl group to the reactant structure. Cytochrome P450 cannot do this process without the use of two key ingredients: molecular oxygen and the electron donating compound NADPH in the case of type II P450's. Electrons donated from NADPH are sent from the flavin domain of Cytochrome P450 reductace to the heme domain to aid in the reduction of the iron center of the heme active site. Molecular oxygen is then bound to the iron center and a second electron from NADPH is added to reduce the iron center to the 3^+ state. With the addition of hydrogen ions from the surrounding environment a water molecule is formed from one oxygen atom from molecular oxygen while the other oxygen atom forms a double bond with the iron producing a high valent state of iron which is now highly reactive and able to perform hydroxylation chemistry on the substrate. The time period to perform one cycle of this chemistry is remarkably short, and the initial steps in the cycle are relatively slow. This presents somewhat of a challenge when attempting to observe iron intermediates beyond the ferrous complex in the reaction cycle.

Transient Intermediates in the P450 Reaction Cycle

There has been a great deal of interest in the characterization and identification of reactive intermediates in the P450 catalytic cycle for some time now. Cryogenic research has allowed for the study of multiple P450 isoforms[15,16,17] which, as a result of this low temperature studies, has allowed for the observation of catalytic species beyond the second electron transfer in the proposed catalytic cycle of P450.[18,19] These studies have, in turn, raised the question of what precise catalytic species is involved in the hydroxylation or if there may be more than one species responsible for the biochemistry occurring in P450 enzymes. Stated previously, it is quite difficult to study reactive intermediates in the P450 cycle because the rate of the reactions after formation of the oxyferryl intermediate are very fast when compared to the slower rates of formation of intermediates before this in the catalytic cycle. A way to "by pass" this slow step is to present a molecule to the enzyme that can donate an oxygen atom directly to the iron and generate, in a single step, the "ultimate oxidant" in

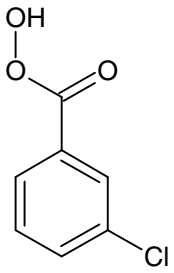
catalysis. Identifying the "ultimate oxidant" is the key component of my research.

Research Goals

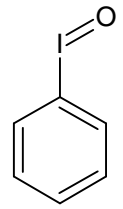
In an attempt to directly detect potential reactive intermediates in the P450 cycle, a technique known as stopped-flow spectrophotometry will be used. It has been discovered through research from various peroxidase enzymes and heme containing enzymes such as horseradish peroxidase[20], cytochrome c periodase[21], and horse heart metmyoglobin[22] that an unstable species known as compound I is formed on the heme active site by the introduction of oxidants such as meta-chloro perbenzoic acid, or m-CPBA. Compound I consists of an oxyferryl, porphyrin π-cation radical intermediate that forms on the porphyrin ring system of these proteins/enzymes. The result is a highly reactive intermediate, which is responsible for initiating oxidative reactions through electron transfer. As a result of this π-cation radical being so unstable, it may react with a neighboring residue, in most cases a tryptophan or tyrosine, resulting in an oxidized amino acid radical at or near the active site. Through diode array

spectrophotometry and electron paramagnetic resonance spectroscopy (EPR) at the appropriate pH it is possible to observe these transient intermediates. Combining these methods, an attempt to determine the exsistance of compound I and related intermediates in Cytochrome P450-BM3 has been implemented in this research endeavor. Oxidants such as meta-chloroperbenzoic acid (m-CPBA) along with iodosobenzene, sodium m-periodate and peracetic acid were used in this study to show the ability to form high valent heme intermediates in P450-BM3. The oxidants m-CPBA and peracetic acid have been used in related kinetic studies involving P450Cam and evidence for the occurrence of high valent, transient intermediates in this enzyme have been reported.[23,24] Schunemann et. al. showed in their studies of P450cam that EPR radical signals produced by reacting peracetic acid with P450Cam were removed by the mutation of tyrosine residues located near the active site.[23] Stopped flow and EPR analysis of P450Cam by Spolitak and Ballou at pH levels greater than 7.0 have also shown formation of compound I with the use of m-CPBA and peracetic acid as oxidants.[24] In the current study, the oxidants are introduced to wild type BM3 as well as various site directed mutants of this enzyme in an attempt to determine

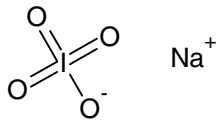
which key residue(s) is/are responsible for the removal of the π-cation radical from the porphyrin ring system of the heme in the active site. Since the oxygen atom donor bypasses the electron transfer step, the flavin domain is theoretically unnecessary for the experiments. Therefore, further mutants of BM3 comprising solely of the heme domain are also used containing complementary residue mutations. Diode array spectrophotometry coupled with stopped-flow mixing has been used to observe the transient intermediates that form, while EPR analysis is used to show the existence of residue radicals that form from the removal of the radical cation from the porphryin.



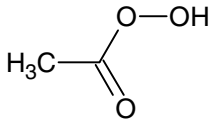
m-CPBA



iodosobenzene



sodium m-periodate



peracetic acid

Figure 5: Oxidant compounds used in this study.

CHAPTER II

MATERIALS AND METHODS

Site-Directed Mutagenesis of P450BM3/BMH and Mutagenesis Screening

To produce the appropriate mutant enzymes of Cytochrome P450BM3/BMH primers were constructed containing the appropriate codon sequence that coded for the desired amino acid residue mutation. For the full length protein, P450BM3, two single site mutations were made. Phe87 was mutated to a glycine in order to allow bulkier oxidant compounds used in this study to enter the active site. Trp96 was mutated to a histidine in an attempt to determine if this residue was responsible for removal of the cation radical from the porphyrin ring after formation of compound I. For the heme domain, P450BMH, a single mutation of Phe87 to glycine and a double mutation of Phe87 to glycine and Trp96 to histidine were made. Wild type plasmids were muatated by use of polymerase chain reaction (PCR). One microliter of plasmid was added to 1μL of reverse and forward primer, 12.5μL of phusion polymerase master mix

solution containing deoxyribo-nucleotides and 9.5μL of deionized water to give a final reaction volume of 25μL. Phusion polymerase was used for mutagenesis reactions because of its' proofreading ability during replication. Samples were then set to run in a PCR thermal cycler for 30 cycles with a denaturing temperature of 95°C for one minute, an annealing temperature of 62°C for one minute and a replication temperature of 72°C for five minutes. To ensure that the desired mutation was obtained in this new plasmid a screening test was implemented. A similar solution was prepared for this test containing 1μL of mutated plasmid, 1μL of positive screening primer, 1μL of reverse primer, 12.5μL of taq polymerase master mix containing deoxyribo-nucleotides and 9.5μL of deionized water. A duplicate sample was also prepared containing 1μL of negative screening primer containing the unmutated sequence at the 3' end. Taq polymerase was used in this reaction since no proofreading of the sequencing was required. Samples were then reacted in the PCR thermal cycler for 30 cycles under similar conditions with the replication portion of the cycle reduced to one minute. Once this reaction was

completed 2μL of loading dye was added to each sample and they were then loaded onto a 3% agarose gel suspended in 10% TAE buffer. The gel was allowed to run for one hour at 120V. The gel was then suspended in deionized water containing 50μL of ethidium bromide for 10 minutes to allow viewing of bands under UV light.

Production of P450-BM3/BMH and Mutants

To properly grow and express Cytochrome P450-BM3 the plasmid that allowed for over expression of this enzyme had to be placed inside an *E. Coli* strain capable of producing the T7 RNA polymerase, in this case the strain of *E. Coli* BL-21 DE3. Transformation of this expression vector was done by introduction of 1μL of plasmid to approximately 100μL of competent *E. Coli* cells while on ice. Samples were left on ice for 10 minutes and then heat shocked at 42° Celsius for 1 minute. Samples were then placed on ice for an additional 2 minutes, 900μL of LB media was added to allow for reproduction of *E. Coli* and this was placed in a 37°C incubator for approximately 1 hour. After incubation was completed 100μL of sample was applied to an agar plate

with ampicillin resistance and left overnight at 37°C. A single colony that grew on the agar plate was removed and introduced to approximately 30mL of *Terrific Broth* (TB) media and placed in a 37°C incubator overnight to grow. Several liters of TB media were prepared for the introduction of the starter culture by the combination of 12.0g of tryptone, 24.0g of yeast extract, and 4.0mL of glycerol per one liter of solution. The media was then autoclaved at 140°C for 35 minutes and allowed to cool. After the media had reached room temperature 100.0mL of phosphate buffer at pH 7.4, which was also autoclaved, and 100mg of ampicillin were added to the TB media to prevent unwanted growth of contaminants in the media. Approximately 1.0mL of starter culture was then added to the TB media and growth was allowed to occur for 24-48 hours at room temperature while shaking at approximately 180rpm. Once optimal growth had been reached, 150mg of IPTG was added to the media to induce the expression of BM3/BMH enzyme. Expression was then allowed to continue at room temperature for 24-48 hours at room temperature while shaking at approximately 80rpm.

Testing of Expression Levels of P450-BM3/BMH

To test for optimal expression of BM3/BMH approximately 3.0-5.0mL of TB media containing the *E.* Coli cells was centrifuged and the pellet re-suspended in half volume of equilibration buffer, which comprised of 15% glycerol by volume, 50mM potassium phosphate buffer at pH 7.4 and 0.1mM EDTA. Sodium dithionite was then added to the sample to reduce the heme iron to the ferrous state. The sample was divided into two cuvettes and a baseline spectrum was recorded using a Cary UV-Vis Spectrophotometer. One of the two cuvettes was then removed and carbon monoxide was bubbled through the sample for approximately 30 seconds. Spectra were recorded every 30 seconds to observe any increase in absorbance at 450nm, the λ_{max} corresponding to a reduced CO heme complex of Cytochrome P450.

Purification of P450-BM3 and Mutants

Cultures containing the P450-BM3 and mutant samples were first centrifuged at 10,000rpm for 20 minutes using a JA-10 rotor for the Beckman J Series Centrifuge. Pelleted

samples were then homogenized in equilibration buffer and the cell walls lysed using lysozyme while mixing for one hour. Samples were then placed on ice and sonicated at high frequency for one minute to break down the cell membrane, which released all cytosolic material from the cells. Finally, samples were centrifuged again at 18,000rpm for one hour using a JA-20 rotor and the supernatant that contained the unpurified BM3, was prepared for liquid chromatography purification.

A DEAE cation exchange column was equilibrated using 3-4 bed volumes of equilibration buffer as defined previously. Raw enzyme samples were then diluted 3:1 in equilibration buffer and loaded onto the column. Samples were then eluted and collected with equilibration buffer containing 0.2M NaCl. This sample was again diluted 3:1 in equilibration buffer and loaded on a Q-Sepharose cation exchange column, also equilibrated using 3-4 bed volumes of equilibration buffer. Purified enzyme samples were eluted using a 0.1M-0.30M step gradient of potassium phosphate buffer at pH 7.4 in 15% glycerol by volume. After purification a spectrum in the 400-500nm region of each sample was taken to ensure contaminants were properly removed. Purification was ensured by a rise in absorbance

at 418nm with no other changes in absorbance in that region of the spectrum.

Alternative Purification Method

In an attempt to purify P450-BMH and mutants an alternative method of liquid chromatography was used. Buffers used in this purification process are as follows: buffer A, 30mM potassium phosphate at pH 7.4, 2mM dithiothreitol (DTT), 0.1M EDTA; buffer B, 100mM potassium phosphate at pH 7.0, 1mM DTT, 0.1M EDTA. Cell pellets were homogenized in buffer A and were then lysed and sonicated as done previously. Supernatant samples were then diluted 3:1 in buffer A and loaded onto a Q-Sepharose cation exchange column previously equilibrated in buffer A. Samples were eluted by passing 100mL of buffer A containing 0.2M KCl and then concentrated to a volume of 5mL. This sample was then loaded onto a Sephacryl S-200 size exclusion column equilibrated in buffer B. Remaining contaminants were separated from the enzyme sample in this final step and a similar spectrum was taken of the sample to test for purity, stated previously. A solution of 50% glycerol was then added to the sample for cold storage purposes.

Diode Array Spectrophotometry

Purified P450-BM3/BMH samples were combined with oxidant solutions and then examined using a High Tech SF-18 double mixing Stopped Flow system with diode array detection. Oxidant and enzyme samples were prepared prior to injection by the addition of 500μL of potassium phosphate buffer at a specified pH, to give a final buffer concentration 250mM. A zero point spectrum was first recorded by combining the prepared enzyme sample against a blank, 250mM potassium phosphate solution. A spectrum was recorded for one second and used as a means of comparison for later addition with oxidant solutions. All experiments, including the zero point spectrum, were preformed at a constant temperature of 15°C. Oxidant solutions were prepared at varying, optimal concentrations as follows: m-CPBA, 125μM; iodosobenzene, 125μM; sodium m-periodate, 5mM; peracetic acid, 5mM. In an attempt to observe compound E-S formation, enzyme and oxidant solutions were prepared at pH 6.8 while to observe compound I formation solutions were prepared at a pH of 7.4. Spectra were recorded in triplicate for 1 second, 10 second

and 50 second reaction times at pH 6.8 to observe the spectral shift from 418nm to 406nm. Spectra were recorded from 1 second to 4 seconds to observe compound I formation at 370nm.

Electron Paramagnetic Resonance (EPR) Spectroscopy

In order to observe a clear radical signal, a higher concentration of enzyme sample was prepared at approximately 100μM. Enzyme and oxidant solutions were prepared in 100mM phosphate buffer at pH 7.4. Oxidant concentrations varied from 125μM to 400μM. After preparation of initial samples were completed they were combined in a 4mm clear fused quartz EPR tube and allowed to react for approximately 10 seconds before freeze quenching with liquid nitrogen. Spectra were then recorded on a Bruker Instruments EMX 6/1 EPR spectrometer at a microwave power of 50.4μW and a frequency of 9.48GHz. The temperature was held constant at 10K while recording spectras by using an Oxford Instruments ESR 900 cryostat.

CHAPTER III

RESULTS AND DISCUSSION

Plasmid Screening

Using polymerase chain reaction and agarose gel electrophoresis a positive screen was observed for the double mutation of Cytochrome P450BMH-F87G:W96H. It should be noted that this double mutated plasmid was obtained by a single mutation of the Trp96 codon sequence on a previously mutated Phe87 plasmid that screened positive for the mutation. Positive screening primers for both mutations annealed to the plasmid at 65°C. After suspension of the agarose gel in an ethidium bromide solution bands were observed under UV light in the positive mutation lane for each residue mutation while no band was observed in either negative mutation lane. This screening ensures that the properly mutated plasmid is being used throughout this study.

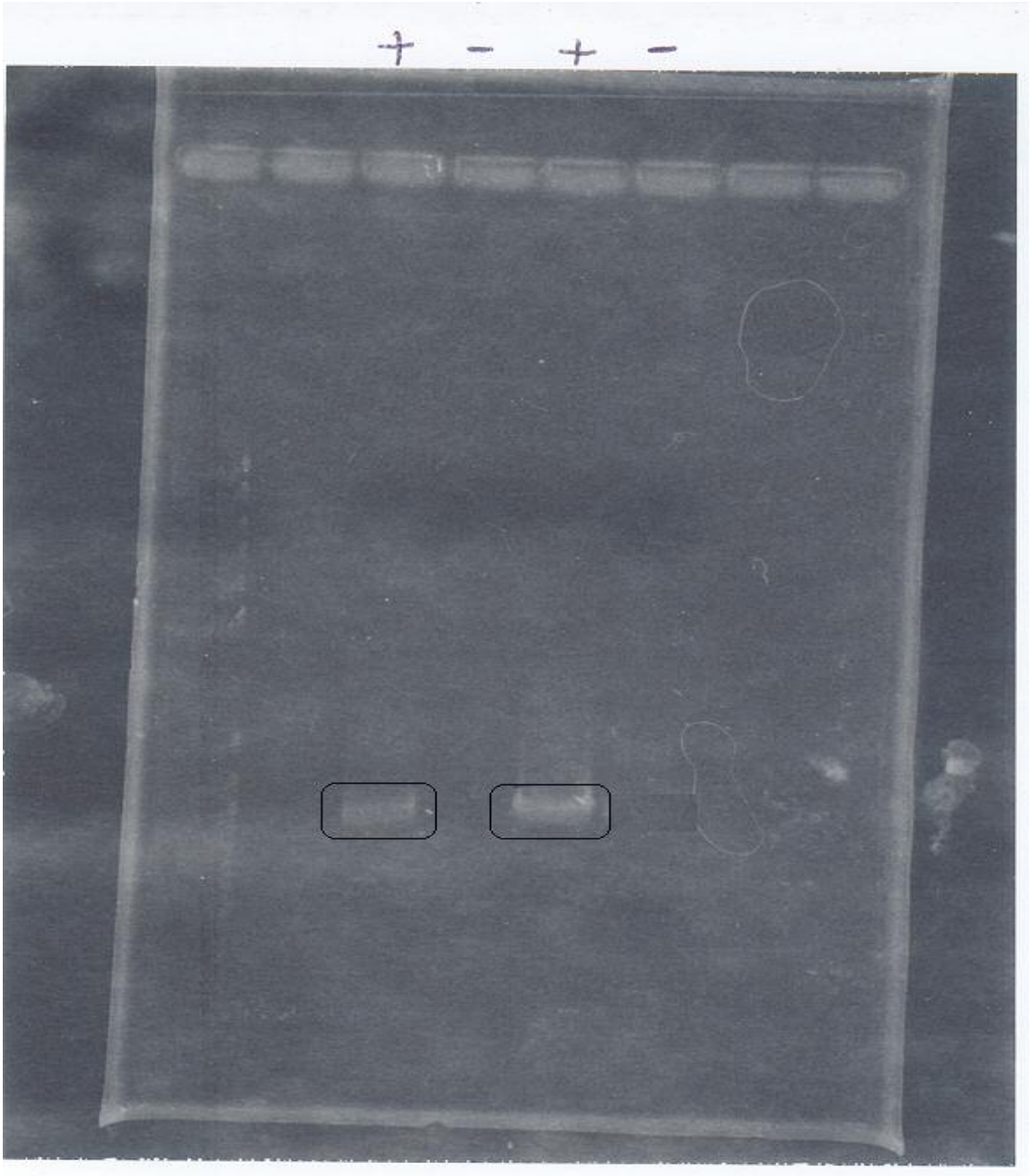


Figure 6: Agarose gel illustrating a positive screen for both F87G and W96H mutations. Lane 3 positive shows positive screening for F87G while Lane 5 positive shows positive screening for W96H.

P450 Expression Levels

CO reduced spectra of both F87G and F87G:W96H mutants of P450BMH revealed a significant drop in expression levels compared to that of P450BM3. Samples (5mL) of each mutation were centrifuged and resuspended in half volume of equilibration buffer for each spectrum. A maximum absorbance difference of ~.01-.03 over a 5 minute time course was observed for the BMH-F87G mutation while a maximum absorbance change of .008-.02 was observed for the BMH-F87G:W96H mutation which corresponded to the expression levels of each enzyme in culture. A possible reason for this lowered expression level could be the resulting instability of the heme active site by removal of the tryptophan residue. Hydrogen bonding is known to occur between this residue and the porphyrin ring in order to anchor the heme active site in place. Mutation of this residue alters this bonding scheme. Other possibilities for a lowered expression level in these heme domain mutations are also possible.

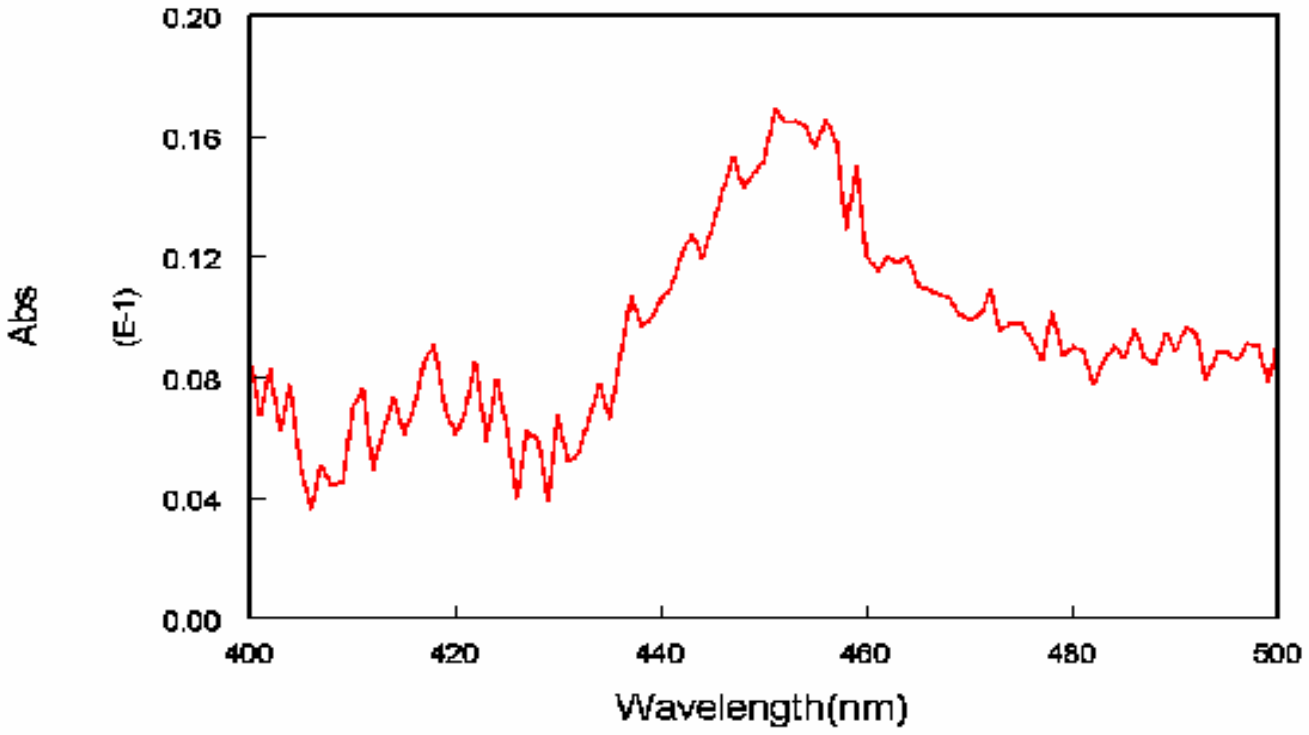


Figure 7: CO reduced spectrum of P450BMH-F87G:W96H. Spectrum shown is for maximum absorbance obtained over a 5 minute time course.

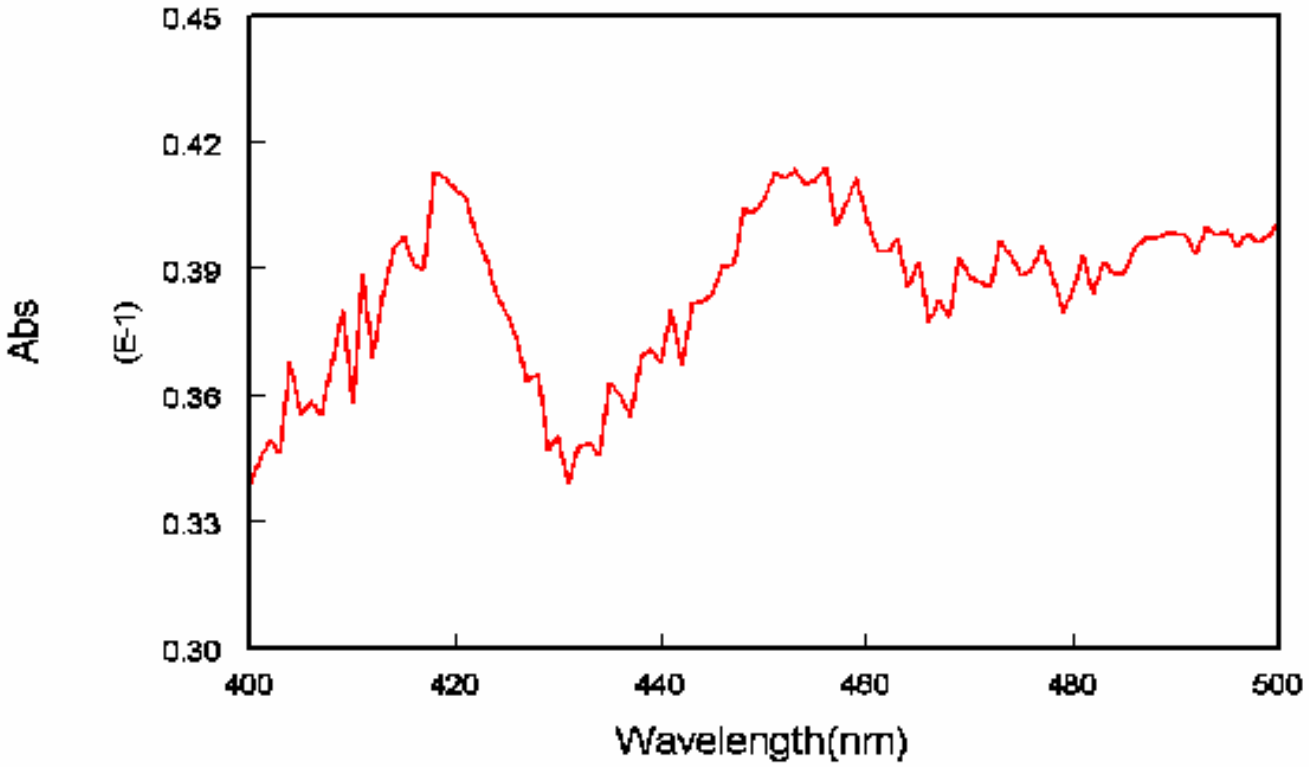


Figure 8: CO reduced spectrum of P450BMH-F87G. Spectrum shown is for maximum absorbance obtained over a 5 minute time course

Diode Array Spectrophotometry

Within milliseconds of the addition of peracetic acid to Cytochrome P450BM3-W96H at pH 7.4 a shoulder was observed in the enzyme spectra at 370nm which corresponded to the formation a π cation radical intermediate on the porphyrin ring of the heme active site, or compound I. This trend was also observed with reaction of P450BM3-F87G with m-CPBA as the oxidant at pH 7.4. This shoulder rapidly decayed in P450BM3-F87G at pH 6.8 to produce a strong Soret peak at 406nm which corresponded to the removal of this cation radical from the porphyrin ring to a neighboring tryptophan residue, or compound E-S (also referred to as compound II). This spectral shift to 406nm gradually came back to its' original position at 418nm with moderate bleaching at an optimal oxidant concentration. Other spectral changes at 575nm and 625nm were evidence of the formation of the transient compound E-S intermediate where the oxidant compound has bound to the heme active site. A spectral change was observed within the first few milliseconds in this region with a decrease in absorbance at 575nm with a coincidental increase in absorbance at 625nm.

A spectral shift to 406nm at pH 6.8 was observed with all oxidants used in this trial when reacted with P450BM3-F87G, however, the timeline to observe this shift was significantly longer with sodium m-periodate at optimal oxidant concentration. An additional feature was also observed in the reaction with sodium m-periodate. A rapid increase in absorbance below 350nm was seen, which may reflect the formation of a tyrosine dimer between Tyr278 and Tyr 429. These residues are approximately 3.8Å from one another, which supports the formation of a dimmer complex. The dimer complex has a maximum absorbance of ~340nm which is observed in the spectra of P450BM3-F87G within milliseconds of addition of sodium m-periodate. A proposed reaction mechanism for the formation of this dimer complex is illustrated in Scheme I and II. The other oxidants produced a spectral shift to 406nm within a time frame of 1-10s while sodium m-periodate took up to 30s.

Reaction of Cytochome P450BMH-F87G with m-CPBA generated a spectrum nearly identical to that of P450BM3-F87G with a spectral shift to 406nm at pH 6.8 followed by a shift back to 418nm with moderate bleaching at an optimal oxidant concentration. Other oxidants reacted in a similar manner as with P450BM3-F87G, however, an identical spectra

could not be produced with the oxidant iodosobenzene. The probable cause for this could be degradation of oxidant stock as a result of its photosensitivity. An increase in absorbance at 300nm with reaction of sodium m-periodate with BMH-F87G showing the presence of a tyrosine dimer complex as before with BM3-F87G. While the BMH-F87G:W96H mutant was successfully expressed, analysis of this enzyme when reacted with any oxidant used in this study could not be performed. Low expression levels of this double mutation along with instability of this enzyme during the purification process as a result of the mutation did not allow for the analysis. An overall scheme of the formation and decay of these transient intermediates is illustrated in Scheme III.

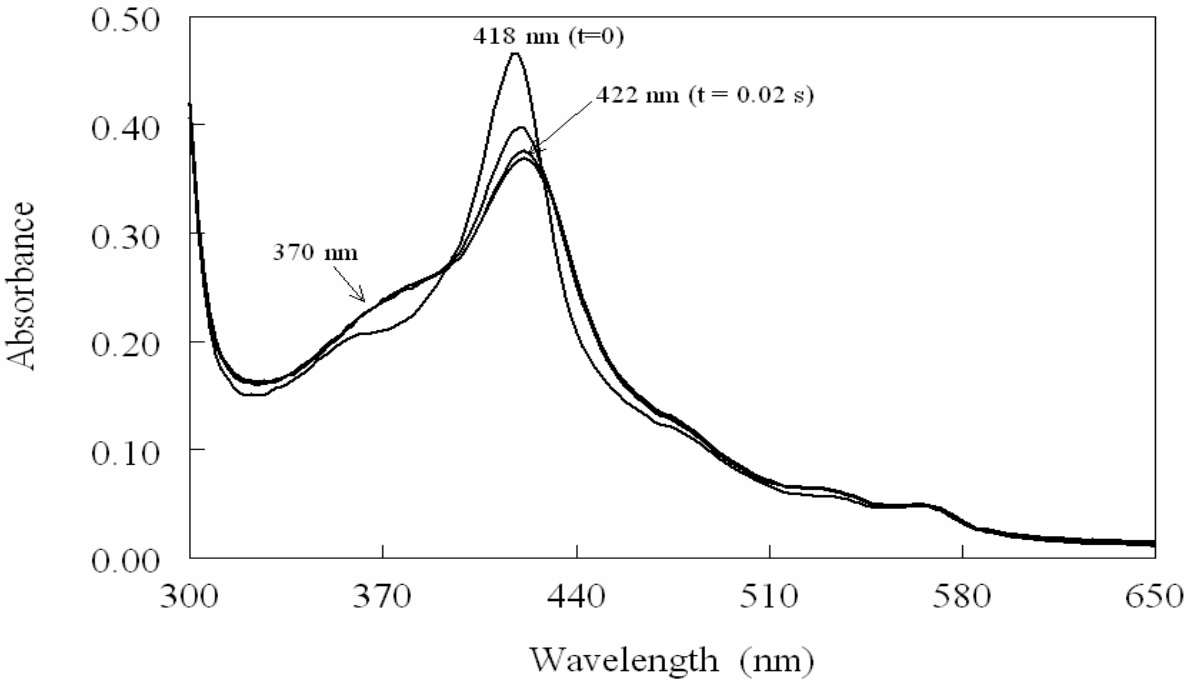


Figure 9: UV-Vis spectrum of P450BM3-F87G reacted with m-CPBA at pH 7.4. Enzyme concentration ~500µM, oxidant concentration 125µM, buffer concentration 250mM. Reaction temperature held constant at 15°C.

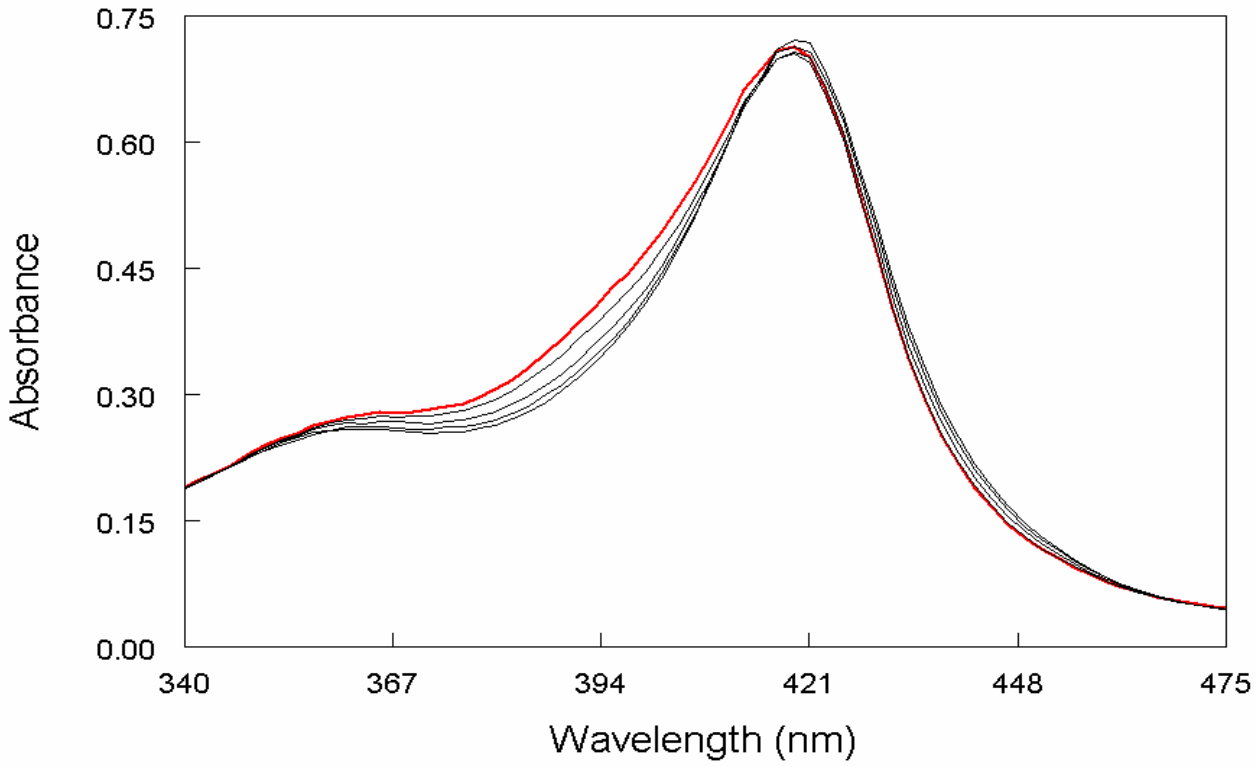


Figure 10: UV-Vis spectrum of BM3-W96H reacted with peracetic acid at pH 7.4. Enzyme concentration ~750µM, oxidant concentration 5mM, buffer concentration 250mM. Time frame of reaction from t = 0s (red) to t = 2s, reaction temperature held constant at 15°C.

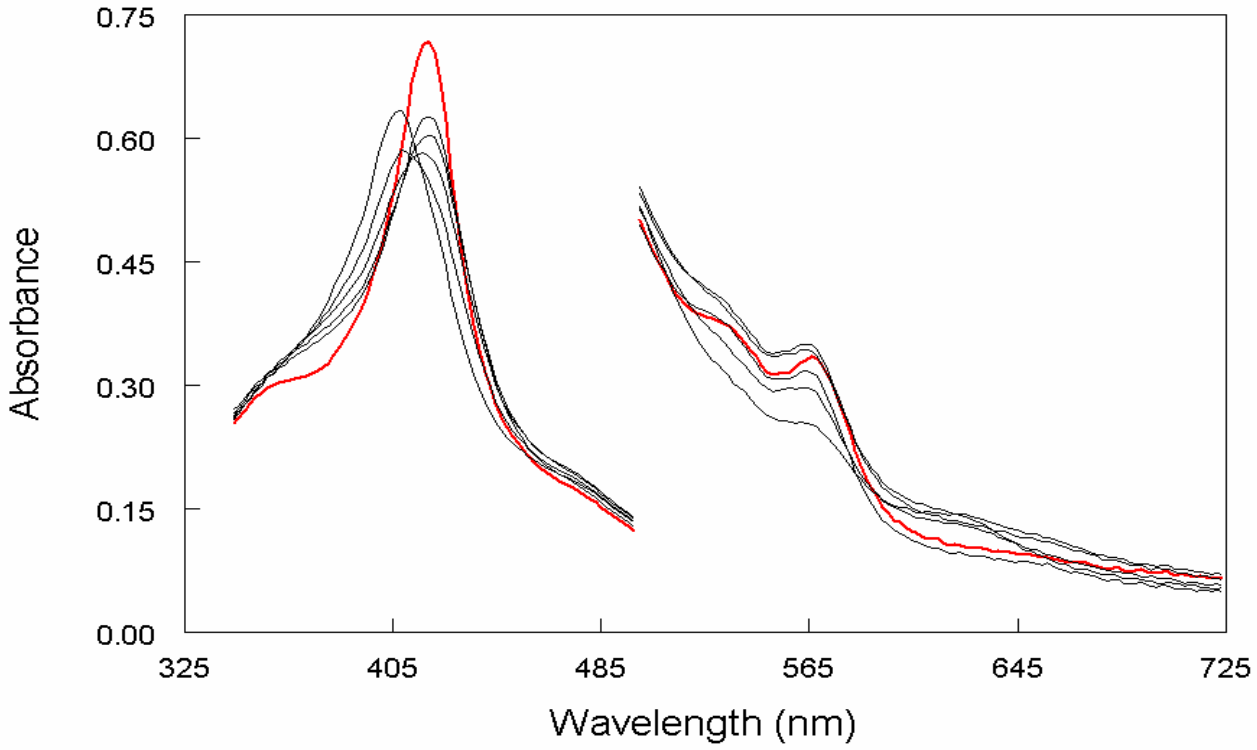


Figure 11: UV-Vis spectrum of m-CPBA reacted with BM3-F87G at pH 6.8. Enlarged region of P450BM3-F87G spectra illustrating formation of compound E-S intermediate. Enzyme concentration ~700μM, oxidant concentration 125μM, buffer concentration 250mM; time frame from 0s(red) to 1s, temperature held constant at 15°C.

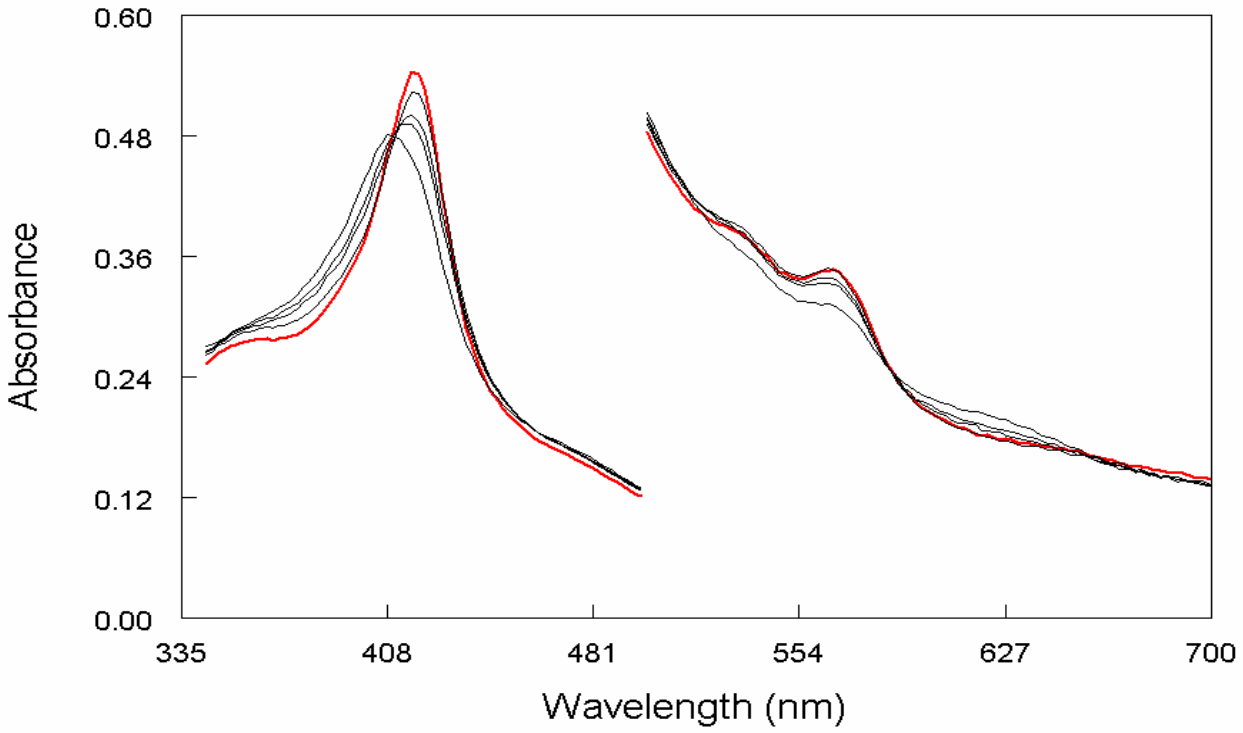


Figure 12: UV-Vis spectum of BM3-F87G reacted with iodosobenzene at pH 6.8. Enlarged region of P450BM3-F87G spectrum illustrating formation of compound E-S intermediate. Enzyme concentration ~700μM, oxidant concentration 125μM, buffer concentration 250mM Time frame from 0s(red) to 10s, reaction temperature held constant at 15°C.

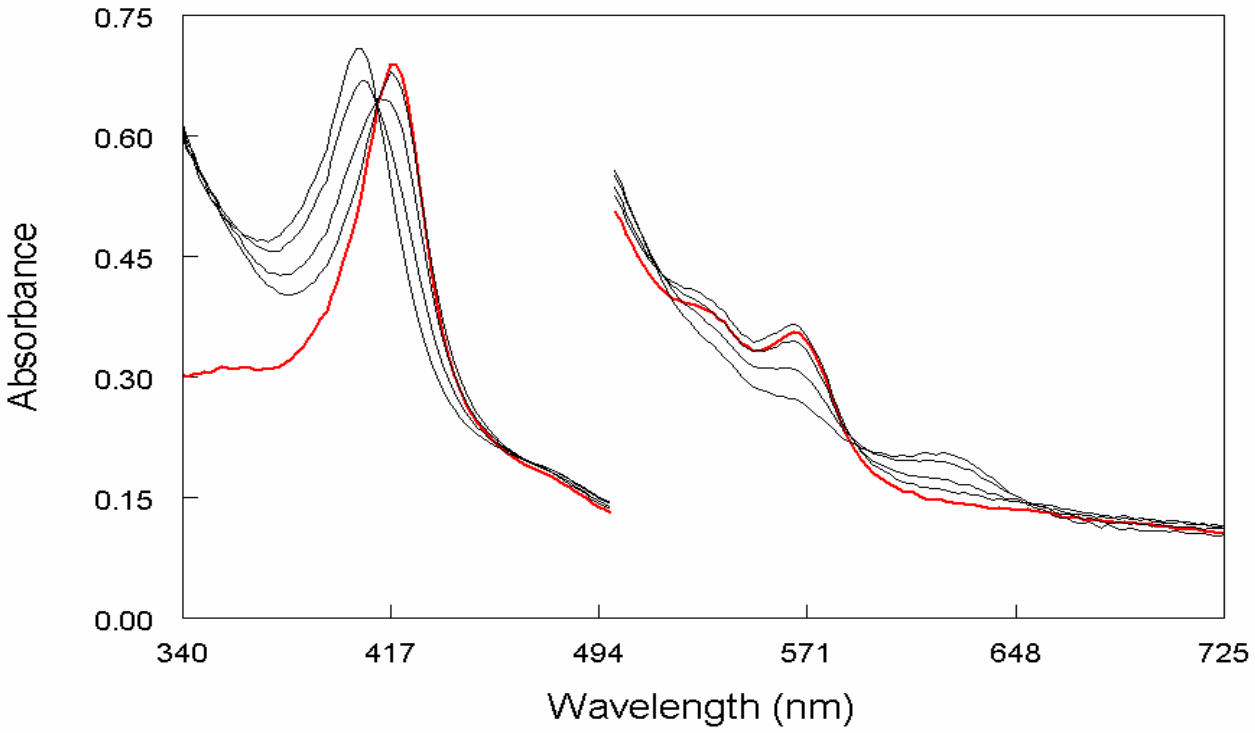


Figure 13: UV-Vis spectrum of BM3-F87G reacted with sodium m-periodate at pH 6.8. Enlarged region of spectrum illustrating formation of compound E-S intermediate. Enzyme concentration ~700μM, oxidant concentration 5mM, buffer concentration 250mM. Time frame from 0s(red) to 10s, reaction temperature held constant at 15°C.

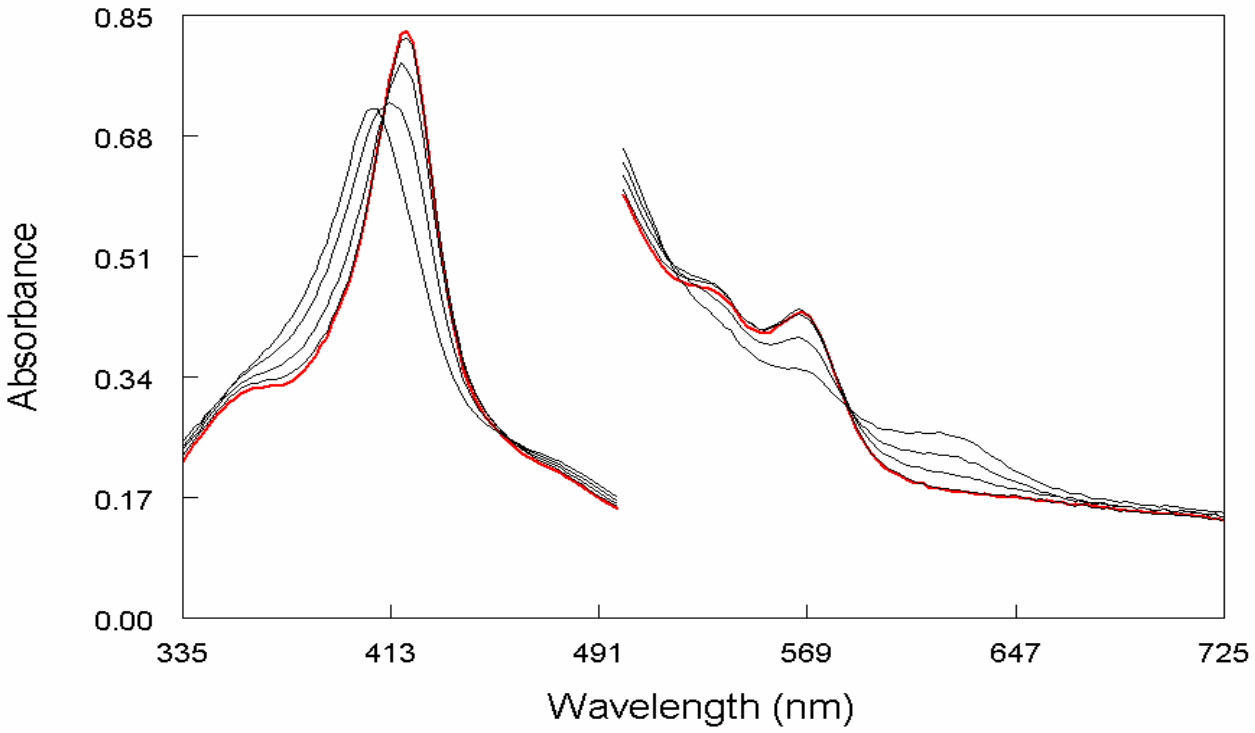


Figure 14: UV-Vis spectrum of BM3-F87G reacted with peracetic acid at pH 6.8. Enlarged region of spectrum illustrating formation of compound E-S intermediate. Enzyme concentration ~800μM, oxidant concentration 5mM, buffer concentration 250mM. Time frame from 0s(red) to 1s, reaction temperature held constant at 15°C.

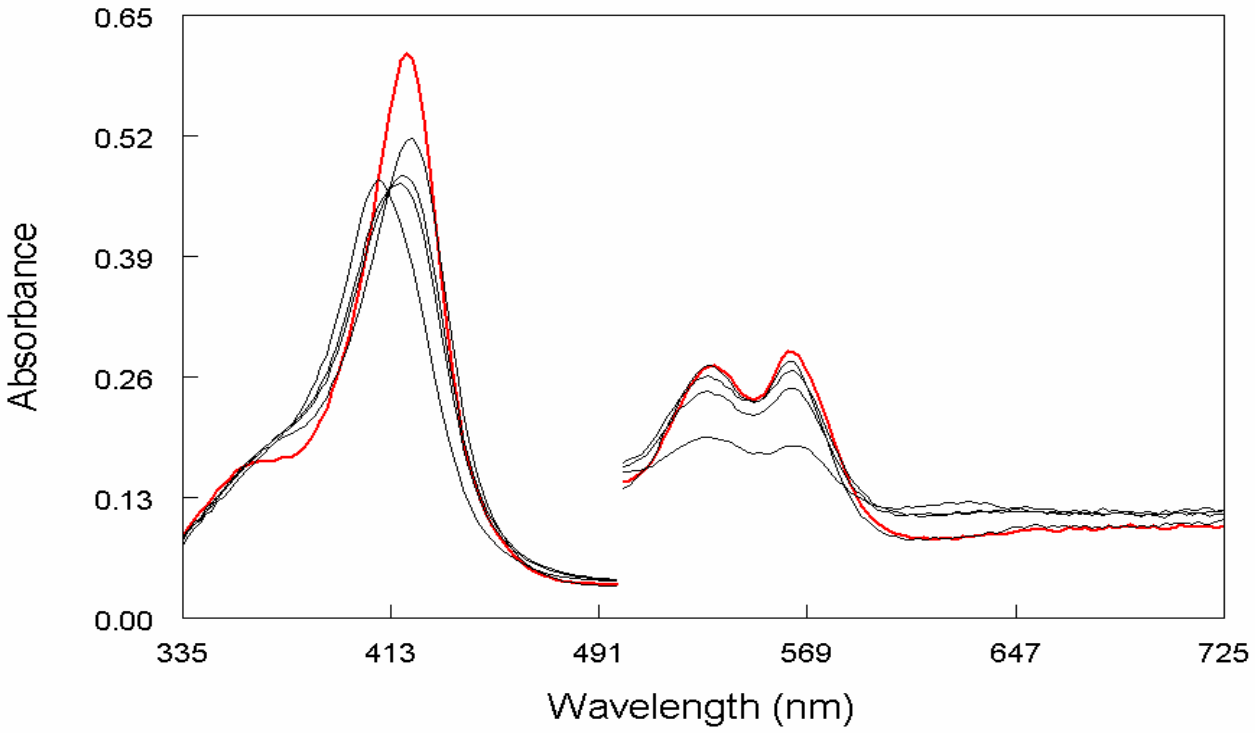


Figure 15: UV-Vis spectrum of BMH-F87G reacted with m-CPBA at pH 6.8. Enlarged region of spectrum illustrates formation of compound E-S intermediate. Enzyme concentration ~600µM, oxidant concentration 125µM, buffer concentration 250mM. Time frame from 0s(red) to 1s, reaction temperature held constant at 15°C.

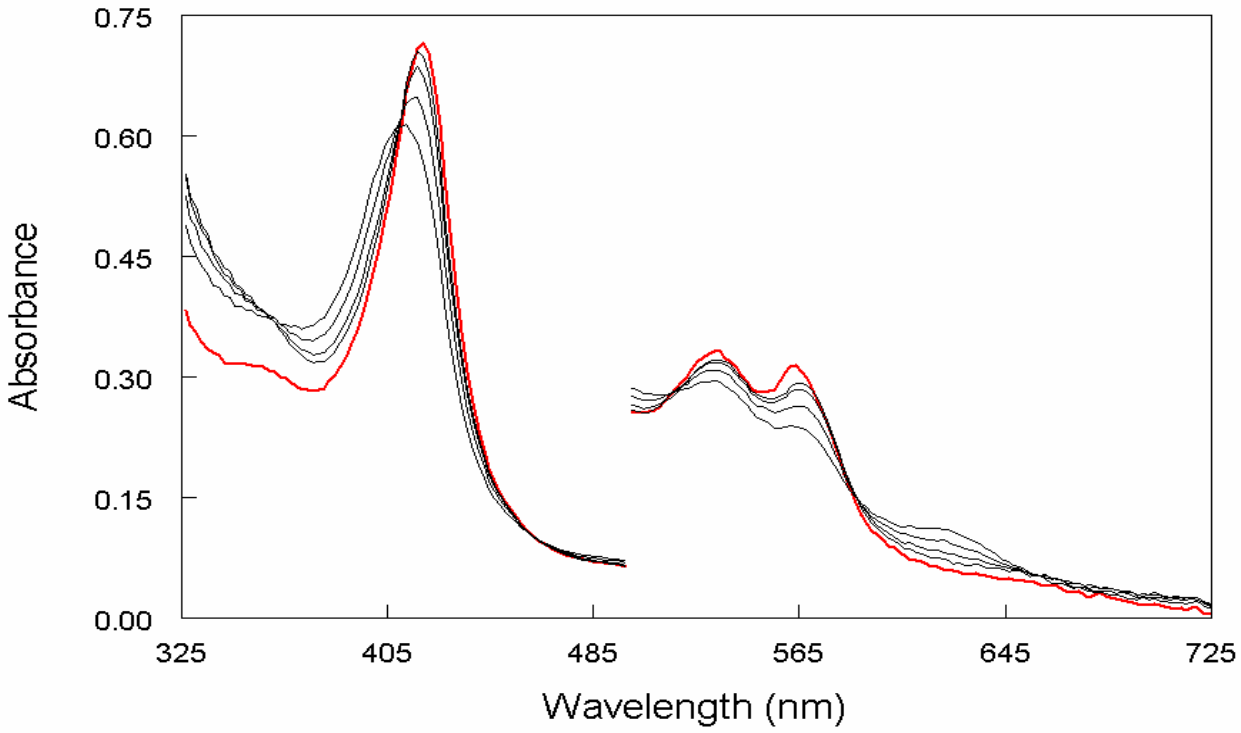


Figure 16: UV-Vis spectrum of BMH-F87G reacted with sodium m-periodate at pH 6.8. Enlarged region of spectrum illustrates formation of compound E-S intermediate. Enzyme concentration ~700µM, oxidant concentration 125µM, buffer concentration 250mM. Time frame from 0s(red) to 10s, reaction temperature held constant at 15°C.

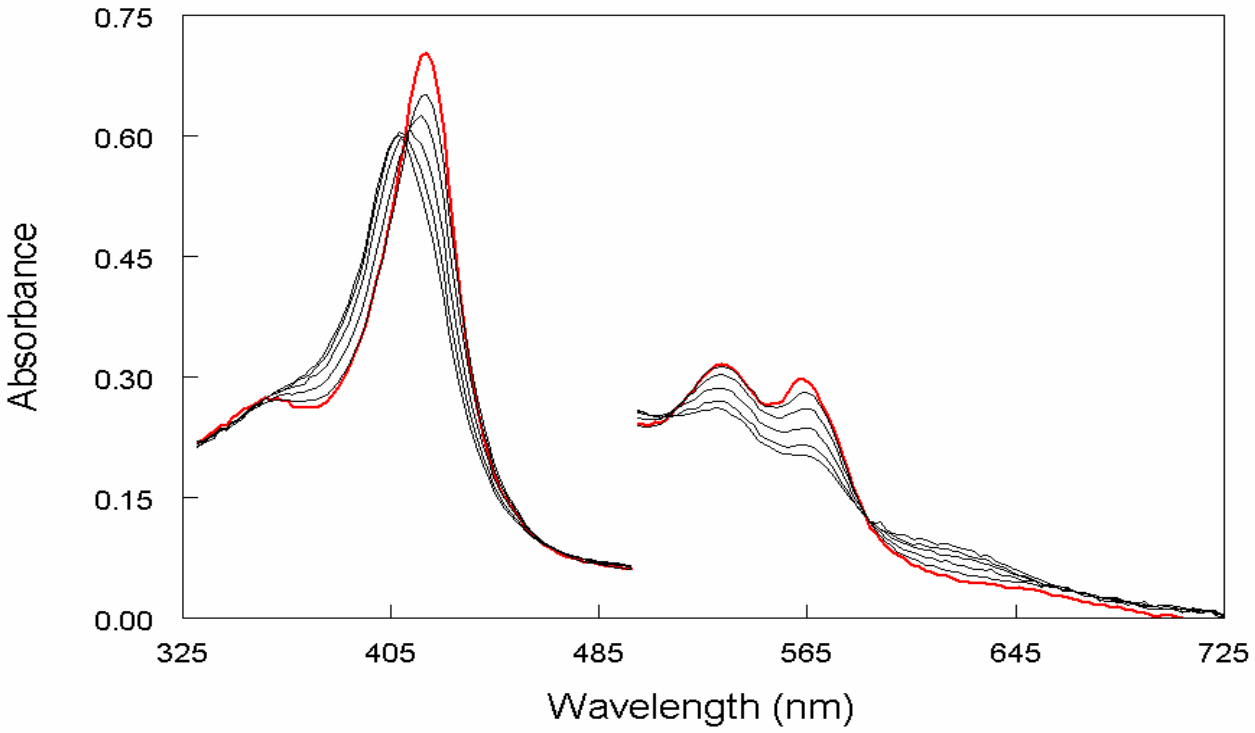


Figure 17: UV-Vis spectrum of BMH-F87G reacted with peracetic acid at pH 6.8. Enlarged region of spectrum illustrates formation of compound E-S intermediate. Enzyme concentration ~700µM, oxidant concentration 5mM, buffer concentration 250mM. Time frame from 0s(red) to 1s, reaction temperature held constant at 15°C.

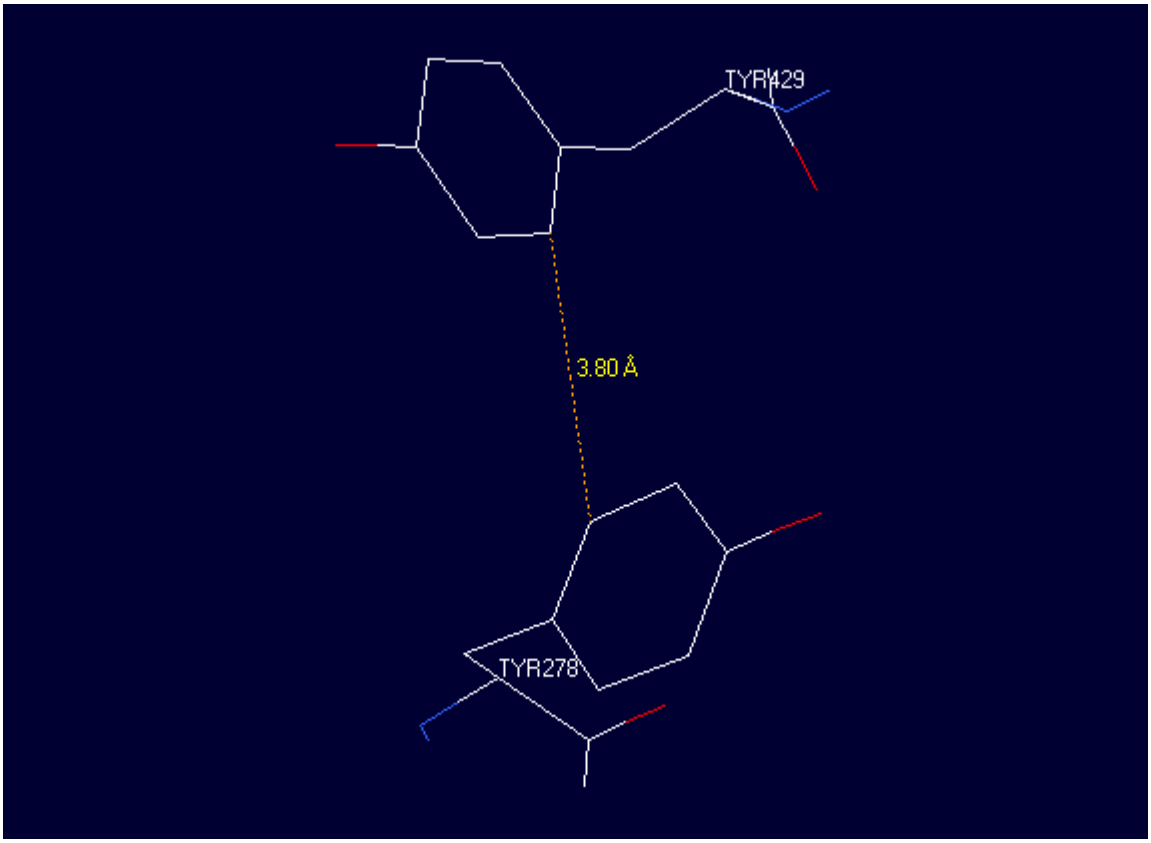
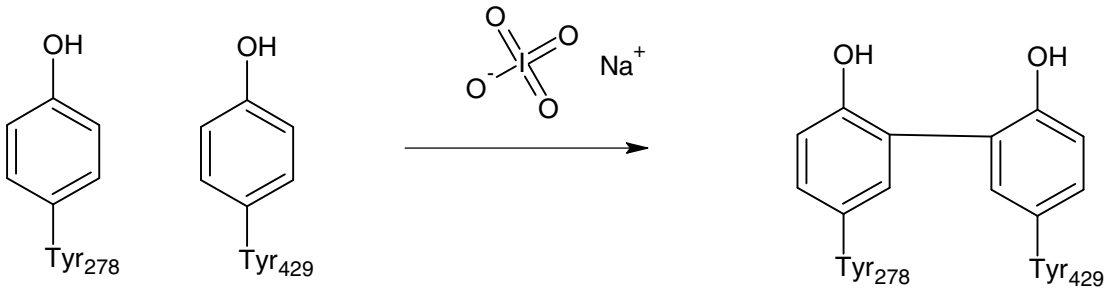
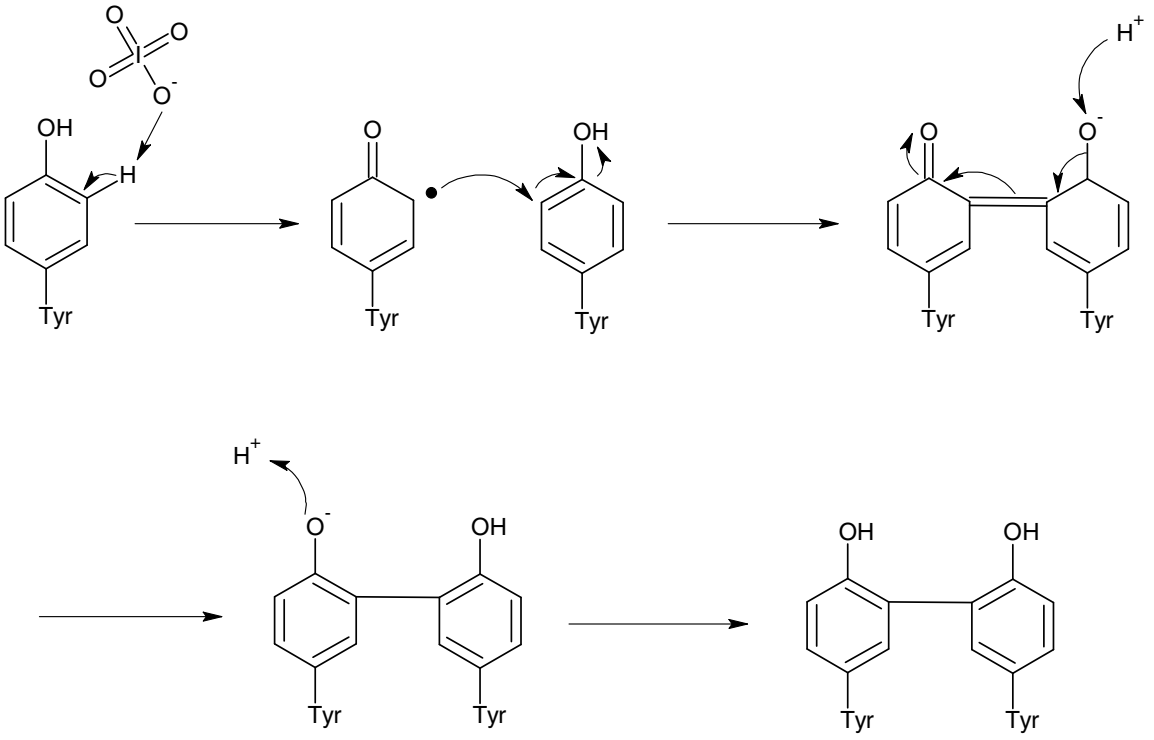


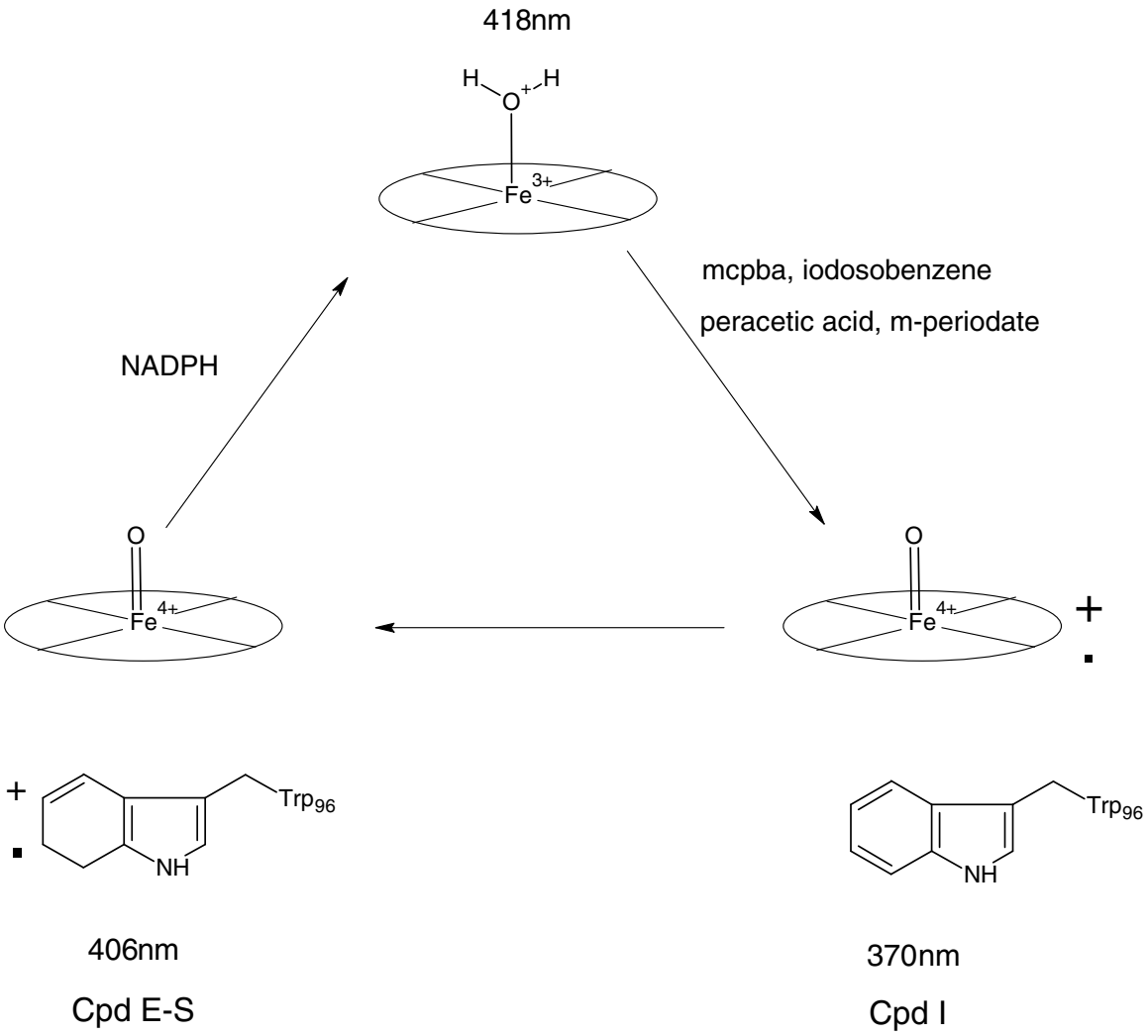
Figure 18: Deep View representation of distance between outlying tyrosine residues of P450BM3. All other outlying tyrosine residues greater than 10Å in distance.



Scheme I: Overall reaction of tyrosine dimer complex formation from outlying, neighboring tyrosine residues of P450BM3 when reacted with sodium m-periodate.



Scheme II: Proposed reaction mechanism for the formation of dimer complex between outlying tyrosine residues of P450BM3 and P450BMH.



Scheme III: Formation of compound I and compound E-S intermediates from reaction of P450BM3-F87G with alternate oxidants.

EPR analysis

Several EPR spectra with varying concentrations of m-CPBA and iodosobenzene were recorded at pH 6.8 reacting with P450BM3-F87G over a time course of ~5s; reaction time varied between +/- 2s as a result of sample freezing time. By increasing the oxidant concentration a more prominent signal was produced in the g = 2 region of the spectra which corresponded to an amino acid radical. This signal coincided with a decrease in low spin ferric heme signal which registered at g = 1.92, 2.25 and 2.41. The region around g = 2 was analyzed at microwave powers ranging from 5 μW to 20 mW for reactions with both mCPBA and with iodosobenzene. The spectrum obtained from the reaction with mCPBA appeared to show two different radical species, one at g = 2.005 with an isotropic line shape, and the other a multiple-lined signal represented by distinct components at g = 2.006, 2.017 and 2.026. The microwave power saturation behavior of the two signals differed noticeably, with the features at g = 2.006, 2.017 and g = 2.026 more easily saturated. The spectrum obtained from the reaction with iodosobenzene showed similar signals, but they were much more easily distinguished by varying the

microwave power. The signal at g = 2.005 was very difficult to saturate at high powers, while the multiple lined signal was the dominant feature at low power. At 5 μW power, the latter signal comprised three features (g = 2.026, 2.017, 2.006) separated by 14-14.5 gauss, suggestive of an amino acid radical. Additional preliminary EPR data were acquired using 165 μM BM3-F87G and 1.7 mM mCPBA. With this high mCPBA:P450 ratio, a broad low intensity feature in the EPR spectrum at g= 1.78 emerged along with a sharp g = 2 peak, and the ferric P450 signals at g = 2.41, 2.25 and 1.92 were weak.

Stopped-flow data suggested that the ES-like intermediate in P450BM3 was considerably more stable than it was in P450CAM. This could be due to the neighboring tryptophan residue in the active site region of P450BM3 and, as a result, this compound ES-like intermediate could be trapped without the need for a rapid-quench system. The saturation behavior of the radical signal and the observed hyperfine splitting at low power were consistent with the presence of a protein radical; however, different components of the EPR signal responded differently to changes in the microwave power, indicating the likely presence of multiple radical species. This became more

apparent when iodosobenzene was used as an oxidant and the EPR spectrum was recorded. The three signals observed in the m-CPBA reaction were all present, but their relative abundance was very different. The main species present in the iodosobenzene reaction was characterized by a multicomponent EPR signal with g-values listed previously of 2.026, 2.017 and 2.006. Although these peaks were also present in the mCPBA reaction, they were minor compared to the main peak at g = 2.005. While we are unable to assign the observed radical to a specific amino acid residue in this intermediate, the behavior of the radical signal is consistent with either a tyrosine or a tryptophan, or possibly a combination of both.[25] A possible explanation for this is that the two radical species originate on different amino acid side chains, and that the two oxidants react differentially with these residues. Assuming that at least one of the two surface tyrosine residues discussed earlier may be oxidized, this is a definite possibility. However, it is possible that the two oxidants produce different ratios of the intermediates represented in Scheme II. For example, the g = 2.005 signal that dominates the mCPBA reaction may represent a small amount of compound I (porphyrin π-cation radical), whereas the multicomponent

signal dominating the iodosobenzene spectrum would represent a protein radical, possibly Trp96. Rutter reported the 9.5 GHz EPR spectrum for compound I of chloroperoxidase (CPO) under several different conditions, and in each case the signal consisted of g ~ 2 and g = 1.73 components.[26] As with CPO, the preliminary EPR spectrum of BM3-F87G, when mixed with mCPBA for ~ 1 s, using a 10:1 mCPBA:BM3 ratio, contained a sharp g ~ 2 component and a broad weak signal at g = 1.78 (data not shown). The experiment with BM3 was carried out at 10K using 20 mW power, in contrast to the CPO experiment, where the microwave power was between 50 μW and 1 mW, and the temperature was between 3.6K and 30K. Although the similarity of the CPO and BM3-F87G EPR spectra alone does not prove that compound I is present in the P450 experiments, the combination of the EPR and the stopped-flow data argue more strongly that a compound I-like intermediate and a compound ES-like intermediate both are produced using mCPBA as an oxidant, whereas with iodosobenzene, the compound ES-like intermediate is generated almost exclusively.

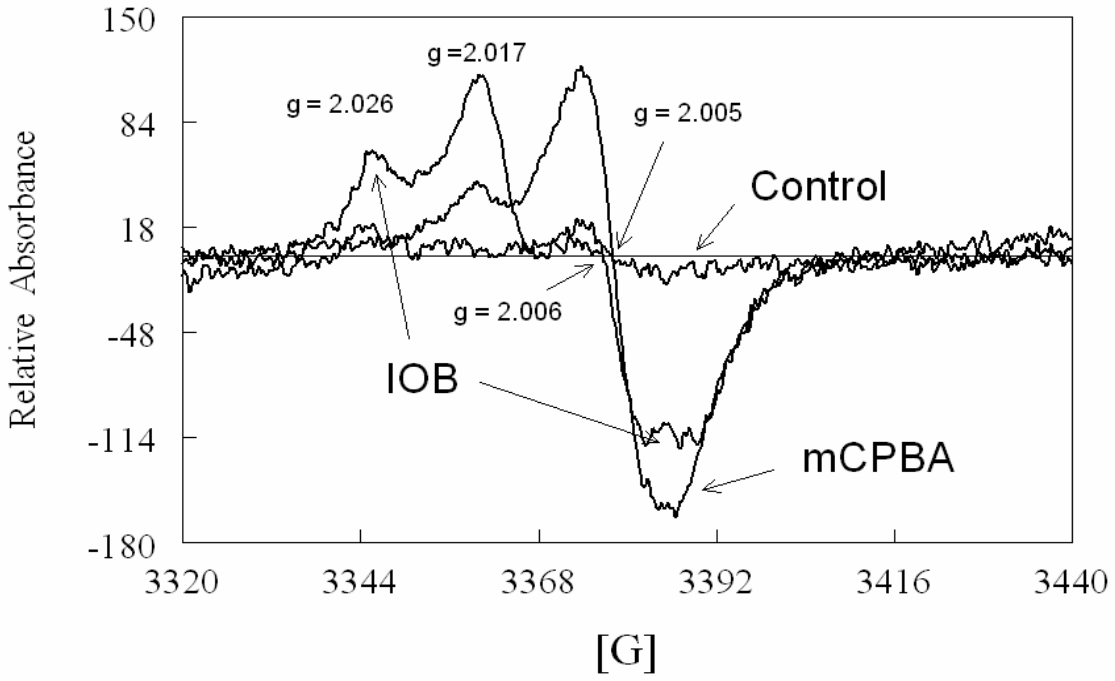


Figure 19: EPR spectra of P450BM3-F87G reacted with high concentrations of m-CPBA and iodosobenzene. Reaction time with oxidant before freeze quenching in liquid N_2 was ~5s.

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