MANJUNATH, SANDHYA C., M.S. Isotopic Probe for Spectroscopic Studies of Heme. (2009)
Directed by Dr. Gregory M. Raner. pp. 59

Cytochrome P450 enzymes (P450s) are heme containing monooxygenases with great potential in applications focused in biotechnological areas. An understanding of the precise chemical process behind this enzyme is essential in research in the areas of pharmaceuticals and biochemistry. In order to get a better understanding of P450s, it is important to provide spectroscopic characterization of the electron spin distributions within the heme macrocycle. Paramagnetic NMR has been shown to be a powerful tool for probing electronic distribution and spin-densities within the heme macrocycle. Selective incorporation of ^{13}C may facilitate the use of ^{13}C-NMR in the study of P450 monooxygenase. Furthermore, selective labeling with ^{15}N or ^{2}H at specific positions within the heme group maybe also be useful in the application of other spectroscopic techniques when applied to P450 enzymes. All of these labeling studies could be accomplished using a recombinant expression system involving a strain of *E.coli* which cannot produce aminolevulinic acid (ALA), a biosynthetic heme precursor. By synthesizing ^{2}H, ^{13}C or ^{15}N labeled ALA, incorporation of these isotopes into the heme of dehaloperoxidase, a model heme protein, via the biosynthetic pathway of the heme cofactor maybe accomplished. A successfully method was developed to make ^{2}H-ALA in a way that is both economical and time efficient, and the relative ability of this expression system to incorporate ^{2}H into the heme was evaluated, relative to ^{13}C or ^{15}N.

ISOTOPIC PROBE FOR SPECTROSCOPIC STUDIES OF HEME

by

Sandhya C. Manjunath

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

Greensboro
2009

Approved by

_____
Committee Chair

To my Amma and Appa,

Thank you for your love, encouragement, and support,

and to Madhura,

for always believing in me and for your love and affection.

Without you three, I would not be where I am today.

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 _____

_____

_____
Date of Acceptance by Committee

_____
Date of Final Oral Examination

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to Dr. Greg Raner for his guidance and support through my graduate work at UNCG. I would also like to thank my other committee members Dr. Alice Haddy and Dr. Nadja Cech for their valuable comments and assistance through my graduate work. Thanks also goes to Dr. Liam Duffy for letting me work in his lab for a year and for letting me play with liquid helium. I would also like to thank all the Professors who have helped me professionally and also as a student at UNCG.

I would like to thank my lab mates, and my fellow graduate students for their support. Special thanks to Molly, Jackie, Lauren and Brian for helping me in every step of the way.

Without my family and friends, I would never have been able to come this far. First, thanks to both of my Ajja and Ajji, to all my aunts and uncles, and my cousins. To Suganthi, Srikanth, Bala and Kalai, thank you for your encouragements, your kind words and support. To Komal, Amanda, Kim and Laura, thank you for just being there when I needed you and for being great friends.

Finally, I could not have done this work without Vamsi. Thank you for running my MS samples, for your support, and most of all for having faith in me.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

I.A. General overview of cytochrome P450

Iron serves important functions in the human body; therefore many important proteins contain heme as a prosthetic group, which means it contains an iron atom in the center of a large heterocyclic ring called a porphyrin. Cytochrome P450 (CYP450) is a diverse family of a metalloproteins containing a heme prosthetic group that is bound to the protein via thiolate ligation of a protein Cys residue[1]. CYP450 belongs to a group of monoxygenase enzyme that catalyzes many biological reactions in bacteria, plants and animals. It is also responsible for the metabolism of many endobiotic metabolisms such as testosterone, progesterone and they also play an important role in the xenobiotic metabolism in humans.

There are the two general classes that P450 enzymes can be split into, class I, which consists of most bacterial P450s and class II, which includes mammalian P450s. Class I P450's are cytosolic in nature with a lot of applications focused in the field of biotechnology. Studies on microsomal P450s tend to aim at understand xenobiotic metabolism in humans which is a major component in the field of pharmacology. CYP450 has a big role in metabolism of pharmaceuticals and contribute to the

biotransformation of a biologically active parent compound[2]. This process generally leads to less active metabolites that are more easily eliminated from the body.

The name cytochrome P450 has been given to this class due to the fact that these proteins all have reduced-CO complexes with an absorption peak with a λ max at 450 nm[1,2,3]. The CO binding pigment has a red shift when reduced and bound to CO whereas most heme proteins have a blue shift when reduced and bound to CO. In the reaction mechanism of P450 containing system, the active site of CYP450 contains a heme iron center. The reaction mechanism of the P450 family is a cyclic reaction that involves the reduction and oxidation of the central heme iron by incoming electrons, donated from nicotamide adenine dinucleotide phosphate (NADPH). The catalytic cycle of CYP450 is shown below in Figure I.1. The reaction takes place with the binding of a substrate to the active site which causes the heme to go from its ferric hexa-coordinated (low-spin) state to the ferric penta-coordinated (high spin) state. The second stage in the cycle is the reduction of the heme iron to the ferrous state by a single electron transfer from NADPH. In the third step, molecular oxygen binds rapidly to the metal center forming a Fe^{2+}-O_2 complex, which undergoes a slow conversion to a more stable Fe^{3+}-$O_2^{\circ-}$ complex. In the fourth step, there is a second reduction in which a second electron is accepted from the reductase and forms a ferric-peroxide complex, $(Fe\text{-}O)^{3+}$. This is a very unstable complex so in the next step, it is rapidly protonated and forms a hydroperoxo-ferric intermediate, which then is protonated again to release water and produces a reactive oxo-ferryl intermediate. In the next step, a ferryl-oxo species reacts with substrate to form a

hydroxylated product. This product is released from the active site and replaced by the water[2].

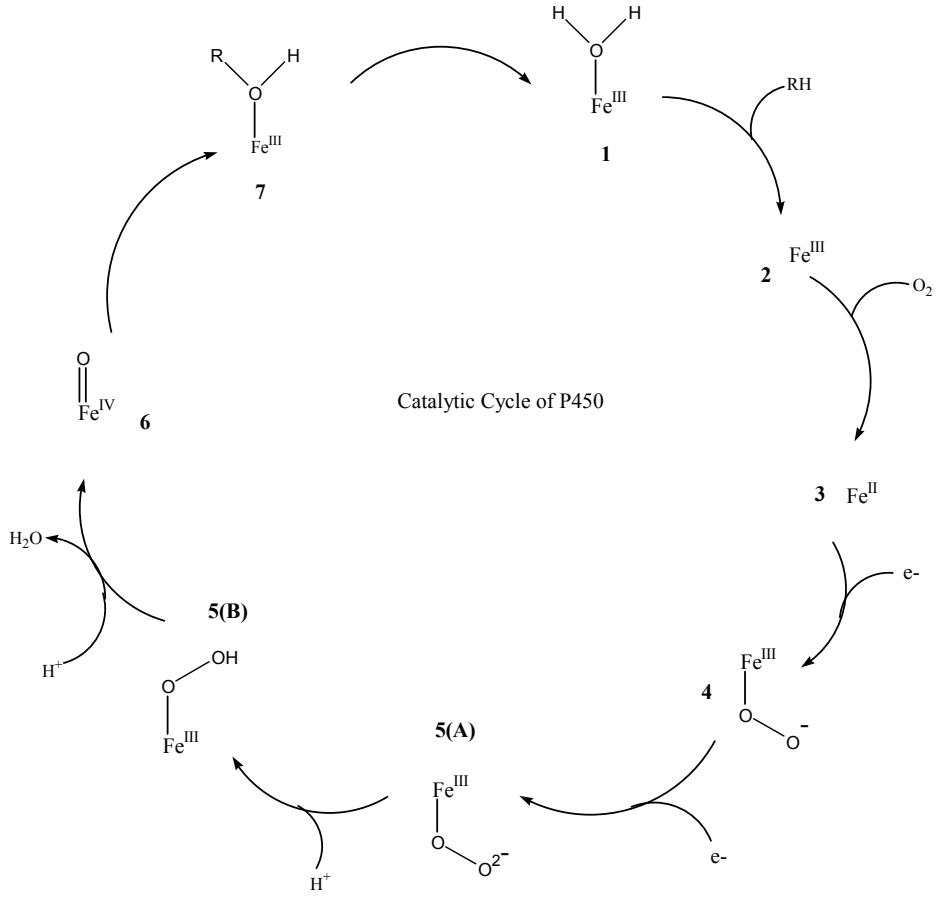


Figure I.1: The catalytic cycle of cytochrome P450[2]

As indicated previously, the active site of P450 contains an iron protoporphyrin IX group where oxygen is activated and substrate oxidation occurs. The numbering system of heme and its structure is found below in Figure I.2. The dots on the figure represent the four meso positions and are labeled with Greek letters α, β, γ, and δ. The core carbons (located in the pyrrole rings) have a separate system of numbering. For

example, the first carbon away from the meso carbon (C_m) is called C_α and the second carbon away from the meso carbon is C_β[4]. Shown in figure below is an iron atom in center of protoporphyrin ring bound to four nitrogen atoms from the pyrrole rings. In the current work an attempt was made to label various positions on the heme with stable isotopes.

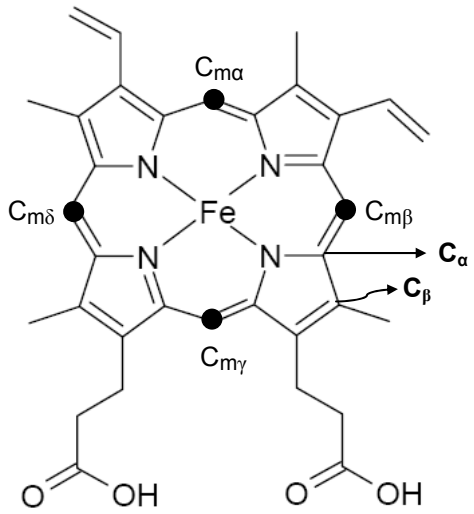


Figure I.2: Structure and numbering of protoheme IX[4]

I.B. *Escherichia coli* Hu227 and dehaloperoxidase

Our attempts to label the heme in P450 involve utilization of a specialized strain of *E.Coli.* Hu227 strain is a mutant strain which contains a mutation in the HemeA gene, which codes for the enzyme glutamyl-tRNA (GTA) reductase[11]. GTA reductase is responsible for the reduction of glutamyl-tRNA to glutamyl-1-semialdehyde (GSA). The next reaction by GSA – aminotransferase converts GSA to aminolevulenic acid (ALA), which is the biosynthetic precursor to heme. Hu227 strains cells cannot synthesize ALA

naturally, so the cultures of this strain must be supplemented with ALA to produce and grow. Previously in our lab, 4-^{13}C and 5-^{13}C ALA were synthesized and included in growth media for Hu227 cells instead of regular ALA. The predicted pattern for ALA using the ^{13}C labeled in positions 4 and 5 is shown below in Figure I.3. The asterisk represents the ^{13}C labeled carbons.

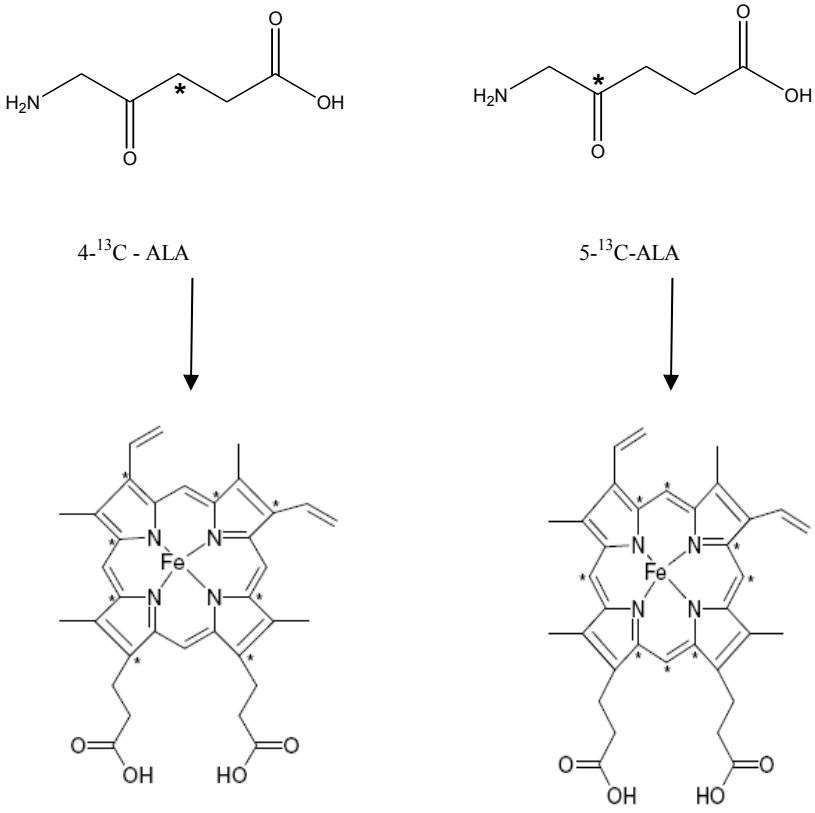


Figure I.3. Expected labeling patterns of 4 and 5-^{13}C-ALA

Initial attempts to express heme containing proteins using this recombinant system focused on an enzyme from a related class of heme proteins. Dehaloperoxidase (DHP) is an enzyme found in a marine worm, *Amphitrite ornate.* DHP is a globular

heme-containing enzyme that provides the marine worm with the ability to catalyze the oxidative dehalogenation of halogenated phenols[12]. Studies using UV-Visible absorption have demonstrated that exogenous ligand-ferric DHP is hexa-coordinated with water and has a proximal neutral histidine as axial ligand. It was proposed that this state activates the peroxide through an electron push-effect mechanism[12]. By synthesizing deuterium labeled ALA, incorporation of ^{2}H into the heme of dehaloperoxidase via the biosynthetic pathway of the heme cofactor may be accomplished. This will open up the door for production of P450 enzymes to be used in biochemical studies involving P450 deuterium isotope effects aimed at understanding the deformylation of aldehydes.

In *E.coli* the biosynthesis of heme starts with the condensation of one glycine and one succinyl-CoA to form δ-aminolevulinic acid (ALA). Next is the condensation of two ALA molecules, catalyzed by ALA dehydratase enzyme, also called Porphobilinogen Synthase which forms porphobilinogen (PBG), as shown in Figure I.4[13]. The four PBG molecules are then condensed by PBG deaminase (by elimination of the amino group) to form a 1-hydroxymethylbilane (HMB). This is then converted to uroporphrinogen III (Uro'gen) by uroporphyrinogen III synthase. The conversion from Uro'gen to protoporphyrin IX happens in several steps. First, the four acetyl side chains on Uro'gen are decarboxylated by Uro'gen decarboxylase which converts them to methyl groups and yields coproporphyrinogen III (Copro'gen). Two of the four propionyl groups undergo oxidative decarboxylation converting them to vinyl groups by Copro'gen oxidase which yields protoporphyrinogen IX. In the next step there is an oxidation of protoporphyrinogen IX, which adds more double bonds by removing six hydrogen atoms

to give protoporphyrin IX catalyzed by protoporphyrinogen oxidase. In the next step, Fe^{2+} is inserted to protoporphyrin IX by ferrochelatase.

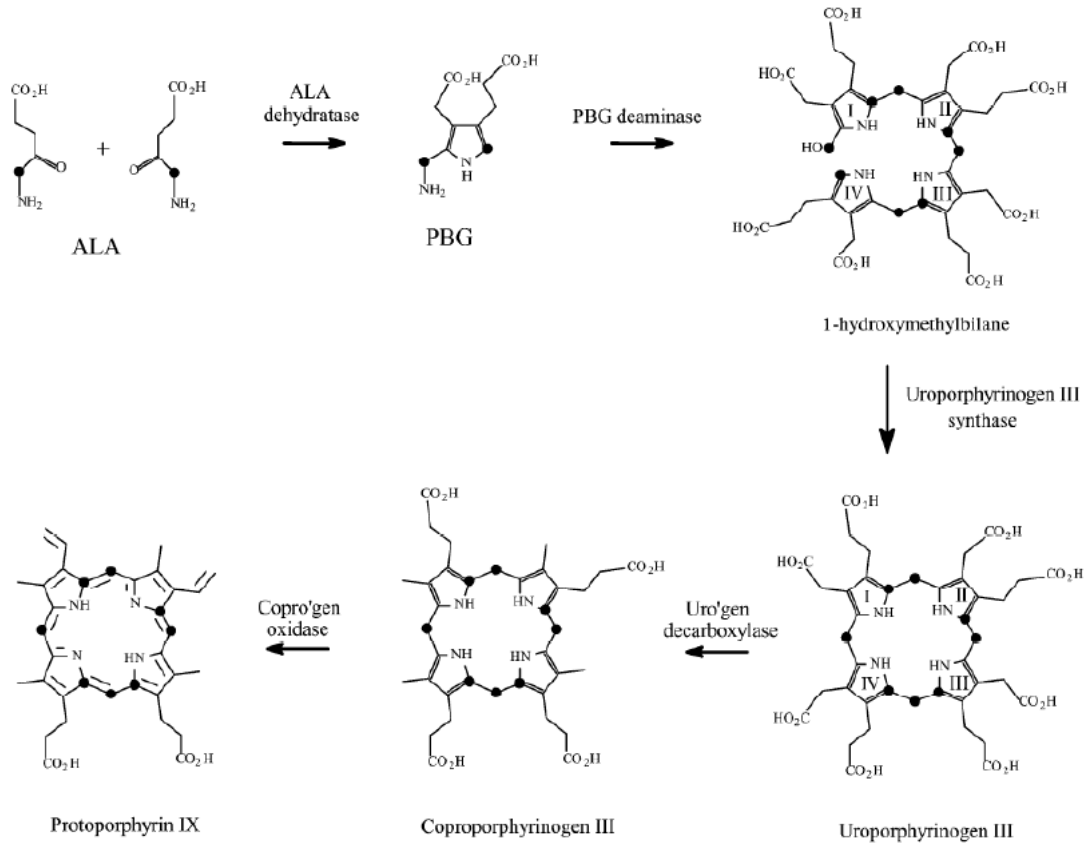


Figure I.4. Biosynthetic pathway of heme[14]. The highlighted atom represents ^{13}C

I.C. Active site of $CYP450_{BM3}$ and transient intermediates

As mentioned before, CYP450 is a class of monoxygenase enzyme, and studies relevant to these particular enzymes have been designed to gain a better mechanistic understanding of this human drug metabolizing systems. $CYP450_{BM3}$ isoform is a fatty acid hydroxylase that uses NADPH and one molecule of molecular oxygen which

oxidizes long chain fatty acids. It is a member of the class II P450s, making it an important model for the human drug metabolism of P450's. The BM3 enzyme is a single polypeptide, has a deep nonpolar pocket leading to heme center. The x-ray crystal structure of BM3 active site shows that there is a phenylalanine residue at position 87 and that iron at the heme center is bound to protein by a cysteine-thiolate linkage. Removing this Phe-87 from the active site and replacing it with any other amino acid with a smaller nonpolar side chain enhances the BM3 enzyme in the oxidation of aromatic substrates[9]. Li and colleagues replaced the Phe-87 with alanine and glycine and noticed that the hydroxylation of p-nitrophenoxydodeconoic acid (12-pNCA) was markedly increased.

Prior studies have shown that $P450_{BM3}$ with a F87G mutant modification of the heme using aromatic aldehydes results in altered catalytic activity rather than inactivation of the enzymes[10]. Raner *et al*. also performed a similar study using stopped-flow experiment to determine different intermediates in the heme reaction involving CYP_{BM3} F87G with 3-phenylpropianaldehyde (3-PPA) or 3-phenylbutyraldehyde (3-PBA) as substrates[8]. This study showed that the aldehydes lost the carbonyl carbon and the heme cofactor was alkylated at the γ meso position, in the course of this reaction as shown in Figure I.5.

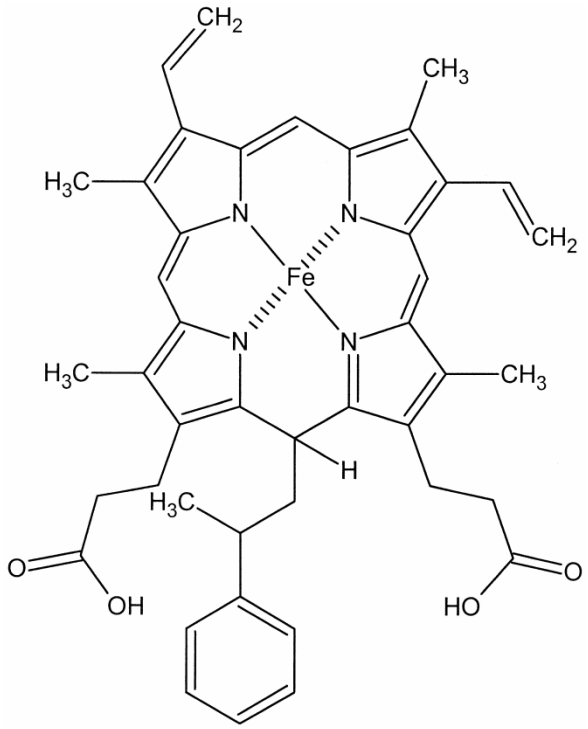


Figure I.5. Proposed isoporphyrin intermediate in the reaction of 3-PBA[8]

One of the long term goals for this project was to develop technology that would be useful in the identification of transient intermediates in the peroxo-pathway for $P450_{BM3}$-F87G. The hypothesis is that in the presence of hydrogen peroxide and an aldehyde substrate, $P450_{BM3}$-F87G will form a transient peroxy-hemiacetal intermediate, which decomposes to form an isoporphyrin intermediate. Heme alkylation occurs in this reaction which proceeds with the formation of γ-meso isoporphyrin intermediate that must decay with the deprotonation at the γ-meso carbon. This deprotonation should show a fully expressed deuterium isotope effect in the stopped-flow experiment. To address this issue, deuterium (^2H) labeled aminolevulinic acid (ALA) was synthesized for the incorporation of deuterium into the meso heme positions. Experiments described in this

thesis were designed to evaluate the ability of *E.Coli* to utilize ALA, synthesized with ^{2}H-labeles to produce a deuterated heme cofactor in order to probe.

I.D. NMR studies of isotopically labeled heme

Isotopic labeling with ^{13}C or ^{15}N may also be useful for NMR studies. The spectroscopic properties of any heme-protein usually result from the heme cofactor, which are related in turn to the electronic state of the central iron and the porphyrin itself. A low spin state exists, for example, when water is coordinated to the central iron in the axial position. A high spin state occurs when this water is removed, leaving iron existing as a pentacoordinated species[1]. These two spin states have distinct spectral patterns which allow the use of visible absorption spectrophotometry to monitor changes. Different forms of spectroscopy are also useful, along with UV/Vis, to probe minor changes in axial ligation and/or oxidation states of the heme group.

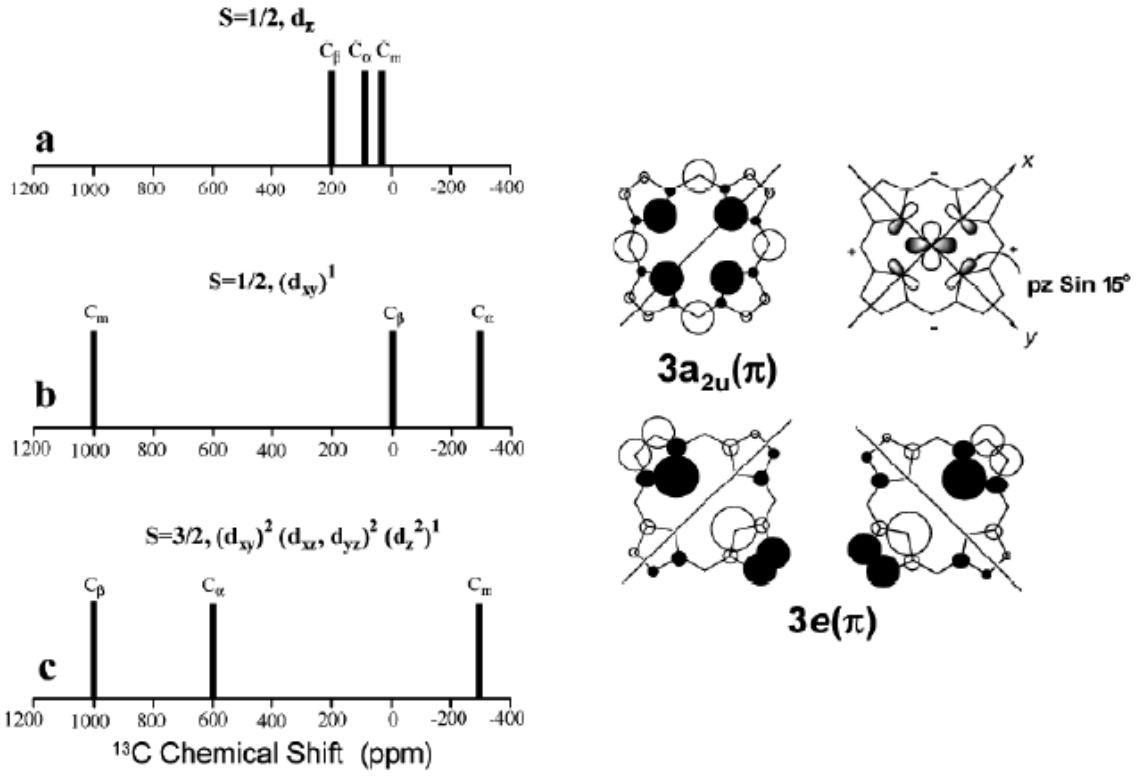


Figure I.6. Left: Typical porphyrin core ^{13}C chemical shifts. (a) Fe^{III}-porphyrinates with the S = ½, d_π electron configuration, (b) Fe^{III}-porphyrinates with the S = ½, $(dxy)^1$ electron configuration, and (c) Fe^{III}-porphyrinates with the S = ³/₂, $(dxy)^2(dxz,dyz)^2(d_z^2)^1$ electron configuration. Right: Schematic representation of the $3a_{2u}(\pi)$ and $3e(\pi)$ porphyrin orbitals. Relative sizes of the circles at each atom are proportional to the calculated electron density[5].

Caignan and his collegues conducted a study with hydroxide complex of *Pseudomonas aeruginosa* heme oxygenase (HO) containing ^{13}C labled heme, in which an OH served as a model for the OOH ligand of the Fe^{III}-OOH intermediate. In this study, they conclude that ^{13}C NMR chemical shifts can be used to determine the heme electronic structure[5]. As shown in Figure I.6.(a), the C_β has the furthest downfield shift, indicating that the major electron density is located on C_β in S = ½, d_π electron configuration have

spin delocalization into porphyrin 3e(π) orbital. Due to a very small shift, C_α has a small amount of unpaired density and C_m has no unpaired density. Ferrihemes with S = ½, $(d_{xy})^1$ electron configuration, on the other hand, have majority of their spin delocalization in the porphyrin $3a_{2u}(\pi)$ orbital, suggesting in Figure I.6(b), that most of the electron density is located on the C_m due to the substantial downfield shift. Ferriheme with S = ³/₂, $(dxy)^2(dxz,dyz)^2(d_z^2)^1$ electron configuration has a significant amount of electron density on C_β and C_α as indicated by the downfield shift, and C_m has no electron density.

Since there are many questions concerning the electronic structure of heme in P450, conducting studies that will probe spin distribution using ^{13}C-NMR paramagnetic spectroscopy will be useful in future applications. In particular, ^{13}C-labeling of heme cofactor will provide a method of exploring the electronic spin density across the porphyrin backbone. One of the drawbacks is that using ^{1}H-NMR is not helpful when probing paramagnetic hemoproteins and spin states. Previously, our lab successfully synthesized ^{13}C-labeled aminolevulinic acid (ALA), a heme precursor, at positions 4 and 5 and ^{15}N has also been incorporated at the pyrrole nitrogens. *E.Coli* Hu227 bacterial cell line was used due to the inability of these cells to produce ALA. Using mass spectrometery, it was demonstrated that the mass of ^{13}C-labeled heme increased by 8 mass units and ^{15}N-labeled heme increased by 4 mass units, as predicted based on the known biosynthetic pathway in *E.Coli*. However, the ^{13}C MS data was slightly ambiguous. In the current study, the goal was to use ESI-MS to clearly show ^{13}C incorporation in the heme. This technology will allow for the evaluation of electronic distribution within the heme cofactor of P450 enzymes using ^{13}C paramagnetic NMR.

I.E. Synthesis of deuterium labeled aminolevulinic acid

For synthesizing deuterium-labeled aminolevulinic acid (^{2}H-ALA), the procedure of Wang and Scott[6] was chosen with slight modification. The reaction scheme of the synthesis is shown in Figure I.7A. The synthesis of ^{2}H-ALA starts with labeled glycine and is protected by phthalic anhydride to yield phthalylglycine. Phthalylglycine is then treated with thionyl chloride to yield phthalimidoacetyl chloride. The next step requires that the phthalimidoacetyl chloride be coupled with zinc homoenolate. A palladium catalyst is used in order to form phthalimolevulic acid ethyl ester. But to get to the last step, the zinc homoenolate had to be synthesized by reacting [(1-ethoxycyclopropyl)-oxy]trimethylsilane with zinc chloride in dry ether, which can be seen in Figure I.7B The zinc homoenolate then reacts with phthalimidoacetyl chloride to yield phthalimolevulic acid ethyl ester. In the next step, acid hydrolysis of phthalimolevulic acid ethyl ester with hydrochloric acid yields labeled aminolevulinic acid (ALA). For the pupose of this project, 2,2-D_2 Glycine was used as the starting material to ultimately generate ^{2}H labeled ALA.

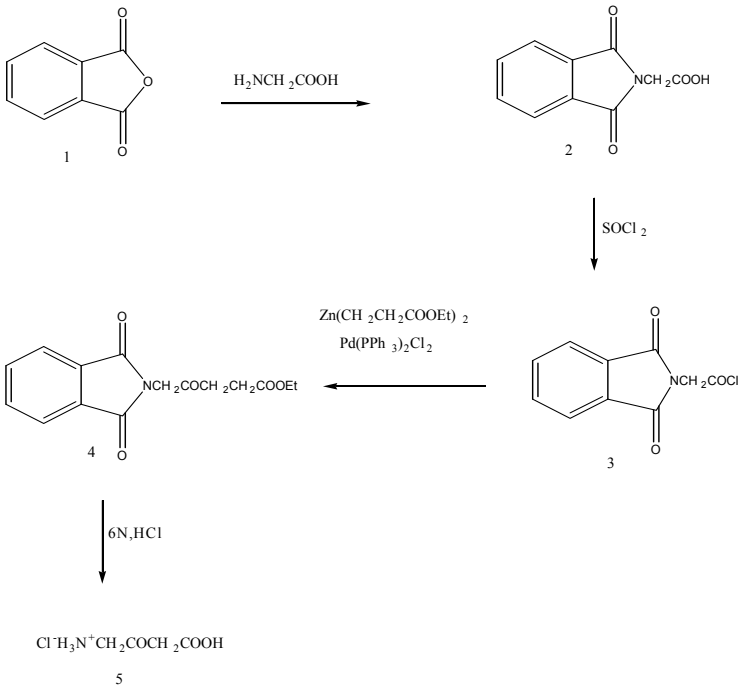


Figure I.7A: Synthetic procedure for the generation of ALA[6]

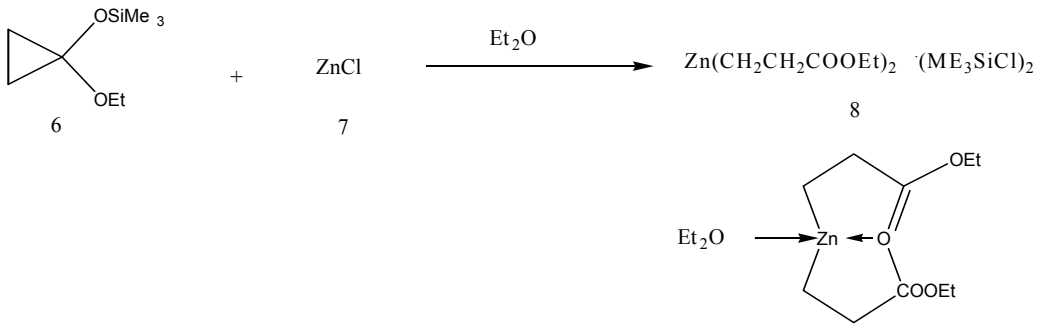


Figure I.7B: Synthesis of zinc homoenolate[6]

CHAPTER II

EXPERIMENTAL

II.A. Preparation of Luria - Bertani broth (LB) media, $CaCl_2$ solution and LB-Agar plates

The LB media was prepared by adding 4 g of LB powder mixture to a total volume of 200 mL of dH_2O. A 80mM $CaCl_2$ solution was prepared by adding 1.18 g of Calcium Chloride to 100mL of dH_2O. LB-Agar solution for the plates was prepared by adding 3.75 g of agar and 5 g of LB powder to 250 mL of dH_2O, and a magnetic stir bar. All 3 solutions were sterilized for 15 min and allowed to cool at room temperature. Once the solutions were sterilized, the LB-Agar solution was stirred room temperature on a stir plate, and when it was cool enough to touch, 100 mg/L of ampicillin and 10 mg/mL of ALA was added. Only 10 mg/mL of ALA was added to the LB media, with no ampicillin. After that, the LB-Agar solution was poured in to the agar plates and once it solidified, it was then placed in a 20°C refrigerator. The preparation of the media and the sterilization of the media were done to grow non-virulent strain of E.Coli cell, Hu227.

II.B. Growing Hu227 untransformed cells in LB media

Sterile techniques were employed through the whole procedure when growing Hu227 cells. Using a 25-mL pipette, a 25 mL aliquot of LB/ALA media was transferred to a 50 mL Falcon tube. The media was then inoculated with the untransformed Hu227 cells and they were than shaken at 200 rpms at 37° C overnight. The following day, the media was thick with growth.

II.C. Procedure for making competent Hu227 cells

E.Coli Hu227 cells were made competent with sterile techniques throughout the preparative steps. 10 mL of the Hu227 thick media was then transferred to another 50 mL falcon tube, along with 15mL of plain LB media. It was grown again for 30 min at 200 rpm, 37°C. The cell cultures were transferred over to 6 of the 1.5 mL microcentrifuge tubes and were centrifuged for 1 min at 10,000 rpm. The supernatant was discarded and 1 mL of ice-cold sterile 80 mM$CaCl_2$ was added to the pellet and the cells were then suspended, very gently. Using chilled $CaCl_2$ increases the permeability of the membranes of the cells. The suspended pellets were centrifuged again for 1 min at 10,000 rpm. The supernatant was discarded and the pellets were suspended in chilled $CaCl_2$ solution and placed on ice for 10 min. The tubes were centrifuged again for a min at 10,000 rpm and resuspended in 200 μL of chilled $CaCl_2$ and placed in -70°C for at least 3 hours before taking it out for transformation. Using the protocol for Plasmid DNA Purification, the QIAprep Spin Miniprep kit and a Microcentrifuge, both 102A2/BL-21 and

BM3F87G/HB101 were purified from overnight cultures of the respective cell lines grown in the presence of appropriate antibiotic to ensure plasmid maintenance.

Another alternative method from Kotchoni et. al. was also used to make Hu227 cells competent[15], since p102A2 was not expressing well with Hu227 competent cells from the method above. For this experiment three seperate solutions had to be made in advance. Solution I (50mM Glucose, 25mM Tris-HCl – pH 8.0, 10mM EDTA – pH 8.0) was prepared by adding 4.5g of glucose, 1.5g of Tris, and 1.9g of EDTA to a 500 mL deionized water. The pH of the solution was taken down to 8.0 with HCl and than autoclaved. 4µg/mL lysozyme was also prepared in order to add into the solution I just before use. Solution II (0.2M NaOH, 1% (w/v) SDS) was prepared by first making a stock solution of 2M NaOH and a stock solution of 10% (w/v) SDS. The solution was than prepared just before use by diluting to 0.2M NaOH and 1% (w/v) SDS and was autoclaved. Solution III (8M Ammonium Acetate) was prepared by adding 61.6 g of ammonium acetate to 100mL of deionized water and was also autoclaved.

An overnight culture was setup with 2mL of 102A2/BL-21and pT7po1 in a 2.5mL eppendorf tubes. The cells were centrifuged at 5000 g for 5 min at room temperature. The pellets were then suspended in 200µL of Solution I, which contained the freshly added lysozyme. The solution was incubated for 5 min in room temperature. After 5 min of incubation, 400µL of freshly prepared Solution II was added and inverted gently 6 times. Immediately, 200µL of Solution III was added and mixed gently. It was then incubated on ice for 5 min and a white precipitate had formed. This was centrifuged

for 5 min at 10000 g and the supernatant was transferred to a new tube. To that supernatant, 600µL of isoproponal was added and mixed gently and kept at room temperature for 10 min. It was then centrifuged again for 5 min at 10000 g, but this time the supernatant was discarded and the pellet was washed with 400µL of 70% (v/v) ethanol. It was again centrifuged at 10000 g for 3 min, after which the supernatant was discarded and the pellet was left to air dry for 30 min to get rid of the residual ethanol. The pellet was resuspended in 30µL of 10mM Tris-HCl (pH 8.0) and was stored in -70°C freezer.

II.D. Plasmid purification of 102A2 from BL-21; BM3F87G from HB101

Plasmid DNA Purification using the QIAprep Spin Miniprep kit and a Microcentrifuge was used to purify both 102A2/BL-21 and BM3F87G/HB101. 102A2 in BL-21 and BM3F87G in HB101 cells were centrifuged at 10000 rpms for 5 min in 1.5mL centrifuge tubes. The supernatant was discarded and the procedure was repeated two more times. After the third time, the pelleted bacterial cell was resupended in 250 µL of Buffer P1 and transferred it to a microcentrifuge tube provided by the kit. Also added 250 µL of Buffer P2 and inverted the tube 6 times, and 350 µL of Buffer N3 and also inverted the tube 6 times. Once everything was mixed properly, the solution was centrifuged for 10 min at 13,000 rpms. The supernatant from last step was transferred to QIAprep spin column by decanting. It was then centrifuged for a minute to discard the flow-through. It was then washed further to remove trace nuclease activity by adding 500 µL of Buffer PB and centrifuging for a minute. The solution was again washed by adding 750 µL of

Buffer PE and centrifuged another minute. After discarding the flow-through, the solution was centrifuged for an additional minute to remove the residual wash buffer, so the residual ethanol will not inhibit subsequent enzymatic reactions. The spin column was then transferred to a clean 1.5 mL microcentrifuge tube, to elute DNA by adding 50 µL of Buffer EB. After letting the solution stand for a minute, it was then centrifuged for an additional minute, and the purified 102A2 and BM3F87G plasmid was stored in -70°C freezer.

II.E. Procedure for transformation of Hu227 cells with p102A2, and pBM3F87G plasmids

Both Hu227 competent cells and plasmids 102A2 and BM3F87G were thawed out on ice, and 200 µL of the competent Hu227 cells were transferred over to two of the sterile 1.5 ml microcentrifuge tubes. Purified 102A2 plasmid (25µL) or BM3F87G plasmid (25µL) was added to the 200 µL of Hu227 cells in separate tubes. The tubes were flicked rapidly by hand several times to increase the efficiency. The tubes were then incubated on ice for 30 min. After the incubation on ice, the solutions were transferred over to a hot water bath with temperature of 42°C for 50 seconds. This was done in order to heat shock the cells to increase the pores in the cells so it will be easier for the plasmids to enter the cells. After the heat shock, the solutions were again placed on ice for 2 minutes. After the incubation on ice, 900 µL of LB/ALA/Amp was added to the mixture and incubated for an hour at 37°C shaking at 200 rpms. After an hour, 100 µL of

each growth mixture was streaked onto the already made LB/ALA/Amp plates. The plates were then allowed to incubate overnight at 37°C.

II.F. Preparing and sterilization of Terrific Broth (TB) media and expression and harvesting of BM3F87G cells

The TB media was prepared by mixing 47.6 g of TB powder to 1L of dH_2O, in a wide-mouth culture flask. The six flasks were then placed in an autoclave for sterilization. Once the media was cool enough, 100 mg/L of ampicillin and 10 mg/L of ALA was added to the media. After that, a single colony of BM3F87G/Hu227 cell was used to inoculate all the cultures. The cultures were mixed in a gyroshaker at room temperature with a shaking rate of 200 rpms. After 40 hours of agitation of the cultures, the cells were induced with 150 mg/L of IPTG, for expression of the recombinant P450 and the speed was reduced to 70 rpms and left overnight. The cells were then harvested by centrifuging the cells at 10,000 rpms for 10 min in the Beckman-Coulter centrifuge. The supernatant was then discarded and the pellets were then suspended in 25 mL of 50 mM phosphate buffer (pH 7.4) and 15% glycerol. The solution was homogenized using a glass homogenizer and 10 mg of lysozyme was added to the solution for cell lysis. It was stirred for an hour at room temperature and the cell membranes were then broken by using a sonicator. The solution was centrifuged at 18,000 rpm for 45 minutes, and the supernatant stored in -70°C freezer.

II.G. Synthesis of deuterated aminolevulinic acid (^{2}H- ALA)

Glycine-2,2-D_2 was purchased from Cambridge Isotope Laboratories, Inc. ^{d}ALA was prepared by the procedure Wang and Scott described in their paper[6]. The scheme used for this synthesis of ALA was presented in the Background, Figure I.7 (A) and Figure 1.7 (B). A more detailed procedure for the synthesis of ALA is now presented. Most of the reactions used were very sensitive to moisture, hence they were carried out under an atmosphere of argon gas.

Glycine (1.0005g) was combined with phthalic anhydride (2.0087g) in a 50 mL round bottom flask. Indirect heat was used to fuse the solids using a Bunsen burner. Heating the solids for approximately 3 minutes caused the white powders to liquefy. Soon after, solid crystals began forming on the inside of the flasks. The product was then recrystallized from water and stored in the refrigerator. After 30 minutes, it was filtered and the yield of the resulting product, phthalylglycine was 2.2548 g, 74.9% yield. It was then stored in a desicator at room temperature.

Dried phthalylglycine (2.2541 g) and 12 mL of thionyl chloride was refluxed overnight in a 25 mL round bottom flask. The reaction was carried out under continuous argon gas flow in a 80°C oil bath. The liquid was then cooled and the thionyl chloride was removed with a rotorvapor hooked to a vacuum. The resulting yellow powder was analyzed by NMR and found to be the desired product phthalimidoacetyl chloride. The overall yield of this product was 2.0632 g, 91.5% yield

In the subsequent step, zinc chloride (2.01g) was fused in a 100 mL round bottom flask with indirect heat from Bunsen burner under vacuum, and cooled under vacuum to a white solid. Approximately 30 mL of diethyl ether was added and the zinc chloride was stirred at room temperature under argon gas flow. It was then refluxed at room temperature for 2 hours to obtain a homogenous mixture. After that, 6.0 mL of dry [(1-ethoxycyclopropyl)-oxy]trimethylsilane was added into the reaction drop wise with a syringe. After 5 minutes of adding it slowly, the solution turned cloudy solution, and the stirring was continued at room temperature for 1 hour. That was then followed by additional reflux in a hot oil bath for 30 min to obtain the zinc homoenolate.

This solution was stirred further in an ice bath under argon gas. To this solution Tetrakis (triphenylphosphine) palladium (155 mg) and phthalimidoacetyl chloride (2.0 g) was added. After waiting for 30 min to obtain a homogenous mixture, 3 mL of N, N-dimethylacetamide was added via a syringe very slowly. The solution was stirred at 0°C for 1 hour and then stirred at room temperature for additional 2 hours under argon gas. The solution was then evaporated using a Rotorvap, under vacuum. The light brown residue was dissolved in 100 mL of dichloromethane and the solution was transferred to a 250 mL separatory funnel and washed with 50 mL of water. The aqueous fraction was discarded and the dichloromethane fraction was again washed with 50 mL saturated NaCl – water. The organic fraction was saved and dried overnight under vacuum. The oily-orange solution that resulted was dried again using a Rotorvap under vacuum in an attempt to attain the solid product, but each time, only an orange oil resulted. To obtain a solid product, recrystallization from ethanol was attempted but again, no crystals were

produced. The speed-vac was again used to remove the solvent and the oily material was again produced. An NMR spectra of the sample was taken, confirming the presence of the product.

Phthalimidolevulinic acid ethyl ester (1.8134g) and 1:1 mix of hydrochloric acid: acetic acid solution was placed in a 50 mL round bottom flask to reflux for 6 hours at 120°C. The deep yellow solution was evaporated using a Rotorvap under vacuum. To remove excess acid, the solution was washed with 20 mL of water. The solid was then taken up with 40 mL of water and transferred to a 125 mL seperatory funnel. To remove phthalic acid, the aqueous fraction was washed with 25 mL of ethyl acetate four times. The aqueous fraction was evaporated under vacuum to a brown-oily residue. To obtain solid product, recrystallization from ethanol and diethyl ether was attempted, but no crystals were produced. Recrystallization with acetone was also tried, but again no crystals were produced. Finally, drop by drop of cold diethyl ether was used and kept to freeze dry overnight to get 1.6613 g of white solid crystals. These were then stored at -20°C in a glass vial.

II.H. Growing Hu227/DHP using synthesized ^{2}H -ALA

Hu227 and DHP were grown according to what was described previously. The media contained LB/^{2}H-ALA/Amp and the DHP harboring *E. Coli* was used to inoculate and cells were grown at 30°C at 200 rpms overnight. Once IPTG was added to induce protein expression, the solution was centrifuged for 10 min at 10,000 rpm. The pellets were then reconstituted with 50 mM, pH 7.4 phosphate buffer (PO_4^{3-}). Then the cells

were lysed by adding 50 mg of Lysozyme and used a homogenizer to homogenize the solution. It was then stirred at room temperature for an hour and was sonicated at full speed for a minute, two times. The solution was again centrifuged for 45 min at 18,000 rpm. 1:1 ratio of 15% glycerol was added to the ^{2}H-DHP supernatant and stored in -80°C freezer.

II.I. Extraction of heme from DHP for LC-MS data

Once ^{2}H-DHP supernatant was thawed, 3 mL of the supernatant was transferred to a screw top test tube and 2M HCl (200 μL) was added to each of the test tube, and was incubated at room temperature for 30 min, after which 2 mL of ethyl acetate was added to one of the test tube, 2 mL of petroleum ether for 2nd of the 3 test tube, and 2 mL of Isobutanol was added to the last test tube. Even though, a clear organic and aqueous layer could be seen, they were all centrifuged for further clarity. Using a Pasteur pipette, the organic layer was drawn out and some of the sample was evaporated under vacuum for 2 hours. The rest of the sample was saved for subsequent HPLC analysis and isolation. The reddish pellet obtained was reconstituted with 60:40 Acetonitrile: water (1% TFA) for both absorption spectra and HPLC chromatographic analysis. For LC-MS, the pellets were treated either with the mobile phase (50:50 ACN: H_20, 1% TFA), or with Methanol.

Since the LC-ESI results had a lot of impurities, the samples were processed by HPLC and the fractions were collected between 1-15 minutes. The organic layer that was saved in the above step was transferred to a HPLC glass sample vial. This was then placed in the autosampler rack of a Shimadzu HPLC system equipped with a C8 column.

The flow rate for the column was 1.0 mL/min and the absorbance of the eluent was observed at 398 nm. The mobile phase used was 60:40 mixture of ACN:H_2O with 0.1% TFA. The largest peaks were collected at retention times from 4 – 9 minutes and the samples were evaporated under vacuum. The pellets were treated with methanol and the data were obtained from LCQ Advantage Thermo Finnigan ion trap mass spectrometer with electrospray ionization coupled to an Agilent HP1100 HPLC.

II.J. Purification of ^{2}H-DHP by anion exchange DEAE column

The column was first washed with 20% EtOH and washed again with 20 mL of 50 mM phosphate buffer (PO_4^{3-})(pH 7.4), and was loaded with the crude cell lysated, diluted 1:4 in 15% glycerol. The column was washed with 20 mL of 50 mM PO_4^{3-}, 100 mM PO_4^{3-}, 150 mM PO_4^{3-}, and 200 mM PO_4^{3-} buffer to generate a stepwise elution gradient. The DHP protein, which adhered to the column during the 50 – 100mM phosphate wash was eluted out. For both 150 mM PO_4^{3-} and also 200 mM PO_4^{3-} elution, visible absorption spectra was taken and samples were stored in -80°C. For the absorption spectrum, the sample was treated with CO gas and absorption between 400-500 nm was recorded. After the background correction, a pinch of sodium dithionate was added for both the sample and background, and an absorption spectrum was then taken. This represents the reduced vs. reduced-CO difference spectrum for the enzyme.

II.K. Preparation of EPR samples

Previously in our lab, [4-^{13}C] ALA DHP, [5-^{13}C] ALA DHP, ^{15}N-ALA DHP, and pure DHP were prepared. In order to run an EPR spectrum on these samples, the enzyme was oxidized to the ferric form using potassium ferricyanide to ensure a homogeneous sample. Once the samples were thawed, they were treated with potassium ferricyanide at which point the sample turned a reddish-yellow color. This sample was then passed through a G-25 Sephadex column to remove excess ferricyanide. The red fraction was extracted out and the yellow fraction (ferricyanide) was discarded. Once absorbance spectra were taken for all four samples, the concentration of all of them had to be normalized. After adjusting all of them to the same concentration, the samples were put in clear fused quartz EPR tube and frozen in liquid nitrogen by immersing slowly over a period of 15 to 20 seconds.

II.L. Extraction of heme from DHP for MALDI data

The DHP, ^{13}C-DHP and ^{2}H-DHP supernatant from the sample (3 ml) was transferred to a screw top test tube and 1.5 mL of 1M HCl was added to each of the test tube, so the heme could be released into the solution. It was incubated at room temperature for an hour which produced a pinkish slurry solution. To this solution, 3 mL of methyl ethyl ketone was added and each of the tubes was centrifuged for 30 min at 2500 rpm. After centrifugation, the organic layer was drawn out and the solution was made ready for MALDI analysis. The matrix used was 12 µL of α-cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile with 0.1% trifluoroacetic acid (TFA).

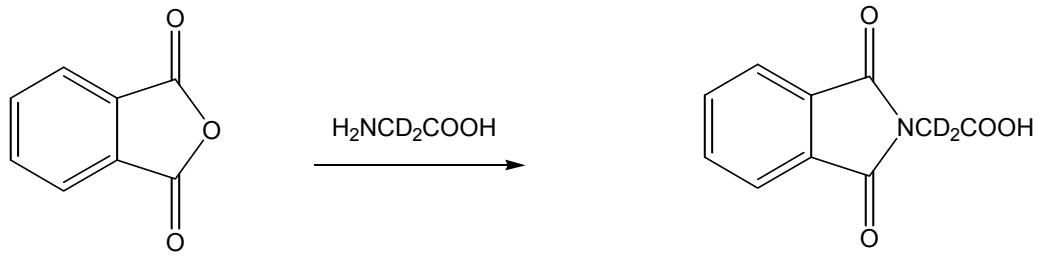
To spot the solution, 1µL of the DHP, ^{13}C-DHP and ^{2}H-DHP samples were added to 12µL of the matrix diluent (50% acetonitrile with 0.1% TFA) and a 12µL aliquot of the matrix solution. From this sample, 0.5µL was spotted on a MALDI plate for analysis. The samples were analyzed in an Applied Biosystems 4700 Proteomics Analyzer and this analysis was conducted in reflector positive mode.

CHAPTER III

RESULTS AND DISCUSSION

III.A. Synthesis of ^{2}H-aminolevulinic acid (^{2}H-ALA)

The first step of the synthesis was formation of phthalylglycine with the yield of 75%. 2,2-D_2 Glycine was reacted with phthalic anhydride to form ^{2}H-phthalylglycine. Nuclear magnetic resonance spectroscopy was used to characterize the products and was carried out on a JEOL JNM-ECA500 FT NMR system. Figure III.1 shows the synthesis of phthalylglycine from phthalic anhydride and glycine (^{2}H denoted by D). Table III.1. shows the available literature values, the chemical shift assignments and ^{1}H-NMR data for the synthesized ^{2}H-Phthalylglycine. The literature values from Wang and Scott[6] for the phthalylglycine (CH_2COOH) was a doublet at 4.40 ppm. In the ^{2}H-phthalylglycine ($C^{2}H_2COOH$), there should not be any doublet peak, and there was not, if the deuterium had been incorporated since ^{1}H-NMR can only detect protons, not deuterium. The assigned chemical shift of ^{2}H-phthalylglycine at 7.90 ppm was close to the literature value of 7.82 ppm. The peak at 2.50 ppm is DMSO solvent peak.



Phthalic Anhydride

^{2}H-Phthalylglycine

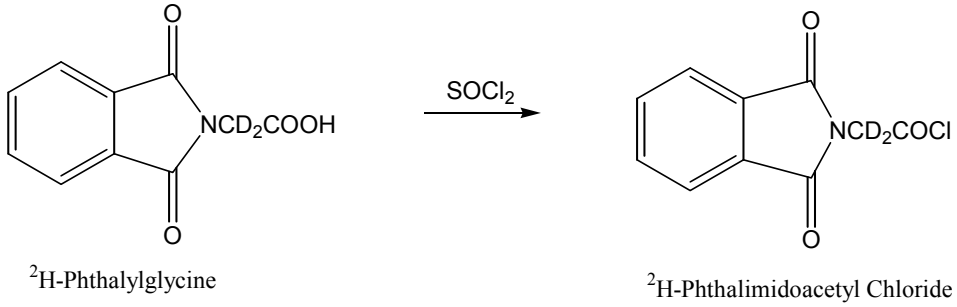
Figure III.1. The synthesis of ^{2}H-phthalylglycine from phthalic anhydride and 2,2-D_2 glycine.

Table III.1. The available literature values, the chemical shift assignments and ^{1}H-NMR data for the synthesized ^{2}H-phthalylglycine.

	Experimental (^{1}H-NMR, ppm)	Literature[6]	Assignment
^{2}H-Phthalyglycine	2.45 No peak 7.85	4.40 7.82	DMSO (solvent) CH_2COOH (lit) AA'BB', 4H

The next step in synthesis of ALA was the formation of phthalimidoacetyl chloride. To do this, the reactant phtalylglycine from the last step had to be completely dry. The dried phthylglycine was reacted with thionyl chloride to yield phthalimidoacetyl chloride. The excess thionyl chloride was evaporated off and the percent yield was calculated to be 92%. Figure III.2. shows the synthesis of ^{2}H-Phthalimidoacetyl Chloride

from ^{2}H-phthalylglycine and thionyl chloride. Table III.2 shows the ^{1}H-NMR data of the synthesized ^{2}H-phthalimidoacetyl chloride. Similar to the last step, there was no peak at 4.80 ppm, indicating there was an incorporation of deuterium. In the literature, there was a doublet at 4.80 ppm (CH_2COCl) and the peaks at 7.78 ppm from the literature were close to the experimental peak also at 7.78 ppm. A singlet peak at 7.24 ppm was indicative of a D-chloroform solvent peak.



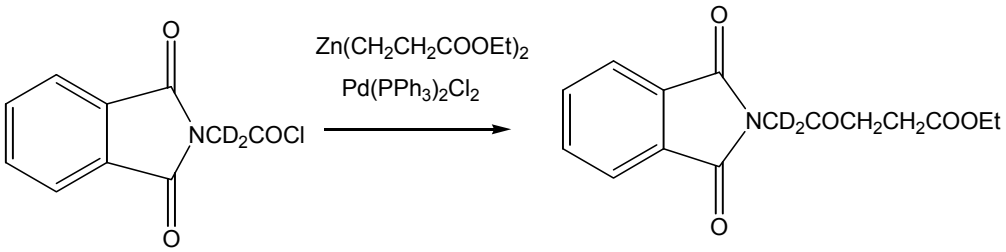
^{2}H-Phthalylglycine ^{2}H-Phthalimidoacetyl Chloride

Figure III.2. Synthesis of ^{2}H-phthalimidoacetyl chloride from phthalylglycine and thionyl chloride.

Table III.2. The available literature values, the chemical shift assignments and ^{1}H-NMR data for the synthesized ^{2}H-phthalimidoacetyl chloride.

	Experimental (^{1}H-NMR, ppm)	Literature[6]	Assignment
^{2}H-Phthalimidoacetyl Chloride	No peak 7.24 7.78	4.80 7.78	CH_2COCl (lit) $CDCl_3$ (solvent) AA'BB', 4H

The third step of this synthesis was the most labor intensive beacause zinc homoenolate solution had to be used in subsequent reaction with ^{2}H-phthalimidoacetyl chloride. It is important to note that all the reagents had to be kept dry at all times and under an atmosphere of argon gas during the synthesis of zinc homoenolate. The overall yield for the synthesis of ^{2}H-phthalimidolevulinic acid ethyl ester was 88%. Figure III.3. shows the synthesis of ^{2}H-phthalimidolevulinic acid ethyl ester from ^{2}H-phthalimidoacetyl chloride and zinc homoenolate. Table III.3. shows the ^{1}H-NMR data for ^{2}H-phthalimidolevulinic acid ethyl ester. All the ^{1}H-NMR data from ^{2}H-phthalimidolevulinic acid ethyl ester was in close agreement with the literature values[6]. Just like the last steps, the deuterium was still intact and incorporated which is shown by the absence of a peak at 4.56 ppm, which is consistent with the results from previous steps. All other protons were observed in the NMR spectra consistent with specific ^{2}H incorporation.



^{2}H-Phthalimidoacetyl chloride

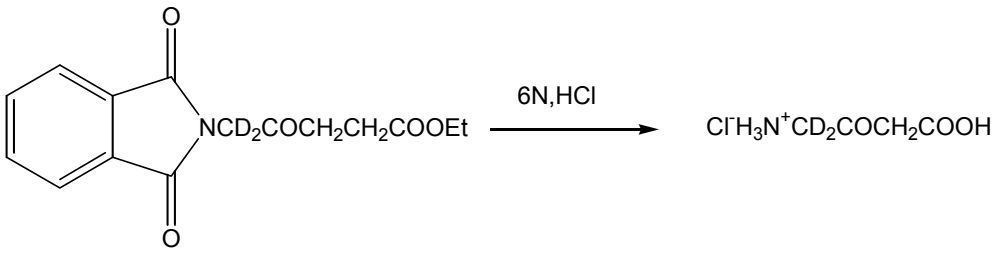
^{2}H-Phthalimidolevulinic acid ethyl ester

Figure III.3. Synthesis of ^{2}H-phthalimidolevulinic acid ethyl ester from ^{2}H-phthalimidoacetyl chloride and zinc homoenolate.

Table III.3. The available literature values, the chemical shift assignments and ^{1}H-NMR data for the synthesized ^{2}H-phthalimidolevulinic acid ethyl ester.

	Experimental (^{1}H-NMR, ppm)	Literature[6]	Assignment
^{2}H-Phthalimidolevulinic acid ethyl ester	1.23	1.26	t, 3H, CH_2CH_3
	2.60	2.65	m, 2H, $COCH_2CH_2$
	2.91	2.84	m, 2H, $COCH_2CH_2$
	4.19	4.14	q, 2H, OCH_2CH_3
	No peak	4.56	NCH_2CO (lit)
	7.24		$CDCl_3$ (solvent)
	7.78	7.80	AA'BB', 4H

The last step of this synthesis was the acid hydrolysis and deprotection of ^{2}H-phthalimidolevulinic acid ethyl ester to yield ^{2}H-Aminolevulinic acid (^{2}H-ALA), the final product. Product yield for this step was calculated to be 92%. Figure III.4 shows the synthesis of ^{2}H-aminolevulinic acid and Table III.4. shows ^{1}H-NMR data of ^{2}H-aminolevulinic acid. The ^{1}H-NMR peak at 4.10 ppm for the last step indicated that the protons had exchanged with deuterium. The other two peaks were in agreement with the literature values, but where there should have been no peaks at 4.13 ppm if deuterium was incorporated; there was a doublet at 4.10 ppm which would indicate 2 protons at the 5th position of ALA. The conclusion is that acid hydrolysis of ^{2}H-phthalimidolevulinic acid ethyl ester converted the deuterium which was incorporated in the last step to exchange back with protons. Due to the conversion between deuterium and hydrogen during acid hydrolysis, another alternative method to make ^{2}H-ALA was chosen. A detailed method to make ^{2}H-ALA is described below in III.B.



^{2}H-Phthalimidolevulinic acid ethyl ester ^{2}H-Aminolevulinic acid

Figure III.4. The synthesis of ^{2}H-ALA from acid hydrolysis of ^{2}H-phthalimidolevulinic acid ethyl ester.

Table III.4. The available literature values, the chemical shift assignments and ^{1}H-NMR data for the synthesized ^{2}H-aminolevulinic acid.

	Experimental (^{1}H-NMR, ppm)	Literature[6]	Assignment
^{2}H-ALA	2.70 3.95 4.10	2.71 2.90 4.13	m, 2H, $_{13}COCH_2CH_2$ m, 2H, $_{13}COCH_2CH_2$ d, 2H, NCH_2CO

III.B. Alternative method to make deuterium-ALA (^{2}H-ALA)

Since in the previous method, the deuterium exchanged with hydrogen, it was necessary to come up with a new method to make ^{2}H-ALA. The last step of the reaction in the synthesis of ALA from Wang and Scott[6] was the acid hydrolysis and deprotection of the phthalimidolevulinic acid ethyl ester. It is probably due to the presence of acid and water that deuterium exchanged with hydrogen. From this logic it was possible to

synthesize ^{2}H-ALA by adding deuterium oxide and 2M HCl to pure ALA. For both regular ALA and for ^{2}H-ALA, ^{1}H-NMR spectra was taken with the same concentration in each of the tubes. Figure III.5 and III.6 shows ^{1}H-NMR spectra of regular ALA and ^{2}H-ALA respectively.

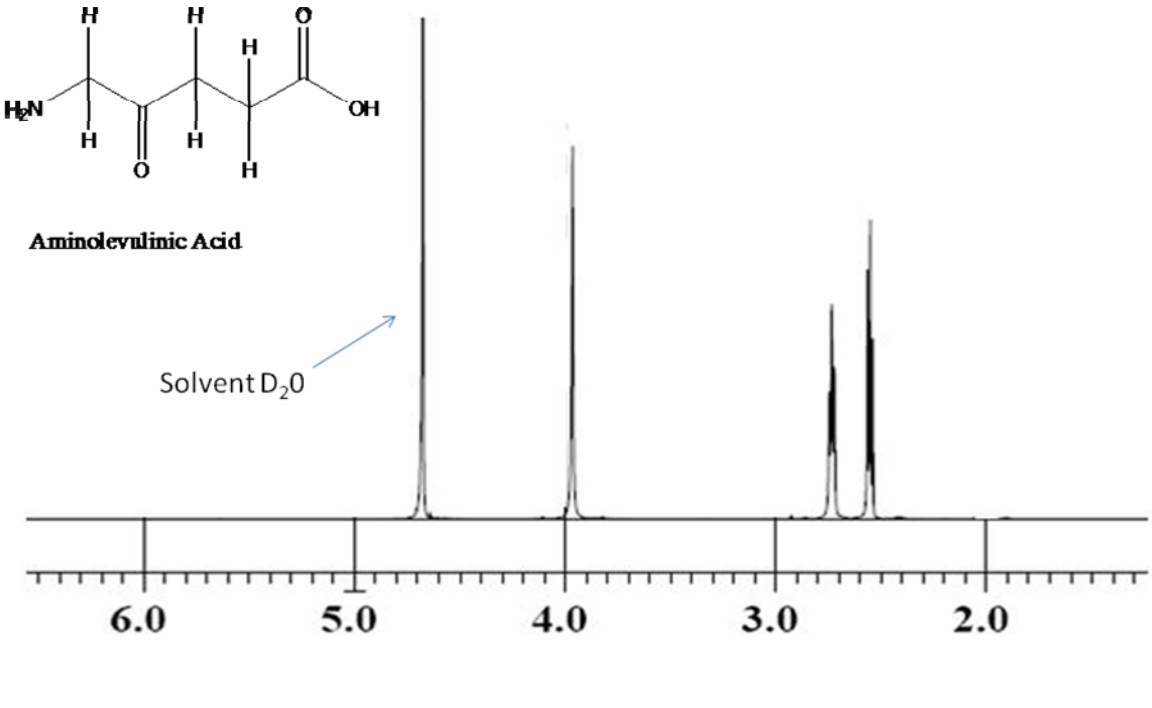


Figure III.5. ^{1}H-NMR spectra of regular ALA.

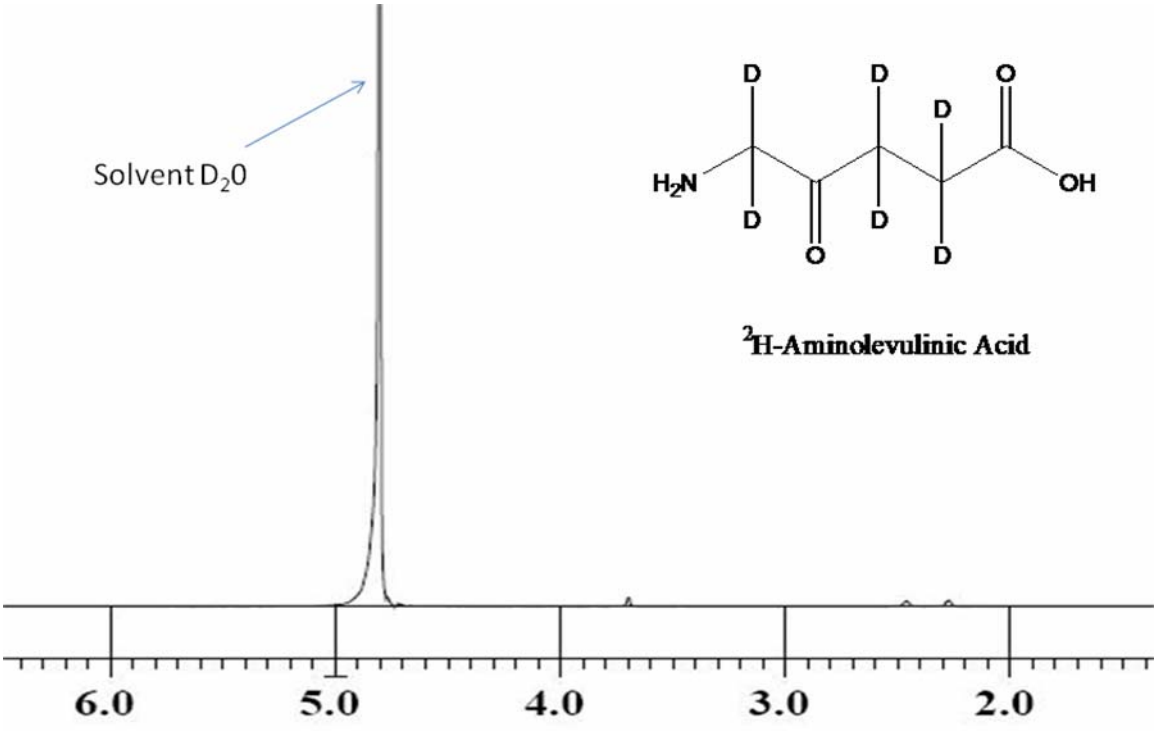
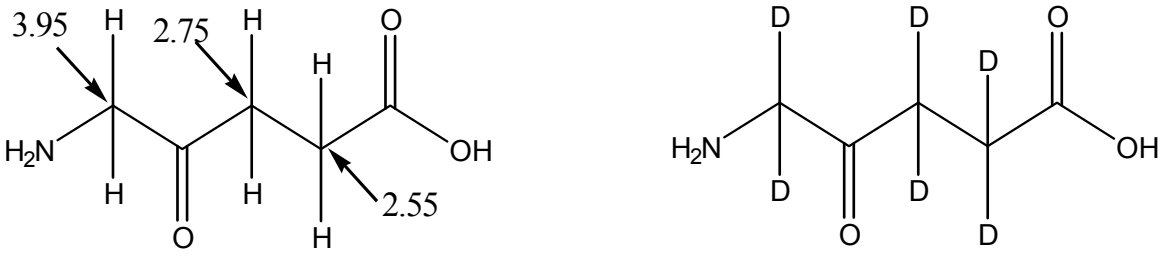


Figure III.6. ^{1}H-NMR spectra of synthesized ^{2}H-ALA.



Aminolevulinic Acid

^{2}H-Aminolevulinic Acid

Figure III.7. Diagram of regular ALA and deuterium-labeled ALA.

In Figure III.5, regular ALA was prepared with solvent deuterium oxide (D_2O). Once ^{1}H-NMR spectra was taken, 25 µL aliquots of 2 M HCl was added to the NMR

tube and each time ^{1}H-NMR spectra was taken. When 150 µL of 2 M HCl, had been added, the peaks at 2.55 pppm, 2.75 ppm and at 3.95 ppm disappeared. Looking at ^{1}H-NMR spectra of regular ALA, the triplet at 2.55 ppm and 2.75 ppm and the singlet at 3.95 ppm is considerably reduced in the ^{1}H-NMR spectra of ^{2}H-ALA. Looking at Figure III.6, the peaks at 2.55 ppm, 2.75 ppm and at 3.95 ppm has very little intensity compared to Figure III.5. Since ^{1}H-NMR can only detect protons and cannot detect deuterium, the absence of the proton peaks in Figure III.6. clearly indicated the incorporation of deuterium into the 2nd, 3rd and the 5th position of ALA.

III.C. Plasmid purification results

One of the goals of this project was to generate enough production of P450 BM3 since this plasmid contained T7 promoter for control of expression, so this recombinant plasmid can be used in ^{2}H – heme incorporation. Since *E.Coli* Hu227 cells do not contain the gene for expressing T7 polymerase, these plasmids cannot be expressed in Hu227 cells. In order to express in Hu227 cells, it is important to transfect the cells with a DNA that will allow T7 polymerase expression. A two step transformation with plasmid T7 polymerase 1 (pT7po1) was attempted. This involved imparting the Hu227 cells with kanamycin resistance and transforming the second plasmid BM3. The QIAGEN kit method to make competent cells was not as successful, so an alternative method from Kotchoni et al[15] was successfully used to make better competent cells. Figure III.8 shows Agarose Gel Electrophoresis of pT7po1 comparing the QIAGEN kit and the method from Kotchoni et al. It was observed that the competent cells using the method from Kotchoni

produced a band with much greater intensity indicating a much higher yield of plasmid, which can be seen on lane 1. The competent cells made using the method from QIAGEN can be seen on lane 2. The problem was that once both plasmids were transformed into Hu227 cells, the expressions of the cells were very low after the induction with IPTG. It appears therefore that the expression system in Hu227 is not effective. Figure III.9 shows the gel in which all the plasmids were purified and transformed. In lane 1 is p102A2, lane 2 is $pBM3_{F87G}$, lane 3 is $pBM3_{W8G7}$ and lane 4 is pBMH. All the plasmids were then expressed in Hu227 cells and induced with IPTG. The $BM3_{F87G}$ in Hu227 was the only one to express fairly well compare to 102A2/Hu227, $BM3_{W87G}$/Hu227 and BMH/Hu227. It correlated to the fact that $pBM3_{F87G}$ had the brightest band as seen in Figure III.9. Once $BM3_{F87G}$/Hu227 was transformed and expressed, an absorbance spectrum of CO-reduced heme complex of $BM3_{F87G}$ was taken as shown in Figure III.10. Looking at the absorbance spectra, it can be seen that the peak is right at 450 nm is typical of the absorbance associated with P450 enzymes. This makes it easier to track the expression of P450 enzymes in whole cells, and is therefore useful in determining whether to harvest the cells based on their expression. The yield of $BM3_{F87G}$ is close to 100 nmoles of BM3/1L, which is typical expression of this particular enzyme in P450's.

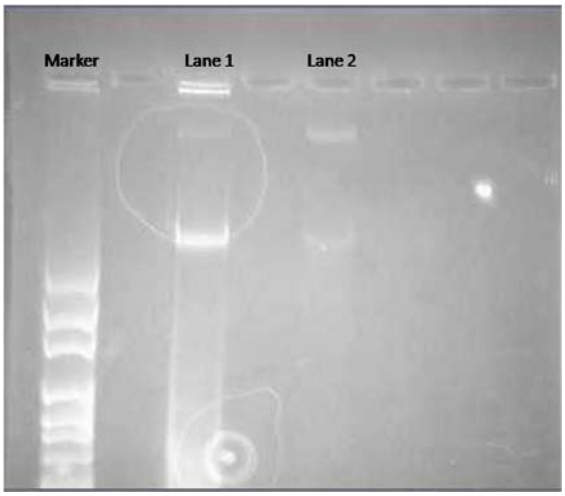


Figure III.8. Agarose gel electrophoresis comparing two different methods to make pT7

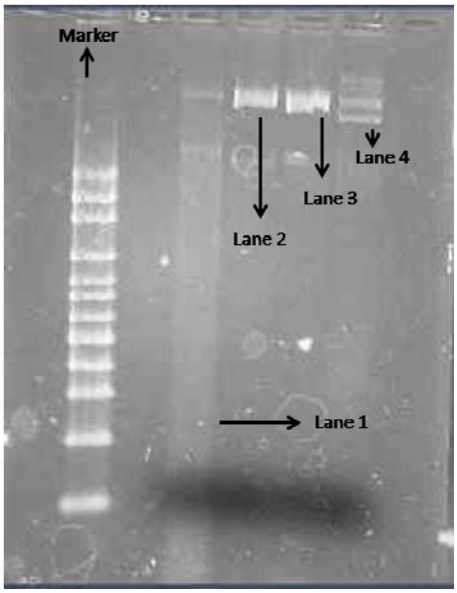


Figure III.9. Agarose gel electrophoresis of plasmid controls

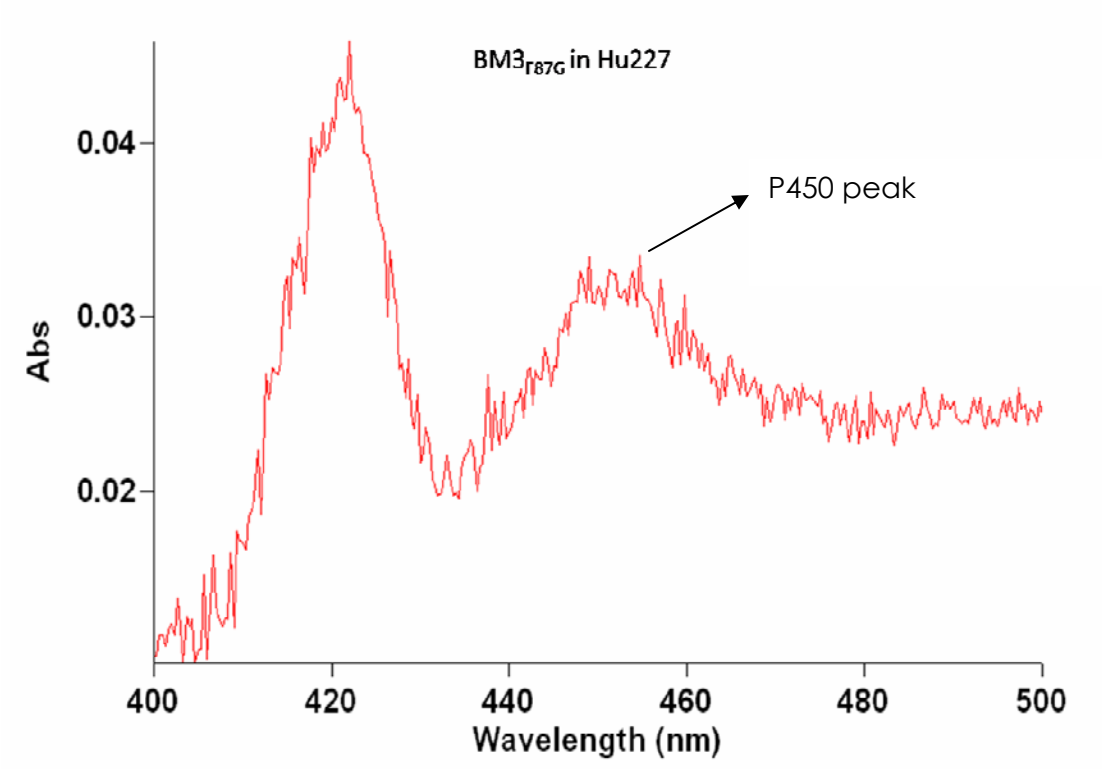


Figure III.10. Absorbance spectrum of CO-reduced P450 $BM3_{F87G}$ in whole cells.

III.D. Dehaloperoxidase expression levels and purification

Dehaloperoxidase (DHP) enzyme was chosen for the ^{2}H incorporation of heme protein, as discussed previously. Aminolevulinic acid (ALA) was required for the cells in order to express since they cannot generate ALA on their own. Therefore, incorporating deuterium labeled ALA (^{2}H-ALA) into the cells ensured the incorporation of deuterium into the heme of DHP. DHP was expressed and purified successfully from *E.Coli* Hu227 cells. These cells were grown in Luria Bertani (LB) growth media containing ^{2}H-ALA. Figure III.11 displays an absorbance spectrum of CO-reduced heme complex of purified DHP. The λ_{max} of purified DHP is right at 420 nm, typical DHP peak, with absorption of

about 0.050 in cultures, which displayed good expression. Even the ^{13}C-labeled dehaloperoxidase and ^{2}H-labeled deahloperoxidase showed decent expression levels. Figure III.12 and Figure III.13 shows an absorbance spectrum of CO-reduced heme complex of [5-^{13}C] DHP and ^{2}H-DHP respectively. The λ_{max} of both purified [5-^{13}C] DHP and ^{2}H-DHP is also right at 420 nm with absorption of about 0.020 in cultures.

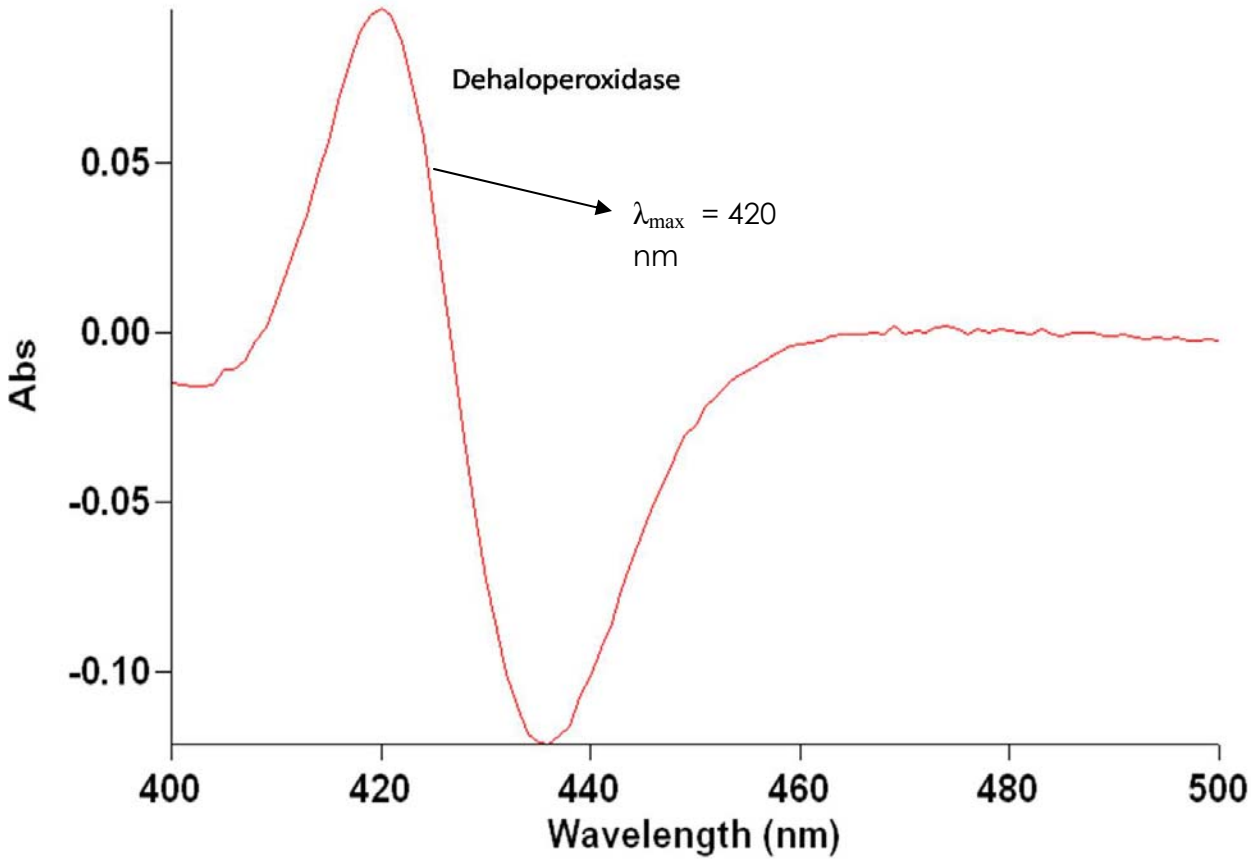


Figure III.11. Absorption difference spectrum of CO-reduced heme complex of purified DHP in whole cells

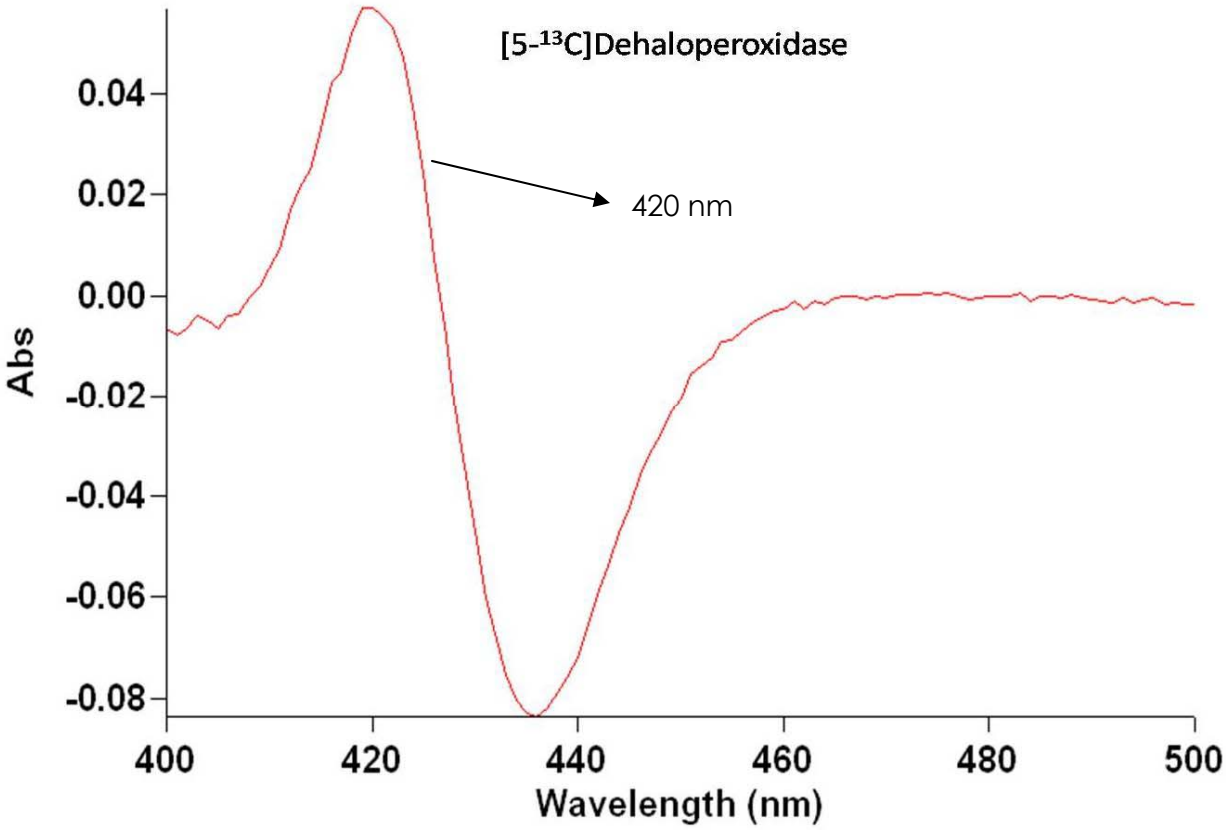


Figure III.12 Absorption difference spectrum of CO-reduced heme complex of purified [5-^{13}C] DHP in whole cells

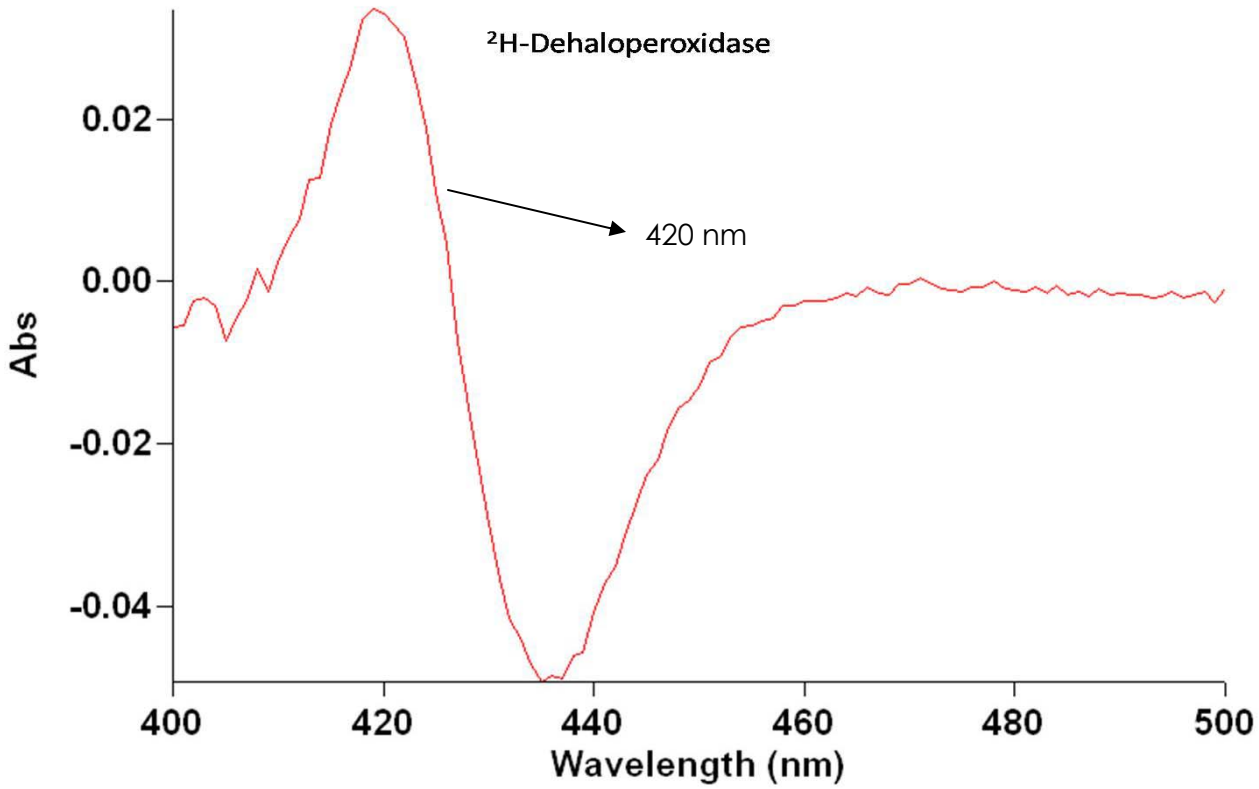


Figure III.13. Absorption difference spectrum of CO-reduced heme complex of purified ^{2}H-DHP in whole cells

III.E. Electron paramagnetic resonance (EPR) spectroscopy

EPR spectroscopy was carried out using a Bruker Instruments EMX EPR spectrometer, equipped with an Oxford Instruments ESR 900 liquid helium cryostat. Currently the EPR results for ^{15}N-DHP, [4-^{13}C] DHP, [5-^{13}C] DHP and pure DHP are inconclusive. The goal of this study is to develop technology that would be useful in the identification of transient intermediates in the peroxo-pathway. If there is incorporation of ^{13}C or ^{15}N in the heme of DHP, the peaks may show broadening or splitting if coupling to these isotopes is significant. During the preparation of the DHP samples for EPR, the

absorbance spectrum was taken in order to get all of them to the same concentration. Figure III.14 shows the absorbtion spectra of all four samples after column purification. Once the concentrations were determined and normalized to the unlabeled sample, the samples were put in a clear fused quartz EPR tubes and frozen in liquid nitrogen over a period of 15 to 20 seconds.

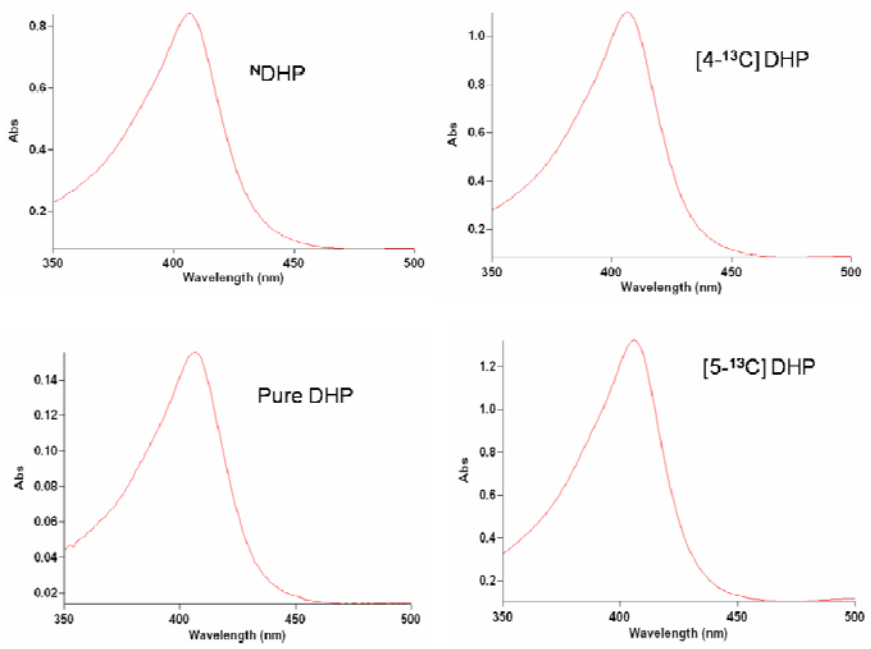


Figure III.14 Absorption spectrum of ^{15}N -DHP, [4-^{13}C] DHP, [5-^{13}C] DHP and pure DHP in preparation for EPR analysis.

Several EPR spectra with varying concentration of isotopically-labeled and unlabeled DHP were recorded and analyzed. The concentration of ^{15}N-DHP, [4-^{13}C] DHP, [5-^{13}C] DHP was approximately 200 µM but the concentration of unlabeled pure DHP was approximately 70 µM. The samples were examined at X-band frequency,

shown in Figure III.15 and Figure III.16. Figure III.15 shows the EPR spectra for DHP samples at g = 2 region. The spectra looks the same for all the samples, but there is a small signal around g = 2.056 for [5-^{13}C] DHP. This could be more likely a disrupted iron species or it could be due to the isotopic effect from [5-^{13}C] DHP as opposed to [4-^{13}C] DHP, or it could potentially also be a contamination from an impurity in the sample. Also, in Figure III.16, at g = 6 region, [5-^{13}C] DHP also shows a little bit of broadening. Though it is not clear whether this is significant, more studies in the future might be worth undertaking.

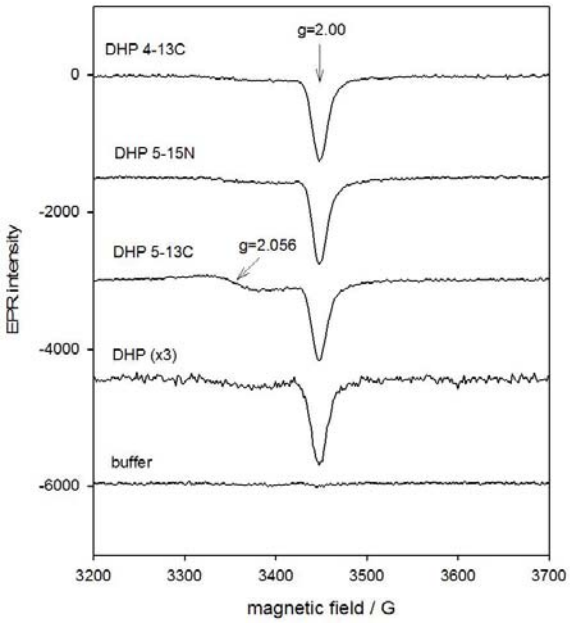


Figure III. 15. EPR spectra of DHP samples at X-band in the g=2 region using 0.5 mW microwave power, a field sweep from 500 to 4000 G modulation amplitude of 15G, and temperature of 10K.

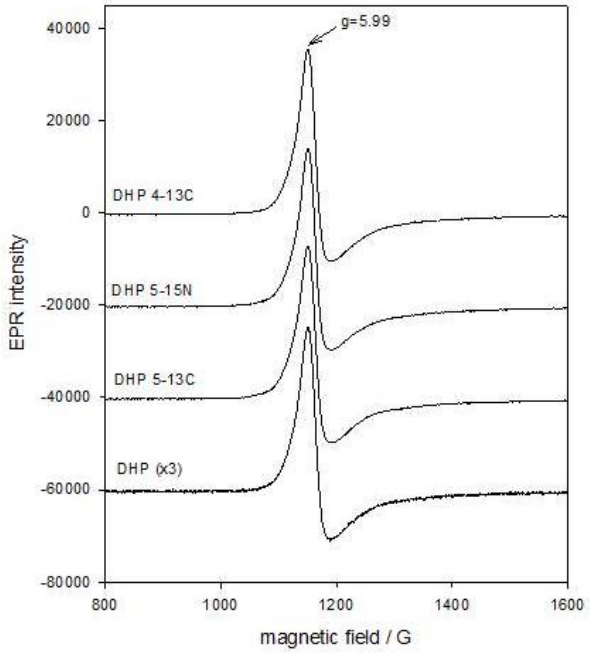


Figure III. 16. EPR spectra of DHP samples at X-band in g=6 region using 0.5mW microwave power, a field sweep from 500-1600 G modulation amplitude of 15G, and temperature of 10K.

III.F. LC-MS analysis of isotopically labeled heme from dehaloperoxidase

High performance liquid chromatography – mass spectrometry with electrospray ionization (LC-MS) was conducted on heme that had been extracted and purified from dehaloperoxidase. Since the MS results had a lot of contaminations and impurities, the samples were further purified using HPLC and the heme fraction at 4.43 min was collected as shown in Figure III.17. The solvent from this fraction was evaporated and resuspended in the mobile phase and subsequently subjected to analysis using LC-MS. The LC-MS chromatograms for unlabeled heme from DHP using unlabeled ALA, ^{13}C heme from DHP using [5-^{13}C] ALA and ^{2}H heme from DHP using ^{2}H-ALA are shown in

Figure III.18. As expected, the blank had no detectable ion at m/Z of 616, but DHP, ^{13}C-DHP and ^{2}H-DHP all had a 616 m/Z. The fact that ^{13}C-DHP had ion at 616 m/Z, not 624 m/Z, as shown in Figure III.19, suggested that eight ^{13}C atoms were not successfully incorporated into the heme. Also the fact that ^{2}H-DHP had a 616 m/Z indicated that deuterium had not fully exchanged with hydrogen. To further clarify the data, esterification of the heme was also done by adding 5% sulfuric acid to methanol. This was done by locking the two free carboxylic acid group to a methyl group so the charge on heme will be +3. The analysis of LC-MS data for the esterification of heme showed that, esterification did not work due to the fact that there was still 616 m/Z on unlabeled DHP instead of expected 205 m/Z (616 mass: 3 charge). Previously, it was shown that ^{13}C-ALA was incorporated into DHP and a clear 624 m/Z peak was shown. But, since there was no 624 m/Z peak in the current sample of DHP but a 616 m/Z was observed, another ionization method, MALDI was used in order to clarify the 616 m/Z of ^{13}C-DHP. As expected, there was a clear 624 m/Z peak, but also a 616 m/Z peak on MALDI. A possible reason for this could be that the cell cultures of DHP/Hu227 were contaminated. If there was no contamination, there should have only been a clear 624 m/Z peak, but because there was a higher ion peak at 616 m/Z and not 624 m/Z was probably due to contamination, as shown in Figure III.20. Re-plating DHP/Hu227 on a LB/ALA/Amp plate and growing fresh cultures out of that eliminated the possibility of contaminants. The absence of 624 m/Z peak of ^{13}C-DHP on LC-MS (ESI) and presence of 624 m/Z by MALDI is probably a dilution issue because LC-ESI the samples molecule have to travel

through the mobile phase before they are ionized in which they are already diluted. In MALDI there is no mobile phase it has to travel through.

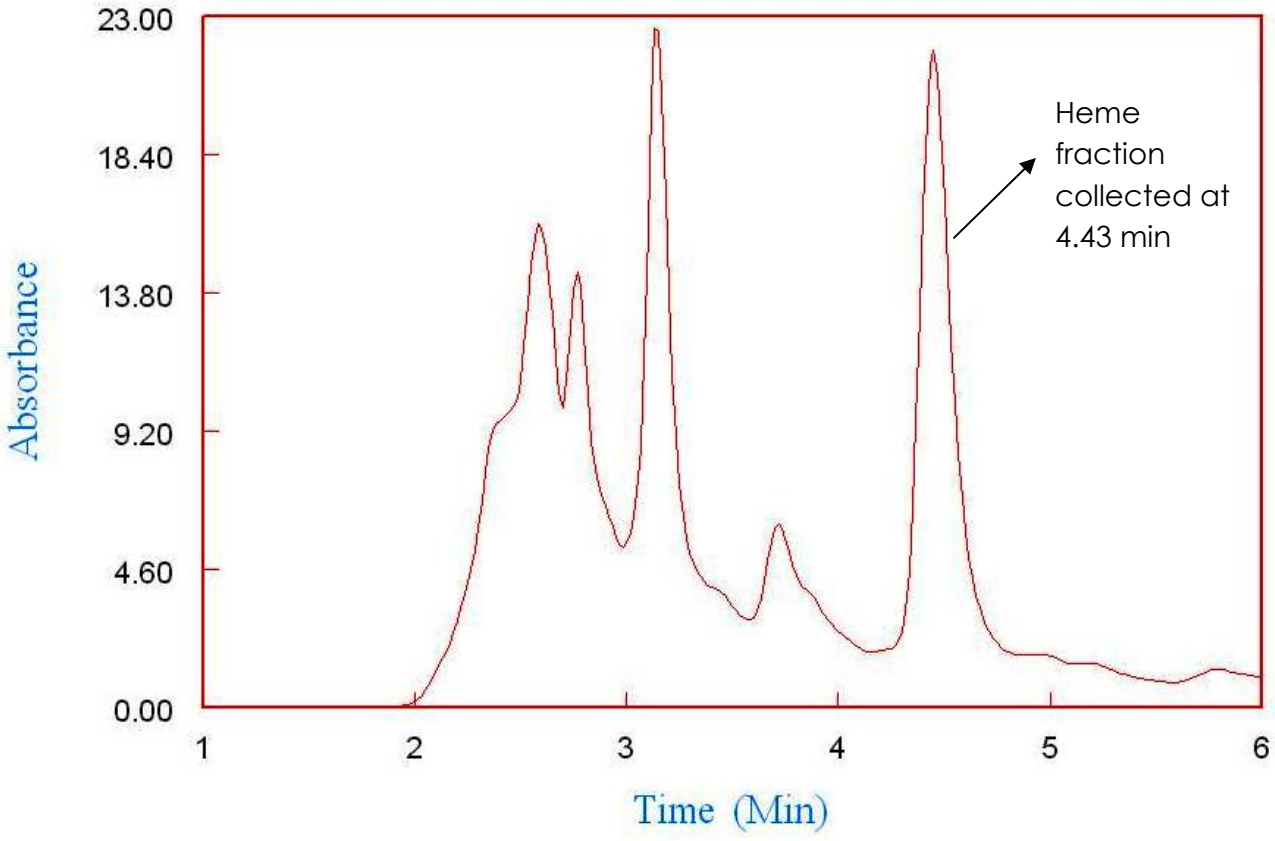


Figure III.17. HPLC purified heme from dehaloperoxidase observed at 398 nm.

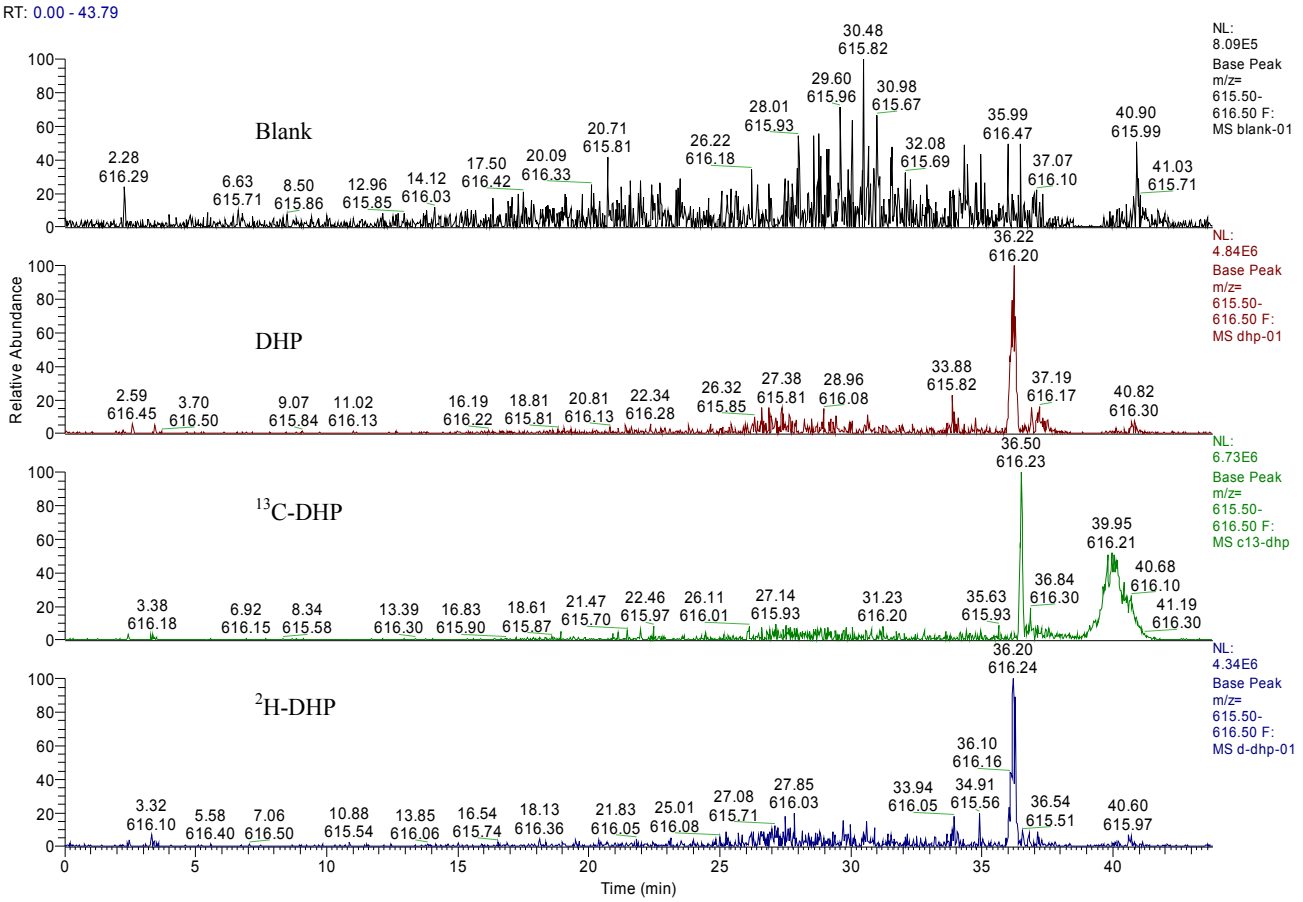


Figure III.18. LC-MS selective ion chromatogram (615.5 - 616.5) of purified isotopically labeled heme from DHP for blank, DHP expressed in unlabeled ALA, ^{13}C-DHP overexpressed in [5-^{13}C] ALA and ^{2}H-DHP overexpressed in ^{2}H-ALA.

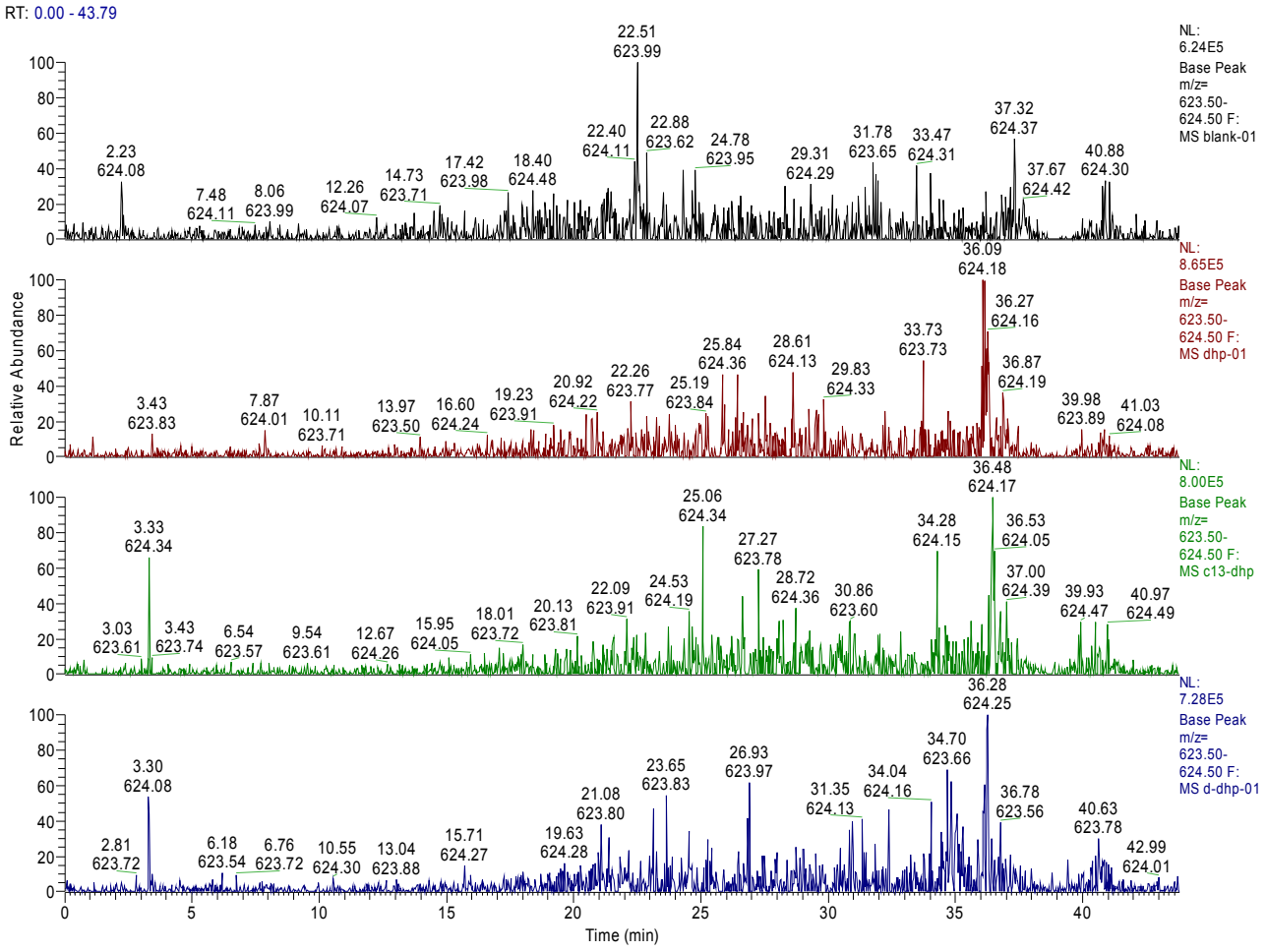


Figure III.19. LC-MS selective ion chromatogram (623.5 - 624.5) of purified isotopically labeled heme from DHP for blank, DHP expressed in unlabeled ALA, ^{13}C-DHP overexpressed in [5-^{13}C] ALA and ^{2}H-DHP overexpressed in ^{2}H-ALA.

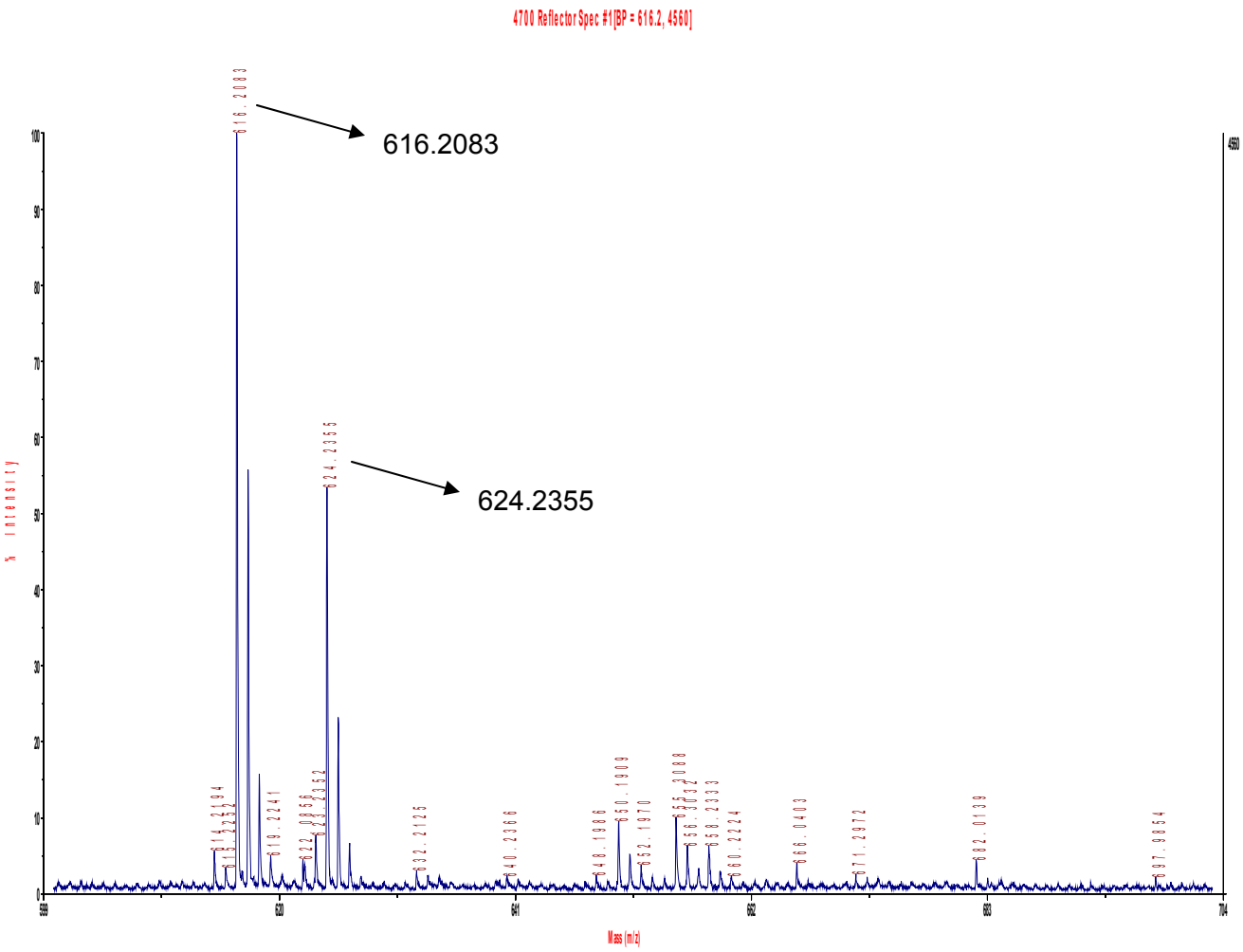


Figure III.20. MALDI-TOF spectrum of ^{13}C labeled heme from DHP, displaying a major peak at 616 m/z and a minor peak at 624 m/z.

III.G. MALDI-TOF analysis of isotopically labeled heme from dehaloperoxidase

Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF) was used for analysis of heme from DHP because it proved to be a better ionization technique, as discussed previously in III.F. Unlabeled heme from DHP using unlabeled ALA, ^{13}C heme from DHP using [5-^{13}C] ALA and ^{2}H heme from DHP using

^{2}H-ALA were analyzed by MALDI to test for successful incorporation of isotopically labeled atoms in to the heme. The spectral data of this analysis is shown in Figure III.19, Figure III.20 and Figure III.21 respectively. When comparing the MALDI-TOF spectrum of unlabeled heme sample (Figure III. 19) to the ^{13}C-labeled heme sample (Figre III. 20), there is an increase in mass from 616 m/z to 624 m/z which suggests that eight ^{13}C atoms were incorporated into the heme as expected. However, when comparing the MALDI-TOF spectrum of unlabeled heme sample (Figure III.19) to the ^{2}H-labeled heme sample (Figure III.21), there is no increase in mass 616 m/z to 624 m/z, which clearly indicates there was no incorporation ^{2}H-labeled atoms in the heme. These data concludes that the deuterium was not incorporated into the heme of DHP. The reason for this could be due to the hydrogen in water environment replacing deuterium in heme. Another reason that deuterium could not be incorporated could also be because of a slow exchange of deuterium and hydrogen during enzymatic reaction of ALA to heme as shown in the biosynthetic pathway of heme in Figure I.4.

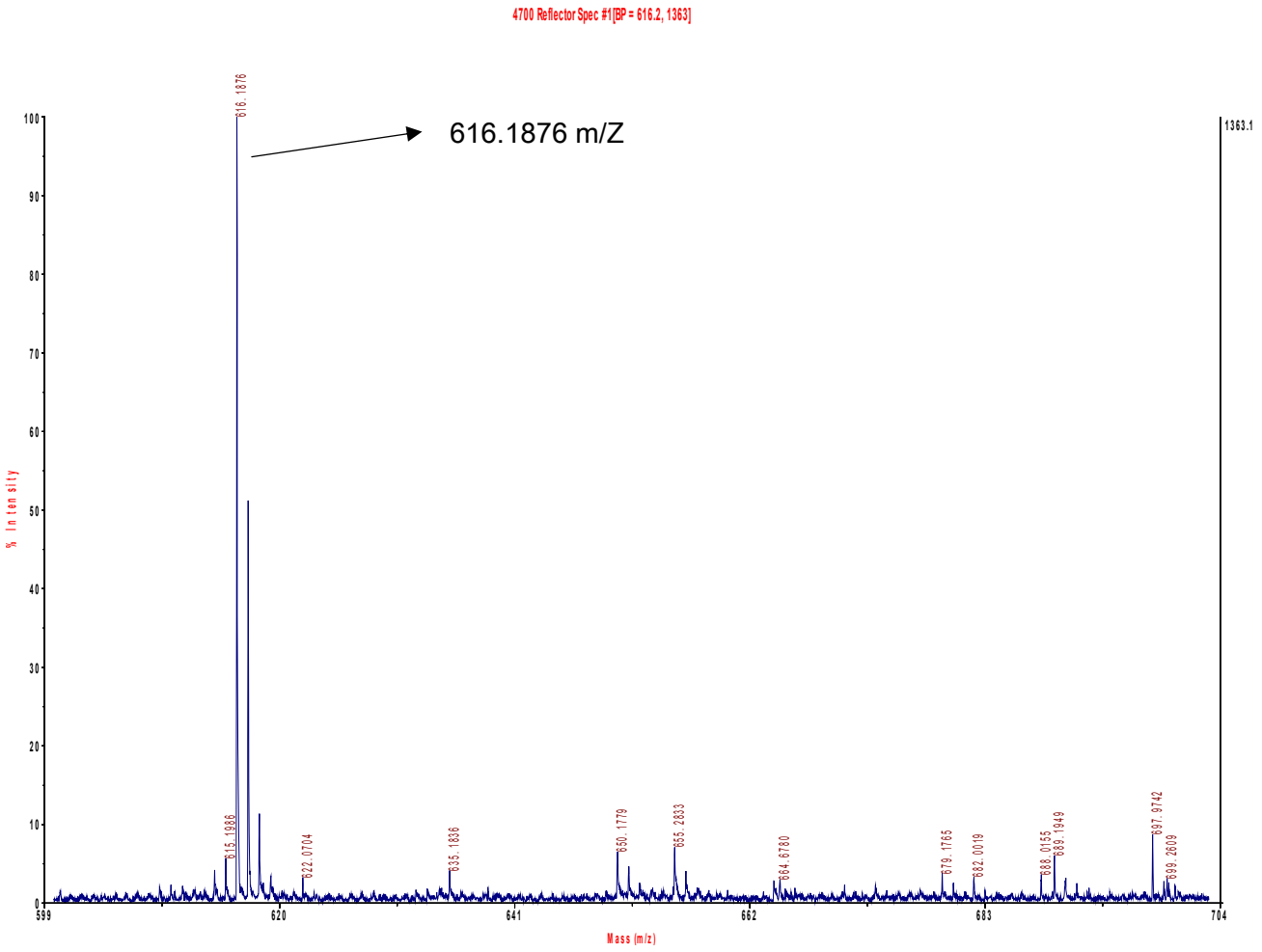


Figure III.21. MALDI-TOF spectrum of unlabeled heme from DHP, displaying a major peak at 616 m/Z.

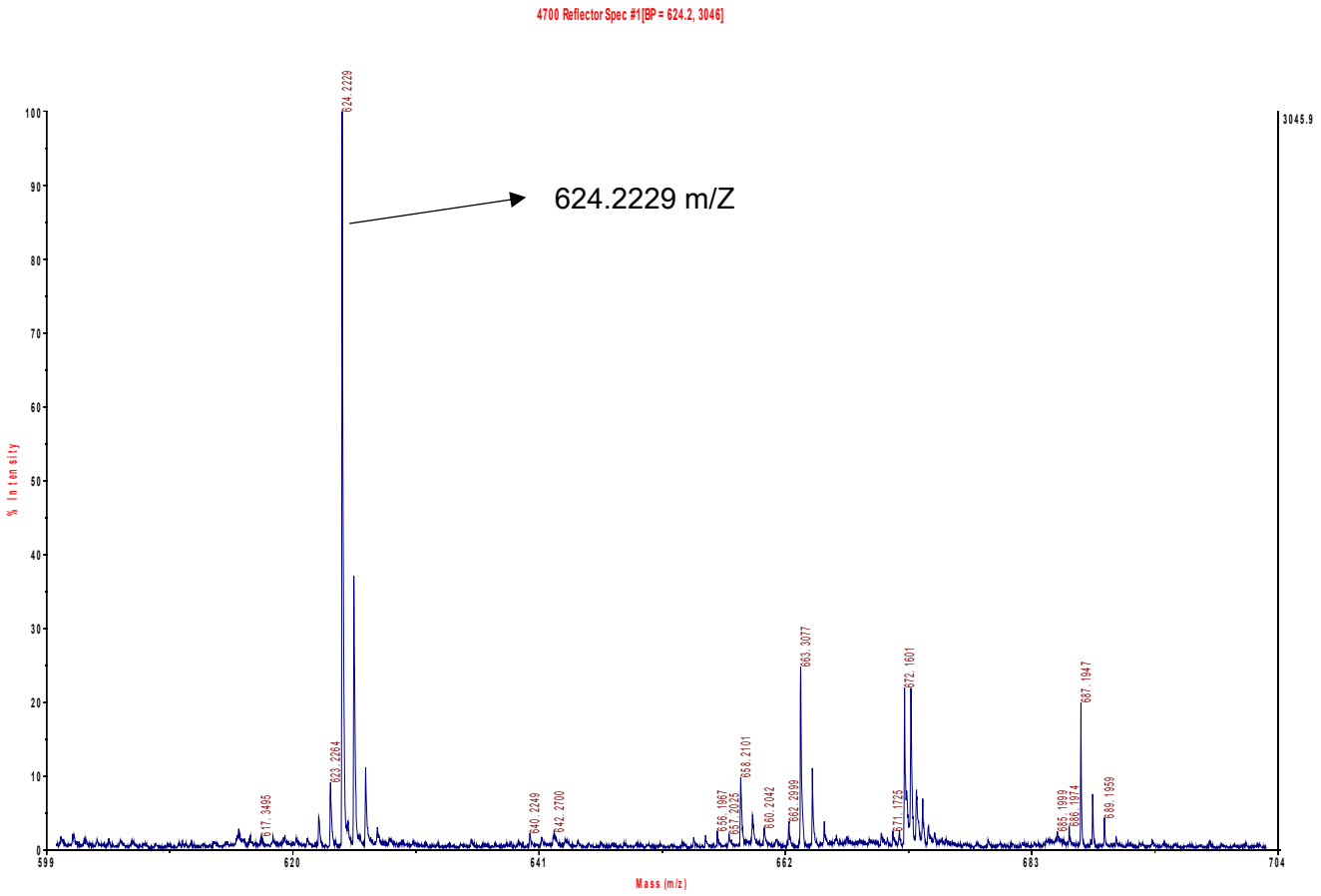


Figure III.22. MALDI-TOF spectrum of ^{13}C labeled heme from DHP, displaying a major peak at 624 m/Z.

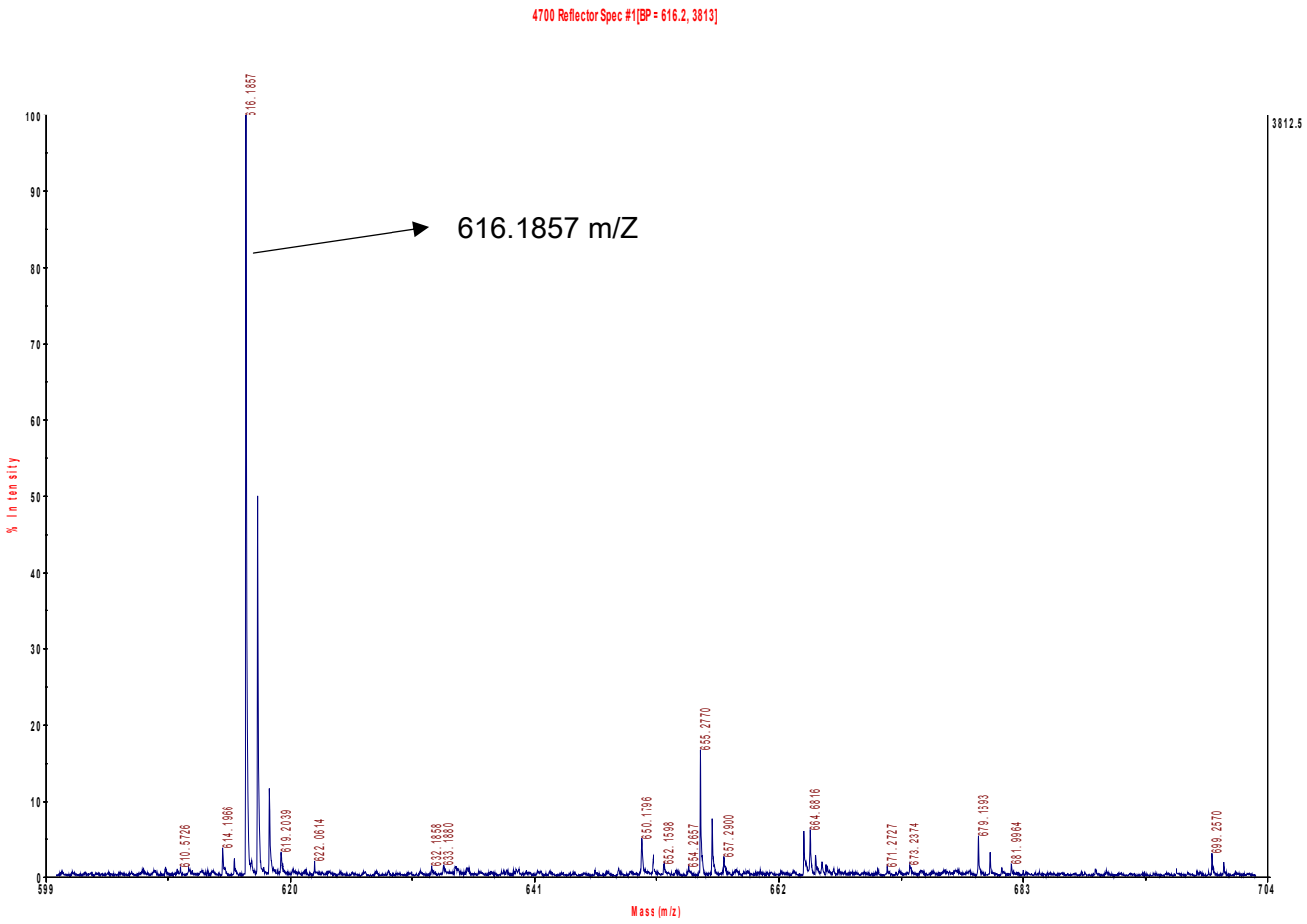


Figure III.23. MALDI-TOF spectrum of ^{2}H labeled heme from DHP, displaying a major peak at 616 m/Z.

CHAPTER IV

CONCLUSIONS

Paramagnetic NMR can be a powerful tool for probing electronic distribution and spin-densities within the heme macrocycle. A significant limitation to its use in cytochrome P450 studies is that natural abundance of ^{13}C is relatively low, and P450 enzymes have proven resistant to heme reconstitution. Therefore, the availability of appropriately labeled ^{13}C enzyme is severely limited. A step in the direction toward utilizing paramagnetic NMR as a tool for studying P450 is the development of a recombinant system whereby the P450 can be expressed with isotopically labeled heme incorporated, thus eliminating the need for inefficient reconstitution procedures.

A strain of *E.coli*, Hu227 was selected because of its inability to produce aminolevulinic acid (ALA), a heme precursor in the biosynthetic pathway. Since *E.coli* Hu227 cannot synthesize their own ALA we can selectively label heme with isotopes by using isotopically labeled ALA in the media. In past studies, Cytochrome P450 has been expressed under the control of T7 promoter using *E.Coli* BL-21 cells. These cells have a T7 polymerase encoded in their genome, under the control of a lac promoter. Thus, IPTG addition involves T7 polymerase production which, in turn, induces the target gene. Hu227 cells have no such T7 polymerase, so expression of P450 from one of the readily available plasmids in Hu227 would be expected to be very inefficient.

Dehaloperoxidase (DHP), an enzyme which also contains heme has been a model protein because it was under the control of lac promoter and expresses very well in these cells. An additional goal of this research, however remained improving expression of the $P450_{BM3}$ enzyme in the Hu227 cells. Initially there was an attempt at a multi step synthesis of ^{2}H-aminolevulinic acid from glycine, but the deuterium apparently exchanged with hydrogen from water in the last step. However, an alternative method was used to make ^{2}H-aminolevulinic acid and it was characterized using ^{1}H-NMR. Additionally, the generation of good expression level for both DHP and $BM3_{F87G}$ was conducted successfully. Also, the expression level for both unlabeled DHP and also isotopically labeled DHP was generated in a high yield.

High performance liquid chromatography – mass spectrometry with electrospray ionization (LC-MS) was conducted on heme that had been extracted and purified from dehaloperoxidase. Previously, it was proven that ^{13}C-ALA was incorporated into DHP and a clear 624 m/Z peak was shown. But, since there was no 624 m/Z peak in the current sample of DHP but a 616 m/Z was observed, another ionization method, MALDI-TOF was used in order to clarify the 616 m/Z of ^{13}C-DHP. After eliminating the possibility of contamination, there was a clear 624 m/Z peak for ^{13}C-DHP on MALDI. However, when comparing the MALDI-TOF spectrum of unlabeled heme sample to the ^{2}H-labeled heme sample, there is no increase in mass 616 m/Z to 624 m/Z, which clearly indicates there was no incorporation ^{2}H-labeled atoms in the heme. This might be due to hydrogen in

water environment replacing deuterium in heme, or a slow exchange of hydrogen and deuterium through the enzymatic reaction of ALA to heme. Although it was not possible to incorporate ^{2}H into the heme, successful incorporation of ^{13}C and ^{15}N into the heme will be useful in the application of the spectroscopic techniques when applied to P450 enzymes.

REFERENCES

1. Danielson, P.B. *Current Drug Metabolism*, **2002**, *3*, 561.

2. Montellano, P. R. O. d. *Cytochrome P450 Structure, Mechanism, and Biochemistry,* 2nd ed. Plenum Publishers: New York, 1995.

3. Guengerich, F. P. *J. Biol. Chem.* **1991**, *266*, 10019.

4. Alontaga, A. Y., Bunce, R.A., Wilkis, A., Rivera, M. *Inorg. Chem.* **2006**, *45*, 8876.

5. Caignan, G.A.; Deshmukh, R.; Zeng, Y.; Wilks, A.; Bunce, R.A.; Rivera, M., *J. Am. Chem. Soc*. **2003**, *125*, 11842.

6. Wang, J., Scott, A.I. *Tetrahedron Lett.* **1997**, *38*, 739.

7. Nakamura, E., Aoki, Kouichi, S., Oshino, S.H., Kuwajima, I. *J. Am. Chem. Soc.***1987**, *109*, 8056.

8. Raner, G.M., Hatchell, A.J., Morton, P.E., Ballou, D.P., Coon, M.J. *J. Inorganic Biochemistry* **2000**, *81*, 153.

9. Li, Q., Ogawa, J., Shimizu, S. *Biochem and Biophys Res Comm,* **2001**, *280*, 1258.

10. Raner, G.M., Hatchell, A.J., Dixon, M.U., Joy, T.L., Haddy, A.E., Johnstone, E.R. *Biochem,* **2002**, *41*, 9601.

11. Li, J., Deslouches, B., Cosloy, S.D., Russell, C.S. *Biochem et Biophys Acta,* **2003**, *1626*,102.

12. Osborne, R. L., Sumithran, S., Coggins, M.K., Chen, Y.P., Lincoln, D.E., Dawson, J.H. *J. Inorg. Biochem.* **2006**, *100*, 1100.

13. Rivera, M., Walker, F.A. *Anal. Biochem.* **1995**, *230*, 295.

14. Rivera, M., Caignan, G. A., *Anal. Bioanal. Chem.* **2004,** *378*, 1464.

15. Kotchoni, S.O., Gachomo, E.W., Betiku,E., Shonukan, O.O. *African J. of Biotech.* **2003**, *2(4)*, 88.

16. Budde, M., Morr, Michael, Schmid, Rolf. D., Urlacher, Vlada B. *Chem. Bio. Chem.* **2006**, *7*, 789.

17. Roach, M. P., Chen, Y.P., Woodin, S.A., Lincoln, D.E., Lovell, C.R., Dawson, J.H. *Biochemistry* **1997**, *36*, 2197

18. Svistunenko, D.A. Biochem. Biophys. Acta. **2005**, *1707*, 127-55.

19. Schunemann, V. J. Biol. Chem. **2004**, *279(12),* 10919- 10930.

20. Hiner, A. Biochemistry. **2003**, *42*, 8799-8908.

21. Niehaus, K., Deng, P., Belyea, J., Franzen, S., Niehause, G.U. *J. Phys. Chem. B.* **2006**, *110*, 13264.

22. von Weymarn, L. B., Blobaum, A. L., Hollenberg, P.F. *Arch. Biochem. Biophys.* **2004**, *425*, 95.

23. Spolitak, T. J. Biol. Chem. **2005**, *280(21),* 20300-20309.