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Brian R. Baer

Autocatalytic Mechanism and Functional Consequences of
Covalent Heme Attachment in CYP4B1

Brian R. Baer

A dissertation submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy

University of Washington
2005

Program Authorized to Offer Degree: Pharmacy - Medicinal Chemistry

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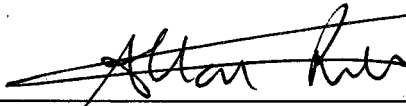
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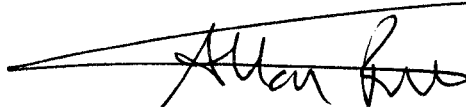
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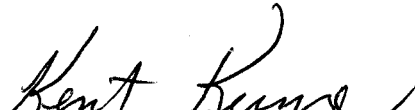
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Abstract

Autocatalytic Mechanism and Functional Consequences of Covalent Heme
Attachment in CYP4B1

Brian R. Baer

Chair of the Supervisory Committee:
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The studies presented in this dissertation establish the precise structure and sites of attachment of the covalent linkage that binds heme to CYP4B1, reveal a viable autocatalytic mechanism for its formation, and evaluate functional consequences on enzymatic activity and physical properties of this novel, structurally modified P450. The site of protein attachment in CYP4B1 was identified as Glu310 in the I-helix, *via* mass analysis of heme-containing tryptic peptides, sequence alignments, and site-directed mutagenesis. Two-dimensional NMR and chromatographic analysis of base-hydrolyzed hemes revealed that native CYP4B1 heme was modified solely at the C-5 methyl, but recombinant CYP4B1 heme was also modified at the C-8 methyl. These results are indicative of two heme orientations in the active site of recombinant P450s, yet further experiments demonstrated that this microheterogeneity of heme orientation may not affect *in vitro* substrate kinetics. The CYP4B1 E310D mutant produced free hydroxymethylheme, suggesting that the mechanism for the formation of the ester bond involved a heme methyl carbocation intermediate. To investigate the mechanism further, the carboxylates of glutamic acid residues were labeled with ^{18}O in the wild type enzyme and the fate of the heavy isotope was traced into base-hydrolyzed monohydroxyhemes by LC/ESI-MS. These data support a mechanism that parallels the stepwise reaction

proposed for the covalent attachment in the mammalian peroxidases, namely initial hydrogen abstraction from the carboxylate of Glu310 to form a carboxyl radical, hydrogen abstraction from the heme methyl, intramolecular electron transfer to generate a heme methyl carbocation, and finally, quenching of the carbocation with the same carboxylate of Glu310. CYP4B1 wild-type and the E310 mutants, devoid of the covalent heme link, were compared in functional assays with lauric acid, various hydrocarbons, 4-ipomeanol, pyrogallol, and 1,2,4,5-tetramethoxybenzene, but no obvious distinction emerged. CYP4B1 was comparable to the E310A mutant in terms of heme retention and thermostability, but polar substitutions were not well tolerated in the thermostability assay. Therefore, during the evolution of the CYP4B1 gene, a random A310E mutation may not have unduly perturbed the selective pressures imposed on the P450 because the resulting polar glutamic acid could be masked by incorporation into the ester bond.

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LIST OF ABBREVIATIONS

2-AA	2-aminoanthracene
2-AAF	2-acetylaminofluorene
2-AF	2-aminofluorene
2-NA	2-naphthylamine
3-MeO-AAB	3-methoxy-4-aminoazobenzene
3-MI	3-methylindole
11-DDYA	11-dodecynoic acid
11,12-EET	11,12-epoxyeicosatrienoic acid
12-HETE	12-hydroxyeicosatetraenoic acid
12-HETrE	12-hydroxyeicosatrienoic acid
17-ODYA	17-octadecynoic acid
20-HETE	20-hydroxyeicosatetraenoic acid
amu	atomic mass units
BSTFA	<i>N,O</i> -bis-(trimethylsilyl)trifluoroacetamide
CAR	constitutive androstane receptor
CYP	cytochrome P450
δ -ALA	δ -aminolevulinic acid
DBC	3,3'-dichlorobenzidine
DHET	dihydroxyeicosatrienoic acid
DLPC	L- α -dilauroylphosphatidylcholine
DMAB	3,2'-dimethyl-4-aminobiphenyl
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EET	epoxyeicosatrienoic acid
GC/FID	gas chromatography flame ionization detection
GC/MS	gas chromatography mass spectrometry
HRP	horseradish peroxidase
HSQC	heteronuclear single quantum correlation

HPLC	high-pressure liquid chromatography
IPO	4-ipomeanol
IPTG	isopropyl-beta-D-thiogalactopyranoside
LPO	lactoperoxidase
LC/ESI-MS	liquid chromatography electrospray ionization-mass spectrometry
MS	mass spectrometry
<i>m</i> -CPBA	<i>m</i> -chloroperoxybenzoic acid
NAC	N-acetyl cysteine
NAL	N-acetyl lysine
NADPH	nicotinamide adenine dinucleotide phosphate
NDBA	N-nitrosodibutylamine
NMR	nuclear magnetic resonance spectroscopy
rLPO	recombinant lactoperoxidase
RCE	rabbit corneal epithelial
ROESY	rotating frame overhauser spectroscopy
RXR	retinoid X receptor
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SIM	single ion monitoring.
TCDD	2,3,7,8-tetrachlorodibenzo- <i>p</i> -dioxin
TFA	trifluoroacetic acid
TOF/MS	time-of-flight mass spectrometry
UV	ultraviolet
VPA	valproic acid

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ACKNOWLEDGMENTS

The author wishes to express sincere appreciation to those individuals who have helped make this effort possible through their contributions:

To Dr. Allan E. Rettie, for his careful guidance throughout this project, encouragement for independent thinking, enlightening scientific discussions, and the occasional game of golf;

To Drs. Kent L. Kunze and William M. Atkins for helpful scientific discussions and for serving on the reading and supervisory committees;

To Drs. Sidney D. Nelson and Shiu-Lok Hu for serving on the supervisory committee;

To Mr. William Howald and Dr. Ross Lawrence, for their help and guidance in the mass spectrometry facility;

To Drs. Kirk Henne, Matthew Cheesman, Byron Kneller, and Yi-Min Zheng for their contributions to this work;

To fellow graduate students Jed Lampe, Josh Pearson, Dustin Smith, Han Ho, Guoying Tai, Bob Lyon, Jason Schuman, Kyle Allen, Cathy Yeung, Clara Hsia, Carrie Mosher, and Dr. Matt McDonald for their support and friendship;

To Sandy Koppenol for encouraging me to enter graduate school and for introducing me to the UW Department of Medicinal Chemistry;

To the Department of Medicinal Chemistry and Pharmacological Sciences Training Grant Program (NIH) for their financial support;

Finally, to my wife Nicole, my son Dylan, my parents Dick and Sherry and my sister Heather for their love, support, and encouragement.

DEDICATION

To my wife Nicole,
For her love and support;

And

To my son Dylan,
Who was born on July 2nd, 2005.

Chapter 1

Enigmatic CYP4B1: An Extrahepatic P450 at the Interface between Endobiotic and Xenobiotic Metabolism

1.1 Overview

CYP4B1 is a predominately extrahepatic P450 responsible for the bioactivation of a wide range of protoxins that often exert tissue-specific toxicological effects. The most notable examples include the activation of aromatic amines to bladder carcinogens and 4-ipomeanol (IPO) to pneumotoxic species. In addition, CYP4B1 selectively hydroxylates the terminal position of certain fatty acids, a reaction that is perhaps indicative of an endogenous role similar to other members of the CYP4 family that participate in the catabolism of eicosanoids. Therefore, CYP4B1 can be classified as a drug metabolizing enzyme like the CYP1-CYP3 families, but also may be categorized along with families 5-51 (14 families) that perform specific physiological functions involving metabolism of specific endobiotics. These seemingly disparate functional roles place CYP4B1 at a unique interface between endobiotic and xenobiotic metabolism (Figure 1.1). This chapter will review the tissue specificity, metabolic and structural characterization, and possible regulatory mechanisms, of various animal orthologous forms of CYP4B1. The significance of the human form of CYP4B1 will then be addressed, as well as the potential use of rabbit CYP4B1 in gene therapy to treat human cancers. Finally, this chapter will introduce the recent identification of the covalently attached heme to CYP4B1, which is the focus of the dissertation research.

1.2 Discovery of CYP4B1

CYP4B1 was originally identified as P450_{II} in the rabbit lung, based on chromatographic separation from the other major pulmonary isoform, P450_I (subsequently termed CYP2B4) (1). These two P450s eluted in nearly equal amounts, indicating similar levels of expression in the rabbit lung. Based on a total recovery of

70%, CYP4B1 was calculated to comprise at least 35% of the total microsomal P450 (1). CYP2B4 and CYP4B1 were distinguished based on differences in molecular weight, CO-ferrous cytochrome P450 UV-visible spectra (1, 2), immunochemical characterization (3), amino acid composition (4), and substrate specificities in reconstituted systems (1, 2). Early preparations of purified CYP4B1 were subsequently found to contain significant amounts of P450 form 6 (later designated CYP1A1), which contributes 1-3% of the total rabbit pulmonary P450 (5). Pulmonary CYP4B1 was shown to be identical to the phenobarbital-inducible hepatic form 5, based on molecular weight, immunochemical characterization, amino acid composition, and metabolic profiles (6, 7). Eventual isolation and sequencing of cDNAs encoding for rabbit and rat CYP4B1 revealed close similarity to rabbit P-450p2 (54% identity) and rat P-450LA ω (51% identity) in gene family IV, and led to its placement in a previously unrecognized subfamily, IVB (8). Throughout the remainder of the chapter, form 5 and P450_{II} are referred to as CYP4B1. CYP4B clones have since been isolated from the rabbit cornea (9), mouse kidney (10), goat lung (11), human lung (12) and human placenta (13).

1.3 Tissue Distribution: Species and Gender Differences

Although CYP4B1 makes up greater than 35% percent of the total P450 in the rabbit lung, relatively low concentrations are found in the uninduced liver (6). In fact, the relative inhibition of aromatic amine activation by anti-CYP4B1 indicated that CYP4B1 makes up only 1-2% of total hepatic P450 (14). Homologues to CYP4B1 in other species are also expressed dominantly in the lung as compared to the liver. CYP4B1 protein has been detected by immunoblotting in pulmonary tissues of rabbit, guinea pig, mouse, monkey, hamster, and rat, and of the five animals examined, only rabbit and hamsters had detectable levels of CYP4B1 protein in hepatic tissues (15). Expression levels in each animal correlated well with the effects of anti-CYP4B1 on the metabolism of 2-aminofluorene (2-AF), indicative of active CYP4B1 in these tissues (15). Similar experimental methods were utilized to show that the rabbit bladder contains CYP4B1, representing approximately 20% of the total P450 in that organ (16). Northern blot

analysis of mRNA extracted from rabbit tissues, hybridized with an oligonucleotide probe to CYP4B1, demonstrated relatively high levels of transcripts in the lung compared to the liver and all other extrapulmonary tissues examined, including kidney, heart, and brain (17). CYP4B1 mRNA and protein also has been detected throughout the rabbit small intestine and colon (18). Furthermore, all rabbit gastrointestinal tissues other than stomach possess a high capacity for the activation of 2-aminofluorene that correlates with CYP4B1 expression levels. More recently, CYP4B1 mRNA has been found in the rabbit cornea, as detected by Southern hybridization (19).

Although CYP4B1 appears to be dominantly expressed in the lung as compared to the liver of many species, the relative distribution of this P450 isoform in various tissues is not conserved between species, or even between sexes. Immunoblots of mouse renal, pulmonary, and hepatic microsomes revealed the presence of CYP4B1 protein in the kidney of male mice, but relatively little expression in females (10). Pulmonary microsomes of both male and female mice also contained relatively low levels of CYP4B1, and the protein could not be detected in hepatic microsomes (10). This expression profile in the mouse is vastly different from that observed with the rabbit. CYP4B1 has also been detected in the mouse bladder by immunoblotting with anti-CYP4B1, at levels that were calculated to be 2.5% of total protein in the kidney (20). Choudhary *et al.* also noted species specific patterns when the authors quantitated CYP4B1 transcript levels in eight mouse and human tissues, including heart, brain, spleen, testis, lung, liver, skeletal muscle, and kidney (21). Mouse transcript levels were similar in the brain, lung, and kidney (~20% distribution for each), and also detected in the spleen, testis, liver, and skeletal muscle (21). Humans transcribed CYP4B1 dominantly in the lung (70% of distribution), with low levels in the heart, skeletal muscle and kidney making up the remainder 30% distribution. A complete list of tissues with detectable CYP4B1 protein and/or mRNA in rabbit, rat, mouse, and human is presented in Table 1.1. In sum, these localization studies demonstrate that CYP4B1 is predominately an extrahepatic P450, but there are significant species and sex differences regarding tissue distribution and expression levels.

Induction of CYP4B1 expression levels by xenobiotics has been demonstrated to be tissue specific as well as species dependent. In the rabbit, phenobarbital (administered in drinking water or intraperitoneally) does not affect CYP4B1 synthesis in pulmonary tissues, but it increases the hepatic microsomal content of CYP4B1 by approximately 11-fold (6). This was demonstrated by quantitative analysis of CYP4B1 by single radial immunodiffusion, western blotting, and anti-CYP4B1 inhibition of the mutagenic activity of 2-AF (6). Another study demonstrated that intraperitoneal administration of Aroclor 1260 or phenobarbital, but not 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), could induce synthesis of CYP4B1 in the liver, and none of these compounds affected pulmonary CYP4B1 levels (22). Induction of CYP4B1 in the rabbit bladder was not observed with intraperitoneal administration of phenobarbital or TCDD (16). Vanderslice *et al.* compared phenobarbital-dependent induction in the lung and liver between rabbit, guinea pig, mouse, monkey, hamster, and rat, and found that only rabbit *hepatic* CYP4B1 levels are elevated in the presence of the inducer (15). In the rabbit cornea, there is some evidence that clofibrate and phenobarbital induce CYP4B1 (19). Corneas incubated with clofibrate showed elevated levels of CYP4B1 transcripts in a Southern blot with CYP4B1 probes, and both clofibrate and phenobarbital treatment resulted in increased metabolism of arachadonic acid to 12-hydroxyeicosatetraenoic acid (12-HETE), a reaction that correlates with CYP4B1 expression (19). Overall, these data indicate that in addition to wide inter-species differences in the tissue distribution of the enzyme, induction of CYP4B1 expression by xenobiotics is also highly variable.

1.4 Xenobiotic Metabolism/Bioactivation

Similar to other members of the CYP4 family, CYP4B1 can preferentially hydroxylate the thermodynamically disfavored ω -position of fatty acids, a reaction that may be indicative of an endogenous role similar to that elaborated for related CYP4A sub-family members. In contrast to other CYP4 family enzymes, CYP4B1 can biotransform a wide variety of xenobiotics, either directly to reactive intermediates, or to

metabolites that can undergo further bioactivation by other enzymes. Reactive metabolite intermediates can form adducts with DNA or proteins and result in carcinogenesis or cytotoxicity, respectively. The bioactivation of valproic acid (VPA) appears to be related to the ω -hydroxylase activity, but other reactions, such as epoxidation of IPO and heteroatom oxidation of 2-AF, mechanistically distinct. A comprehensive list of known CYP4B1 substrates are presented in Table 1.1 and the structures of representative substrates, as well as the relevant metabolites are shown in Figure 1.2. This chapter section will review the variety of reactions catalyzed by this extrahepatic P450.

CYP4B1 was originally characterized as an ω -hydroxylase when the Masters' laboratory was searching for the P450 responsible for ω -hydroxylation of prostaglandins in the lungs of pregnant rabbits (23). These authors found that P450_{PG- ω} (CYP4A4), which is upregulated in pregnancy, was responsible for this specific reaction, but that reconstituted CYP4B1 preferentially hydroxylated lauric acid at the ω -position, albeit with little positional selectivity ($\omega/\omega-1 = 1.4$) (23). Rabbit CYP4B1 was later found to hydroxylate VPA, a branched, medium-chain, fatty acid anticonvulsant drug, at the terminal position, with even greater regioselectivity ($\omega/\omega-1 = 49$) (24). Fisher *et al.* proposed that the CYP4A, CYP4B, and CYP4F sub-families might all be functionally related as selective fatty acid ω -hydroxylases, but with different chain-length specificities (25). This hypothesis was tested by comparing the metabolite profiles generated by rabbit CYP4B1 from a series of short-medium chain fatty acids. While the turnover numbers were approximately 11 nmol/nmol P450/min for octanoic, nonanoic, and decanoic acid, the selectivity for hydroxylation at the ω -position increased with decreasing chain length. The shortest chain compound tested, heptanoic acid, was a relatively poor substrate, with a ω -hydroxylation rate of 0.8 nmol/nmol P450/min. Therefore, an eight carbon fatty acid is the optimal length for regioselective ω -hydroxylation by CYP4B1, in contrast to the twelve carbon fatty acid (lauric acid) that is preferred by CYP4A1. The authors also demonstrated that the carboxylate functional group is *not* required for the observed regiospecificity, by comparing the metabolite

profiles of the corresponding hydrocarbons. This feature also distinguishes catalysis by CYP4B1 from CYP4A enzymes. As noted for the fatty acids, regioselectivity for the ω -position of the hydrocarbon derivatives increased with decreasing chain length. Heptane, the seven carbon hydrocarbon equivalent to heptanoic acid, was metabolized at a comparable rate to octane and nonane, and displayed a surprisingly high degree of regioselectivity ($\omega/\omega-1 = 23$). Chain branching in the fatty acids (VPA) and hydrocarbons (4-methylheptane) resulted in elevated rates of ω -hydroxylation and further increases in regiospecificity. Fisher *et al.* concluded that an intact seven methylene unit skeleton is the minimum requirement for regioselective ω -hydroxylation (25). The significance of CYP4B1-dependent ω -hydroxylase activity in relation to endogenous metabolism will be discussed later in this chapter.

CYP4B1 has been most extensively studied for its role in the bioactivation of xenobiotics to reactive intermediates that exert a toxicological effect *via* protein or DNA binding. The bioactivation phenomenon observed with CYP4B1 may be associated with the same active site restrictions that permit preferential ω -hydroxylation, at least in the case of VPA activation. VPA is an anticonvulsant drug that can be metabolized by P450s to the terminal olefin, 4-ene-VPA (26), which likely plays a role in the idiosyncratic, sometimes fatal, hepatotoxicity caused by administration of VPA (27). Rettie *et al.* first demonstrated that anti-CYP4B1 inhibited the majority of 5-hydroxy-VPA and 4-ene-VPA production in rabbit lung microsomes, whereas anti-CYP2B4 inhibited the majority of 4-hydroxy-VPA formation (24). The association between ω -hydroxylation and terminal desaturation of VPA was reinforced by analysis of the VPA metabolite profile generated by cDNA-expressed CYP4B1. CYP4B1 rapidly metabolized VPA to 5-hydroxy VPA, yet production of 4-hydroxy VPA was only 2-3 times greater than 4-ene-VPA formation. To determine the underlying mechanism that links these two metabolic pathways, Rettie *et al.* analyzed the intramolecular deuterium effects for CYP4B1 mediated metabolism of VPA (24). Relatively large isotope effects were obtained for 4-ene-VPA and 4-hydroxy-VPA, but not 5-hydroxy-VPA, derived from [4,4- $^2\text{H}_2$]VPA. In

addition, high isotope effects were seen for 5-hydroxy-VPA, but not 4-ene-VPA and 4-hydroxy-VPA, derived from [5,5,5-²H₃]VPA. Similar isotope effects were observed for the CYP2B1 isoform, despite its preference for ω -1 hydroxylation. In all experiments, the isotope effects for 4-ene-VPA and 4-hydroxy-VPA co-segregated, suggesting that the rate-limiting step in formation of 4-ene-VPA is hydrogen atom abstraction from the C-4 position, regardless of the regioselectivity of the isoform involved. It was further proposed that the propensity for ω -hydroxylation influences the second half-reaction regarding the extent of terminal destaruation (4-ene-VPA) versus oxygen rebound (4-hydroxy-VPA) from the C-4-centered radical of VPA (24). An analogous mechanism for terminal desaturation has more recently been demonstrated for lauric acid (28).

Extrahepatic P450s have long been studied for their ability to bioactivate compounds that elicit organ specific toxicities, despite systemic exposure. CYP4B1, specifically, has been strongly associated with activation of the pneumotoxins, IPO and 3-methylindole (3-MI), as well as various aromatic amines associated with bladder cancer. IPO is a furan derivative produced naturally by the common sweet potato, *Ipomoea batatas*, in the presence of *Fusarium solani*, a typical mold (29). Cattle that consume mold-damaged sweet potato crops experience extreme respiratory distress, including pulmonary edema and congestion, often leading to death. This toxic response, first observed in cattle, also occurs in other animal species, notably rabbits (30, 31). In the lung, relatively high levels of IPO alkylating metabolites are covalently bound to nonciliated bronchiolar epithelial (Clara) cells (32), where pulmonary P450 enzymes are expressed at their highest levels (33). Rabbit CYP4B1 was first implicated in the bioactivation of IPO when Slaughter *et al.* demonstrated that anti-CYP4B1 could inhibit covalent binding of radiolabeled IPO to protein in pulmonary and hepatic microsomal preparations (34). Moreover, when purified CYP4B1 was analyzed in a reconstituted system with [³H]IPO, radioactivity was associated with the P450 (34). In addition to protein adducts, covalent binding of IPO to cellular DNA has been observed in human hepatocellular carcinoma cells (Hep G2) infected with rabbit CYP4B1 cDNA-containing vaccinia virus (35). This study also

demonstrated that rabbit CYP4B1 can activate IPO to DNA-binding metabolites at an exceptional rate relative to the 12 human P450s screened (35).

The cytotoxic effects of IPO bioactivation have been examined in a mouse cell line, in which retroviral vectors were used to transfer and express the cDNA encoding rabbit CYP4B1 (36). CYP4B1 expression levels in various clones coincided with their sensitivity toward IPO, whereas the parental cells were completely resistant to IPO-induced cytotoxicity (36). The role of rat CYP4B1 in IPO bioactivation has been examined *in vivo* by evaluating the protective effects of CYP4B1-selective inhibitory substrates on toxicity (37). Pretreatment of rats with the CYP4B1 substrate, *p*-xylene (38), increased the LD₅₀ from 18 mg/kg to 150 mg/kg (37). Additionally, decreased covalent binding of [¹⁴C]IPO metabolites to lung protein was observed in microsomes obtained from rats after chemical modulation of P450 activity with *p*-xylene, as compared to microsomes without treatment (37). These studies provide a link between CYP4B1-mediated bioactivation of IPO, covalent binding of metabolites to protein, and resulting cytotoxicity.

Recently, a mechanism for IPO bioactivation has been proposed based on the structural characterization of an IPO-adduct, generated in incubations with recombinant CYP4B1, IPO, N-acetyl cysteine (NAC) and N-acetyl lysine (NAL) (39). The product was unambiguously identified as an N-substituted cysteinyl pyrrole derivative of IPO (39), analogous to that characterized previously in model chemical studies conducted with cis-2-butene-1,4-dial (40). Baer *et al.* deduced that CYP4B1 initially oxidized the furan ring and subsequent rearrangement led to a reactive enedial intermediate. This electrophilic intermediate, in turn, could then react with the NAC and NAL nucleophiles to produce the observed IPO adduct (39). If this mechanism holds true *in vivo*, the reactive enedial may be responsible for the observed covalent binding and resulting cytotoxicity.

Lung-specific toxicity has also been observed in cattle, goats, and, to a lesser extent, in mice and rabbits, following systemic exposure to 3-MI, a compound produced by fermentation of tryptophan by ruminal and intestinal microorganisms (41). The reactive intermediate responsible for toxicity is likely a methylene imine, formed by a one-electron abstraction from the indole nitrogen and subsequent hydrogen abstraction from the methyl group (41). Early studies with lung tissues showed that covalent binding of radiolabeled 3-MI to protein could be inhibited by SKF 525A, indicating that P450s played a role in bioactivation of 3-MI (42). In addition, 1-aminobenzotriazole, a suicide substrate inhibitor of P450, inhibited covalent binding of 3-MI to goat lung microsomes (43). It was speculated that the species-selective and organ-specific toxicity caused by 3-MI was due to the expression of particular extrahepatic P450 isoforms in the affected tissues (41). CYP4B1 was an ideal candidate for 3-MI bioactivation, given the fact that it is highly expressed in the lung of many species, and it was known to be involved in the activation of other protoxins. Indeed, CYP4B transcripts were readily identified by Northern blotting in goat pulmonary tissues, which show high levels of covalent binding of 3-MI (44). Furthermore, vaccinia-expressed rabbit CYP4B1 was found to metabolize 3-MI to 3-methyloxindole, indole-3-carbinol, and 3-methyleneindolenine, the suspected reactive intermediate responsible for covalent binding and subsequent toxicity (45). Mouse CYP1A2 and the human forms CYP2A6 and CYP2F1, also formed 3-methyleneindolenine, but bioactivation rates were higher for rabbit CYP4B1 than any other P450 screened (45). The authors point out that even though rabbit CYP4B1 can rapidly convert 3-MI to a covalent binding species, rabbits are the least susceptible to 3-MI-induced pneumotoxicity when compared to cattle, goats, and rodents. Thornton-Manning *et al.* propose that the species selectivity of 3-MI toxicity may be due to competing bioactivation and detoxification processes in the pulmonary tissue (45). Recently, CYP4B2 has been cloned from the goat lung and the resulting purified protein was able to bioactivate 3-MI to the reactive 3-methyleneindolenine (11). Therefore, this isoform seems likely to be responsible for the highly organ-specific toxicity observed in goats (11).

The arylamines, 2-AF, 2-aminoanthracene (2-AA), and 2-acetylaminofluorene (2-AAF) were among the first substrates identified for CYP4B1 in the rabbit lung (14). Robertson *et al.* established that rabbit pulmonary microsomal fractions could efficiently activate these promutagens (14) by using the Salmonella test of Ames *et al.* (46). The authors then demonstrated that of the two major P450s in the rabbit lung, purified and reconstituted CYP4B1, but not CYP2B4, could activate these promutagens. Furthermore, it was shown that anti-CYP4B1 inhibited mutagenic activation of 2-AF, 2-AAF, and 2-AA by greater than 85% in pulmonary microsomal fractions (14). In hepatic microsomal fractions, mutagenic activation of 2-AF, 2-AAF, and 2-AA was inhibited with the anti-CYP4B1 by 55, 69, and 48%, respectively. Additionally, liver microsomes from phenobarbital treated rabbits, which have increased levels of CYP4B1, show a corresponding increase in the mutagenic activation of these aromatic amines (6). Czerwinski *et al.* later demonstrated that vaccinia virus-expressed rabbit CYP4B1 could effectively metabolize 2-AF to DNA-binding metabolites (35). The bioactivation of various aromatic amines has also been investigated in a mammalian cell line, in which retroviral vectors were used to transfer and express the cDNA encoding rabbit CYP4B1 (36). In this experiment, only exposure of 2-AA gave a clear cytotoxic response in the CYP4B1-expressing cells, compared to only marginal toxicity in control cells. The lack of cytotoxicity for 2-AF and 2-AAF in this expression system may be due to lack of sufficient sulfonylation and deacetylation activity involving the further bioactivation to reactive intermediates (36).

P450s can bioactivate 2-AF, 2-AAF, and 2-AA to DNA-binding agents by initial heteroatom oxidation to an N-hydroxy intermediate (47, 48). The N-hydroxy derivatives of 2-AF or 2-AA can form DNA adducts either directly, in an acid-catalyzed reaction, or by a PAP-dependent sulfonylation that creates an electrophilic N-sulfonyloxy ester derivative (47, 48). Hydroxylation of 2-AA at C-1 can also generate an mutagenic intermediate (47). Following N-hydroxylation of 2-AAF, deacetylation can lead to the N-hydroxy-2-AF intermediate described above (49). In addition, sulfonylation of 2-AAF results in a reactive intermediate that can form guanine:acetylaminofluorene adducts (49).

The carcinogenicity of aromatic amines in the bladder of some species, including rabbit (50), was proposed to be due to bioactivation by P450s in the target organ (16). More specifically, Vanderslice *et al.* suspected that CYP4B1 was involved, because this isozyme is present in the liver and lung and is highly active in the metabolism of aromatic amines to mutagenic products (16). After demonstrating with immunoblotting that CYP4B1 was present in the microsomal fraction of the bladder, anti-CYP4B1 was added to microsomal incubations to evaluate their effect on 2-AF metabolism. The production of organic-extractable 2-AF metabolites, which were detected by HPLC-UV and scintillation counting of radiolabeled compounds, was inhibited by 85% by anti-CYP4B1 and 86% by carbon monoxide ($\text{CO}:\text{O}_2 = 4:1$) (16). Metabolites identified from bladder microsomal incubations included a ring-hydroxylated derivative of 2-AF, N-hydroxy-2-AF, 2-AAF, 2-nitrosofluorene, and 2,2'-azoxybisfluorene. Formation of protein-bound products and water soluble products were also inhibited by anti-CYP4B1, although total production was low relative to the organic soluble fraction (16). In sum, these experiments support a role for P450s, specifically CYP4B1, in the activation of 2-AF to reactive intermediates, although a contribution to carcinogenesis *in vivo* remains to be determined.

Mouse renal CYP4B1 has been implicated in the activation of the aromatic amine, 3-methoxy-4-aminoazobenzene (3-MeO-AAB), a procarcinogen that can cause hepatic tumors in rats (10). Following purification of P450 from male mouse renal microsomes, isolated fractions were analyzed for the ability to activate 3-MeO-AAB to mutagenic compounds detected by an *umu* gene expression assay (10). The N-terminal sequence of the purified microsomal protein with this activity was similar to that of rat CYP4B1, suggesting that a CYP4B1 homologue was present in the mouse kidney. In addition to activation of 3-MeO-AAB, the purified protein was able to convert 2-AF and 2-AA to mutagenic metabolites. Antibodies generated from the purified protein inhibited the activity of these three procarcinogens in male renal microsomes. A CYP4B1 gene was cloned and sequenced from a mouse renal cDNA library that had high sequence identity

to CYP4B1 orthologs in the rat, rabbit, and human (10). Therefore, mouse CYP4B1 can bioactivate the procarcinogen 3-MeO-ABB in the kidney, but the relevance of this reaction to hepatic toxicity, which has been observed in rats, is undetermined.

Imaoka *et al.* also demonstrated that CYP4B1 could activate 3,3'-dichlorobenzidine (DCB), a potent bladder procarcinogen in rats and humans, to mutagenic metabolites (20). Mouse renal microsomes and purified CYP4B1 had high activity toward DCB, as determined by activation of *umu* gene expression. Furthermore, anti-CYP4B1 completely inhibited mutagenic activation in mouse renal microsomes. The low activity of the mouse bladder toward DCB did not permit evaluation of anti-CYP4B1 inhibition, but lauric acid, a competitive substrate for CYP4B1, was shown to effectively inhibit DCB activation. A panel of purified rat P450s was subsequently screened, and rat CYP4B1 was found to have relatively high activity toward DCB, as well as 3,2'-dimethyl-4-aminobiphenyl (DMAB) and 2-naphthylamine (2-NA). The authors also demonstrated the presence of both CYP4B1 mRNA and expressed protein in the rat bladder (20). In sum, CYP4B1 in mice and rats can activate DCB to reactive intermediates that may potentially contribute to the observed bladder carcinogenesis.

Additional substrates for CYP4B1 that have been noted in the literature include *p*-xylene, N-nitrosodibutylamine (NDBA), N-hydroxyamphetamine, 11-dodecynoic acid (11-DDYA), 17-octadecynoic acid (17-ODYA), and 7-ethoxycoumarin. *p*-Xylene is oxidized by purified and reconstituted rabbit CYP4B1 to form *p*-methylbenzyl alcohol (38). Smith *et al.* suspected that *p*-methylbenzyl alcohol was subsequently oxidized by an alcohol dehydrogenase to *p*-tolualdehyde, which could, in turn, be converted by the monooxygenase system to a product that destroys pulmonary cytochrome P450 (38). Schulze *et al.* have shown that CYP4B1 can preferentially ω -hydroxylate N-nitrosodibutylamine (NDBA) ($\omega/\omega-1 = 2$) to N-nitrosobutyl-4-hydroxybutylamine (NB-4-HBA). Although this metabolite is not reactive itself, it is the first step in a sequence of reactions that lead to the ultimate carcinogen responsible for urinary bladder tumors (51). The metabolite, NB-4-HBA can be further oxidized, to N-

nitrosobutylcarboxypropylamine (BCPN), not necessarily catalyzed by CYP4B1(51). It is possible that BCPN is activated in the bladder *via* α -oxidation (52), with subsequent spontaneous decomposition to reactive aldehydes and alkyl diazonium ions (53). N-hydroxyamphetamine is presumably oxidized by CYP4B1 to the nitroso species that subsequently forms a metabolic intermediate (MI) complex with the heme iron (54). The acetylenic compounds 11-DDYA and 17-ODYA have been shown to inactivate CYP4B1 *via* mechanism based inhibition, as determined by pre-incubation of these compounds and subsequent quantitation of lauric acid turnover in rabbit lung microsomes and reconstitutions with purified enzyme (55). Finally, purified rat lung CYP4B1 has been reported to O-dealkylate 7-ethoxycoumarin (56).

1.5 CYP4B1 Active Site Topography

Evaluation of the range of chemically diverse substrates accommodated by the CYP4B1 active site suggests that i) the active site is restricted directly above the heme group to favor oxidation of terminal carbons over oxidation of internal or adjacent aromatic carbons ii) distal regions of the active site are more expansive and/or flexible to accommodate larger substrates. The topographical features of the active site of CYP4B1 have been further probed with a variety of aromatic ligands (57). First, hydroxylation of cumene by CYP4B1 was compared to that of CYP2B1 and CYP102, to assess the enzyme's relative capacity to catalyze ω -hydroxylation versus benzylic hydroxylation. Cumene was predominately hydroxylated at the terminal position by CYP4B1, whereas CYP102 and CYP2B1 preferentially formed the benzylic metabolite, 2-phenyl-2-propanol. CYP4B1 regioselectivity for ω -hydroxylation was large, yielding an $\omega/\omega-1$ ratio of >27 , and the enzyme exhibited significant prochiral selectivity with an (S)/(R) ratio of 3:1. Therefore, active site constraints in the immediate vicinity of the CYP4B1 perferryl species can overcome thermodynamically preferred benzylic hydroxylation in favor of ω -hydroxylation and also can distinguish between the substrates two chemically equivalent methyl groups (57). Secondly, intramolecular deuterium isotope effect

profiles for the P450-catalyzed benzylic hydroxylation of a series of selectively deuterated xylene and dimethylbiphenyl substrates were evaluated to assess the steric restrictions in the active site (57). This study revealed that the CYP4B1 active site provides less space for substrate rotation prior to oxidation, as compared to CYP2B1. Finally, phenyldiazene was reacted with CYP4B1 to produce an σ -complex with the heme iron, but upon chemical oxidation, the phenyl group did not migrate to any of the four nitrogens on the heme (57). This experimental result was interpreted to signify that the active site directly above the heme plane was spatially restricted, but the subsequent finding that the CYP4B1 heme is covalently bound to the protein may also help explain the lack of detectable migration products (58). Collectively, the results indicate that the active site of CYP4B1 is considerably restricted, at least relative to CYP2B1, promoting the observed ω -regioselectivity (57).

1.6 Endogenous Role of CYP4B1

The CYP4 family is reported to be one of the oldest P450 families, having appeared about 1.25 billion years ago following the formation of the steroid biosynthetic genes (59). Since the CYP4 family is evolutionarily related to the cholesterol metabolizing enzymes, it has been proposed that together these two families may have been involved in maintaining the membrane integrity in early eukaryotes (59). The specific role of CYP4B1 is unknown, but it is predicted to be physiologically important because the gene sequence has been conserved throughout evolution. The high sequence identity of CYP4B1 orthologs in rabbit, rat, mouse, and human is suggestive of a functional constraint on this gene (60). Nelson *et al.* has predicted that orthologous genes are highly specific for endogenous substrates, whereas the non-orthologous genes are more likely to act on foreign substrates (60). When expression patterns of mouse and human P450 orthologs during development were analyzed, Choudhary *et al.* found that CYP4B1 first appeared on day 11 of embryonic development and continued to be expressed through the remaining ontogenic stages (21). The authors suggested that the specific temporal appearance of CYP4B1, as well as other orthologous genes, indicated a precise

developmental role that is unrelated to their ability to metabolize xenobiotics (21). Although the antiquity, the evolutionary conservation, and the pattern of developmental expression of the gene are indicative of a physiological functional role, there is currently no direct evidence for a relevant CYP4B1-mediated reaction.

Rabbit CYP4B1 will preferentially ω -hydroxylate lauric acid (28), which has traditionally been used as a model substrate to probe for ω -hydroxylase activity toward physiologically relevant substrates, but lauric acid itself is found in very small quantities in the hepatic and renal endoplasmic reticulum (59, 61, 62). Terminal hydroxylation activity is predicted to be relevant to the metabolic inactivation of physiologically or pathophysiologically important fatty acid derivatives which are not required as energy sources (63). CYP4B1 has also been shown to ω -hydroxylate short-medium chain fatty acids and hydrocarbons (C7-C10), with increasing ω -hydroxylation regionselectivity toward the shorter carbon chain lengths (25). However, inhibition studies performed with the C-18 acetylenic fatty acid, 17-octadecynoic acid (17-ODYA), indicate that CYP4B1 is also active toward long chain fatty acids as well (55). The relevance of these fatty acid oxidations to the metabolism of endogenous substrates such as arachidonic acid, prostaglandins, and leukotrienes is unknown. Rabbit CYP4B1 displayed no activity toward prostaglandin E₁ (23), and rat CYP4B1 did not metabolize prostaglandin A₁ (56), suggesting that metabolic inactivation of prostaglandins is not an endogenous function as proposed for CYP4A4 (P450_{PG- ω}) and other members of the CYP4A family (59). To date, studies have not been conducted to examine CYP4B1-mediated metabolic inactivation of leukotrienes, for which the CYP4F family shows a high degree of substrate selectivity (63, 64). Another possible role for the ω -hydroxylase capabilities of CYP4B1 may be to metabolize lipids during fatty acid overload of the mitochondrial β -oxidation system, as proposed for CYP4A1 (59). It is believed that after CYP4A1 hydroxylates fatty acids at the terminal position, further cytosolic oxidation occurs to produce long chain fatty dicarboxylic acids, which are then taken up by peroxisomes for β -oxidation (59).

Recently, induction of CYP4B1 in the rabbit cornea under hypoxic conditions has been correlated with increased conversion of arachidonic acid to 12-HETE and 12-hydroxyeicosatrienoic acid (12-HETrE) (19, 65-67). 12-HETE is a Na,K-ATPase inhibitor with chemotactic activity, and 12-HETrE is a powerful proinflammatory mediator that has been shown to stimulate vasodilation, increase anterior chamber proteins, chemoattract polymorphonuclear leukocytes, and elicit angiogenesis (68). Originally, Vafeas *et al.* demonstrated that incubation of corneal organ cultures under hypoxic conditions increased the corneal epithelial synthesis of 12-HETE and 12-HETrE by 2- to 3-fold over the control normoxic conditions (69). The increase in synthesis of these inflammatory mediators was linked to P450s by showing that the reaction was inhibited by 17-ODYA (69). To identify the hypoxia-induced P450 isoform, Mastuygin *et al.* extracted mRNA from the rabbit corneal organ cultures and hybridized the mRNA with a panel of P450 cDNA probes using low-stringency conditions (19). A signal was observed in a Northern analysis with a CYP4A1 probe and the intensity was higher with the mRNA from the hypoxia-treated corneas. Subsequent cloning and sequencing of the CYP4A PCR fragments revealed that the mRNA was closely related to CYP4B1 (95-98.8%). Inhibition studies with anti-CYP4B1 and 10 μ M lauric acid provided further evidence for the involvement of CYP4B1 in 12-HETE and 12-HETrE production (19). To further implicate CYP4B1 in hypoxia-induced inflammation, Mezentsev *et al.* demonstrated that cultured rabbit corneal epithelial (RCE) cells transfected with a CYP4B1 plasmid metabolize arachidonic acid to 12-HETrE at a 5-fold higher rate than control cells (67). Activity was increased with the addition of NADPH and was inhibited by 17-ODYA, confirming that the reaction is P450 dependent. Corneal epithelial transfection of CYP4B1 *in vivo* also resulted in increased 12-HETrE levels and neovascularization. These experiments seem to provide a strong link between CYP4B1 levels, 12-HETrE production, and angiogenic activity in the rabbit cornea (67).

Despite the convincing evidence for CYP4B1-mediated 12-HETE and 12-HETrE production, this internal oxidation is suspect given the propensity for CYP4B1 to

preferentially hydroxylate at the terminal position. Mezentsev *et al.* reports CYP4B1 dependent production of 20-hydroxyeicosatetraenoic acid (20-HETE) from arachidonic acid in transfected RCE cells, but the rates are 7-fold lower than for 12-HETrE production (67). There is evidence for a shift toward oxidation at the penultimate carbon with increasing fatty acid chain length (25), but there is no other evidence for CYP4B1 catalyzed ω -9 oxidations of fatty acids, as proposed for arachidonic acid. Based on the typical CYP4 oxidations, we would expect the preferential formation of 20-HETE, which in fact is formed by recombinant CYP4As (70, 71) and CYP4Fs (64, 72-74). CYP4A2 has been shown to epoxidize the 11,12 double bond of arachidonic acid to form 11,12-epoxyeicosatrienoic acid (11,12-EET), yet this is a minor metabolite compared to 20-HETE formation (75). In contrast to the correlation studies done in rabbit corneas that indicate CYP4B1 involvement in arachadonic acid metabolism, Zeldin *et al.* found that CYP4B1 did not contribute to arachadonic acid in the rabbit lung (17). Anti-CYP4B1 polyclonal antibodies did not inhibit formation of any arachidonic acid-derived metabolites in rabbit lung microsomes, whereas anti-CYP2B4 polyclonal antibodies completely inhibited epoxyeicosatrienoic acid (EET) and dihydroxyeicosatrienoic acid (DHET) production (17). In addition, arachidonic acid metabolites were not detectable in incubations with purified CYP4B1 reconstituted with cytochrome P450 reductase, and cytochrome b₅ in the presence of DLPC and NADPH (17). The contradictory results between CYP4B1 activity in the rabbit lung and cornea might be attributable to the amino acid sequence differences, I263V and M289L in the cornea, although the substitutions are conservative (19). A more likely explanation is that 12-HETE and 12-HETrE production in the rabbit cornea is indirectly associated with CYP4B1 levels.

1.7 Gene Regulation

As described earlier, phenobarbital substantially induces expression of CYP4B1 in rabbit liver. Although the molecular mechanisms for xenobiotic induction of CYP4B1 have not been studied, it seems likely that phenobarbital induction is mediated by the CAR (constitutive androstane receptor) nuclear receptor, based on characterized

regulatory mechanisms in other P450s (76). Once phenobarbital has activated CAR, probably by displacing inhibitory androstanes, a CAR/RXR (retinoid X receptor) heterodimer may bind to a conserved phenobarbital-responsive enhancer sequence in the promoter region and enhance transcription, as suggested for CYP2B genes (76). In contrast, studies have been conducted regarding mechanisms of regulation of CYP4B1 in response to endogenous compounds and hypoxia. A potential role for CYP4B1 in 12-HETE and 12-HETrE production and the inflammatory response prompted Ashkar *et al.* to investigate CYP4B1 gene regulation in the rabbit cornea (77). The retinoids were evaluated specifically because they are known to mediate wound-healing processes in many tissues and are integral components of the inflammatory response. Analysis of the promoter region of the CYP4B1 gene revealed sequences that could potentially bind heterodimers of the retinoic acid receptors including RAR/RXR, VDR/RXR, and PPAR/RXR. Incubations with 9-*cis* and *all-trans* retinoic acid, but not vitamin D, demonstrated significant transcriptional activation in HepG2 cells transiently transfected with luciferase reporter vectors containing the CYP4B1 promoter. In addition, there was a corresponding increase in 12-HETE and 12-HETrE production under normoxic conditions. The authors suggest that the effect of retinoic acid on CYP4B1-mediated 12-HETE/12-HETrE levels may provide a linkage between wound healing and inflammation in the ocular surface (77). The promoter region for rabbit corneal CYP4B1 also contains DNA binding sequences for factors known to activate gene transcription in response to hypoxia, namely HIF-1, NFκB, and AP-1 (9). Mastuygin *et al.* demonstrated that RCE cells, which were transiently transfected with luciferase reporter vectors containing the CYP4B1 promoter, had increased luciferase activity under hypoxic conditions. To determine if HIF-1, NFκB, and/or AP-1 were involved in hypoxia-induced transcription, electrophoretic mobility shift assays were performed on nuclear extracts from RCE cells exposed to hypoxia using ³²P-labeled probes representing the putative DNA binding sequences for each transcription factor (9). Binding activity was increased under hypoxic conditions for HIF-1 (5-fold), NFκB (4-fold), and AP-1 (1.5-fold). The authors suggest that these results provide a molecular mechanism for the induction of CYP4B1 and,

therefore, the production of 12-HETE and 12-HETrE in response to hypoxic injury in the rabbit cornea (9).

Androgen regulation of CYP4B1 has been investigated due to the observed sex differences in bladder carcinogenesis (78). Imaoka *et al.* utilized competitive reverse transcription polymerase chain reaction to show that CYP4B1 mRNA levels increased during development in the bladders of male rats, but that there was no change in female bladders at any age. Castration of male rats decreased levels of CYP4B1 mRNA, which could be restored partly by administration of testosterone, suggestive of a role for androgens in the regulation of the gene (78). A similar experiment was conducted to evaluate whether sex steroids mediate sex-differentiated CYP4B1 gene expression in the mouse kidney (79). Northern blot analysis revealed the CYP4B1 mRNA decreased after castration (79), in agreement with previous work that demonstrated renal sex differences in CYP4B1 expression and activity (10). Analysis of the 1.8-kb CYP4B1 5'-flanking region did not reveal an androgen response element (ARE) motif, but the authors suggested that androgens might activate the promoter indirectly (79). The effect of 5 α -dehydrotestosterone (DHT) on promoter activity was examined in a renal proximal tubule cell line transfected with both a luciferase reporter vector containing the CYP4B1 promoter and an androgen receptor encoding plasmid (79). Treatment with DHT for 48 hrs resulted in only minimal changes in luciferase induction. The molecular mechanism underlying androgenic regulation of CYP4B1 in the kidney remains unclear.

1.8 The Role of CYP4B1 in Humans

With a clear connection between CYP4B1 and the bioactivation of protoxins in animals, attention was directed toward toxicological implications in humans. Historically, there has been confusion surrounding the activity of human CYP4B1. The cDNA for human P450 IVB1 was first isolated from a λ gt11 library constructed from human lung mRNA using a cDNA probe to rat CYP4A1 (12). Nhamburo *et al.* reported the recombinant expression of human CYP4B1 in human cells lines, *via* a vaccinia virus

expression vector containing CYP4B1 cDNA, and observed the typical P450 absorption spectrum of the reduced CO-bound complex with a λ_{max} at 450 nm. However, the expressed P450 did not metabolize substrates prototypic for rabbit CYP4B1, such as 2-AF or IPO (12, 35). In addition, Waxman *et al.* reported human CYP4B1-mediated 6 β -hydroxylation of testosterone, androstenedione, and progesterone (80), which are reactions more characteristic of CYP3A5. Their metabolic profiles initially suggested large interspecies differences existed between the animal and human forms of CYP4B1.

The abnormal metabolism demonstrated by human CYP4B1 was further addressed with the expression of human placental CYP4B1 in insect cells *via* infection with a recombinant baculovirus (81). Although human CYP4B1 was expressed, as determined by SDS-PAGE analysis, there was no observable peak at 450 nm in the spectra when the membrane preparations were reduced and bubbled with carbon monoxide. In accordance with this result, neither lauric acid nor testosterone, were metabolized by membrane preparations when supplemented with coenzymes. Findings from these studies contrasted with the activity measurements determined previously with the human lung CYP4B1 (12, 35, 80). When the original vaccinia virus for human lung CYP4B1 was purified and subsequently expressed in HepG2 cells, the ability of the preparation to bind carbon monoxide in the reduced state was lost. The discrepancies were ultimately attributed to contamination with a virus encoding CYP3A5 in the original preparation (81).

After establishing that the recombinant human CYP4B1 enzyme expressed in insect cells was inactive, studies were conducted to determine which residues may confer the loss in function, as compared to the animal forms of CYP4B1. When human CYP4B1 cDNA was isolated from the human placenta and sequenced, Yokotani *et al.* noted a serine (position 427) was substituted for a proline residue in the conserved region near the putative heme-binding cysteine (13). Zheng *et al.* generated the human CYP4B1 S427P mutant with site directed mutagenesis in the baculovirus expression system, under the same conditions as the native P450 (81). Interestingly, this substitution restored

activity to the human placental enzyme. Additionally, a complementary experiment was performed where the conserved proline residue present in native rabbit CYP4B1 was exchanged for the serine residue of the “wild-type” human form. As expected, the rabbit CYP4B1 P422S mutant lost the ability to bind carbon monoxide and could no longer metabolize lauric acid. It was proposed that the serine found at position 427 in human placental CYP4B1 disrupts the ordered framework of the structure imposed by a conserved ERR motif and results in defective heme incorporation (81).

Evidence that human CYP4B1 in bladder microsomes could oxidize 2-AF was provided by Imaoka *et al.* (82). This work was instigated based on the fact that CYP4B1 has been detected in bovine and rabbit bladders, and that animal forms of CYP4B1 are known to bioactivate bladder carcinogens. CYP4B1 protein levels, as detected by Western blotting with an anti-mouse CYP4B1 antibody, and CYP4B1 mRNA, as determined by RT-PCR, were higher in the bladder microsomes from patients with bladder tumors than in controls. Increases in CYP4B1 protein levels and mRNA correlated with 2-AF bioactivation in bladder microsomes from two patients, although control reactions were not provided to determine background activity in the *umu* gene expression assay (82). These researchers also utilized a high throughput RT-PCR assay on a larger population, to demonstrate that patients with bladder tumors have significantly higher levels of CYP4B1 mRNA, in normal and tumor tissues, than do healthy patients (82). Although definitive experiments are required to make the association between human CYP4B1 levels and the observed activity, these results do make a case that human CYP4B1 might be active in its native environment.

Additional work supporting a functional role for human CYP4B1 *in vivo* was provided by the generation of a transgenic mouse model expressing human CYP4B1 in the liver (83). The human CYP4B1 gene was successfully expressed in the transgenic mouse liver, as determined by Western Blot analysis of hepatic microsomes. Rates for lauric acid hydroxylation were measured and it was found that microsomes from the transgenic mice had 25% more activity than microsomes from control mice. In addition,

2-AF was bioactivated by transgenic mouse liver microsomes at a rate twofold above control microsomes. These metabolic activities were not greatly elevated above background measurements, but the fact that a CYP4B1-specific antibody inhibited activity in both cases provides more compelling evidence for *in vivo* activity. No inhibition was observed in the control mice for either assay.

The same laboratory also reported heterologous expression of active human CYP4B1 in yeast cells, but the P450 was unstable and displayed no activity toward lauric acid and 2-AF (82). Yet, when CYP4B1 - cytochrome P450 reductase fusion protein was constructed, the CO-reduced difference spectra of the yeast microsomes revealed a peak at 450 nm. The microsomes containing the fusion protein also metabolized lauric acid and 2-AF, providing the first evidence for an active heterologously expressed human CYP4B1 protein (82). The authors note that the cDNA used to generate human CYP4B1 encoded a serine insertion at position 207. More rigorous assays, using purified protein from these microsomes, would assist in confirming human CYP4B1 activity.

Despite the ongoing debate over human CYP4B1 activity *in vivo*, work has continued in the area of tissue specificity, regulation, and genetic polymorphisms. Human CYP4B1 was identified as an extrahepatic P450 when CYP4B1 mRNA was initially detected in the lung, but not in the liver (12). Yokotani *et al.* found CYP4B1 mRNA in the human placenta as well as various human cancer tissues (13). When human gastrointestinal tissues were analyzed by immunohistochemistry or *in situ* hybridization, CYP4B1 was not detected in the duodenum, jejunum, or ileum, but was present in six of twelve colon samples (18, 84, 85). Human CYP4B1 mRNA has also been detected in seminal vesicles (86), prostate (87), breast (88), heart, pancreas, skeletal muscle, kidney, spleen, thymus, and small intestine (21).

Attempts have been made to associate human CYP4B1 mRNA levels to extrahepatic tumor development, anticipating that the human form may bioactivate procarcinogens as does the animal ortholog. Czerwinski *et al.* quantified CYP4B1 mRNA in the human

lung and lung tumors and found 2.3-fold less mRNA in tumors (89). There was also a larger variation of mRNA levels in the lung tumor samples (64-fold) than the normal lungs samples (33-fold). Similar levels of CYP4B1 mRNA were found in human breast tumors and surrounding normal breast tissue (88). This indicates that CYP4B1 is not induced in human tumor tissues, but mRNA levels in breast tissue of tumor-free patients were not compared to determine if a correlation exists between mRNA levels and cancer development.

To identify functional human CYP4B1 regulatory domains, Poch *et al.* prepared a series of reporter constructs in which CYP4B1 sequences containing nested deletions of the 5'-upstream region, directed luciferase expression (90). After transient expression of these constructs in A549 lung carcinoma cells and HepG2 liver carcinoma cells the relative luciferase activity was measured. They authors identified a proximal positive regulatory element located between -118 and -73, a liver-selective negative regulatory element located between -457 and -216, and a distal, lung-selective positive element located between -1052 and -1008. Analysis of the sequences in the proximal region revealed three possible binding sites for the Sp/XKLF family of transcription factors. Subsequent electrophoretic mobility shift assays with the addition of Sp/XKFL consensus sequences or Sp-monospecific antibodies implicated a role for the Sp1 and/or Sp3 transcription factors in CYP4B1 regulation through the proximal regulatory element. The transcription factors involved in the distal, lung-selective positive element could not be identified. These studies provide insight to the molecular mechanisms controlling CYP4B1 lung-selective expression, regardless of the activity of the final protein product.

Genetic polymorphisms of human CYP4B1 were first analyzed in 190 French Caucasians, using single-strand conformation polymorphism analysis of PCR products (91). Lo-Guidice *et al.* identified four variants, CYP4B1*2, CYP4B1*3, CYP4B1*4, and CYP4B1*5 in addition to the wild type gene designated CYP4B1*1. The CYP4B1*3, CYP4B1*4, and CYP4B1*5 alleles encode missense mutations Arg173Trp, Ser322Gly, and Met331Ile, respectively. The most interesting variant is CYP4B1*2, which harbors

three missense mutations (Met331Ile, Arg340Cys and Arg375Cys) and a double nucleotide deletion (AT881-882del) that causes a frameshift and resultant premature stop codon. This truncation eliminates the conserved cysteine residue that forms the coordinate covalent bond with the heme iron, and also eliminates substrate recognition sites, SRS-4, 5, and 6 (92). Genotyping studies on 2082 French Caucasians revealed that 2% of the population was homozygous for the two base-pair deletion (91). This presents the possibility that pathologic processes may be disrupted in these individuals if CYP4B1*1 is active, and involved in the metabolism of endogenous substrates such as arachidonic acid. In addition, this polymorphism could result in interindividual differences in susceptibility to chemically-induced diseases, if CYP4B1*1 is active and responsible for the bioactivation of protoxins, such as IPO. Subsequent pharmacogenetic studies in a Japanese population revealed two more human CYP4B1 alleles, CYP4B1*6 and CYP4B1*7. The CYP4B1*6 allele contained point mutations in exons 5, and 8, encoding the both missense mutations Arg173Trp and Val345Ile (93). The CYP4B1*7 allele was truncated like CYP4B1*2 (AT881-882) and also encoded the missense mutations Met331Ile and Arg340Cys. Unfortunately, the functional effects of the human CYP4B1 SNPs cannot be evaluated in the absence of a heterologous expression system that readily permits expression of functional CYP4B1*1 holoenzyme.

1.9 CYP4B1 and Gene Therapy

The rabbit CYP4B1 gene has been examined for potential use as gene therapy in humans to treat cancer. The goal behind prodrug activated gene therapy is to specifically deliver the cDNA *via* vector-mediated transfer, to the site of the tumor, and subsequently administer the protoxin. Ideally, toxicity would only occur in the cells containing the exogenous gene. Rabbit CYP4B1 was considered an ideal candidate for gene-therapy because it bioactivates 2-AA and IPO to toxic metabolites, and the equivalent human isozymes have low activity against these compounds. Rainov *et al.* first tested the feasibility of the gene therapy system in rat 9L gliosarcoma and human U87 glioblastoma cells (94). Cells transfected with plasmid DNA containing the rabbit CYP4B1 gene, were

found to exhibit high sensitivity to 2-AA, demonstrating greater than 90% cell death within 2 days exposure of 5 μ M 2-AA. Toxicity was not observed in the control cells over 6-day exposure of 5 μ M 2-AA. The LD₅₀ of 2-AA was approximately 2.5 μ M over 2 days for both cell lines. Western blot analysis of the 9L cells demonstrated a direct correlation between CYP4B1 protein concentration and sensitivity to the prodrug in culture. The authors also noted a marked bystander effect where nontransduced neoplastic cells in close proximity to the transgene-expressing cells are killed, thereby enhancing the antitumor efficiency (94). To show that CYP4B1 gene therapy was relevant *in vivo*, control and 9L-4B1 cells were implanted subcutaneously in nude mice and tumor growth was measured over time following intraperitoneal administration of 200 μ g of IPO daily (94). Tumor growth was arrested within 2 days from the start of IPO application. Subsequent experiments demonstrated that this gene therapy system may be useful in the treatment of hepatocellular carcinoma (95). Additional work has been performed that correlates toxicity due to IPO metabolism with rabbit CYP4B1 expression in cancer cell lines by fusing the P450 to enhanced green fluorescent protein and measuring fluorescence (96, 97). More recently, the rabbit CYP4B1 gene has been fused to the EGR1 promoter, which is activated by ionizing radiation, and transfected into human embryonic kidney cells (98). The cells bearing CYP4B1 exhibited a decrease in the relative survival fraction (survival with IPO / survival without IPO) with increasing ionizing-radiation dose, whereas no effect was observed in control cells (98). This system offers precise control over the expression of rabbit CYP4B1 in cancer tissues and therefore, the organ-specificity for toxicity resulting from prodrug activation.

In summary, these studies demonstrate that CYP4B1 gene therapy, in combination with IPO or 2-AA administration, causes toxicity in rat and human cancer cell lines, as well as in tumor cells of mice. The effectiveness *in vivo* on *human* tumors remains to be investigated. A major concern here is the relative rates of bioactivation by rabbit CYP4B1, introduced by transgene therapy, compared to bioactivation by human P450s, which may lead to undesired toxicity in normal tissues. Recently, a panel of recombinant human P450s were screened for their ability to bioactivate IPO to reactive

metabolites (39). The proposed dialdehyde intermediate was trapped with the nucleophiles, N-acetylcysteine and N-acetyllysine and quantitated in an HPLC-UV assay. Of the 14 P450s evaluated, CYP1A2, CYP2C19, CYP2D6, and CYP3A4 all exhibited substantial rates of IPO bioactivation (>100 nmol/nmol/30 min), suggesting that IPO can be activated in the human liver. Despite the reported low doses required for cell death *in vitro* using gene therapy, bioactivation of IPO by human P450s *in vivo* could still potentially lead to hepatotoxicity, as observed in patients dosed with IPO for the treatment of lung cancer (99).

1.10 Covalent Heme Attachment

The newest development in the CYP4B1 saga is the identification of a novel covalent linkage that attaches the protein backbone to the heme catalytic center (58). Henne *et al.* found that the CYP4B1 heme did not dissociate from the protein under typical LC-MS conditions, as observed for other families of P450s (58). This conclusion was based on the fact that the protein peak eluting from a POROS R2 column had significant absorbance at 400 nm, and the experimentally determined protein mass was 616 Da larger than the predicted mass for CYP4B1 apo-protein. The tight association between heme and protein was further demonstrated by staining heme bound to CYP4B1 with tetramethylbenzidine/H₂O₂, following SDS-PAGE. Base-treatment prior to LC-MS analysis released a monohydroxyheme from the protein, indicating that an ester bond had been hydrolyzed. Evidence for an ester bond linkage between the heme and protein was also provided for members of the CYP4A subfamily (100). Mass analysis of heme-containing peptides released from CYP4A3 by Pronase treatment, led to the identification of Glu318 as the site of heme attachment (100). Alignment of the sequence of CYP4A3 with other members of the CYP4 family revealed a conserved glutamate at this position in the I-helix, whereas members of other P450 families do not have a corresponding acidic residue (58). In CYP4B1, the equivalent glutamate involved in covalent heme attachment would be at position 310. The authors speculated that the heme adduction

may restrict conformational flexibility in the P450 active site and, as a result, enable the ω -hydroxylase specificity observed in the CYP4 isoforms (58).

The proposed ester linkage between CYP4B1 and the heme group closely resembles the heme-protein interactions characterized previously in several mammalian peroxidases. The crystal structure of myeloperoxidase (101), and NMR studies of the heme released upon proteolysis of lactoperoxidase (102), reveal that their hemes are bound at both the C-1 and C-5 methyl positions *via* ester bonds to glutamic acid and aspartic acid residues, respectively. Heme attachment can be initiated in recombinant lactoperoxidase with the addition of H_2O_2 , indicative of an autocatalytic oxidation carried out by Compound I (103). Therefore, DePillis *et al.* proposed that this reaction involved protein-mediated heme co-oxidation, wherein heme methyl groups were oxidized by the enzyme during nonproductive turnover events to cationic species, subsequently quenched by appropriately positioned carboxylate groups (103). Because the mammalian peroxidases are thought to have a catalytic cycle similar to P450s, *via* formation of an iron-oxo species at the heme center, inferences can be made about the mechanism of covalent bond formation in the CYP4 enzymes.

The goal of this thesis is to delineate structural, functional, and mechanistic aspects of the covalently attached heme in CYP4B1. First, I will address the role of Glu310 as the site of attachment to CYP4B1 *via* mutagenesis studies and mass analysis of CYP4B1-derived heme-containing peptides (Chapter 2). Next, the corresponding site of attachment on the heme will be identified by co-chromatography of the base-hydrolyzed heme with biologically-derived monohydroxyheme standards and 2D NMR analysis (Chapter 3). To investigate mechanisms of covalent bond formation, oxygen incorporation from the glutamic acid into the ester bond will be traced using ^{18}O (Chapter 4). Finally, to evaluate functional consequences of the heme-ester link, E310 mutants that lack covalently bound heme will be compared to the wild-type enzyme regarding their ability to ω -hydroxylate hydrocarbons, bioactivate IPO, thermostability, heme retention (Chapter 5).

Table 1.1. Essential characteristics of CYP4B1. Molecular weight, amino acid sequence length, $\lambda_{\max}$ for the reduced CO-bound spectra, tissue locations, and identified substrates for rabbit, rat, mouse and human CYP4B1, as discussed in chapter text. ^a Average apo-protein MW calculated from amino acid sequence. ^b Reported for CYP4B1-cytochrome P450 reductase fusion protein only (83). ^c Detected presence of mRNA only. ^d There is some evidence for activity toward lauric acid and 2-AF in transgenic mice microsomes and with recombinant CYP4B1-cytochrome P450 reductase fusion protein (83), but other systems do not produce functional protein (81).

Characteristic	Rabbit	Rat	Mouse	Human
MW (Da) ^a	58,605	58,937	58,900	59,019
Amino Acids	506	511	511	511
$\lambda_{\max}$ (nm)	450	451	Not reported	450 ^b
Tissue Location	Lung Bladder Cornea Small intestine Colon Liver	Lung Bladder	Lung Kidney (male) Bladder Brain ^c Spleen ^c Testis ^c Liver ^c Skeletal muscle ^c	Lung ^c Heart ^c Pancreas ^c Placenta ^c Skeletal muscle ^c Kidney ^c Spleen ^c Thymus ^c Prostate ^c Colon ^c Small Intestine ^c Seminal Vesicles ^c Breast ^c
Substrates	Lauric acid C7-C10 fatty acids C7-C10 hydrocarbons valproic acid IPO 3-MI 2-AF 2-AAF 2-AA <i>p</i> -xylene NDBA 11-DDYA 17-ODYA	Lauric acid 7-ethoxycoumarin DCB DMAB 2-NA	Lauric acid 3-MeO-AAB 2-AF 2-AA DCB	Unknown ^d

Figure 1.1. Interface between endobiotic and xenobiotic metabolism.

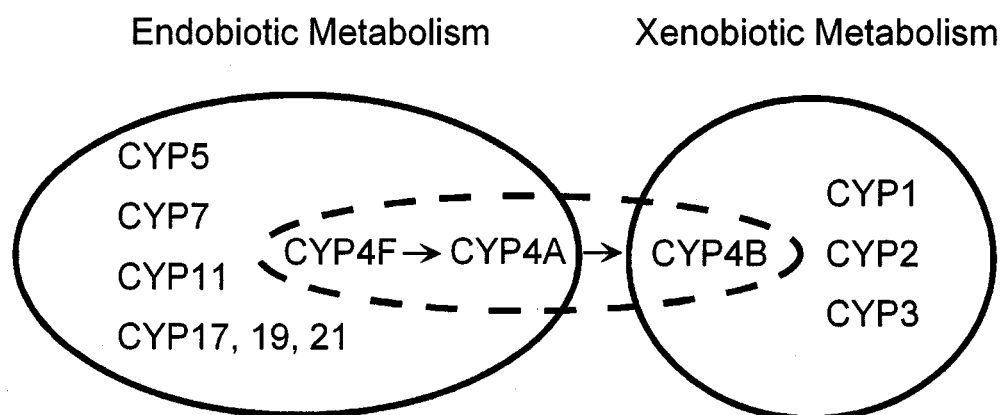
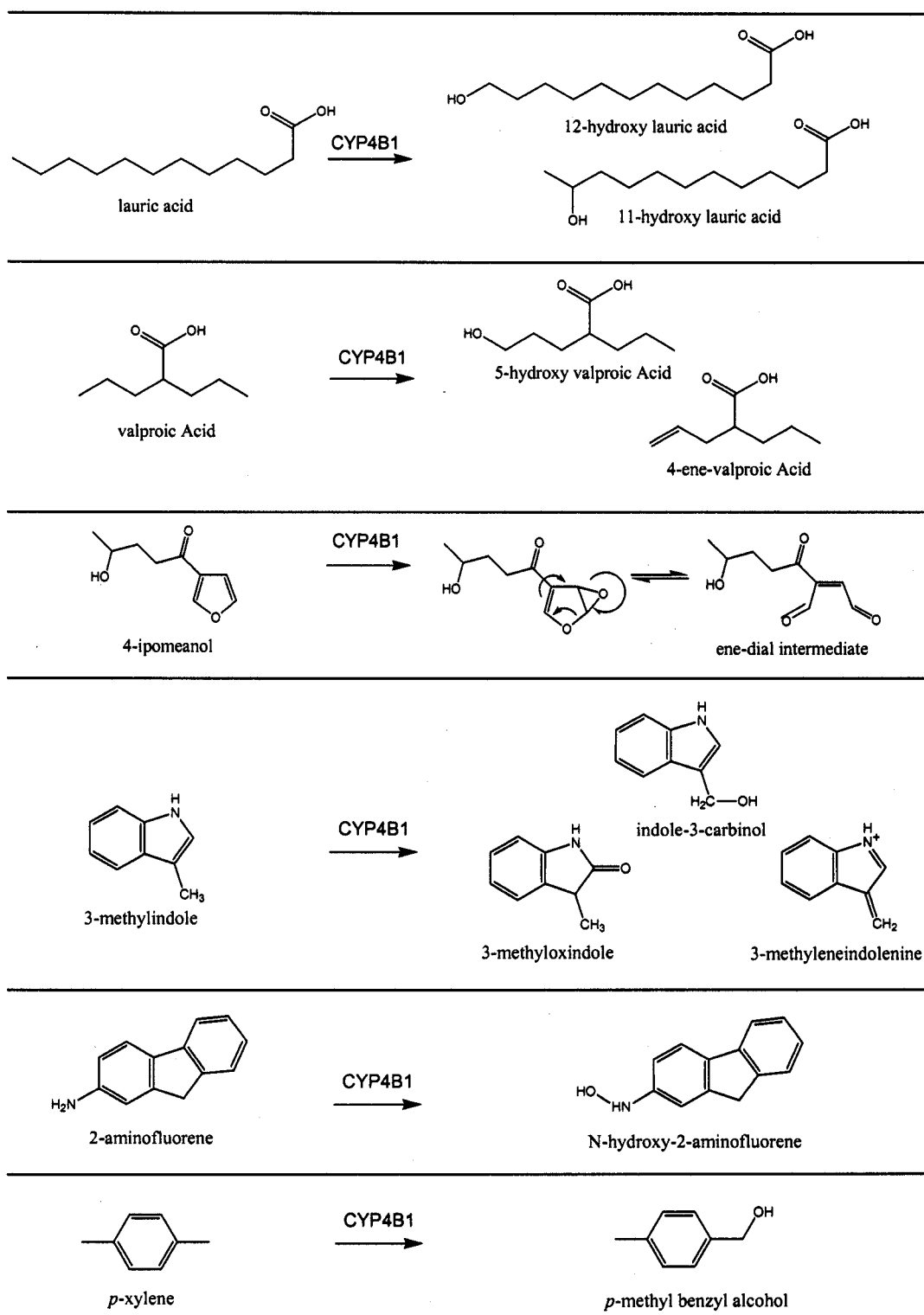


Figure 1.2. CYP4B1 substrates and relevant metabolites.

1.11 Notes to Chapter 1

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Chapter 2

Covalent Heme Binding to CYP4B1 via Glu310 and a Carbocation Porphyrin Intermediate

2.1 Site of Heme Attachment on the I-helix of CYP4 Enzymes

Cytochrome P450s are a superfamily of oxygen activating enzymes that carry out an enormous range of metabolic reactions on endogenous and exogenous substrates in processes both beneficial (detoxification) and deleterious (bioactivation) to the organism (1). It has long been recognized that P450s are *b*-type hemoproteins and that the heme prosthetic group represents the catalytic center of these enzymes where activation of molecular oxygen to the perferryl oxygen complex occurs (2). In general, the heme of P450 is attached noncovalently through a thiolate coordinate bond between the Fe atom and a completely conserved cysteine residue in the C-terminal portion of the protein.

However, we have recently presented evidence that certain members of the human and rabbit CYP4A, CYP4B, and CYP4F subfamilies also bind 60-80% of their heme group covalently through a base-labile ester linkage (3). Ortiz de Montellano and co-workers have shown that a similar situation exists for several rat and human CYP4A and several rat CYP4F isoforms (4-6). These workers were able to isolate a heme-linked peptide from CYP4A3 following Pronase digestion that implicated a conserved I-helix glutamate residue, E318, as the protein site of covalent attachment to this isoform. Subsequent site-directed mutagenesis studies by this group confirmed the importance of this residue in autocatalytic heme binding to CYP4A3 (6) and further suggested variable effects of the covalent link on substrate metabolism and binding to the enzyme. In rabbit CYP4B1, E310 is the amino acid residue that corresponds to the I-helix glutamate that forms the heme ester link in CYP4A3 (3). These acidic residues are located within a substrate recognition site of the enzyme (SRS-4) (7), near the oxygen-binding pocket in the active site.

The primary goals of the present study were to (i) determine the effect of the rabbit CYP4B1 I-helix mutants, E310G, E310A, and E310D, on covalent heme binding, (ii) confirm that the heme is attached to Glu310 by mass analysis of tryptic peptides and (iii) demonstrate that covalent heme attachment is an autocatalytic event in the CYP4 enzymes. The mutagenesis studies support our hypothesis, based on sequence alignments, that Glu310 is the site of the heme ester link in CYP4B1. Unexpectedly, we found that the E310D mutant released a monohydroxylated heme upon mild acid treatment. Therefore, stable isotope studies were conducted to probe the origin of the additional oxygen atom in the modified heme obtained from this mutant. These latter studies shed light on the mechanism of formation of the covalent heme link to CYP4 enzymes.

2.2 Experimental Procedures

Materials.

NADPH, lauric acid, and C- α -dilauroylphosphatidylcholine (DLPC), dimethyl sulfoxide (DMSO), acetic acid, H₂¹⁸O (95 atom %), and heme and were purchased from Sigma-Aldrich (St. Louis, MO). Trypsin was purchased from Promega (Madison WI).

Protein Expression and Purification.

Generation of recombinant baculoviruses and mutant proteins in suspension culture was performed by Dr. Yi-Min Zheng as described previously (8). Dr. Yi-Min Zheng also purified each mutant to near homogeneity following solubilization with sodium cholate and column chromatography on Octyl-Sepharose 4B and hydroxyapatite (8). The E310D mutant was also expressed on plates (10 mL) in the presence or absence of H₂¹⁸O that had been filtered and added to the culture to give final concentrations of $6.79 \pm 0.06\%$ H₂¹⁸O (~1 g of 95% H₂¹⁸O added to ~9 mL of media), or $14.0 \pm 0.14\%$ H₂¹⁸O (~2 g of 95% H₂¹⁸O added to ~8 mL of media) as determined by GC-MS analysis of the filtered expression media. Cells were harvested at 48 h, and membrane fractions were prepared for analysis of polar heme by mass spectrometry. P450 reductase and cytochrome *b*₅

were purified from *Escherichia coli* expression systems as previously described (9). The rat CYP4A8 gene was amplified from cDNA (synthesized with PCR using oligo dTs and rat kidney total RNA) with the following primers: forward primer, 5'-GGCATGAGTGGCTCGGCTCTGAG-3', reverse primer, 5'-CGAGATAGAAAGACTGACAGACAAGG-3'. The gene was then ligated into PCRblunt (Invitrogen). A single colony was grown up overnight, the plasmid was isolated, and the gene was sequenced. The sequence was found to be identical to the sequence published on PubMed. The following primers were then used to modify the N-terminus and introduce *NdeI* and *SalI* restriction sites as reported previously (10): forward primer, 5'-GGAATTCATATGGCTCTGTTATTAGCAGTTTTCTGCTTGCTCACTGTGCTCCTG-3', reverse primer, 5'-ACGCGTCGACTTAATGATGATGATGATGATGTCCTTGGAGCTTTTGAGACG-3'. The PCR product was digested with *NdeI* and *SalI*, ligated into pcWori+. CYP4A8 was expressed in *E. coli* in Terrific Broth for 48 hrs. Cells were lysed into a solubilization buffer (50 mM KPi, pH 7.4, 500 mM KCl, 20% glycerol, 1% Emugen 911) by using a French Press with the chamber pressure at 10,000 psi. Lauric acid was added at a concentration of 100 μ M to help stabilize the P450. The broken cells were then spun at 150,000 x gravity for 1 hr. The supernatant was loaded directly onto Ni-NTA resin (Qiagen) and purified as described for CYP4B1 (11).

HPLC-Vis and LC/ESI-MS Analysis of Intact CYP4B1 Proteins.

Proteins were injected onto LC columns in storage buffer (100 mM potassium phosphate, 0.1 mM EDTA, 20% glycerol, pH 7.4). Heme was dissociated from the CYP4B1 holo-enzyme by treatment with 0.25 M sodium hydroxide. Enzyme aliquots in storage buffer were mixed 1:1 with a freshly prepared 0.5 M sodium hydroxide solution and left to sit on ice for 10-20 min. Loss of the heme moiety from CYP4B proteins was monitored by following absorbance of HPLC eluates at both 280 and 400 nm. LC analysis of P450 proteins was performed on a self-packed 2.1 X 250 mm POROS R2 column (Applied Biosystems, Foster City, CA). The mobile phase used in conjunction with the R2 packing consisted of 0.05% TFA (A) and acetonitrile containing 0.05% TFA (B). Initially, the flow-rate was set at 1 mL/min with a solvent composition of 20% B. At

3 min, a linear gradient was established that increased from 20 to 60% B over 20 min. Under these conditions, polar heme eluted at 8.5 min, free heme at 11 min, and P450 proteins eluted between 16 and 18 min. Resolution of monomeric and dimeric mutant CYP proteins was accomplished using a linear gradient that increased from 42 to 50% B over 12 min. Under these conditions, protein monomer eluted at ~6.5 min and protein dimer at ~9 min. Protein dimers were reduced by adding DTT to a final concentration of 20 mg/mL. For LC/ESI-MS analysis, 60% of the flow was diverted to waste. LC/ESI-
<KEYWORD>Hydrogen

rformed using a Micromass Quattro II tandem quadrupole mass spectrometer (Micromass, Ltd., Manchester, UK) coupled to a Shimadzu LC instrument. The mass spectrometer was operated in electrospray ionization mode (ESI) at a cone voltage of 45 V, source block temperature of 180 °C, and a desolvation temperature of 350°C. Data analysis was carried out using Windows NT based Micromass MassLynxNT 2.2 and MaxEnt software.

LC/ESI-MS Analysis of Monohydroxyheme from the E310D Mutant.

The membrane fractions containing the E310D mutant expressed in H₂¹⁸O were diluted 1:1 with a 50:50 solution of DMSO and acetic acid. The acid released the heme from the P450, and the DMSO helped to solublize it. The sample was then spun down in a centrifuge to remove insoluble components. LC separation of the supernatant was performed on a 2.1 X 250 mm C4 protein column (Vydac, Hesperia, CA). The mobile phase consisted of 0.05% TFA (A) and acetonitrile containing 0.05% TFA (B). Initially, the flow-rate was set at 0.2 mL/min with a solvent composition of 40% B. A linear gradient was established that increased from 40 to 55% B over 15 min, then from 55 to 100% B from 15 to 18 min, and held at 100% B from 18 to 22 min. Under these conditions, polar heme eluted at 15.5 min. Perturbation of the unmodified heme isotope ratios due to ¹⁸O incorporation into polar heme from the E310D mutant was examined by monitoring individual ions (*m/z* 670-681) in the envelope corresponding to the acetonitrile adduct (M +41), using a dwell time of 0.15 s and a cone voltage of 60 V. Integrated ion intensities for the acetonitrile adduct were an order of magnitude greater than those of the M + H ions. Fractional intensities were calculated for each ion over the

m/z 671-676 envelope. Uncertainties were determined from the standard deviation of three measurements and were propagated throughout the calculations.

Analysis of $H_2^{18}O$ Content by GC-MS.

To determine accurately the fraction of ^{18}O in the $H_2^{18}O$ enriched cell culture, the media was filtered through a 0.2 μm membrane, and 0.05 μL of the sample was injected manually into the GC-MS system. The gas chromatograph (Agilent 6890, Agilent Technologies, Palo Alto, CA) was equipped with a 30 m X 250 μm capillary DB-5 column. The carrier gas was helium with a constant pressure of 5.0 psi. The injection port temperature was 70 $^{\circ}C$, the purge flow was 50 mL/min, and the purge time was 0.1 min. The temperature gradient was increased from 40 to 60 $^{\circ}C$ over 2 min, then from 60 to 120 $^{\circ}C$ in 1 min, and held at 120 $^{\circ}C$ for 7 min. Water eluted from the column at 3.4 min. The column was connected to an Agilent 5973 mass spectrometer. Ions from m/z 16-21 were monitored in SIM mode with a dwell time of 75 ms. Data were analyzed on using Chemstation G1701CA version C.00.00 software (Agilent Technologies, Palo Alto, CA). The two samples of media were determined to contain $6.79 \pm 0.06\%$ $H_2^{18}O$ and $14.04 \pm 0.14\%$ $H_2^{18}O$. The percentages were calculated from the fraction of $M+2$ ion intensity relative to the $M+$ ion intensity after correction for the natural abundance isotopes. Uncertainties were determined from the standard deviation of four measurements and were propagated throughout the calculations.

Spectroscopic Characterization of CYP4B1 Proteins.

P450 spectra (reduced-CO complex versus reduced enzyme) were taken with an Agilent 8453 UV-vis spectrophotometer (Agilent Technologies, Palo Alto, CA) following addition of methyl viologen (1.2 μM) and a few grains of sodium dithionite to insect cell membrane suspensions (1 mg/mL) or purified cytochrome P450s in 50 mM potassium phosphate, 20% glycerol, 1 mM EDTA, pH 7.4 that had been bubbled with carbon monoxide for 30 s. P450 concentrations were estimated using an extinction coefficient of $100 \text{ mM}^{-1} \text{ cm}^{-1}$ for ΔAbs (450-500) nm.

CYP4B1 digest and LC/ESI-MS Analysis of Tryptic Peptides

Approximately 1 nmol of each protein was diluted 1:4 with 50 mM NH_4HCO_3 (pH 7.8) and 1% trypsin was added. The digest was allowed to proceed for 2 hr at 37°C. Peptides were chromatographed on a C18 column (Vydac), using the LC/MS system described above. Initial conditions were 95% Solution A (0.05% TFA in H_2O) and 5% Solution B (0.05% TFA in acetonitrile) at a flow rate of 0.3 ml/min. The linear gradient increased B from 5-65% between 0 and 20 min. The mass spectrometer was operated in the electrospray ionization positive (ESI+) mode at a cone voltage of 45 V, source block temperature of 150 °C, and a desolvation temperature of 380 °C. The detector was in scan mode and monitored ions ranging from m/z 500-2500. Data analysis was carried out using Windows NT based Micromass MassLynxNT® 3.2 software.

Autocatalysis Incubations.

CYP4B1 or CYP4A8 (100 pmol) was combined with cytochrome P450 reductase (500 pmol), cytochrome b_5 (100 pmol), 100 U of catalase, and 20 μg of DLPC. After incubating for 5 min at room temperature, 100 μM lauric acid and buffer (50 mM Tris pH 7.5, 250 mM NaCl, and 10% glycerol) was added to a total volume of 450 μL . The reaction was initiated with the addition of 50 μL of 20 mM NADPH (2 mM NADPH final) and the reconstituted P450 was incubated at 37 °C for 0 to 20 min. At each time point, 150 μL was removed and the reaction was quenched with 50 μL of 10% HCl. The sample was then analyzed by HPLC-Vis to assess the distribution of heme products and the extent of heme “bleaching.”

2.3 Results

Apo-P450 Versus Holo-P450 Content of E310 Mutant Proteins.

Chromatography of wild-type CYP4B1 on a POROS R2 column in the presence of 0.05% TFA resulted in the appearance of two analyte peaks with absorbance at both 280 and 400 nm (Figure 2.1A). These correspond to acid dissociable heme (R_t = 10.8 min)

and intact holo-CYP4B1 ($R_t = 16.5$ min) that exist in a ratio of ~1:4, respectively, as judged by the integrated heme absorbances at 400 nm. This ratio is indicative of ~80% covalently bound heme in native CYP4B1, as previously reported (3). In marked contrast, the E310G, E310A, and E310D mutants exhibited practically no absorbance at 400 nm for the later eluting protein peak (Figure 2.1B-D), and mass analysis of the earlier eluting peak (Table 2.2) confirmed its identity as either native heme (E310G and E310A) or monohydroxymethyl heme (E310D, see below). When combined with the molecular weight data in Table 2.1, it is clear that heme is not significantly bound in a covalent manner to the E310G, E310A, or E310D mutant forms of CYP4B1.

Monohydroxy Heme from the E310D Mutant.

Intriguingly, the heme peak obtained from the E310D mutant upon chromatography on POROS R2 was shifted to an earlier retention time ($R_t = 8.5$ min, Figure 2.1D). This heme species co-chromatographed with the polar heme released from wildtype CYP4B1 upon base treatment (data not shown) and exhibited the same 16 Da increase in mass observed previously (3) (Table 2.2). These data indicate that monohydroxylated heme is bound to the E310D mutant in a noncovalent manner.

Source of Oxygen in Monohydroxylated Heme Released from the E310D Mutant.

Small-scale expression of the E310D mutant on plates was optimized (1.5-4 nmol P450/ plate) to perform experiments with H_2^{18}O enriched media. E310D membranes were prepared following expression in H_2^{16}O , $6.79 \pm 0.06\%$ H_2^{18}O and $14.0 \pm 0.14\%$ H_2^{18}O , and analyzed directly by LC/MS because of the limiting sample size. Fractional ion intensities for monohydroxyheme were measured over the ion envelope corresponding to the polar heme-acetonitrile adduct (base peak at m/z 673). The fractional intensity of the $M + 2$ ion at m/z 675 increased from 0.063 following expression in H_2^{16}O to 0.088 in 6.79% H_2^{18}O and to 0.121 in 14.0% H_2^{18}O , with corresponding decreases in the fractional ion intensities of the base peak (Table 2.3). These data demonstrate incorporation of ^{18}O from H_2^{18}O into the monohydroxylated heme released from the E310D mutant. The relative abundance of the $M + 2$ ion from

polar heme generated in the presence of 6.79% H_2^{18}O indicated that $5.10 \pm 0.25\%$ of the $M + 2$ peak was due to incorporation of ^{18}O from water. Similarly, the relative abundance of the $M + 2$ ion generated in the presence of 14.0% H_2^{18}O indicated that $11.2 \pm 0.7\%$ of the polar heme contained ^{18}O incorporated from the labeled water. The comparisons show that the heavy isotope was incorporated at a molar ratio of 0.75-0.80:1. To investigate the potential for ^{18}O exchange into the propionate groups, the experimental analysis was repeated with heme and 8-hydroxy-heme that had been exposed for 48 h to the recovered H_2^{18}O -enriched cell culture media. These control experiments showed no detectable exchange of ^{18}O into either heme or monohydroxylated heme (data not shown).

Dimer Formation in E310 Mutants of CYP4B1.

The asymmetry of the protein peak detected at 280 nm during HPLC analysis of each of the I-helix mutants suggested a heterogeneous enzyme composition that was most conspicuous for the E310G and E310D mutants (Figure 2.1B,D). Further ESI-MS analysis of the resolved proteins using an expanded mass window demonstrated that the later eluting peaks had molecular weights of 117,122 Da and 117,230 Da for the E310G and E310D preparations, respectively. These values are in close agreement (<0.05% error) to the masses expected for the apo-dimers of E310G and E310D. However, treatment of these two enzyme preparations with DTT prior to chromatography resulted in a single protein peak that ESI-MS analysis confirmed as monomer (data not shown). These data suggest that these CYP4B1 I-helix mutants have been purified, at least partially, as disulfide dimers.

Heme-bound Tryptic Peptide.

Peptides resulting from the digestion of CYP4B1 by trypsin were analyzed by HPLC/ESI-MS in search for the site of heme attachment. Trypsin cleaves at the C-term side of the basic residues arginine and lysine, and is predicted to produce the peptide, 302-AEVDTFMFEFHDTTTSGISWFLYCMALYPEHQQR-335, containing Glu310 (underlined E). The calculated molecular ion $[M+H]$ for this peptide plus 614.5 Da for the covalently

attached heme is 4625 Da. All heme containing peptides eluted from the POROS R2 column between 16 min and 20 min, as determined by absorbance at 400 nm (Figure 2.2). The major 400 nm absorbing peak at 18.1 min aligned well with peaks in the m/z 1157 trace and m/z 1542 trace, representing the $[M+3H]$ and $[M+4H]$ ions for the predicted heme-bound tryptic peptide (Figure 2.2). The deconvoluted mass spectrum for the peak at 18.1 min gives a m/z value of 4626 for this peptide, in close agreement with the predicted mass of 4625 Da (Figure 2.3). None of the minor heme-peptides in the 400 nm trace could be identified by mass spectrometry. Other proteolytic enzymes such as AspN and chymotrypsin were used to digest CYP4B1, but the resulting heme peptide m/z values could not be matched with predicted mass values (data not shown). This may have been because the hydrophobic peptides did not ionize well in the mass spectrometer, or there were multiple missed cleavage sites that resulted in larger peptides than predicted.

Autocatalysis.

To attempt to demonstrate that the covalent heme attachment is an autocatalytic event, CYP4B1, which was expressed in insect cells and has 75% of the heme bound, was incubated under turnover conditions. The change in the amount of free heme and protein bound heme was monitored by HPLC-Vis every 2 min over 10 min. There was an increase in the area under the curve for the 400 nm trace, representing the protein-bound heme, at the first time point following the addition of NADPH (Figure 2.4A). This increase indicates that CYP4B1 bound an additional 15% of the heme. After 2 min, the absorbance for both the free heme and protein-bound heme decreased, with half-lives of 55 min and 105 min, respectively. The calculation for the remaining free heme assumes that there is initially 25% of free heme and that the heme from cytochrome b_5 and catalase does not decrease. Yet, when the spectrum for the protein peak is compared between the 0 min and 2 min time point, there is no commensurate increase in the absorbance at 400 nm relative to 280 nm (Figure 2.5A). This indicates that there may be similar amounts of heme relative to CYP4B1 protein, although we can't rule out that changes in NADPH or $NADP^+$ may alter the absorbance at 280 nm. The absorbance at

280 nm is high relative to that of 400 nm because reductase co-elutes with CYP4B1. To provide more convincing evidence for autocatalysis of covalent heme binding, we examined CYP4A8, which can be isolated with a much lower percentage of heme initially bound covalently than can CYP4B1. LeBrun *et al.* have already shown that CYP4A8 covalently binds heme under turnover conditions (6). However, our preparation of CYP4A8 is 100% P450 with no trace of P420, unlike the CYP4A8 preparation reported by LeBrun *et al.*, which contains a ratio of 60:40 for P420:P50. For CYP4A8 we were able to show an increase in protein-bound heme following addition of NADPH, with a simultaneous decrease in the observable free heme (Figure 2.4B). In this case, there is an obvious increase in the absorbance of 400 nm relative to 280 nm in the spectrum of the eluting protein (Figure 2.5B). The increase in peak area representing the protein-bound heme, and the relative increase of absorbance at 400 nm relative to 280 nm, indicates a 1.5-fold increase in covalently bound heme. This result, equates to an increase from 39% bound heme to 56% bound heme under turnover conditions. CYP4A8 eluted from the POROS R2 column with a longer retention time, did not co-elute with co-enzymes, and therefore the spectrum for CYP4A8 has reduced absorbance at 280 nm when compared to the CYP4B1 spectrum. After 2 min, the absorbance for both the free heme and protein-bound heme decreased, with half-lives of 23 min and 51 min, respectively. Only a small amount of P420 is observed in the reduced CO-bound spectra after 5 min of incubation. The total observable enzyme content (P450 + P420) is slightly lower, in agreement with the absorbance decrease observed in the HPLC-Vis 400 nm trace (Figure 2.6).

2.4 Discussion

In addition to CYP4B1, covalent heme binding has been demonstrated for several members of the CYP4A and CYP4F subfamilies (3-6). CYP4A1 isolated from rat liver, CYP4A5/7 isolated from rabbit kidney, and CYP4B1 isolated from rabbit lung exhibited high levels of heme covalent binding indicating that this phenomenon is not an artifact of the heterologous expression systems used to obtain the recombinant proteins (3, 4, 12).

The finding that numerous CYP4 proteins covalently bind their heme prosthetic group through an ester linkage parallels earlier observations made for the mammalian peroxidases myeloperoxidase, lactoperoxidase, thyroperoxidase, and eosinophil peroxidase (13-16).

We reported previously that base treatment of rabbit CYP4B1 released a polar heme species (3) consistent with the presence of an ester linkage formed with a backbone acid residue. Hoch and Ortiz de Montellano (4) originally identified a conserved I-helix glutamate residue, E318, as the likely site of covalent attachment to CYP4A3 following MALDI-TOF analysis of a heme-peptide digest, and recently confirmed that assignment by site-directed mutagenesis (6). The corresponding amino acid position in rabbit CYP4B1 is E310, and it is clear from the mutagenesis studies reported herein that this acidic residue serves the same critical function in CYP4B1 because replacement of the glutamate residue with glycine, alanine, or aspartate abolished heme covalent binding in the resulting mutants. In addition, mass analysis of CYP4B1 tryptic peptides provided evidence for a heme-bound peptide that corresponds to the fragment of the I-helix on which Glu310 resides. The identified peptide does contain two additional glutamic acid residues and two aspartic acid residues that could potentially form an ester linkage to the heme, but these residues are not predicted from a homology model (See Figure 3.5) to be in close proximity to the heme in the active site of CYP4B1.

A new finding from the present studies is the observation that some of the I-helix mutants of CYP4B1 were isolated as two chromatographically distinguishable protein populations. Mass spectrometry analysis demonstrated the existence of a dimeric species that was sensitive to treatment with reducing agents for the E310G, E310A, and E310D mutants. Protein-protein cross-linking of hemoproteins, such as lactoperoxidase and myoglobin, is a well-documented phenomenon that involves the generation of protein radicals during catalysis that can be propagated to the protein surface to generate both homo- and hetero-dimers (17-19). The formation of a disulfide bridge in P450_{cam} has been shown to decrease activity by only 14%, and the C334A mutant has similar optical

spectra, camphor binding, and activity as the wild type enzyme (20). The effect of a disulfide dimer in P450 BioI (CYP107H1), from *Bacillus subtilis*, on activity has not been evaluated, but does help to stabilize the enzyme (21). In the present work, dimer formation in CYP4B1 was not clearly associated with disruption or augmentation of the heme covalent link.

Valuable mechanistic information is provided by the CYP4B1 E310D mutant that contains a monohydroxylated heme that is noncovalently bound to the enzyme. Thus, shortening the acidic amino acid side chain at position 310 by one methylene unit still permits heme activation, but diverts the fate of the activated heme away from ester bond formation. These observations are most easily rationalized by a heme carbocation intermediate that is quenched by water in the absence of a suitably positioned carboxylate group (see Figure 2.7A, B). Similar findings have been reported recently for the E318D mutant of CYP4A3 (6) and for the E375D and D225E mutants of lactoperoxidase (22). To test the hypothesis that a carbocation is featured on the reaction pathway for CYP4B1 heme activation, the E310D mutant was expressed in the presence of H_2^{18}O and the resulting polar heme species was analyzed by LC/ESI-MS. This experiment demonstrated that the monohydroxyheme incorporated ~ 0.8 mol equiv of the heavy isotope from labeled water, indicating participation of a carbocation intermediate in the heme activation process. In two separate experiments, the extent of ^{18}O incorporation was less than 100%, which may reflect competition between H_2^{18}O and excess H_2^{16}O in the active site of the enzyme. One obvious source of additional active site H_2^{16}O is the autocatalytic process itself, as outlined mechanistically by Ortiz de Montellano *et al.* for heme covalent binding to lactoperoxidase (14).

The data support the participation of a carbocation intermediate in heme skeleton oxidation for the E310D mutant of CYP4B1. The mechanistically attractive inference that a carbocation intermediate is exposed on the reaction trajectory to covalently bound heme for CYP4B1 requires that the mechanism of oxidation for the E310D mutant is the same as wild-type enzyme. In evaluating this, we need to be cognizant of the extensive

dimerization exhibited by the E310D mutant. However, the fact that the chemically reduced E310D mutant retains the characteristic ability of P450 enzymes to bind carbon monoxide provides ancillary support for a single mechanism of heme activation for mutant and wild-type CYP4B1.

Although we could not clearly demonstrate autocatalysis for wild type CYP4B1, we were able to reproduce the reaction reported for CYP4A8 (6). This confirms the hypothesis that the covalent linkage is formed under normal turnover conditions, and is therefore initiated by auto-oxidation by the P450 itself. The inability of the P450s to completely bind free heme under *in vitro* conditions may be due to the presence of residual nonspecifically bound heme that was carried throughout the purification process, or due to free heme in the active site of P420 that cannot be activated. There is also a possibility that the rate of covalent bond formation is slow relative to the rate of heme "bleaching," and therefore the amount of observable protein-bound heme is limited. The "bleaching" of the heme chromophore has been described previously (23), and is observed for both the free heme and protein bound heme in the CYP4 enzymes. For both CYP4B1 and CYP4A8, the apparent rate of free heme "bleaching", following the first 2 min time point, is 2-fold greater than for the protein-bound heme. If it is assumed that both free heme and bound-heme "bleach" at a similar rate, then the CYP4 enzymes could still be undergoing covalent heme attachment at half that rate.

The ability of purified CYP4A8 to covalently bind heme upon the addition of its native coenzymes cytochrome P450 reductase, and cytochrome *b*₅, leads us to question how the CYP4 autocatalytic reaction occurs in the recombinant expression system. The endogenous redox partners in the insect cell expression system have not been identified, but low levels of catalytic activity has been reported for CYP2A6 and CYP3A4 in microsome preparations without exogenous cytochrome P450 reductase (24, 25). Therefore, it is possible that the autocatalysis of heme attachment could occur during expression in *T. ni* cells. Recently, we have expressed recombinant CYP4B1 in *E. coli*, and found that the heme is completely bound to the purified protein (11). There is

evidence for endogenous *E. coli*. redox partners, such as the flavodoxin/flavodoxin reductase system, that are capable of supplying P450c17 with reducing equivalents, and could possibly initiate autocatalysis in the CYP4 enzymes (26-28). Alternatively, it is possible that CYP4 P450s may be able to use endogenous oxygen surrogates, perhaps lipid peroxides or hydrogen peroxide, but this requires further study. It is not known why the extent of covalently bound heme in CYP4B1, CYP4A and CYP4F enzymes varies so widely, ranging from 6% in CYP4A8 to 99% in CYP4B1 (3, 4). Different expression conditions across laboratories may explain this, or it is possible that the efficiency of electron transfer from the endogenous coenzyme varies for the P450s. Also, if a ligand is required to initiate the catalytic cycle, and therefore covalent heme attachment, then it is possible that differences in affinity for an endogenous substrate can explain the varied levels of covalently bound heme between CYP4 isoforms.

In summary, we have shown (i) that E310 serves as the site of covalent attachment of heme to the protein backbone of rabbit CYP4B1; (ii) that the mechanism of formation of the heme-CYP4B1 covalent link most likely involves a carbocation intermediate located on the porphyrin; and (iii) that the heme of CYP4 enzymes are bound *via* an autocatalytic process.

Table 2.1. Spectroscopic and molecular weight characterization of rabbit CYP4B1 I-helix mutants.

enzyme	Fe ³⁺ (nm)	Fe ²⁺ -CO (nm)	MWt (obs)	MWt (calc)	Δ (Da)
wild-type	418	448	59,222.0	59,222.1 (holo)	-0.1
E310G	416	448	58,544.7	58,537.5 (apo)	7.2
E310A	415	447	58,553.3	58,551.5 (apo)	1.8
E310D	419	448	58,595.1	58,594.5 (apo)	0.6

Table 2.2. Covalent heme binding and molecular weight of the acid-dissociable heme of rabbit CYP4B1 I-helix mutants. ^aMean $\pm$ standard deviation of three separate preparations.

enzyme	% covalently bound	Heme (D)
wild-type	75 $\pm$ 4	616.2
E310G	1	616.2
E310A	0	616.2
E310D	2	632.3

Table 2.3. Experimentally determined fractional ion intensities for the polar heme-acetonitrile adduct obtained from CYP4B1 E310D expressed in H₂¹⁶O or H₂¹⁸O.

^a Values in parentheses represent the standard deviation of triplicate measurements.

m/z	expression in H ₂ ¹⁶ O	expression in 6.79% H ₂ ¹⁸ O	expression in 14.0% H ₂ ¹⁸ O
673.3	0.577 (± 0.0023) ^a	0.535 (± 0.0057)	0.513 (± 0.0044)
674.3	0.258 (± 0.0016)	0.243 (± 0.0022)	0.229 (± 0.0020)
675.3	0.063 (± 0.0011)	0.088 (± 0.0007)	0.121 (± 0.0019)
676.3	0.015 (± 0.0006)	0.031 (± 0.0029)	0.044 (± 0.0003)

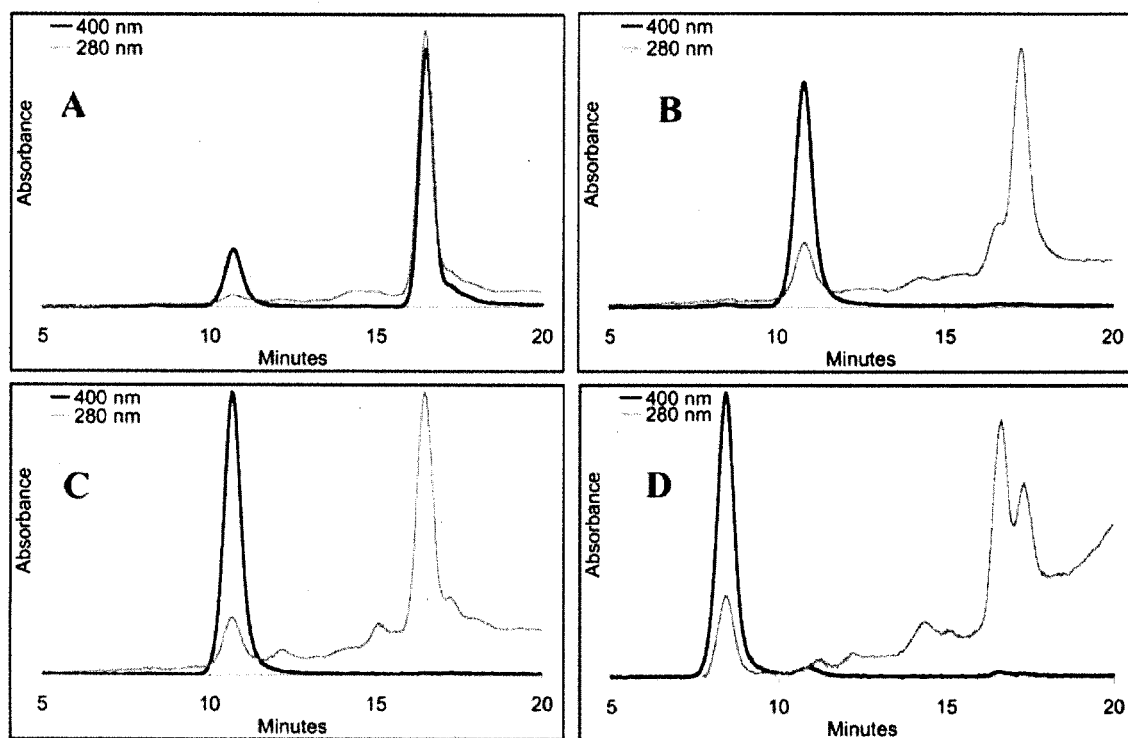


Figure 2.1. POROS R2 chromatography of rabbit CYP4B1 mutants with HPLC-UV detection at 400 nm (dark trace) and 280 nm (gray trace). Panel A: Wild-type CYP4B1; panel B: E310G mutant; panel C: E310A mutant; and panel D: E310D mutant. CYP4B1 protein elutes at 16.8 min, free heme elutes at 10.8 min, and monohydroxyheme elutes at 8.5 min. The later eluting protein peaks in panels B and D exhibited molecular weights of 117 122 and 117 230, respectively, as determined by electrospray mass spectrometry.

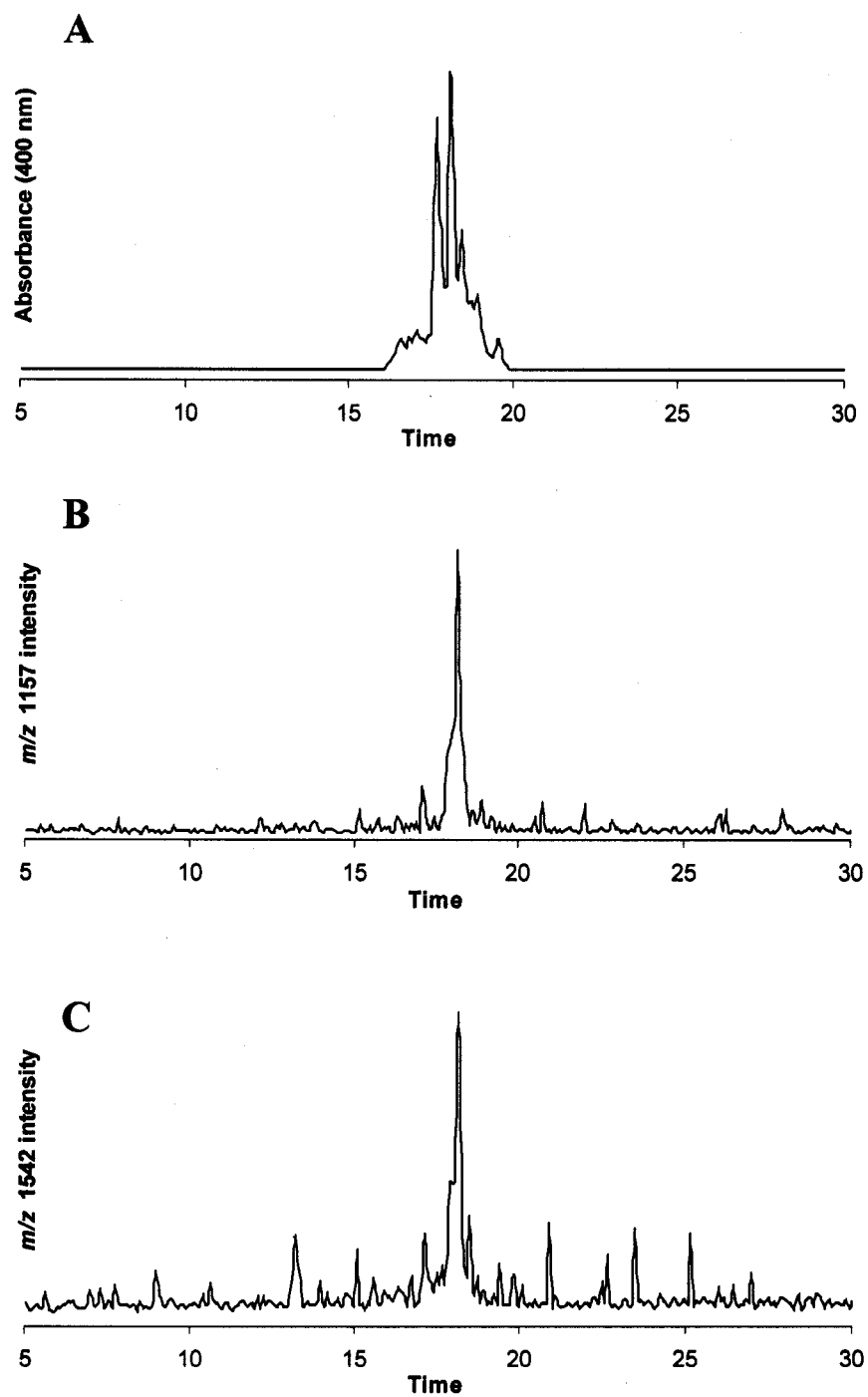


Figure 2.2. Identification of peptide 302-AEVDTFMFEFHDTTTSGISWFLYCMALYPEHQQR-335 with bound heme, by (A) absorbance at 400 nm, (B) ion intensity of m/z 1157, representing the $[M+H]^{4+}$ ion of T40 and (C) ion intensity of m/z 1542, representing the $[M+H]^{3+}$ ion of T40.

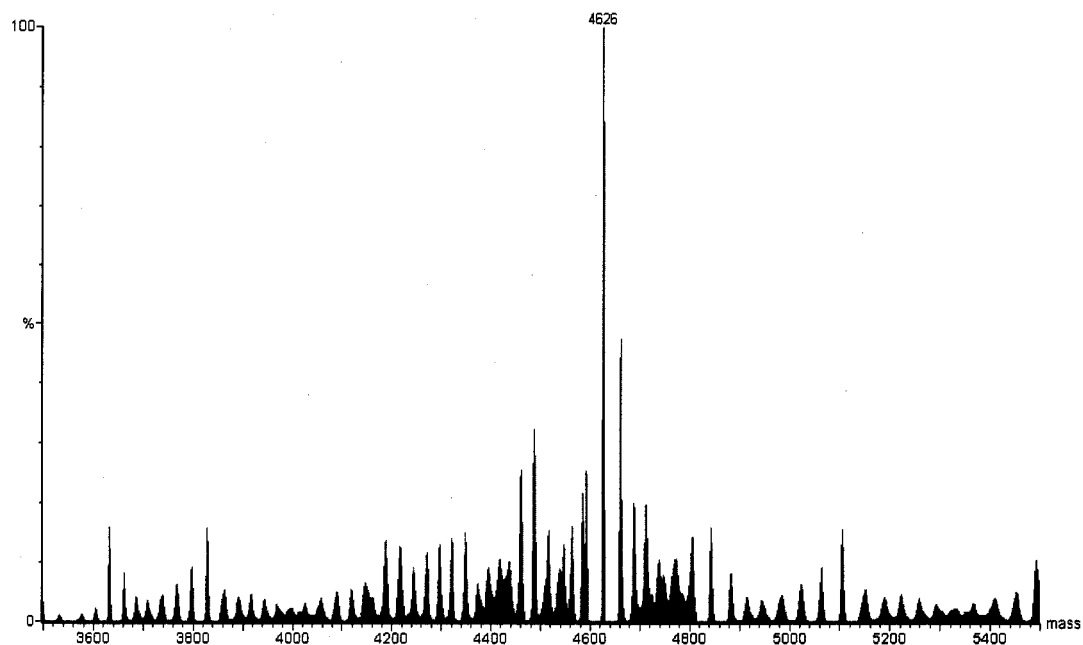


Figure 2.3. The deconvoluted mass spectrum for heme-linked peptide 302-AEVDTFMFEFHDTTSGISWFLYCMALYPEHQQR-335, with a predicted mass of 4625 Da.

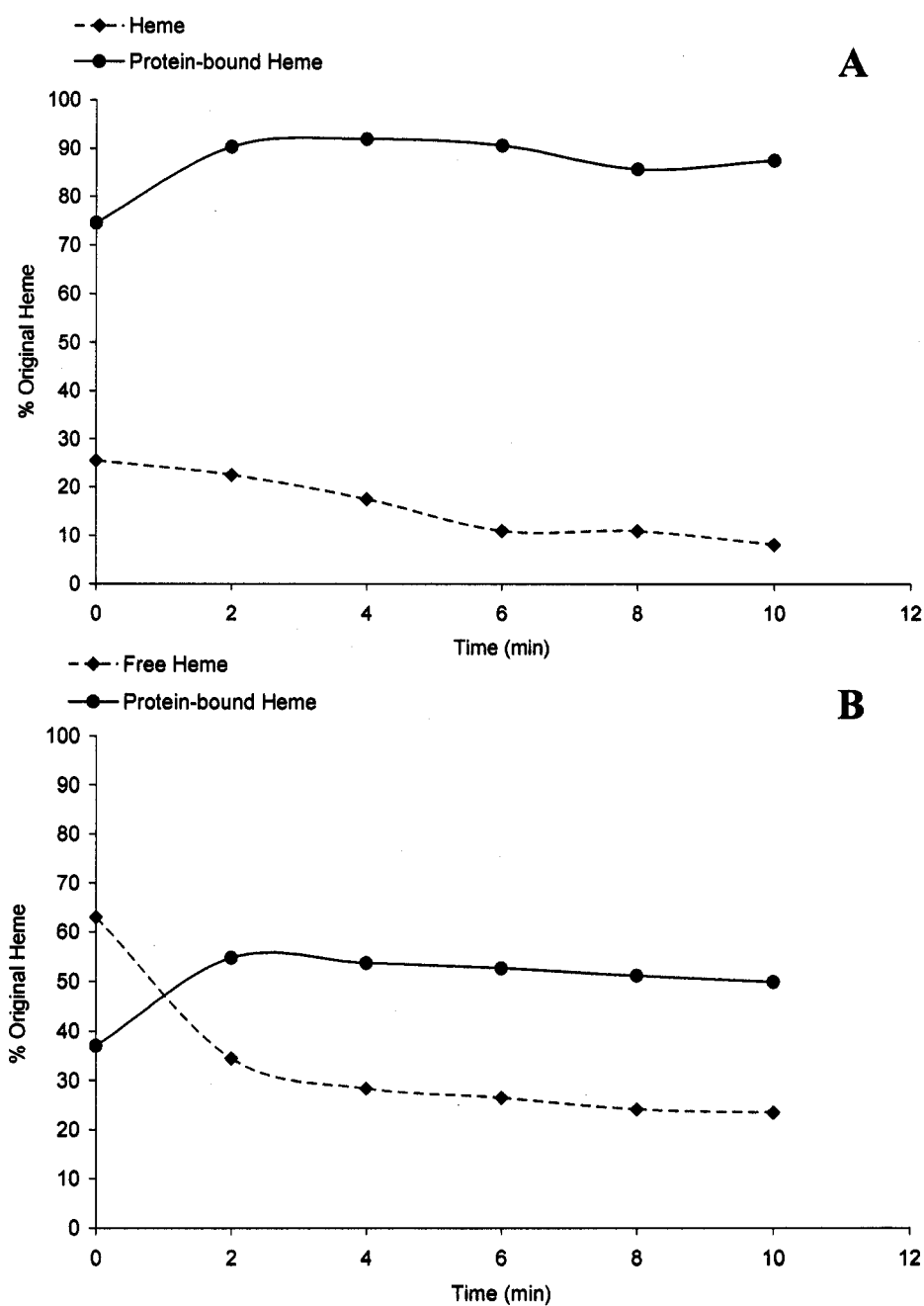


Figure 2.4. Percent peak areas representing free heme and P450-bound heme from the 400 nm trace, versus incubation time under turnover conditions, for (A) CYP4B1 and (B) CYP4A8.

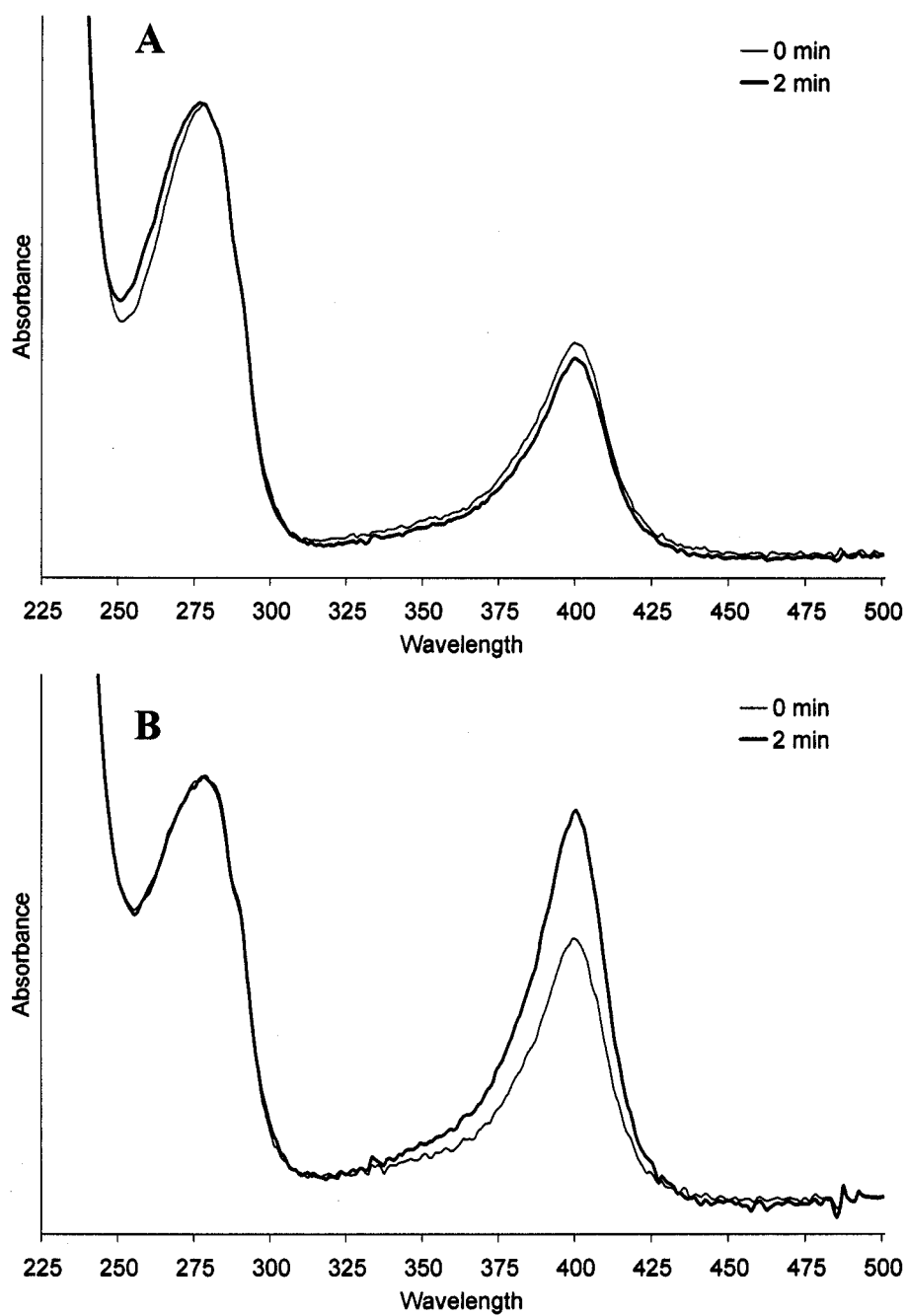


Figure 2.5. Absorbance spectrum acquired during elution of (A) CYP4B1 and (B) CYP4A8 from the POROS R2 column.

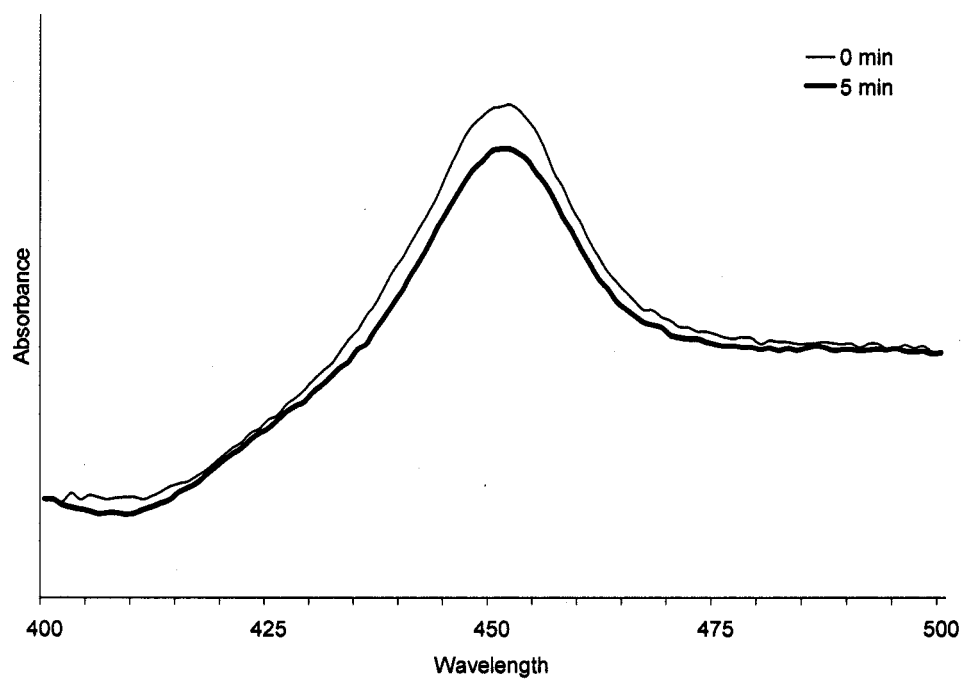


Figure 2.6. Reduced CO-bound spectra for CYP4A8 (A) at 0 min, prior to the addition of NADPH, and (B) at 5 min after the initiation of the reaction.

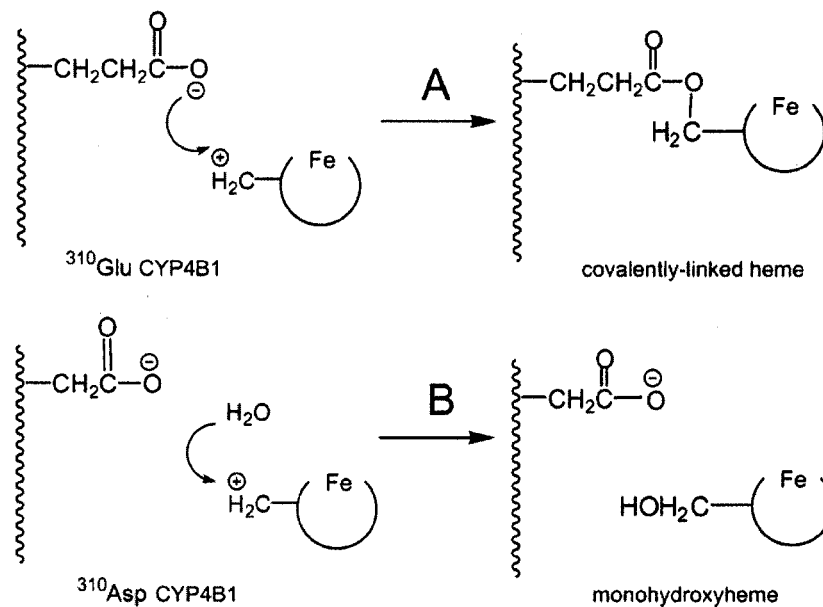


Figure 2.7. Alternative fates of a heme carbocation in CYP4B1 that partitions between (A) reaction with the appropriately positioned carboxylate of E310 to generate an ester bond and (B) quenching by an active site water molecule to generate monohydroxyheme in the E310D mutant.

2.5 Notes to Chapter 2

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Chapter 3

Sites of Covalent Attachment of CYP4 Enzymes to Heme: Evidence for Microheterogeneity of P450 Heme Orientation

3.1 Modification of Heme Group in CYP4 Enzymes

The heme prosthetic group, situated deep within the hydrophobic active site of cytochrome P450 enzymes, serves as the catalytic center for activation of molecular oxygen. Cytochrome P450s employ the activated perferryl species to metabolize a wide range of endogenous and exogenous substrates to both pharmacologically active and inactive products (1). In the vast majority of cytochrome P450s characterized to date, the heme is attached to the protein backbone by strictly non-covalent interactions. The most distinguishing interaction is the thiolate coordinate bond between the iron atom and a completely conserved cysteine residue in the C-terminal portion of the protein. Non-covalent interactions between the propionate groups of heme and basic residues of the protein, as well as hydrophobic interactions between the periphery of the heme and the active site pocket, are also important in heme binding to P450s (2).

In the last few years it has become clear that certain members of the CYP4 family, including CYP4B1, also bind their heme group covalently through an ester linkage with a conserved Glu residue in the I-helix of these enzymes and an oxidized heme methyl group (3-7). A similar phenomenon has been observed in the mammalian peroxidases, including lactoperoxidase, myeloperoxidase, eosinophil peroxidase, and thyroid peroxidase. The crystal structure of myeloperoxidase reveals that the heme is attached to the protein at three locations. Two ester bonds link the C-1 methyl and the C-5 methyl group to the carboxyl groups of Glu242 and Asp94, respectively. In addition, a thioether sulfonium bond is observed between the β -carbon of the 2-vinyl group and Met243 (8). The prosthetic heme group released from lactoperoxidase upon digestion has been identified by NMR and mass spectrometric methods as the 1,5-dihydroxymethyl derivative of heme, implying that two ester bonds link the heme to the protein (9, 10).

Sequence alignments and mutagenesis studies indicate further that Asp255 is bound to the heme C-5 methyl and Glu375 is bound to the heme C-1 methyl of lactoperoxidase (11).

Recently it was concluded that certain rat CYP4A and 4F forms covalently bound their heme at the C-5 methyl position, based on a chromatographic comparison of hydroxymethylhemes released from recombinant forms of the P450s and lactoperoxidase (5, 6). The identities of lactoperoxidase-derived 1- and 5-hydroxymethylhemes were inferred from studies conducted with active site mutants that sequentially disrupted the enzyme's 1,5-diester heme link (11), but no detailed structural analyses in support of these assignments is available. Therefore, the aim of the present study was to unambiguously determine the complementary site of attachment on the heme prosthetic group of rabbit CYP4B1 using chromatography, mass spectrometry, and NMR. Unexpectedly, we found evidence for microheterogeneity in heme orientation in the recombinant, but not the native proteins. Potential reasons for this difference and possible implications are discussed.

3.2 Experimental Procedures

Enzymes.

CYP4B1 was purified from rabbit lung microsomes, *E. coli* (DH5 α F'IQ) cell paste, or insect cells (*Trichoplusia ni* H5B1-4) (12-14). CYP4A3 was expressed in *E. coli* essentially as previously described (6). Recombinant enzyme preparations were harvested at 48 hrs. Horseradish peroxidase (HRP) was purchased from Sigma. Recombinant lactoperoxidase (rLPO) was expressed in CHO cells and purified as reported previously (15).

Heme standards.

An authentic standard of 8-hydroxymethylheme was prepared from phenyldiazene-treated HRP according to published procedures (16). Lactoperoxidase was used as

source of 1-hydroxymethylheme and 5-hydroxymethylheme, based on a report that recombinant forms of lactoperoxidase contain small amounts of these intermediates (10, 17).

Heme extraction.

CYP4B1, CYP4A3 or rLPO was treated with 1 M NaOH for 15 min to hydrolyze the ester linkages followed by addition of equal volume of 16 M urea. HRP did not require treatment prior to extraction. Solutions were acidified with TFA before extracting the heme with a 2X volume of ether. The ether was washed with a 1X volume of distilled water then with a 1X volume of a saturated NaCl solution. Extracts were evaporated to approximately 200 μ l, at which point 200 μ l of methanol was added. The volume was again reduced to 200 μ l, removing the majority of the ether.

Chromatography.

The samples were loaded onto a 4.6 $\times$ 250 mm analytical 5 μ m C-4 column (Vydac, Hesperia, CA) for separation. HPLC analysis was performed on a Shimadzu instrument consisting of two LC-10ADvp pumps, an SPD-M10Avp UV-Vis detector, an SCL-10Avp controller, and an SIL-10ADvp autosampler (Shimadzu Scientific Instruments, Inc., Columbia, MD). During analysis, data were collected using EZSTART chromatography software (v 7.2, Shimadzu Scientific Instruments, Inc.) running on Windows 2000. Initial conditions were 55% Solution A (0.05% TFA in H₂O) and 45% Solution B (0.05% TFA in acetonitrile) at a flow rate of 1 ml/min. The linear gradient increased B from 45 to 55% between 0 and 60 min. Heme elution was monitored by absorption at a wavelength of 400 nm. Under these conditions, dihydroxymethylheme eluted at 11 min, 1-hydroxymethylheme at 23.4 min, 5-hydroxymethylheme at 28.9 min, 8-hydroxymethylheme at 30.7 min, and free heme at 44.7 min.

LCMS conditions.

LC/ESI-MS analysis was performed using a Micromass Quattro II tandem quadrupole mass spectrometer (Micromass, Ltd., Manchester, U.K.) coupled to a Shimadzu LC

instrument similar to that described above. The mass spectrometer was run in electrospray ionization (ESI) mode at a cone voltage of 40 V, source block temperature of 180 °C, and a desolvation temperature of 350 °C. Data analysis was carried out using Windows NT based Micromass MassLynxNT[®] 3.2 software. The total flow into the mass spectrometer was 350 µl/min. The isotopic masses for dihydroxymethylheme (m/z 646-653) were monitored from 5-20 min and the isotopic masses for monohydroxymethylheme (m/z 630-637) were monitored from 20-35 min.

Heme preparation for NMR.

Hydroxymethylheme was extracted from approximately 1 µmol of CYP4B1 and loaded onto a 10 × 250 mm semi-preparative 5 µm C-4 column (Vydac, Hesperia, CA). The Shimadzu HPLC instrument described above was used for purification. Initial conditions were 65% Solution A (0.05% TFA in H₂O) and 35% Solution B (0.05% TFA in acetonitrile) at a flow rate of 3 ml/min. The linear gradient increased B from 35 to 45% between 0 and 20 min. Heme elution was monitored by absorption at a wavelength of 400 nm. Under these conditions, hydroxymethylhememes from CYP4B1 eluted at ~28 min, but 5-hydroxymethylheme and 8-hydroxymethylheme were incompletely resolved on the semi-preparative column. Therefore, the leading edge of the earlier eluting, major HPLC peak was collected from approximately 10 injections, lyophilized, and then dissolved in d₅-pyridine. Approximately 5 mg of SnCl₂ was added to reduce the heme and the sample was transferred to a NMR tube, which was flushed with argon and sealed.

NMR conditions.

NMR data were acquired at 500 MHz on a Varian Inova 500 spectrometer equipped with an actively shielded z-axis gradient and a triple resonance probe. All experiments were conducted at 25°C. 1D ¹H NMR spectra were acquired with a spectral width of 8000 Hz using 8192 acquired points. The ¹H ROESY data set included an 8000 Hz spectral width, 320 transients, 128 increments, and 8192 points along F₂. ROESY experiments were acquired with a mixing time of 300 ms. 2D data sets were processed using MestRe-C software (version 3.5.9, <http://www.mestrec.com>).

Lauric acid hydroxylation activity.

Rabbit CYP4B1 (10 pmol of each purified form) was preincubated with P450 reductase (40 pmol), cytochrome b₅ (10 pmol), catalase, (100 units) and 20 µg of L-α-dilauroylphosphatidylcholine (DLPC). Metabolic reactions were carried out in 0.1 M potassium phosphate buffer, pH 7.4 containing 1-100 µM lauric acid and 0.5 mM NADPH at 37°C. The total volume of the reconstituted mixture was 2 mL. Reactions were terminated after 10 min by the addition of 1 mL of 10% HCl, and spiked with internal standard (2 nmol), 15-hydroxy pentadecanoic acid. The metabolites were extracted with 3 mL of ethyl acetate, dried under nitrogen, and derivatized with BSTFA. Metabolites generated from the incubation of CYP4B1 and lauric acid were quantified using a Shimadzu GC/MS (Shimadzu Scientific Instruments, Inc., Columbia, MD). The gas chromatograph (GC-17A) was equipped with a 30 m × 250 µm capillary XTI-5 column (Restek Corp., Bellefonte, PA). The carrier gas was helium with a constant flow rate of 1 mL/min. The injection port temperature was 250°C. The temperature gradient was increased from 120 to 160°C at a rate of 20°/min, then from 160 to 210°C at a rate of 4°/min, and finally from 210 to 280°C and held at 280°C for 2 min. The column was connected to a Shimadzu mass spectrometer (GCMS-QP5050). The interface temperature was 300°C and the detector voltage was 1.6 V throughout the method. The following metabolites were observed and confirmed with standards: 10-hydroxy lauric acid (11.5 min, *m/z* 131), 11-hydroxy lauric acid (11.8 min, *m/z* 117), 12-hydroxy lauric acid (13.2 min, *m/z* 345). 15-hydroxypentadecanoic (17.1 min, *m/z* 387) was used as an internal standard. Ions were monitored in selected-ion monitoring mode with an interval time of 50 ms. Data were analyzed on using GCMS Solution version 1.10 beta software (Shimadzu Scientific Instruments, Inc., Columbia, MD).

4-Ipomeanol (IPO) bioactivation activity.

Purified P450 (200 pmol) was reconstituted with P450 reductase (400 pmol), DLPC (20 µg), and cytochrome b₅ (200 pmol) in 100 mM potassium phosphate buffer (pH 7.4). *N*-acetyl cysteine and *N*-acetyl lysine were then introduced in 100 mM potassium

phosphate buffer to a final concentration of 10 mM each in order to trap the reactive enedial intermediate (18). IPO was then added to a final concentration of 1 mM (using a 10 mM stock solution made in 10% methanol). The total volume of the reconstituted mixture was 0.5 mL. Metabolic activity was initiated by the addition of NADPH (1 mM final concentration), and incubations allowed to proceed at 37 °C for 30 min. Reactions were terminated by the addition of 50 μ l of a 15% zinc sulfate. The rates of bioactivation of IPO were determined by HPLC analysis of a stable pyrrole adduct as previously described (18).

3.3 Results

Chromatographic separation of monohydroxymethylhemes.

Recombinant LPO-derived 1- and 5-hydroxymethylhemes (Figure 3.1) and HRP-derived 8-hydroxymethylheme were well separated on a Vydac C4 HPLC column (Figures 3.2A and 3.2B). The monohydroxymethylheme released from native rabbit lung CYP4B1 eluted at the same retention time (28.9 min, Figure 3.2C) as the minor and later eluting isomer generated from incompletely processed rLPO, and so was tentatively identified as 5-hydroxymethylheme (11).

NMR analysis of 5-hydroxymethylheme.

In order to unambiguously establish the site of covalent attachment of native CYP4B1 to its heme, ~25 mg of purified recombinant enzyme was prepared and monohydroxymethylheme was released by base hydrolysis for structural elucidation by NMR. The heme that co-eluted with the 5-hydroxymethylheme biosynthetic standard was collected, lyophilized, dissolved in d_5 -pyridine, and treated with tin chloride. A ROESY 2D NMR spectrum of this sample is shown in Figure 3.3. The location of the hydroxyl group of this monohydroxymethylheme was identified by the chemical shift changes compared to unmodified heme (Table 3.1). The most valuable ROE signal used to definitively assign the structure is the cross peak between the β -meso proton (10.59 ppm) and the 5-hydroxymethylene protons (6.31 ppm). The signal for the methylene

protons, to which the hydroxyl group is attached, is shifted 2.83 ppm downfield when compared to the 5-methyl protons in heme (3.48 ppm). The signal for the β -meso proton is 0.46 ppm downfield compared to the β -meso proton in heme (10.13 ppm). Although we did not have sufficient quantities of heme from recombinant lactoperoxidase for NMR analysis, we did obtain 1D and 2D ROESY spectra of the 8-hydroxymethylheme generated from horseradish peroxidase (16). The chemical shift alterations due to the hydroxyl group at the 5-methyl position agree well with the corresponding chemical shift changes observed due to the hydroxyl group at the 8-methyl position (Table 3.1).

Microheterogeneity of heme incorporation into recombinant CYP4 enzymes.

Unlike the native enzyme, recombinant CYP4B1 expressed in insect cells or *E. coli* contained a mixture of hemes (Figure 3.2D and 3.2E). The major heme species was 5-hydroxymethylheme, as observed in the native form of the enzyme. The minor peak - 13% of the total monohydroxymethylheme in CYP4B1 expressed in insect cells and 40% of the total monohydroxymethylheme in CYP4B1 expressed in *E. coli* - co-eluted with the 8-hydroxymethylheme standard. CYP4A3 expressed in *E. coli* exhibited a similar heme profile, with the majority of heme aligning with the 5-hydroxymethylheme peak and 20% of the heme co-eluting with 8-hydroxymethylheme (Figure 3.2F).

Alternative origins of 8-hydroxymethylheme.

To evaluate the possibility that 8-hydroxymethylheme present in recombinant CYP4 preparations derived from the incomplete processing of a 5, 8-diester link, we performed selected-ion monitoring at m/z 648 using lactoperoxidase as a positive control. No evidence was found for any dihydroxymethylheme species from either CYP4B1 or CYP4A3 (Figure 3.4A). Figure 3.4B shows that the m/z 632 traces for both CYP4B1 and CYP4A3 agree with the 400 nm-absorption traces in Figure 3.2. The isotopic patterns for both species match with predicted values (Figure 3.4B, inset). Another possibility to consider is that the minor 8-hydroxymethylheme isomer originated from an ester linkage to an amino acid other than Glu310. To probe this further, we inspected a homology model of rabbit CYP4B1, structured after another fatty acid hydroxylase, P450_{BM3}. The

homology model reveals a single glutamate near the heme periphery (Figure 3.5). The carboxylate group of Glu310 on the I-helix, previously identified as the site of protein attachment, is located just 3.3 Å from the C-5 methyl group of heme. There are no glutamate or aspartate residues within close proximity to the C-8 methyl group, which could potentially form a second ester linkage.

Functional comparisons of CYP4B1 activity.

Lauric acid was hydroxylated at the ω -position by native and recombinant forms of CYP4B1 at maximal rates of 14-25 nmol/nmol/min and with similar K_m values of 26 – 37 μ M (Table 3.2). The $\omega/\omega-1$ regioselectivity was ~ 1.4 for these three CYP4B1 preparations. Likewise, IPO was bioactivated by native CYP4B1 and both recombinant forms at near equivalent rates rate of 560 - 715 nmol/nmol/30min (Table 3.2). These data are not indicative of gross differences in functional activity between the structurally heterogenous forms of CYP4B1.

3.4 Discussion

The covalently attached prosthetic group in CYP4B1 presents a unique opportunity to examine the orientation of the heme relative to the P450 protein structure. After hydrolyzing the ester linkage between the heme and Glu310 of CYP4B1, the resulting hydroxymethylheme isomer retains information about the original position in relation to the I-helix. Chromatography and NMR analysis of heme released upon mild base hydrolysis of native rabbit lung CYP4B1 revealed that the heme species is exclusively the 5-hydroxymethylheme isomer, which places the C-ring of the heme in close proximity to the I-helix. Surprisingly, recombinant preparations of CYP4B1 yielded a second monohydroxymethylheme that co-chromatographed with authentic 8-hydroxymethylheme. Mass spectrometry data argued against a di-ester linkage to CYP4B1 and homology modeling of the active site of rabbit CYP4B1 provided no support for an alternative amino acid partner. Collectively, these observations suggest

that heme is inserted into recombinant CYP4B1 in two distinct orientations that are related by a 180° rotation about the heme α - γ -meso carbon axis.

The current studies also suggest that differential heme insertion is a general phenomenon, at least in recombinant CYP4 enzymes, because *E. coli*-expressed CYP4B1 and CYP4A3 both contained a mixture of the modified hemes following ester hydrolysis. Previous chromatographic analysis of recombinant CYP4A8, CYP4A3/E318D, and CYP4F5/G330E hemes, suggested covalent attachment at only the C-5 position (5, 6). In these earlier studies the extent of covalent heme attachment was generally lower than reported here, and so expression conditions may be an important variable.

Conformationally distinct (A and B) orientations of heme in cytochrome b₅ have been known for 25 years (19). The ratio of the resulting conformers is a function of steric restraints imposed by amino acid side-chains lining the heme cavity, which helps rationalize the vastly differing A:B ratios evident in cytochrome b₅ from different species (20). Much of this information is derived from NMR studies of the protein, as crystal structures have not generally been of sufficient resolution to discriminate between the two isomers (21). However, recent high resolution X-ray crystallography studies of *Mycobacterium tuberculosis* CYP121, expressed in *E. coli*, are indicative of a 70:30 mixture of the two heme orientations posited here for CYP4B1 and CYP4A3 (22). In addition, the structure of CYP154A1 from *Streptomyces coelicolor* A3(2), also expressed in *E. coli*, has revealed that the heme is present in a 180°-flipped orientation (23). While good evidence exists for different heme conformers in cytochrome b₅ and bacterial P450s, the present studies provide the first evidence for multiple heme conformers in mammalian P450.

An alternative method for evaluating P450 heme stereochemistry has been developed that takes advantage of the alkylated heme adducts formed from 3,5-bis(carbethoxy)-2,6-dimethyl-4-ethyl-1,4-dihydropyridine (DDEP) during catalytic turnover (24). This method allows the analysis of heme orientation in mammalian P450s, for which no

sufficiently high resolution structures yet exist to discriminate between heme conformers. Circular dichroism spectra of the C-ring isomer of the N-ethylprotoporphyrin IX hemes formed from native rat P450, revealed optical purity similar to that of hemoglobin, in which the heme is known to be in a single orientation (24). This result agrees with the single hydroxymethylheme stereoisomer that was found in native CYP4B1 purified from rabbit lung. Based on these findings, multiple heme orientations may not be important for P450s expressed in their native environment, but could be a general phenomenon for recombinant P450 enzymes.

The presence of an ester linkage to the 8-methyl group of heme in the recombinant forms of CYP4B1 indicates that the fidelity of the heme insertion process and/or the protein folding process that occurs in the rabbit lung, is not replicated in the bacterial or insect cell expression systems. Bacterial P450s and cytochrome b_5 permit the facile exchange of heme ligands without denaturing the enzyme, in contrast to eukaryotic P450s where the heme insertion process during folding could well be very different. One plausible explanation for the altered heme orientations from native and recombinant mammalian P450 is that protein folding chaperones in the various expression systems (rabbit lung, insects cells, and *E. coli*), are not identical. There is little information in the literature available to date about P450 heme-protein assembly, but there is evidence for the involvement of chaperones in the process. In phenobarbital-pretreated rats, Zgoda *et al.* discovered a possible role for the chaperone GRP94 in heme-protein assembly (25). GRP94 is shown to assist in restoring allylisopropylacetamide-inactivated CYP2B1 by exchanging the N-alkylated heme for unmodified heme, and is described as a “finishing” chaperone in the biosynthesis and/or assembly of ER proteins (25). Inoue *et al.* found that when CYP3A7 was expressed alone in *E. coli* only apo-protein was produced, but when the molecular chaperone GroEL was co-expressed, holo-CYP3A7 was detected. This indicates that the bacteria did not contain the appropriate protein folding / heme insertion machinery for production of the mammalian P450 (26). Therefore, it is possible that heme insertion is facilitated by distinct chaperones in *E. coli*, which do not have the same stringency for stereoselective incorporation of heme as the

mammalian forms. Inefficient heme insertion by insect cell chaperones might also offer an explanation for why recombinant human CYP4B1 holoenzyme is not expressed successfully using the baculovirus expression vector system (14). Possibly, with the appropriate chaperone machinery in mammalian tissues, heme can be correctly incorporated into human CYP4B1 to produce active protein (27).

Microheterogeneity of heme orientation in recombinant P450 enzymes raises the possibility of multiple heme-protein species with altered catalytic activity, because steric interactions between the heme binding cavity in the protein and the repositioned vinyl and methyl groups of the heme may influence active site conformation. This could have important ramifications for the industrial drug discovery process where recombinant P450 enzymes are used routinely to evaluate and make predictions about in vivo drug metabolism (28). Therefore, we examined lauric acid metabolism and 4-ipomeanol bioactivation rates to evaluate the effect of heme orientation in CYP4B1 on substrate turnover. With lauric acid, the regioselectivity of ω / ω -1 hydroxylation of lauric acid was similar for all enzymes, suggesting that the active site conformation is unaffected. Apparent K_m and V_{max} values for laurate varied by less than 2-fold, and IPO bioactivation rates were similar across the three enzyme preparations. No definitive trends could be observed and so these studies do not provide evidence for modified monooxygenase activity due to altered heme orientation in CYP4B1. However, the activity contribution of CYP4B1 with heme bound to the 8-methyl position is difficult to assess because of intrinsic variability in enzyme activities from purified P450 preparations and a lack of knowledge concerning the orientation of the heme component that is not covalently bound. Moreover, electron transfer and oxygen binding could potentially be affected by a re-oriented heme. An altered redox potential has been reported for the two heme conformers of cytochrome b5, but the magnitude of the change is believed to be too small to be functionally significant (29). Additional studies are required to deconvolute this complex system in cytochrome P450 in order to more definitively address the question of functional heterogeneity secondary to promiscuous heme processing by recombinant CYP4 proteins.

In conclusion, chromatography and 2D NMR experiments establish that the structure of the major heme species released from CYP4B1 upon base treatment is 5-hydroxymethylheme. Covalent binding of heme to the C-8 methyl group also occurs to a minor extent with recombinant CYP4 proteins, suggesting that the fidelity of holoenzyme assembly for mammalian P450s is not necessarily recapitulated in heterologous expression systems. The phenomenon of covalent heme binding to CYP4 proteins provides a novel method for assessing microheterogeneity in heme orientation to the mammalian P450s in the absence of high resolution crystallographic data or solution structures.

Table 3.1. Chemical shifts for protons in heme, HRP-derived 8-hydroxymethyl heme (8-OH), CYP4B1 derived 5-hydroxymethyl heme (5-OH), and observed ROE interactions.

Proton	Chemical Shift (δ , ppm)			ROE Interaction
	Heme	8-OH	5-OH	
α -meso	10.30	10.26	10.26	3-methyl, 2 α -vinyl
β -meso	10.13	10.14	10.59	5-methyl (methylene), 4 α -vinyl
δ -meso	9.96	10.43	9.95	1-methyl, 8-methyl (methylene)
γ -meso	10.35	10.42	10.35	6 α , 6 β , 7 α , and 7 β -propionic
1-methyl	3.67	3.54	3.65	δ -meso, 2 β -vinyl (trans)
3-methyl	3.65	3.65	3.61	α -meso, 4 β -vinyl (trans)
5-methyl(ene)	3.48	3.49	6.31	β -meso, 6 α -propionic
8-methyl(ene)	3.51	6.37	3.48	δ -meso, 7 α -propionic
2 α -vinyl	8.54	8.49	8.52	2 β -vinyl (cis), 2 β -vinyl (trans), α -meso
4 α -vinyl	8.48	8.51	8.35	4 β -vinyl (cis), 4 β -vinyl (trans), β -meso
2 β -vinyl (trans)	6.34	6.30	6.35	2 α -vinyl, 1-methyl, 2 β -vinyl (cis)
4 β -vinyl (trans)	6.32	6.32	6.29	4 α -vinyl, 3-methyl, 4 β -vinyl (cis)
2 β -vinyl (cis)	5.98	6.01	6.00	2 α -vinyl, 2 β -vinyl (trans)
4 β -vinyl (cis)	6.00	5.96	5.86	4 α -vinyl, 4 β -vinyl (trans)
6 α -propionic	4.52	4.52	4.64	6 β -propionic, γ -meso, 5-methyl(ene)
7 α -propionic	4.52	4.71	4.48	7 β -propionic, γ -meso, 8-methyl(ene)
6 β -propionic	3.56	3.57	3.67	6 α -propionic, γ -meso
7 β -propionic	3.56	3.72	3.54	7 α -propionic, γ -meso

Table 3.2. Heterogeneity of covalent heme binding and functional characteristics of native and recombinant CYP4B1. The $V_{\max}$ for lauric acid (LA) hydroxylation represents the rate for ω -hydroxylation and is expressed as nmol/nmol P450/min and the K_m is expressed as μM . The rate for 4-ipomeanol (IPO) bioactivation is expressed as nmol/nmol P450/30min.

Expression System	% Bound Heme		% Free Heme	LA Hydroxylation			IPO-Bioactivation
	5-methyl	8-methyl		$V_{\max}$	K_m	$\omega/\omega-1$	
Rabbit Lung	80		20	24.5 ± 2.6	37 ± 10	1.3	683 ± 43
	100	0					
Insect cells	81		19	13.7 ± 0.7	26 ± 3	1.5	715 ± 52
	87	13					
<i>E. coli</i>	100		0	14.6 ± 1.4	35 ± 8	1.4	560 ± 38
	62	38					

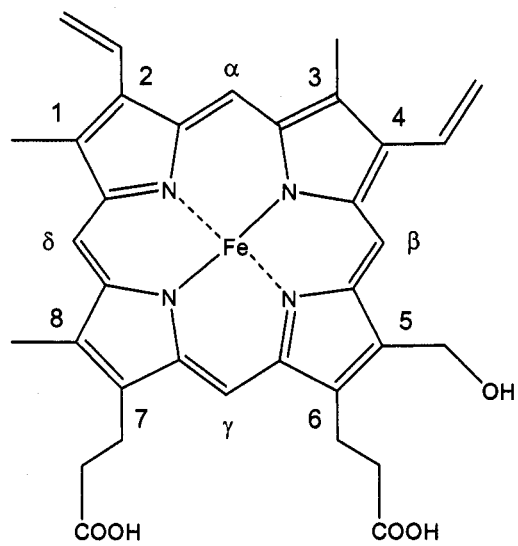


Figure 3.1. Structure of 5-hydroxymethylheme.

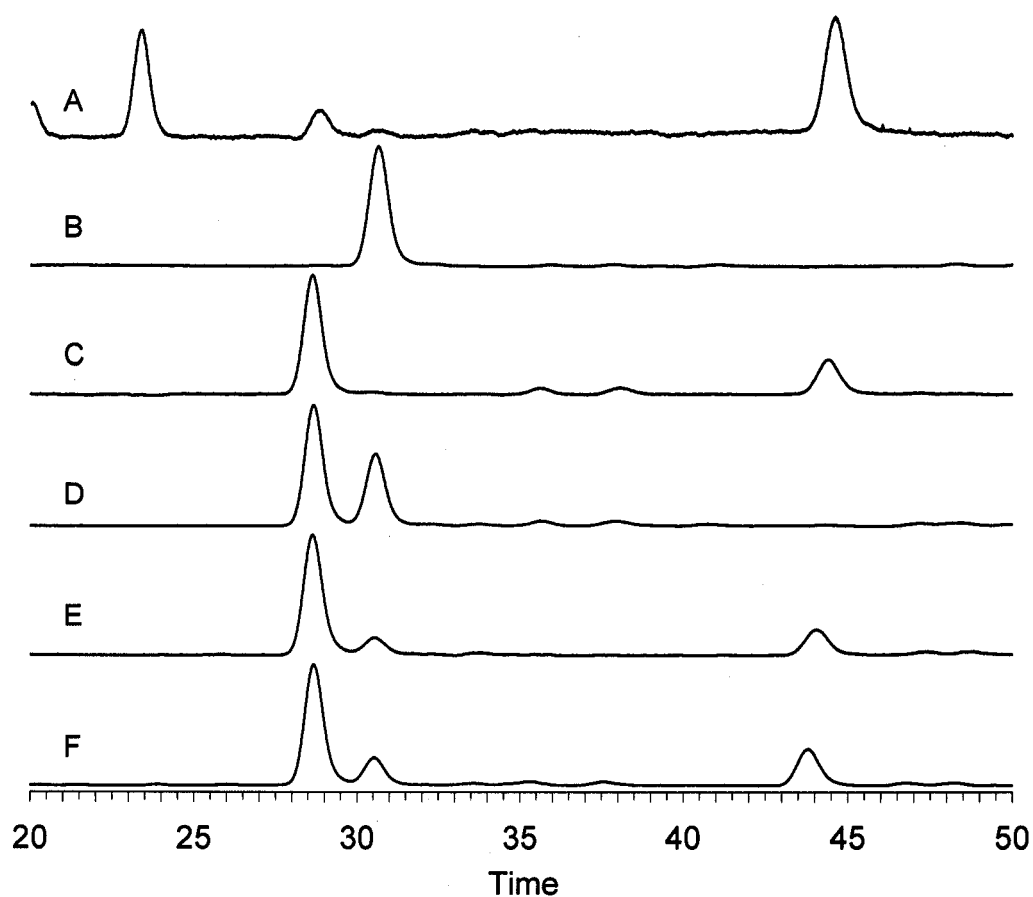


Figure 3.2. Chromatographic analysis of monohydroxymethylhemes. Modified hemes were monitored by absorbance at 400 nm. Trace A: 1-hydroxymethylheme (23.4 min) and 5-hydroxymethylheme (28.9 min) hydrolyzed from rLPO and free heme (44.7 min). Trace B: 8-hydroxymethylheme (30.7 min) from phenylhydrazine treated HRP. Trace C: hemes released by hydrolyzing the ester linkage in native CYP4B1. Trace D: hemes released by hydrolyzing the ester linkage in recombinant CYP4B1, expressed in insect cells. Trace E: hemes released by hydrolyzing the ester linkage in recombinant CYP4B1, expressed in *E. coli*. Trace F: hemes released by hydrolyzing the ester linkage in recombinant CYP4A3.

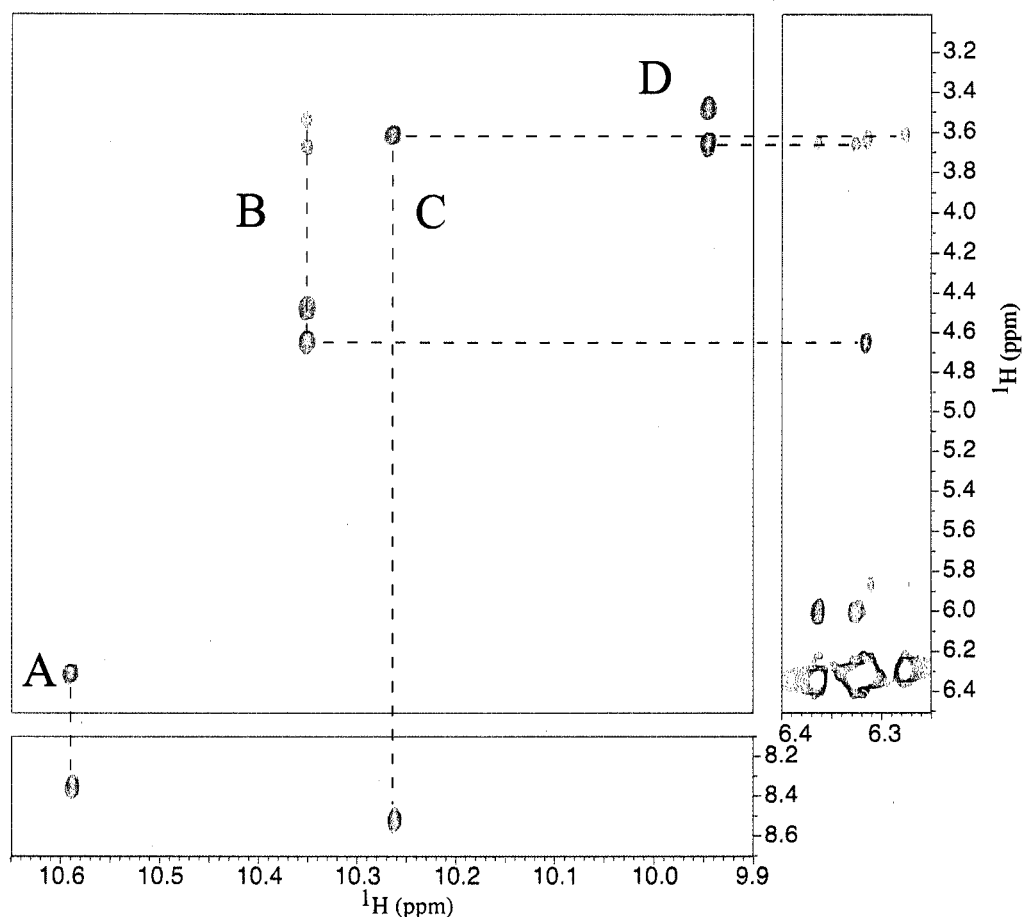


Figure 3.3. 2D NMR ROESY spectrum for the *bis*-pyridine complex of ferrous 5-hydroxymethylheme in d_5 -pyridine. (A) β -meso proton connectivity with 5-methylene and 4 α -vinyl protons. (B) γ -meso proton connectivity with 7 β , 6 β , 7 α , and 6 α propionic protons; the 6 α -propionic protons also show a connectivity with the 5-methylene protons. (C) α -meso. proton connectivity with 3-methyl and 2 α -vinyl protons; the 3-methyl protons also show a connectivity with the trans-4 β -vinyl protons. (D) δ -meso proton connectivity with 8-methyl and 1-methyl protons; the 1-methyl protons also show a connectivity with the trans-2 β -vinyl protons.

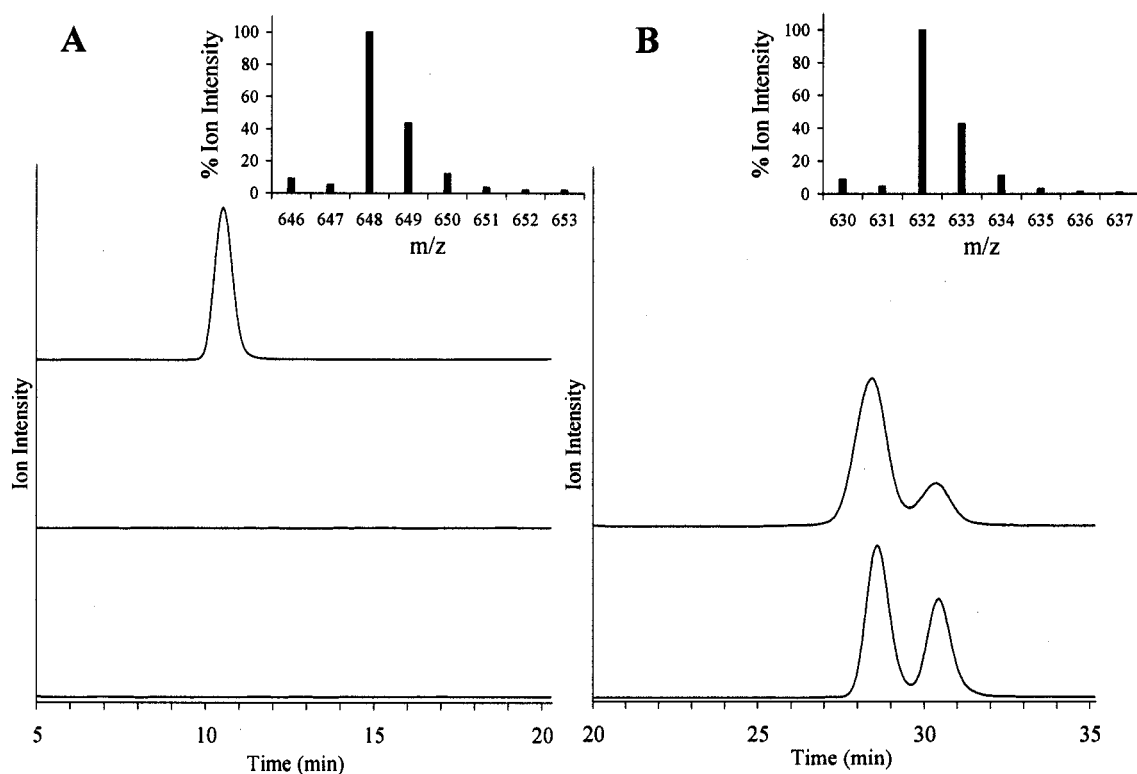


Figure 3.4. Selected ion monitoring chromatograms for CYP4B1, CYP4A3, and LPO hemes. Panel A: The mass for dihydroxymethylheme (m/z 648) was monitored from 5-20 min for CYP4B1 (lower trace) and CYP4A3 (middle trace). The SIR trace for 1-, 5-dihydroxymethylheme from LPO (upper trace) is included as a positive control. Panel B: The mass for monohydroxymethylheme (m/z 632) was monitored from 20-35 min for CYP4B1 (lower trace) and CYP4A3 (upper trace). The relative ion intensities for the isotopic masses of each heme are shown in the inset.

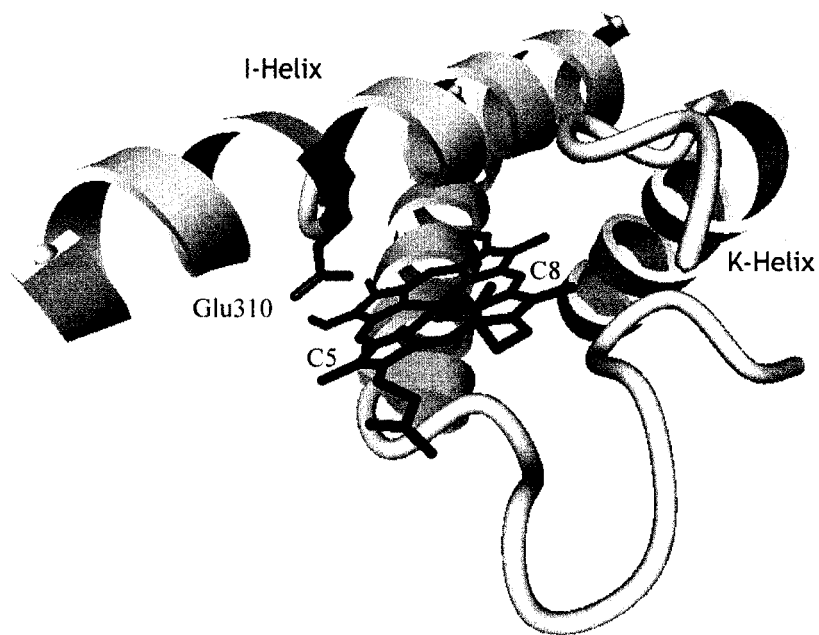


Figure 3.5. CYP4B1 homology model structured after P450_{BM3}. The heme is shown flanked by the L-helix and the I-helix, which contains Glu310. The protein backbone extending from the L- and K-helices is in close proximity to the C8 methyl group, but lacks any aspartate or glutamate residues.

3.5 Notes to Chapter 3

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Chapter 4

Formation of a Carboxyl Radical on Glu310 in the Mechanism of Covalent Heme Attachment to CYP4B1: Hydrogen Abstraction by Iron-oxo Species versus Homolytic Scission of a Perester Intermediate

4.1 Possible Mechanisms of Heme Activation

A unique oxidation performed by the CYP4 enzymes initiates the formation of an ester bond between a conserved glutamate on the I-helix and the C-5 methyl group on the heme cofactor (1-6). The mechanism for this reaction cannot simply be rationalized using the chemistry observed in characteristic P450 reactions, in which an endogenous or exogenous compound is converted to a more lipophilic metabolite *via* hydroxylation, epoxidation, or heteroatom oxidation (7). The reaction does have similarities with the reaction that forges two ester linkages connecting the C-1 and C-5 methyl groups of heme to both an aspartic acid residue and a glutamic acid residue in the mammalian peroxidases (8-10). Mutagenesis studies with lactoperoxidase (LPO) indicate that a properly positioned carboxylic acid group is strictly required for ester bond formation (9). Furthermore, the covalent link can be forged simply by the addition of hydrogen peroxide, suggesting that the mechanism is autocatalytic (11). To accommodate these experimental observations, a mechanism has been proposed which involves initial formation of a compound I-like intermediate, followed by a process that removes a hydrogen atom from each of the two methyl groups involved in cross-link formation (10). Consequently, the heme methyl radical is converted to a carbocation by transfer of one electron to the Fe^{IV} of the ferryl species. The carbocation is then quenched by the properly positioned carboxylate group of the glutamate or aspartate residue to form the ester linkage. The structure of the modified heme has been confirmed with the solved x-ray crystal structure of myeloperoxidase (12). Strong evidence has been presented for a heme carbocation intermediate in the mechanism of covalent bond formation in CYP4B1 (5) and CYP4A3 (3), but the preceding steps have not yet been elucidated for the CYP4 enzymes or the mammalian peroxidases.

To generate the proposed methyl carbocation, heme oxidation is required. A heme iron oxidant, such as the iron-oxo species does not seem suitable for direct oxidation of the methyl group because of large distance from the iron on the rigid heme plane. Therefore, it is more likely that a protein residue functions as an oxidized intermediate. Based on a homology model of LPO, it has been proposed that the carboxylate groups that eventually form the ester linkage are themselves initially oxidized, and are responsible for subsequent hydrogen atom abstraction from the heme methyl groups (10). Additional evidence for the oxidation of carboxylic acids has recently been demonstrated with horseradish peroxidase in the presence of acetate and H_2O_2 (13). Existence of a carboxyl radical intermediate was deduced from the formation of ester-linked heme adducts and the production of 8-hydroxymethylheme. From mutagenesis studies in CYP4B1 (5) and CYP4A3 (3), we can conclude that a carboxylate functional group on the I-helix is required for heme activation. The CYP4B1 E310D mutant maintains the ability to form a heme methyl carbocation, demonstrated by hydroxymethylheme production, whereas E310A, E310G, and E310Q mutants show no indication of heme modification. The Glu310 carboxylate in the wild-type CYP4B1 enzyme is, therefore, important in heme activation, as well as quenching the carbocation to forge the ester linkage. This carboxylate, positioned on the I-helix above the heme (6), may ultimately become the oxidized intermediate that subsequently removes electrons from the C-5 methyl group of heme. A Glu310 carboxyl radical intermediate in CYP4B1 is consistent with all current experimental data.

The mechanism for the generation of the carboxyl radical has not been elucidated, but proposals have been made based on other oxidations performed by the peroxidases. In the mechanism proposed for LPO, compound I oxidizes a carboxylic acid side chain by removing one electron, to produce a carboxyl radical (10). Oxidation by compound I was suggested because typical peroxidase reactions involve single electron oxidations of substrates. The P450 iron-oxo species, which is the equivalent of Compound I in the

mammalian peroxidases, potentially may catalyze the initial oxidation of the carboxylate, but the involvement of precursor P450 oxidants should also be considered

Reaction of *m*-CPBA with CYP2B4 has been reported to result in formation of a heme-ester adduct (14). These authors suggested that the *m*-chlorobenzoyloxy radical was first generated by homolytic scission of a perester oxygen-oxygen bond, followed by addition to the porphyrin, although structural characterization of the heme adduct was not complete. If this reaction does occur as suggested by the authors, then it is possible that homolytic oxygen-oxygen bond scission of a perester plays a role in the mechanism for covalent heme attachment in CYP4B1. It is conceivable that a perester could be formed in CYP4 enzymes by initial attack of the nucleophilic peroxo-iron oxidant on the carboxylate of the suitably positioned glutamic acid. The peroxo-iron species has been implicated in the terminal step of steroid demethylation catalyzed by P450 aromatase (15) and the deformylation of a variety of aldehyde substrates by CYP2B4 (16, 17). In these reactions the peroxo-iron oxidant attacks the aldehyde carbonyl to form a peroxyhemiacetal intermediate, followed by homolytic oxygen-oxygen bond scission to give compound II and a carbon-centered radical. The nucleophilic peroxo-iron species, by analogy, could react with a carboxylate group to produce a perester intermediate, followed by homolytic scission to produce a Compound II equivalent, and a carboxyl radical.

Consideration of the reaction between *m*-CPBA and CYP2B4 prompts us to ask if the presumptive carboxyl radical is formed from homolytic scission of the oxygen-oxygen bond in a perester, instead of radical abstraction by the iron oxo-species as previously proposed for the mammalian peroxidases. The experiments presented in this chapter will first test the validity of the ester linkage formed from CYP2B4 and *m*-CPBA by confirming the structure of the modified heme. Secondly, we will distinguish between mechanisms representing hydrogen abstraction by the typical iron-oxo oxidant and a homolytic mechanism involving initial attack of the peroxo-iron oxidant on the carboxylate. Previous experiments involving incorporation of the oxygen of H_2^{18}O into

the modified heme of CYP4B1 E310D provided evidence for a carbocation intermediate, but gave no indication of the preceding mechanistic steps (5). By labeling the carboxylates of glutamic acid of CYP4B1 with ^{18}O and monitoring the incorporation of oxygen into the heme ester linkage, we will shed light on the nature of the oxidized intermediates involved in formation of the heme methyl carbocation in the CYP4 proteins. Insight into the mechanism for this series of oxidations will provide us with a better understanding of the versatile chemistry conducted by P450s.

4.2 Experimental Procedures

Materials.

The following chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO): DLPC, TFA, urea, SnCl_2 , and all amino acids and nucleic acids used in minimal media preparation. 1,2,4,5-Tetramethoxybenzene was synthesized according to a published procedure (18). Trypsin was purchased from Promega (Madison WI). Pyridine- d_5 and H_2^{18}O were purchased from Cambridge Isotope Laboratories (Andover, MA).

CYP enzymes and co-enzymes.

Recombinant rabbit CYP4B1 was cloned, expressed and purified as described previously by Cheesman *et al.* (19). The gene was amplified and cloned a second time because of a PCR error, translated to D253G, that was discovered in the original clone. The E310D mutant was cloned into pCWori+ in the same manner, using the mutant gene in PFastBac as the template (5). CYP2B4 was cloned into pCWori+ expression vector, expressed, and purified as described previously for CYP4B1 (19). Rat cytochrome b_5 and P450 reductase were expressed and purified as described previously (20, 21).

Incubation of CYP2B4 with m-CPBA.

Incubation mixtures contained 40 nmol CYP2B4, 200 μg DLPC, in 2 ml of 100 mM KPi buffer. Tetramethoxybenzene was added as a substrate that could be oxidized to a

relatively unreactive radical and, in fact, increased the heme adduct yield ten-fold. Thirty equivalents of *m*-CPBA were added to start the reaction. This ratio was determined to maximize the heme adduct yield; additional oxidant led to the disappearance of heme (determined by absorption at 400 nm). The reaction was allowed to proceed at 37°C for 30 min. Modified heme from 15 incubations was extracted for 2D NMR analysis.

NMR and High-Resolution MS of Heme Adduct.

The reaction was quenched with equal volumes of 16 M urea and TFA. The heme adduct was then extracted twice with 3 ml of ether, washed with 3 ml of water, and then 3 ml of a saturated NaCl solution. The extracts were evaporated to approximately 200 μ l, at which point 200 μ l of MeOH was added. When the volume was again reduced to 200 μ l, removing the majority of the ether, the sample was injected onto a 10 $\times$ 250 mm semi-preparative 5 μ m C-4 column (Vydac, Hesperia, CA) for purification of the heme adduct. HPLC-UV analysis was performed on a Shimadzu instrument consisting of two LC-10ADvp pumps, an SPD-M10Avp UV-Vis detector, an SCL-10Avp controller, and an SIL-10ADvp autosampler (Shimadzu Scientific Instruments, Inc., Columbia, MD). During analysis, data were collected using EZSTART chromatography software (v 7.2, Shimadzu Scientific Instruments, Inc.) running on Windows 2000. Initial conditions were 60% Solution A (0.05% TFA in H₂O) and 40% Solution B (0.05% TFA in acetonitrile) at a flow rate of 3 ml/min. The linear gradient increased B from 40-50% between 0 and 15 min. A second gradient was then initiated that increased B to 100% over 5 min, followed by an isocratic period for an additional 2 min. The total run time was 30 min. UV analysis was performed at a wavelength of 400 nm. Under these conditions, heme eluted at ~11.4 min and the *m*-CPBA heme adduct eluted at ~14.6 min. The HPLC fractions were collected from approximately 15 injections, lyophilized, and then dissolved in pyridine-d₅. Approximately 5 mg of SnCl₂ was added to reduce the heme and then the sample was transferred to a NMR tube, flushed with argon, and sealed.

NMR data were acquired at 500 MHz on a Varian Inova 500 spectrometer equipped with an actively shielded z-axis gradient and a triple resonance probe. All experiments

were conducted at 25°C. 1D ^1H NMR spectra were acquired with a spectral width of 8000 Hz using 8192 acquired points. The ^1H ROESY data set included an 8000 Hz spectral width, 320 transients, 128 increments, and 8192 points along F_2 . ROESY experiments were acquired with a mixing time of 300 ms. 2D data sets were processed using MestRe-C software (version 3.5.9, <http://www.mestrec.com>).

High resolution MS experiments to confirm the elemental composition of the heme adduct generated from CYP2B4 was carried out using a PE Biosystems Mariner ESI-TOF mass spectrometer (PE Biosystems, San Jose, CA). Sample was introduced in a methanol solvent delivery system with an integrated syringe infusion pump and a Rheodyne 7125i injector (Rheodyne, Inc., Cotati, CA). Data was collected and analyzed by Windows NT based PE Biosystem Mariner Control[®] software and Biospectrometry Data Explorer[®] 3.0 software.

Labeling Glutamate and Aspartate.

To uniformly label the carboxylate oxygens of glutamate with ^{18}O , 900 mg of glutamic acid hydrochloride was dissolved in 3.6 mL of H_2^{18}O . The solution was heated at 90°C for 4 days at pH 1. To prevent back exchange, the solution was neutralized on ice with 580 μl of 15 M NaOH. The oxygen atoms in the carboxylates of aspartate were labeled similarly, by dissolving 600 mg of the amino acid in 3.6 mL of H_2^{18}O . The pH of the solution had to be adjusted by adding 440 μl of concentrated HCl. After heating for 4 days, the aspartic acid solution was neutralized on ice with 540 μL of 15 M NaOH. The water was evaporated from the dicarboxylic acids using a Rotovap. Either glutamic acid or aspartic acid was then dissolved in 8 ml of water and 1 mL of 1 M NaHCO_3 was added to facilitate solubilization. The two solutions were then sterile filtered with a 0.2 μm filter. Exchange of the label into the carboxylic acids was determined by mass analysis on a Micromass Quattro II tandem quadrupole mass spectrometer (Micromass, Ltd., Manchester, U.K.) coupled to a Shimadzu LC. Each amino acid was diluted 1:10,000 for analysis. A volume of 5 μl was injected into a mobile phase consisting of 50:50

methanol:water at 100 μ L/min. The mass spectrometer was run in negative electrospray ionization mode at a cone voltage of 40 V, source block temperature of 150°C, and a desolvation temperature of 380°C. Data analysis was carried out using Windows NT based Micromass MassLynxNT[®] 3.2 software. Ions spanning the isotopic ranges were monitored with single ion monitoring (SIM), m/z 144-158 for glutamate and m/z 130-144 for aspartate.

Expression of CYP4B1 with labeled glutamic and aspartic acid.

CYP4B1 wild-type and CYP4B1 E310D were expressed in growth media containing defined amounts of salts, nucleic acid, and amino acids, as described previously by Muchmore *et al.* (22). In addition, the media was supplemented with 250 μ L of trace elements solution (23). For the wild-type enzyme, 200 μ L of an overnight culture in Luria-Bertani (LB) media was used to inoculate 200 mL of the complete media in a 2.8 L Fernbach flask and 1 mL of the labeled glutamate solution was added. For the E310D mutant, 300 μ L of culture was used to inoculate 300 mL of media and 2 mL of the labeled aspartate solution was added. These cultures were allowed to shake at 180 rpm at 37°C for approximately 4 hr. At this time, IPTG (1 mM), δ -ALA (0.5 mM), and ¹⁸O-glutamic acid (200 mg) or ¹⁸O-glutamic acid (150 mg) were added. The temperature was reduced to 27°C and the shaking speed was reduced to 140 rpm. The remaining glutamic acid (700 mg) or aspartic acid (450 mg) solutions were added to the appropriate culture at regular intervals over the 48 hr growth period. Control cultures for each enzyme were prepared exactly the same except that unlabeled amino acid was added. All proteins were purified as previously described on a nickel column (19). CYP4B1 wild-type and E310D were not purified on the hydroxyapatite column because of the limited quantities of protein. The yield was between 15 and 18 nmols for all proteins.

Hydroxymethylheme Mass Analysis.

Modified heme was released from wild-type CYP4B1 by hydrolyzing with 0.25 M NaOH for 15 min. The sample was then neutralized with HCl for mass analysis. The

E310D mutant did not require prior treatment since the modified heme is not covalently bound. The hydrolyzed hydroxymethylhemes were analyzed on a Micromass Quattro II tandem quadrupole mass spectrometer (Micromass, Ltd., Manchester, U.K.) coupled to a Shimadzu LC instrument similar to that described above. The hemes were separated from the protein on a POROS R2 column. Initial conditions were 80% Solution A (0.05% TFA in H₂O) and 20% Solution B (0.05% TFA in acetonitrile) at a flow rate of 1 ml/min. The linear gradient increased B from 20-95% between 0 and 15 min. Hydroxymethylheme eluted at 7.3 min and heme eluted at 8.5 min. Ions spanning the isotopic ranges were monitored in SIM mode, m/z 655-665 for the heme-acetonitrile complex and m/z 671-681 for the hydroxymethylheme-acetonitrile complex.

CYP4B1 digest and LCMS analysis.

Approximately 1 nmol of each protein was diluted 1:4 with 50 mM NH₄HCO₃ (pH 7.8) and 1% trypsin was added. The digest was allowed to proceed for 2 hr at 37°C. Peptides were chromatographed on a C18 column (Vydac), using the LCMS system described above. Initial conditions were 95% Solution A (0.05% TFA in H₂O) and 5% Solution B (0.05% TFA in acetonitrile) at a flow rate of 0.3 ml/min. The linear gradient increased B from 5-65% between 0 and 20 min. The mass spectrometer was run in electrospray ionization (ESI) mode at a cone voltage of 45 V, source block temperature of 150 °C, and a desolvation temperature of 380 °C. Ions spanning the isotopic ranges of 14 peptides were monitored in SIM mode. Data analysis was carried out using Windows NT based Micromass MassLynxNT[®] 3.2 software. The tryptic peptides that were used for the calculation of label incorporation were analyzed in triplicate.

4.3 Results

*Characterization of Heme Adduct Generated With CYP2B4 and *m*-CPBA.*

The heme adduct generated in incubations with CYP2B4, utilizing *m*-CPBA as an artificial oxidant, has previously been identified solely by nominal mass. To pursue this reaction as a possible model mechanism for the covalent attachment of heme to CYP4B1,

structural confirmation was required. Approximately 1 μmol of purified recombinant CYP2B4 was reacted with 30 equivalents of *m*-CPBA. Tetramethoxybenzene was included in the incubation mixture to serve as a substrate that could undergo one electron, and this increased yields of the heme adduct by 10-fold. A sample of the purified heme was subjected to high resolution mass spectrometry to confirm the elemental composition of the heme adduct. The observed mass of 770.1623, and isotope distribution agreed well with the predicted mass, m/z 770.1590, and isotope distribution (Figure 4.1).

For NMR analysis, the collected HPLC fractions were lyophilized, dissolved in pyridine- d_5 , and finally reduced with SnCl_2 . The 1D NMR spectrum is shown in Figure 4.2A, and for comparison, the 1D NMR spectrum for heme is shown in Figure 4.2B. Assignments were made based on chemical shift values and ROE interactions (Figure 4.3). The 1D spectrum shows that the γ -meso proton signal is no longer observed, indicating that the chlorobenzoyloxy group is bound at this position. Other evidence supporting this structure includes altered chemical shifts in the nearby protons on the C-8 methyl, C-5 methyl, α -propionates, and β -propionates. Two new signals are observed at 7.7 ppm and 9.0 ppm, which are likely due to the protons on the chlorobenzoyloxy group. These two signals have a large ROE interaction with each other. The signal at 9.0 ppm also shows interactions with the α -propionates and β -propionates, again suggesting the heme adduct is formed at the γ -meso position. A signal due to the third proton on the chlorobenzoyloxy group is not observed, but is likely at the same chemical shift as one of the other two protons on the ring. A similar 1D spectrum was obtained for γ -benzoyloxyprotoporphyrin-IX dimethyl ester in CDCl_3 (24). The signal for *o*-ArH is reported to be at 8.70 ppm, *p*-ArH at 7.88, and *m*-ArH at 7.75. These chemical shifts will be affected by the iron atom in the heme and the presence of a chlorine atom on the aromatic ring.

Source of Oxygen in the Ester Linkage between CYP4B1 and Heme.

Various mechanisms for the covalent attachment of heme can be envisioned in which the oxygen in the ester linkage derives from molecular oxygen, water, or the carboxylates of Glu310. To trace the source of the oxygen in the CYP4B1 ester link, the carboxylates of glutamate were labeled with a heavy isotope and then the hydroxymethylheme, released upon hydrolysis, was analyzed for ^{18}O incorporation. The labeled glutamate was generated by incubating glutamate hydrochloride at 90°C , in H_2^{18}O . After 4 days, the solution was neutralized and analyzed by LCMS for ^{18}O incorporation into glutamate. Unlabeled glutamate had a molecular ion of m/z 146, whereas the labeled glutamate sample showed masses at m/z 150, m/z 152, and m/z 154, representing a mixture of species with multiple ^{18}O oxygen atoms (Figure 4.4A). After accounting for the natural abundance of isotopes, which were based on ion intensities of unlabeled glutamate, the mole percent of each species was calculated (Table 4.1). Overall, $89.0 \pm 0.23\%$ of the oxygen atoms were labeled with the heavy isotope. Recombinant CYP4B1 was expressed in *E. coli*., in a defined medium of salts, nucleic acids, amino acids, vitamins, and minerals. Natural glutamate was omitted from the defined media and replaced with glutamate with ^{18}O -labeled carboxylates. Following purification on a nickel column, both labeled and unlabeled proteins were digested with trypsin and analyzed by LCMS. Fourteen peptides in the control protein digest were identified based on predicted masses and isotopic distribution (Table 4.2). Fortunately, four of these peptides contained glutamic acid residues. The peptide containing Glu310 could be observed, but did not ionize well enough to be analyzed for heavy isotope incorporation. Thirteen ions, spanning the possible isotopic distribution patterns, were monitored for each of the 14 peptides with SIM. After subtracting the intensities due to the natural abundance of isotopes, which were based on ion intensities of the peptides from the unlabeled protein, the mole percent of each species was calculated (Table 4.2). Tryptic peptides T17, T18, T33, and T50 all show significant incorporation of three ^{18}O atoms, which is expected for peptides that contain the labeled glutamic acid. By examining the mole percent of 1 or 2 labels in the remaining peptides, it is clear that the ^{18}O label was transferred to other amino acids during biosynthesis. This phenomenon is not surprising since glutamic acid

is known to be central to the production of several other amino acids. Considerable incorporation of 1 label or 2 labels is observed in peptides containing proline, glutamine, aspartic acid, and asparagine. Tryptic peptides T17 and T18 were used to calculate the mole percent of labeled oxygen atoms because these two peptides contain the fewest additional residues that appear to be labeled with one or two ^{18}O atoms. Mass spectra for peptides T17 and T18 are shown in figures 4.5A and 4.5B. The most accurate method for determining the mole percent of labeled oxygen atoms in this case is to assume that the label distribution in the glutamic acid in the protein is no different than the original amino acid that was added to the expression system, and base the calculation on the mole percent of the triple-labeled species. This method minimizes the contribution of label in other amino acids to the calculated value. When this method is applied to the T17 and T18 peptides, values of $13.4 \pm 0.1\%$ and $10.6 \pm 0.6\%$ ^{18}O incorporation are calculated, respectively (Table 4.3). There is a possibility that the distribution of the label was not maintained during expression. This could occur *via* conversion of amino acids by biosynthetic enzymes. To account for this occurrence, the contribution from 1, 2, and 3 labels was summed to calculate the total mole percent of label in the peptide. This method produces slightly higher values for the T17 ($16.0 \pm 0.2\%$ mole %) and T18 ($14.3 \pm 1.7\%$ mole %) peptides, but reflects the maximum possible levels of label incorporation.

The hydroxymethylheme from CYP4B1 wild-type was analyzed for heavy isotope incorporation after hydrolyzing the ester bond with base (Figure 4.6A). The natural isotope abundance was subtracted from the ion intensities of the labeled hydroxymethylheme-acetonitrile complex and the mole percent of label was calculated from the additional M+2 ion intensity. The hydroxymethylheme derived from CYP4B1, with ^{18}O -labeled glutamate, contained $12.8 \pm 1.9\%$ mole % of the heavy isotope. To ensure that the heavy isotope was not incorporated into the carboxylates of the hemes, free unmodified heme was analyzed by the same method. No incorporation was observed, as evidenced by the calculated value of $-0.7 \pm 1.4\%$ mole percent.

Source of Oxygen in Monohydroxylated Heme Released from the E310D Mutant.

Previous experiments established that the oxygen atom in the hydroxymethylheme from E310D can be derived from water. To confirm this result a complementary experiment was designed to show that water does not originate from Asp310 in the mutant. The labeled aspartate was generated by heating at 90°C in H₂¹⁸O at pH 1. After 4 days, the solution was neutralized and analyzed by LCMS for ¹⁸O incorporation into aspartate. Unlabeled aspartate had a molecular ion of *m/z* 132, whereas the labeled aspartate sample showed masses at *m/z* 134, *m/z* 136, and *m/z* 138, representing a mixture of species with multiple ¹⁸O oxygen atoms (Figure 4.4B). After accounting for the natural abundance of isotopes, which were based on ion intensities of unlabeled aspartate, the mole percent of each species was calculated (Table 1). Overall, 83.4 ± 0.3 % of the oxygen atoms were labeled with the heavy isotope. CYP4B1 E310D was expressed as described for the wild-type enzyme, except that the labeled aspartate was added in place of glutamate. The enzyme was purified, digested with trypsin, and analyzed by LCMS for isotope incorporation. The mole percent of label incorporation was calculated from the ion intensities for the same 14 peptides (Table 4.2). Four of the observed peptides, T8, T13, T23, and T33, contained a single aspartic acid residue. Evidence for incorporation of labeled aspartate can be seen in the triple-labeled species for each of these peptides with ranges from 2.1 to 3.9 mole percent. It is also apparent that the heavy isotope in this experiment has been transferred to other amino acids, similar to the results seen for the labeled glutamate. Peptides containing asparagines, glutamate, and glutamine appear to contain some heavy isotope, as evidenced by the increase in ion intensity of the M+2 and M+4 peaks. Tryptic peptides T8 and T13 were used to calculate the mole percent of labeled oxygen atoms because these two peptides contain the fewest additional residues that appear to be labeled with one or two ¹⁸O atoms. The mass spectra for these two peptides are shown in figures 4.5C and 4.5D. Based solely on the heavy isotope levels in the triple-labeled species, the T8 peptide is 6.0 ± 0.9 mole % labeled and the T13 peptide is 6.5 ± 0.5 mole % labeled. When including the 1-, 2-, and 3-labeled species for these peptides, the T8 peptide is 7.6 ± 0.6 mole % labeled and the T13 peptide is 8.2 ± 0.3 mole % labeled (Table 4.3). Calculations for label content in

hydroxymethylheme ($0.09 \pm 0.7\%$) and unmodified heme ($-0.1 \pm 0.4\%$) from CYP4B1 E310D indicate that no heavy isotope was incorporated (Figure 4.6B).

4.4 Discussion

Mutagenesis studies with CYP4B1 and CYP4A3 have helped to identify mechanistic steps in the formation of the ester linkage that binds heme covalently to P450, including the generation of a heme methyl carbocation and a role for a properly positioned carboxylate in heme activation. When the catalytically relevant glutamate is mutated in either enzyme to an aspartate residue, unbound hydroxymethylheme is produced (3, 5). These data suggest that a heme methyl carbocation has been quenched by water instead of an appropriately positioned carboxylic acid, thereby forming the ester in the wild-type enzyme. Further evidence for this reaction was obtained when CYP4B1 E310D was expressed in media containing H_2^{18}O , purified, and analyzed by mass spectrometry. The label was found to be incorporated into the hydroxymethylheme, suggesting that water had attacked the preformed carbocation during the autocatalytic cycle (5). A heme methyl carbocation has previously been inferred in the reaction of phenylhydrazine and hydrogen peroxide with horseradish peroxidase (25). In this reaction, a phenyl radical abstracts an electron from the methyl group on the C-8 position of heme and the carbocation is formed by subsequent intramolecular electron transfer. Metabolic reactions conducted in H_2^{18}O demonstrate that water attacks the carbocation to form 8-hydroxymethylheme (25). The generation of unbound hydroxymethylheme in the CYP4 mutants suggests either that the catalytic cycle has been disrupted and ester formation between a hydroxymethylheme precursor and glutamate has been prevented, or that the modified heme is a side-product of the normal reaction.

There is strong evidence to support the role of a carboxylate side chain in heme activation, which initiates formation of the heme methyl carbocation and subsequent ester bond formation between heme and protein of CYP4 enzymes and the mammalian peroxidases. As noted above, the CYP4B1 E310D and CYP4A3 E318D mutants still

retain the ability to activate heme and form a methyl carbocation, where alanine or glutamine mutants at these positions do not. These results indicate that the carboxylate functional group is not only required for attacking the preformed carbocation in the wild-type enzyme, but is required for the formation of the heme methyl carbocation itself. To demonstrate that a suitably positioned carboxylate is the only requirement for heme activation Colas *et al.* showed that HRP, a plant peroxidase, can be engineered to covalently bind heme (26). The authors inserted a glutamic acid into the active site of HRP, *via* mutagenesis at Phe⁴¹. When HRP F41E was reacted with H₂O₂, the enzyme covalently linked nearly all of the heme to the protein, presumably by forming an ester bond between Glu⁴¹ and the 3-methyl group. Another mutant, S73E, generated 8-hydroxymethyl heme during catalysis, similarly to the production of 5-hydroxymethylheme in CYP4B1 E310D CYP4A3 E318D. Mutagenesis studies in the active site of the bacterial enzyme P450_{cam}, were successful in demonstrating that a properly positioned carboxylate can provoke the covalent attachment of heme in P450 that normally binds heme with strictly non-covalent interactions (27). When P450_{cam} G248E was reacted with camphor, the P450 covalently bound 10% of the heme at the 5-methyl position. These experiments indicate that no unique structural features in the active site, in addition to the carboxylate side chain, are required for heme activation.

The functional role of the carboxylate in heme activation has been proposed to be a transient oxidized intermediate that can in turn oxidize the heme methyl group to a methyl radical. In LPO it is believed that compound I is responsible for the first single-electron oxidation of the carboxylate (10). This hypothesis has also been extended to the CYP4 enzymes since a P450 compound I species is believed to be the primary oxidant (Figure 4.8). An alternate mechanism for the generation of the carboxylate radical can be considered, in which the peroxo-iron intermediate of the P450 catalytic cycle is responsible for the initial oxidation. This concept originates from the proposed mechanism for the reaction between CYP2B4 and the artificial oxidant *m*-CPBA (14). Kuo *et al.* first described a chlorobenzoyloxy heme adduct in this reaction and suggested that the product must be generated *via* homolytic scission of the oxygen-oxygen bond of

a perester intermediate. Because this ester-linked heme adduct appears to be a model reaction for the heme-protein crosslink in the CYP4 family enzymes, we decided to further characterize the structure in search of mechanistic analogies.

High resolution mass spectrometry and 2D NMR data were used to identify the heme adduct generated when *m*-CPBA reacts with CYP2B4. The results demonstrate that the chlorobenzoyloxy group, derived from the organic peroxide, is bound at the γ -meso position of the heme group. This finding was anticipated because previous experiments have shown that when 3-phenylpropionaldehyde is bioactivated by CYP2B4, a phenylpropional heme adduct is generated at this same position (14). This phenomenon is thought to be a result of steric constraints in the CYP4B1 active site, which leave only the γ -meso heme edge exposed to the substrate. Because the chlorobenzoyloxy group is not bound to an unactivated heme moiety, such as the 5-methyl group, this reaction only poorly models the recombination event in the proposed mechanism for covalent heme attachment in CYP4B1. A simpler mechanism can be envisioned for this reaction wherein the chlorobenzoyloxy radical recombines with an electron on the heme periphery, easily accessible in the conjugated π -system (Figure 4.7). Conjugation would be restored to the system by the intramolecular oxidation of the heme periphery by compound I and the simultaneous reduction of oxygen to water.

Even though the site of attachment on the heme differs between the two systems, the reaction between *m*-CPBA and CYP2B4 does contribute to exploration of the mechanism of heme modification in CYP4B1, as it presents the possibility that a perester intermediate could be involved in the formation of the carboxylate radical. A mechanism can be envisioned in which the peroxo-iron intermediate in the P450 cycle acts as a nucleophilic oxidant and attacks the carboxylate group of Glu310. Subsequent homolytic scission of the oxygen-oxygen bond would result in a carboxylate protein radical and the equivalent of compound I (Figure 4.9). This mechanism could also be applied to the mammalian peroxidases, since hydrogen peroxide addition results in the same initial peroxo-iron species intermediate, preceding the formation of compound I. A mechanism

involving initial oxidation by the peroxo-iron species is appealing because the extended [Fe-O-O⁻] oxidant has the ability to reach a glutamate that is more distant, when compared to the [Fe=O] oxene species. The I-helix would have to be quite flexible to allow the carboxylate group of Glu310 to reach the iron center, as well as the 5-methyl carbon on the heme periphery.

Insight into the mechanism of covalent bond formation can be gained by determining the origin of the oxygen in the ester linkage. An experiment was designed in which the carboxylates of glutamate residues in CYP4B1 were labeled globally with ¹⁸O. Following expression and purification, the amount of label in the glutamate carboxylates of CYP4B1 was determined by the increase in intensity of the [M+6] ions of tryptic peptides. Based on the T17 peptide, the carboxylates were 13.4 ± 0.12 % labeled, and based on the T18 peptide the carboxylates were 10.6 ± 0.6 % labeled. Incorporation of the heavy isotope into the ester bond was evaluated by releasing 5-methylhydroxyheme from the enzyme by base hydrolysis and analyzing the heme for an increase in the [M+2] ion intensity. We found that the modified heme was labeled with 12.8 ± 1.9 % ¹⁸O, representing the mole percentage of label in the ester bond. Calculations from this data indicate stoichiometric (96 ± 15 % based on T17, and 121 ± 20 % based on T18) transfer of the label from Glu310 to the ester bond. The fact that all of the oxygen atoms in the heme originate from the glutamate carboxylates, suggests a mechanism involving initial oxidation by the oxo-iron species (Figure 4.8). If the peroxo-iron species formed the perester intermediate, resulting in homolytic scission of the oxygen-oxygen bond, one of the oxygen atoms in the carboxylate would derive from iron bound dioxygen (Figure 4.9). This reaction pathway would result in the transfer of 50% of the heavy isotope into the heme, or 67% if a tetrahedral intermediate existed on the carbon of the carboxylate. Therefore, these data are inconsistent with a mechanism involving a perester intermediate. We can also rule out a mechanism in which CYP4B1 first hydroxylates the heme and subsequently forms the ester bond *via* acid-base catalysis. The oxygen atom in the hydroxymethylheme could originate from water, as described previously for the E310D mutant, or from the iron-bound oxygen, as observed in typical P450 substrate

hydroxylations. In either case, there would be no incorporation of the heavy label from the carboxylates of Glu310.

The same experimental technique was then applied to the E310D mutant of CYP4B1 in order to confirm previous results, which showed that the oxygen atom in hydroxymethylheme originates from water. Between 6.0 ± 0.9 and 6.5 ± 0.5 mole percent of the carboxylates of aspartic acid residues in CYP4B1 E310D were globally labeled with ^{18}O . Mass analysis of the free hydroxymethylheme from this protein revealed essentially no transfer of the heavy isotope label from Asp310. This result was anticipated because the complementary experiment with H_2^{18}O reveals quantitative incorporation of the heavy isotope.

In conclusion, studies with the labeled glutamate residues demonstrate that 100% of oxygen from Glu310 is transferred to the ester linkage, suggesting that homolytic scission of a perester oxygen-oxygen bond is not a viable mechanism. However, our data are consistent with a mechanism in which the typical oxo-iron P450 oxidant removes an electron from the glutamic acid carboxylate, followed by hydrogen abstraction from the C-5 position of the heme group. Intramolecular electron transfer within the heme forms the methyl carbocation, which is then quenched by Glu310 to form the ester bond (Figure 4.8). These experiments provide the first glance at the unique oxidative chemistry that takes place in covalent heme attachment to CYP4B1.

Table 4.1. Mole % of glutamate and aspartate labeled with ^{18}O . A total of four labels can be incorporated into each dicarboxylic acid. The total contribution is calculated by assuming that the mole percent of the fully labeled amino acid contributes 100%, the amino acid containing 3 labels contributes 75 %, etc.

	Mole % labeled				% Total contribution
	1 label	2 label	3 label	4 label	
Glutamate	0.11	2.79	23.11	62.98	88.98
Aspartate	0.40	5.72	29.07	48.18	83.38

Table 4.2. Calculated percentage of label incorporation for each of 14 identified CYP4B1 wild-type and E310D tryptic peptides.

CYP4B1	Peptide	Sequence	Mole % labeled				
			1 label	2 label	3 label	4 label	5 label
WT ¹⁸ O-Glu	T6	LAR	1.0	0.0	0.0		
	T8	TGSLDK	6.7	3.9	0.3	0.0	
	T13	GLLVLDGPK	16.5	6.5	1.7	0.9	
	T14	WFQHR	8.0	12.3	0.2		
	T17	IMLEK	5.7	7.0	9.5		
	T18	WEK	6.8	6.9	7.5		
	T23	GDSGLNHR	11.7	2.9	0.2	0.0	0.0
	T27	FLR	0.9	0.2	0.0		
	T30	VIR	0.2	0.6	0.1		
	T33	AALWDEK	12.9	14.5	8.7	2.8	1.9
	T36	IQNR	11.6	14.4	1.2	-0.1	
	T50	FSPENSSGR	17.3	7.2	9.2	2.4	
	T55	LPIK	14.0	0.3	0.0		
	T56	LPQLVLR	18.5	10.3	1.7		
E310D ¹⁸ O-Asp	T6	LAR	0.8	0.7	0.0		
	T8	TGSLDK	6.7	2.7	3.4	0.9	
	T13	GLLVLDGPK	6.4	3.5	3.7	0.3	
	T14	WFQHR	7.5	0.8	-0.1		
	T17	IMLEK	7.9	0.5	0.0		
	T18	WEK	7.1	1.4	-0.1		
	T23	GDSGLNHR	6.1	4.1	2.1	1.9	1.8
	T27	FLR	1.1	0.5	0.1		
	T30	VIR	0.8	0.6	0.2		
	T33	AALWDEK	8.1	3.1	3.0	3.2	1.7
	T36	IQNR	8.4	6.2	1.9	0.5	
	T50	FSPENSSGR	11.1	6.1	2.6	0.5	
	T55	LPIK	2.6	-0.1	0.0		
	T56	LPQLVLR	8.4	1.1	-0.2		

Table 4.3. Calculated percentage of ^{18}O -labeled atoms in glutamic acid residues of CYP4B1 wild-type, aspartic acid residues of CYP4B1 E310D, and their respective modified hemes. The total calculated label content in glutamic acid and aspartic acid residues is based solely on the relative intensity of the triple labeled peptide containing these amino acids, assuming that the original glutamate or aspartate was not isotopically diluted.

CYP4B1	Peptide	Sequence	Total label based on 3-label content (%)	Label in hydroxy- methylheme (%)
WT ^{18}O -Glu	T17	IMLEK	13.4 ± 0.1	12.8 ± 1.9
	T18	WEK	10.6 ± 0.6	
E310D ^{18}O -Asp	T8	TGSLDK	6.0 ± 0.9	0.09 ± 0.7
	T13	GLLVLDGPK	6.5 ± 0.5	

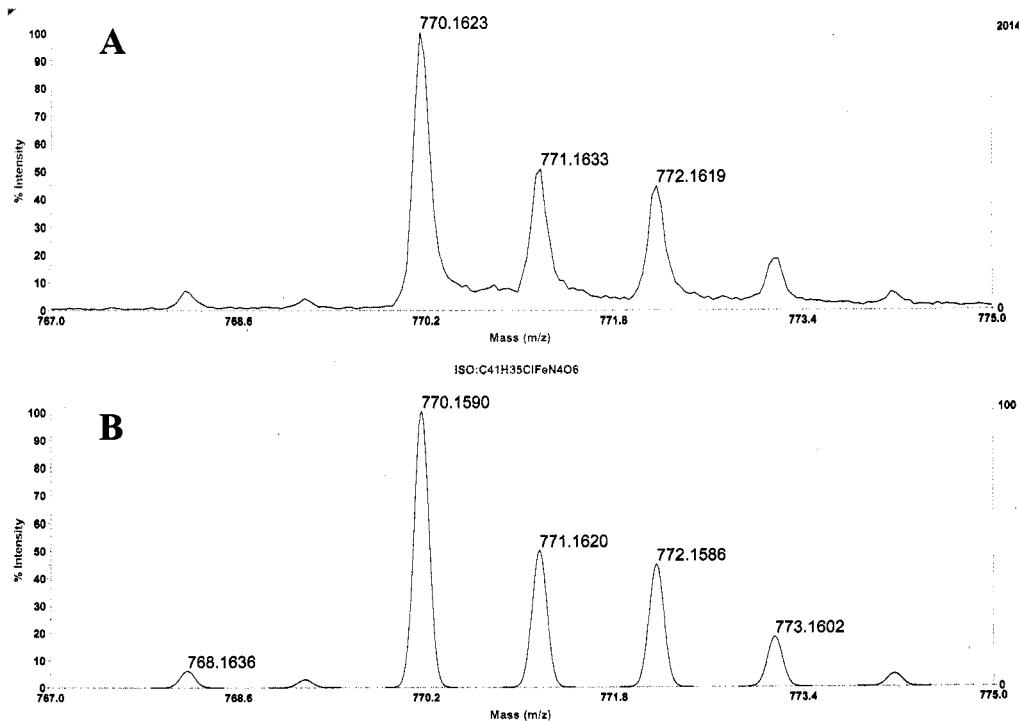


Figure 4.1. High resolution mass spectrum of heme adduct formed in the reaction of CYP2B4 with *m*-CPBA (A) and the predicted mass and isotope pattern (B).

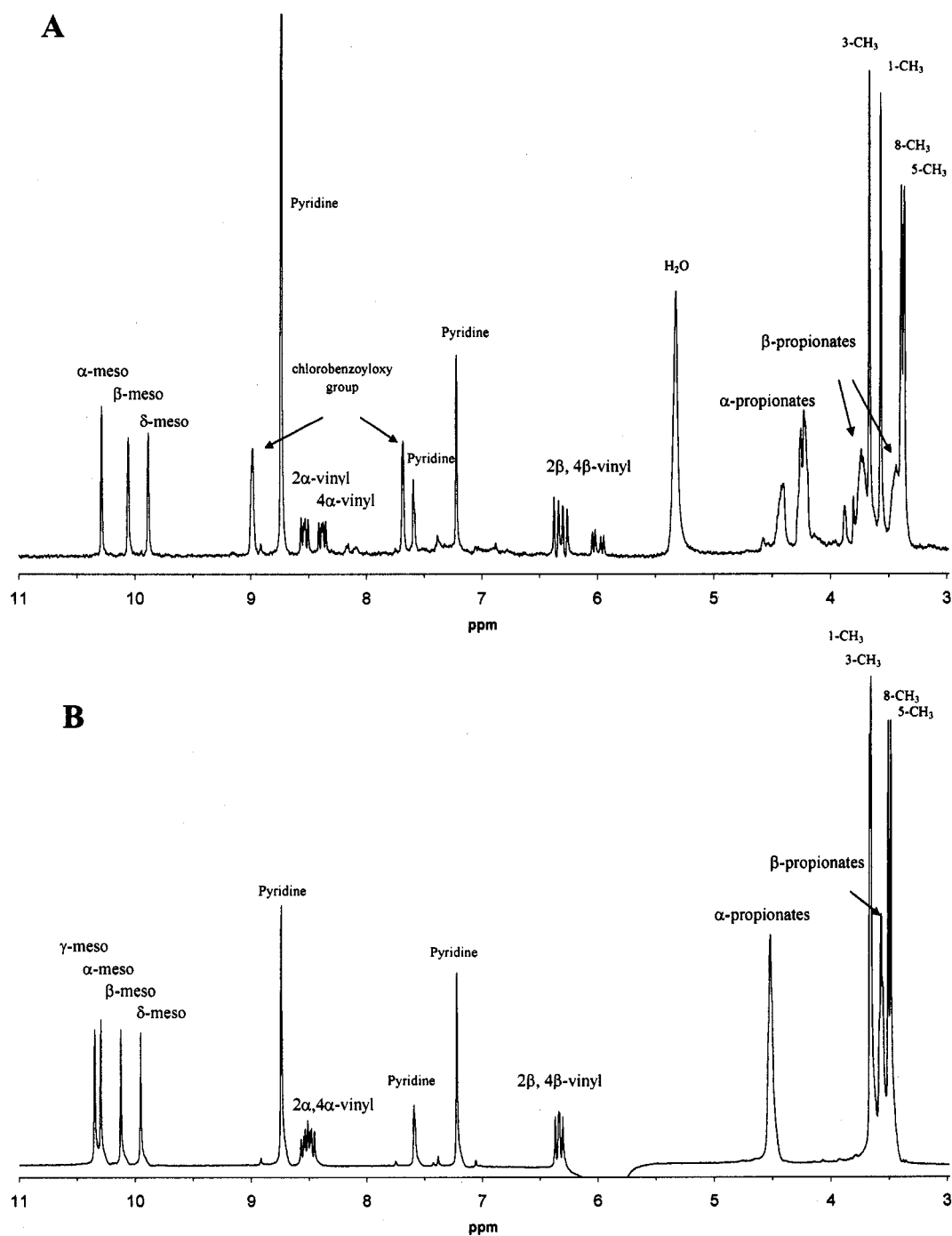


Figure 4.2. 1D NMR spectrum of (A) heme adduct formed in the reaction of CYP2B4 with *m*-CPBA and (B) heme.

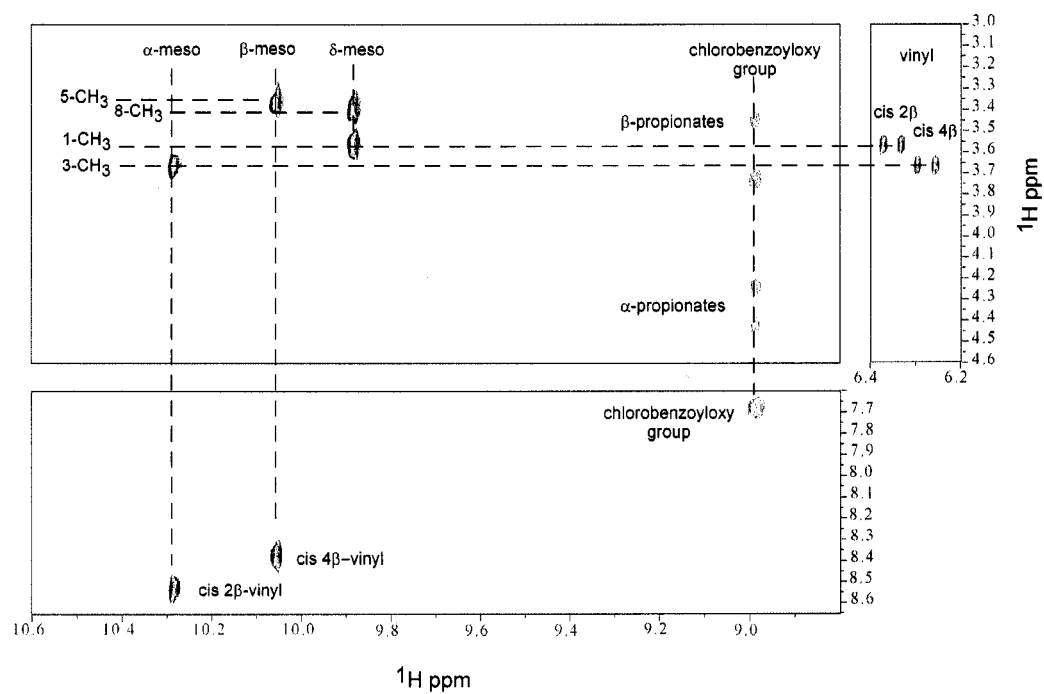


Figure 4.3. 2D NMR ROESY spectrum of heme adduct formed in the reaction of CYP2B4 with *m*-CPBA.

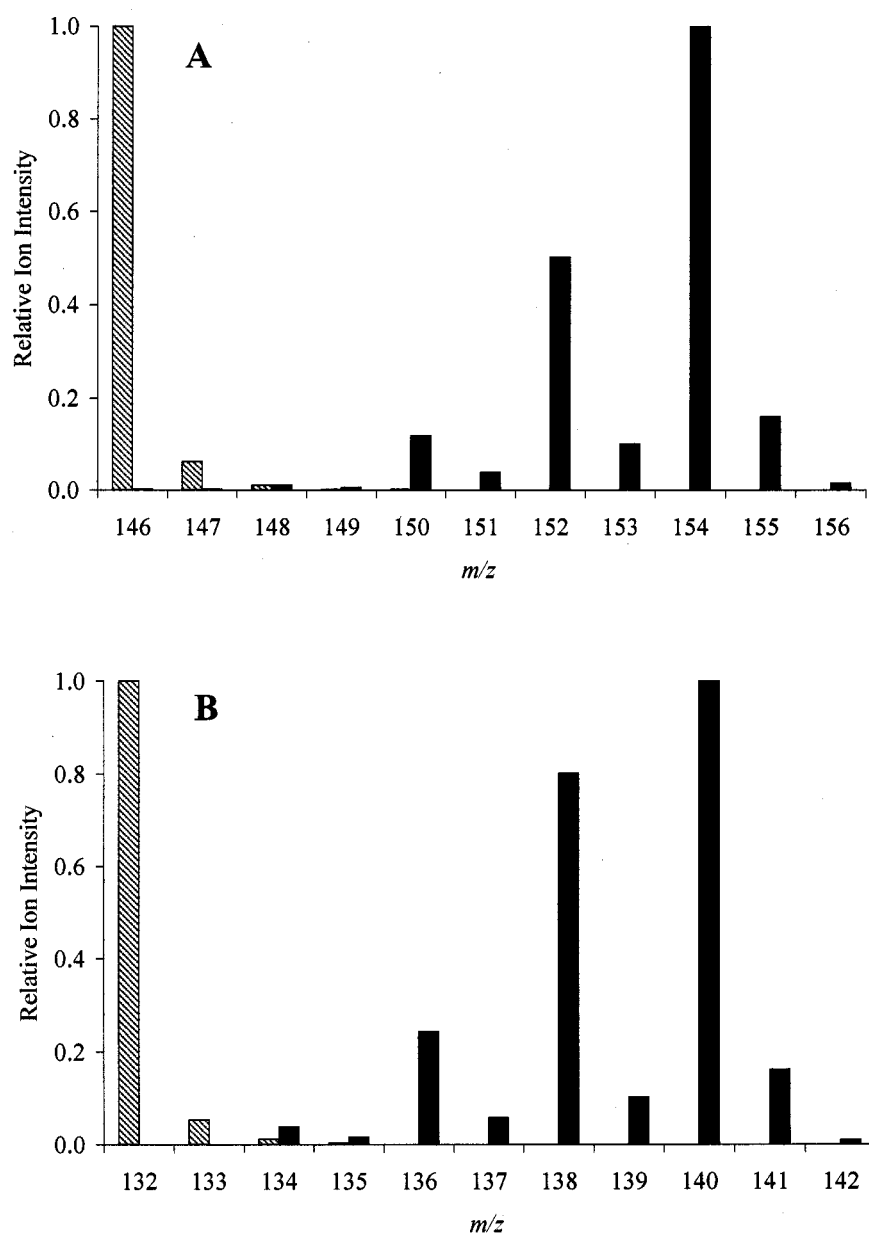


Figure 4.4. Mass spectra of ^{18}O -labeled glutamic and aspartic acid. Relative ion intensities for isotopic masses are shown for glutamic acid (A) and aspartic acid (B). The spectrum for the unlabeled amino acid is shaded grey and the spectrum for the labeled amino acid is shaded black.

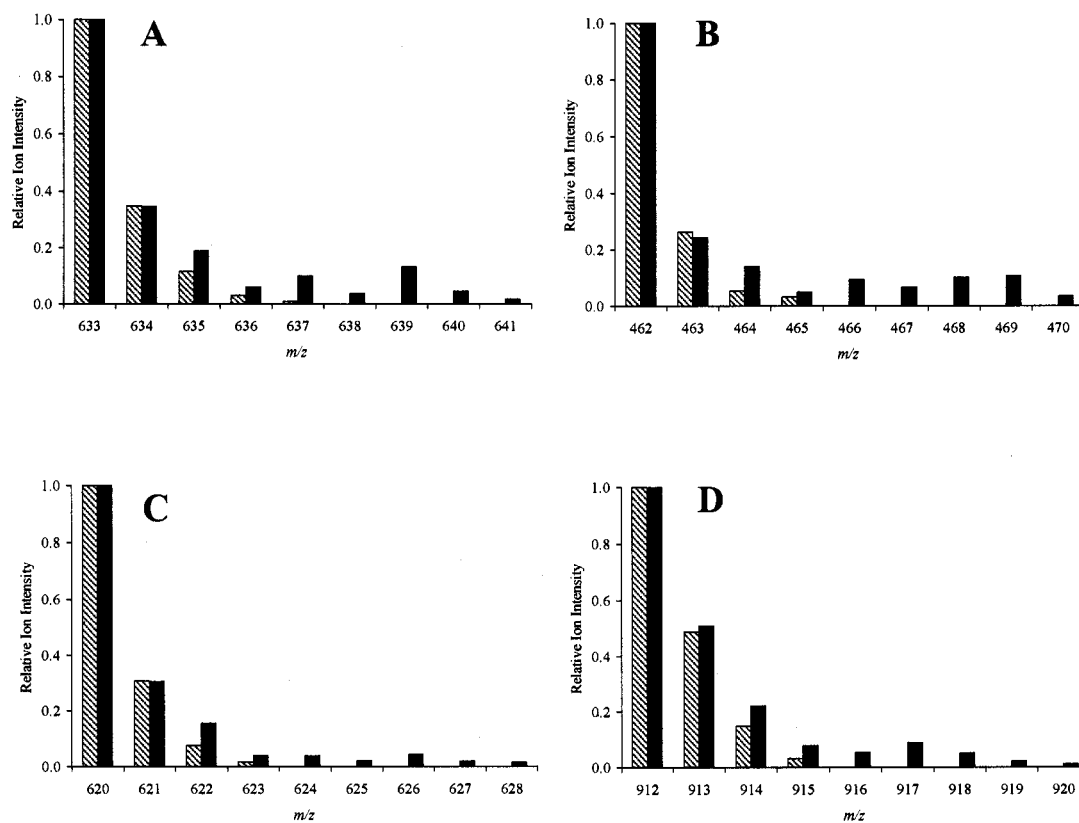


Figure 4.5. Mass spectra of tryptic peptides from wild-type CYP4B1 expressed with ^{18}O -labeled glutamic and CYP4B1 E310D expressed with ^{18}O -labeled aspartic acid. Relative ion intensities for isotopic masses are shown for peptides T17 (A) and T18 (B) from wild-type CYP4B1 and for peptides T8 (C) and T13 (D) from CYP4B1 E310D. The spectrum for the unlabeled peptide is shaded grey and the spectrum for the labeled peptide is shaded black.

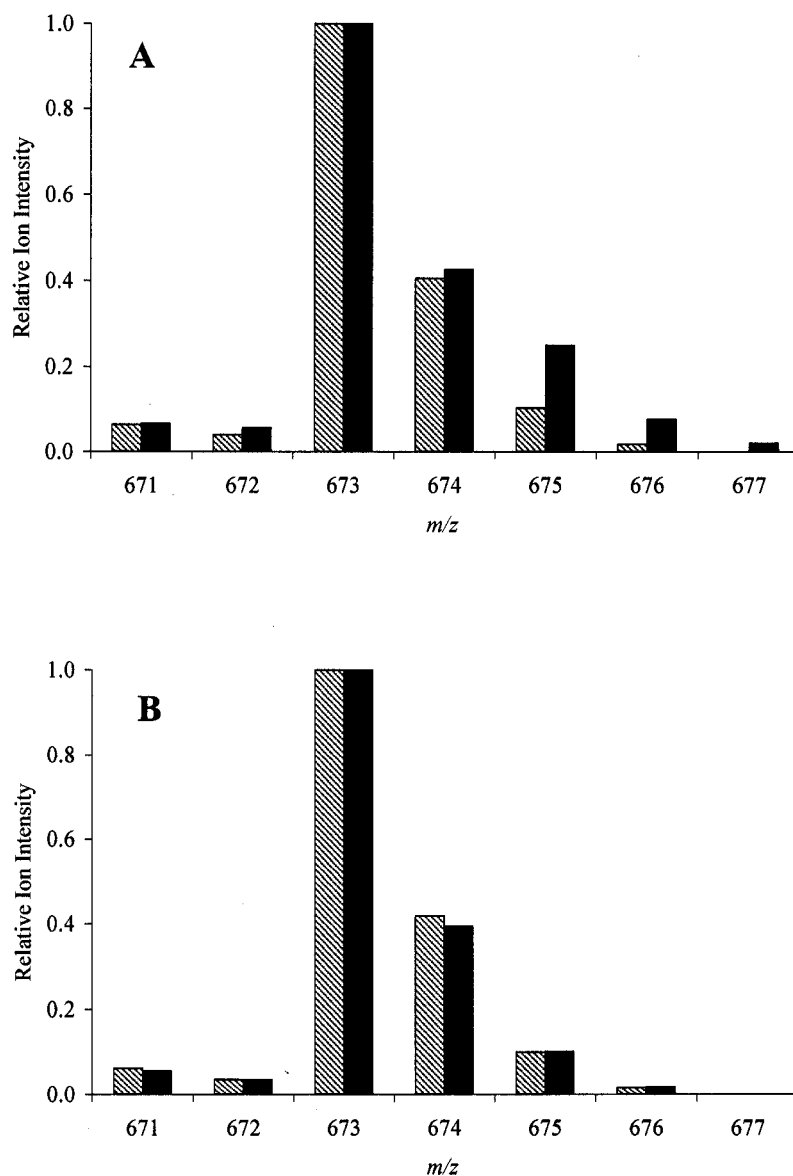


Figure 4.6. Mass spectra of hydroxymethylheme-acetonitrile complex from wild-type CYP4B1 expressed with ^{18}O -labeled glutamic and CYP4B1 E310D expressed with ^{18}O -labeled aspartic acid. Relative ion intensities for isotopic masses are shown for hydroxymethylheme following base-hydrolysis of wild-type CYP4B1 (A) and unbound hydroxymethylheme from CYP4B1 E310D (B). The spectrum for the unlabeled amino acid is shaded grey and the spectrum for the labeled amino acid is shaded black.

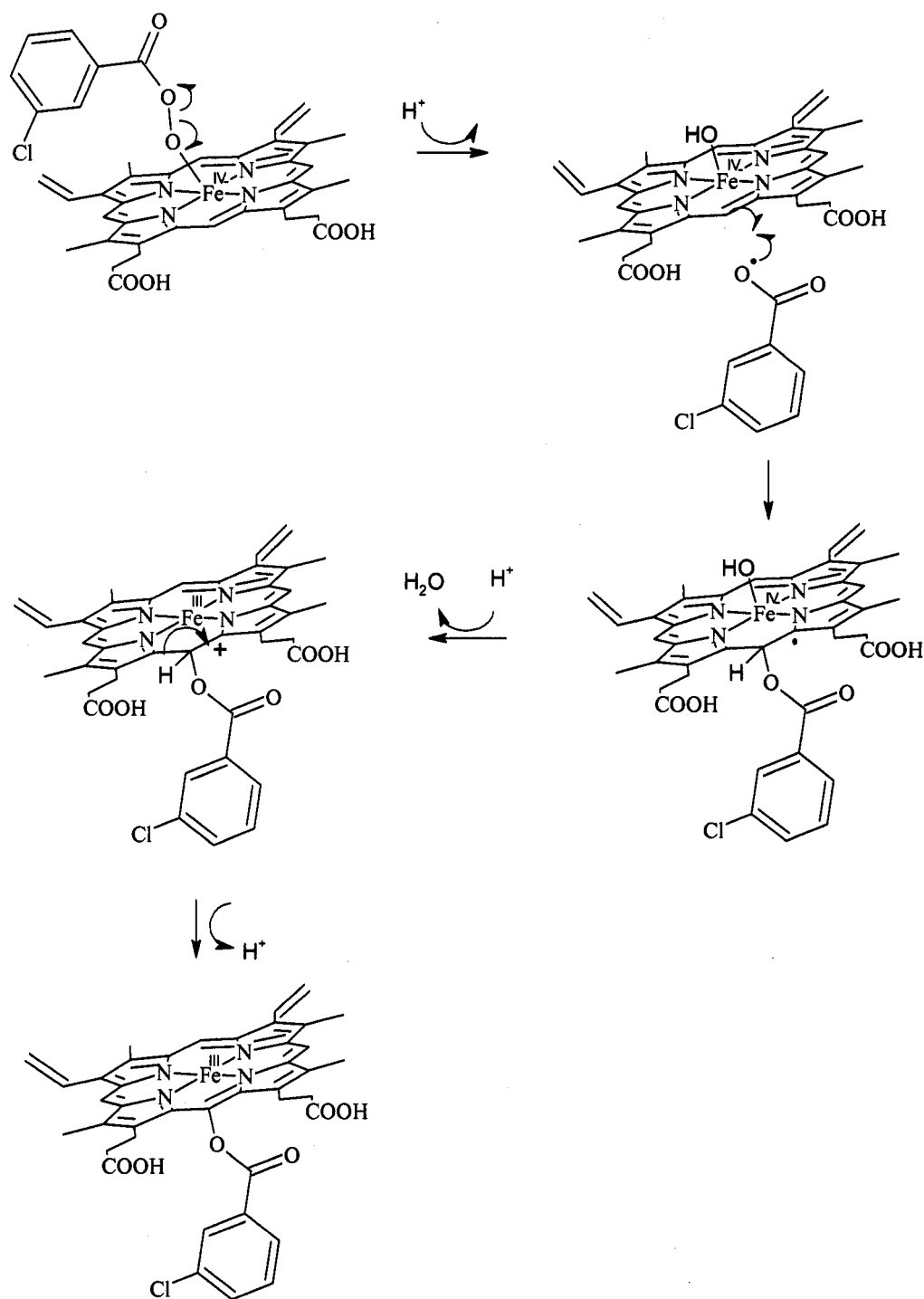


Figure 4.7. Proposed reaction of *m*-CPBA with the heme group of CYP2B4.

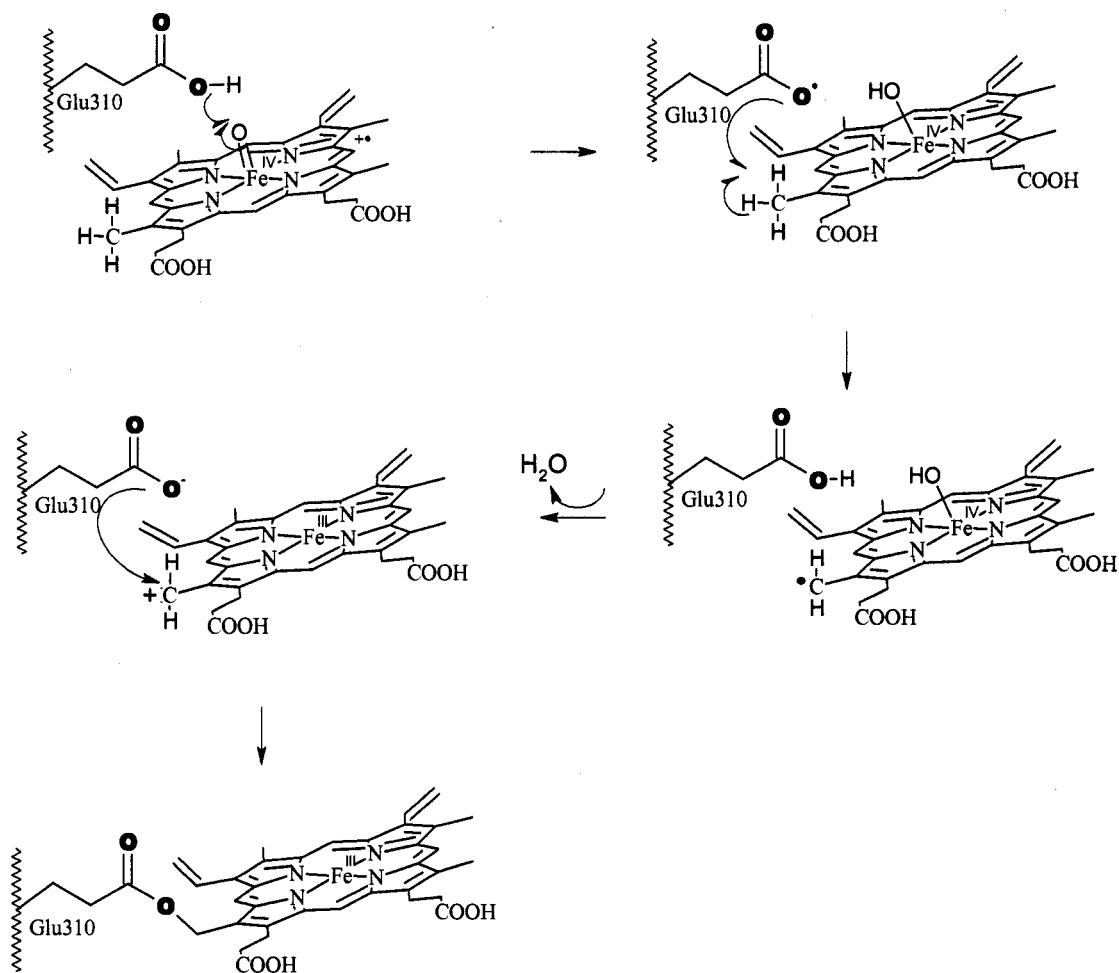


Figure 4.8. Mechanism for covalent heme attachment involving hydrogen abstraction by the iron-oxo species. The labeled oxygens (bold face print) are traced throughout the mechanism. This mechanism would result in 100% incorporation of glutamate oxygen into the ester linkage.

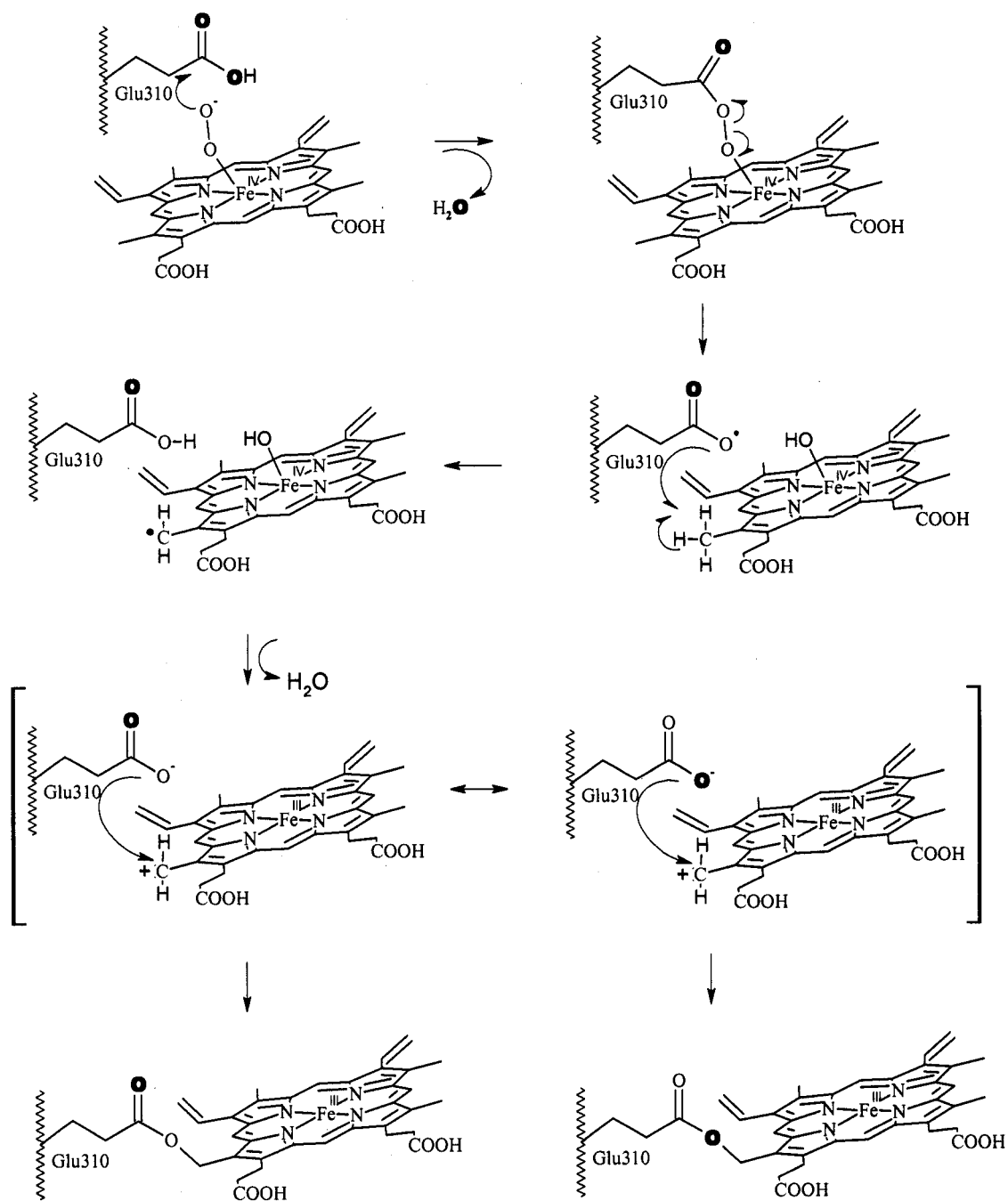


Figure 4.9. Mechanism for covalent heme attachment involving homolytic scission of a perester oxygen-oxygen bond. The labeled oxygens (bold face print) are traced throughout the mechanism. This mechanism would result in 50%, and possibly 67% if a tetrahedral intermediate exists, incorporation of glutamate oxygen into the ester linkage.

4.5 Notes to Chapter 4

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Chapter 5

Functional Consequences of Covalently Attached Heme in CYP4B1

5.1 Potential Functional Effects of Covalent Heme Attachment

The covalent linkage between the heme and protein is unique to the CYP4 family of P450s and so it is logical to suspect that the structural alteration could have functional consequences that are exclusive to this family of P450s. The most distinctive activity of the CYP4 enzymes is preferential hydroxylation at the thermodynamically disfavored ω position of fatty acids, leukotrienes, and hydrocarbons, yet this activity does not depend upon a covalently linked heme. When heme covalent binding in CYP4A3 is eliminated by the E310A mutation, the enzyme is still able to preferentially hydroxylate lauric acid at the ω position, albeit to a lesser extent than the wild type enzyme (1). It is interesting to note that rat CYP4F5 and CYP4F6, which both have a Gly at this corresponding position and do not covalently bind heme, predominantly oxidize leukotriene B₄ at the ω -1 and ω -2 positions (2, 3). Whether this influence on metabolism is due to the covalent link itself or the steric restraints in this region of the active site, remain to be determined. Amongst CYP4 proteins, CYP4B1 is metabolically interesting because of its unique ability to bioactivate a diverse range of protoxins, such as 4-ipomeanol (IPO), 2-aminofluorene, and 3-methylindole. Therefore, CYP4B1 allows the opportunity to study potential associations between bioactivation of xenobiotics and the presence of the covalent bond between heme and protein.

All of the identified mammalian peroxidases also covalently attach the heme group, but no functional advantage has yet been demonstrated for these enzymes compared to plant and fungal peroxidases that do not possess this linkage. Peroxidases, with or without the bound heme, retain the ability to oxidize phenols, iodide ion, and thiocyanate (4). Even the more difficult oxidation of bromine can be carried out by plant and fungal peroxidases that lack the heme covalent bond. Colas *et al.* have suggested that the

covalent heme attachment could assist in binding of the water molecule that is produced by heterolytic scission of H_2O_2 in the formation of compound I (4). The positioning of this water molecule is suspected to be important in differentiating peroxidase and catalase activities. These authors have also suggested that the covalent heme attachment could distort the porphyrin framework, which might facilitate spin-spin coupling between the compound I porphyrin π radical cation and oxoferryl moiety (4). This phenomenon is proposed to occur with the deformation of heme by two cysteinevinyl links in cytochrome *c* (5, 6). In the solved crystal structure for myeloperoxidase, the heme is, in fact, bowed by the ester and cysteinevinyl covalent linkages so that the A and C rings are tilted toward the distal side (7).

Other logical functional consequences of covalent heme attachment include enhanced heme retention and increased structural stability. Although heme bound strictly by non-covalent interactions in bacterial P450s can be exchanged, this process requires stringent conditions such as acid/acetone precipitation and acid/butanone extraction (8, 9). It is generally believed that mammalian P450s require heme for correct folding and heme cannot be removed or reintroduced to the active site. Yet, it is possible that the CYP4 enzymes are an exception, and might easily lose their heme without the additional covalent bond. The ester linkage could also potentially stabilize the enzyme by adding additional structural support, thereby preventing unfolding and/or conversion to P420. Examples of intramolecular bonds that offer such stability include the CYP2C9 R108H mutant in which the histidine side chain ligates with the heme iron and increases the half-life at 48 °C from 16 to 177 min (10). Also, P450 BioI has an intramolecular disulfide bond that stabilizes the enzyme in the presence of the fatty acid palmitoleate (11). The T_m , as determined by differential scanning calorimetry, decreased by 4.3 °C when the disulfide linkage was reduced with DTT. CYP4B1, with the ester linkage between Glu310 on the I-helix and the heme, may conceivably provide similar thermostability.

Therefore the objectives of this chapter were to probe the influence of the heme covalent link in CYP4B1, by comparing the following physical and functional differences between the wild type enzyme and the E310 mutants:

- 1) Enzyme stability, as assessed by monitoring the depletion of P450 content at elevated temperatures;
- 2) Heme retention, as assessed by comparing the relative heme and protein concentrations;
- 3) Regioselectivity of hydrocarbon hydroxylation and chain length preference, as assessed by examining the rates and ratios of hydroxylated metabolites produced from heptane, octane, nonane, and decane;
- 4) Xenobiotic bioactivation, as assessed by quantitating the reactive intermediate formed from 4-ipomeanol, for which a new assay was developed;
- 5) Peroxidase activity, as assessed by measuring the single electron oxidations of pyrogallol to purpurogallin and 1,2,4,5-tetramethoxybenzene to a cation radical (Figure 5.1).

5.2 Experimental Procedures

CYP4B1 expression and purification.

The CYP4B1 E310 mutant genes were amplified from the pFastBac constructs made by Zheng *et al.* (12) and cloned into the pcWori+ expression vector (13). CYP4B1 wild type and E310 mutants were recombinantly expressed in *E. coli.* and purified as described by Cheesman *e. al.* (13).

Haemochromogen assay.

Pyridine haemochromogen assays were performed according to Falk (14). Approximately 1 nmol of P450 in 1.5 mL of buffer was combined with 333 μ L of pyridine and 167 μ L of 1 M NaOH. The solution was split equally into 2 cuvettes. One sample was oxidized with 25 μ M $K_3Fe(CN)_6$ and a baseline spectrum from 500 nm to 600 nm was acquired. The second sample was reduced with dithionite and the difference

between the absorbance at 557 nm and the absorbance at 541 nm was measured. The concentration of heme was determined using an extinction coefficient of $20.7 \text{ mM}^{-1} \text{ cm}^{-1}$.

Lowry Protein Assay.

Protein concentrations were determined using the Folin phenol reagent as described by Lowry *et al.* (15). A standard curve was generated using 0-100 μg of BSA and approximately 50 μg of each P450 mutant was analyzed.

Thermostability Measurements.

CYP4B1 stocks were diluted to 1 μM with storage buffer (100 mM KPi, 20% glycerol). One mL of solution was dispensed into a 1.7 mL cuvette, bubbled with CO, and a baseline spectrum was acquired on an Aminco DW2 Conversion spectrophotometer. The P450 was then reduced with a few grains of dithionate and methyl viologen was added to a final concentration of 1.2 μM to facilitate complete reduction. Spectra were then acquired every 2 min for 40 min, from 390 to 500 nm at 37 °C. Half-lives for the thermally induced conversion of P450 to P420 were determined according to first-order decay kinetics. The half life based on the decrease of the peak at 450 nm for the ferrous CO-bound complex was determined to be identical to the half life of the ferric form. The ferric CO-bound form was used for these experiments because this method uses much less enzyme and more data points could be collected.

Hydrocarbon Metabolism.

Incubations contained 100 pmol P450, 200 pmol cytochrome P450 reductase, 100 pmol cytochrome b_5 , 20 μg DLPC, 1 mM substrate, in 900 μL of 100 mM KPi buffer. The reaction was initiated with the addition of 100 μL of 10 mM NADPH. The reaction was allowed to proceed for 30 minutes at 37°C, and then quenched with 100 μL of a 15% solution of zinc sulfate. Following the addition of internal standard (10 μL of 1 mM 1-octanol in methanol) the metabolites were vortex extracted in 250 μL of ethyl acetate.

The samples were centrifuged to break the emulsion and then 80 μ l of the organic layer was removed and added to 20 μ l of BSTFA.

Metabolites generated in the incubations with CYP4B1 and various hydrocarbons were quantified using a Shimadzu GC/MS (Shimadzu Scientific Instruments, Inc., Columbia, MD). The gas chromatograph (GC-17A) was equipped with a 30 m $\times$ 250 μ m capillary XTI-5 column (Restek Corp., Bellefonte, PA). The carrier gas was helium with a constant flow rate of 1 ml/min. The injection port temperature was 250 $^{\circ}$ C. Hydrocarbon metabolites were injected at an initial temperature of 50 $^{\circ}$ C. After 1 min, the oven temperature was raised at 5 $^{\circ}$ C/min to 115 $^{\circ}$ C. Finally, the temperature was raised rapidly to 280 $^{\circ}$ C. The following hydrocarbon metabolites were observed and confirmed with authentic chemical standards: 1-heptanol (10.97 min, m/z 173), 2-heptanol (9.20 min, m/z 117), 3-heptanol (9.04 min, m/z 131), 1-octanol (13.62 min, m/z 187), 2-octanol (11.75 min, m/z 117), 3-octanol (11.50 min, m/z 131), 1-nonanol (16.24 min, m/z 201), 2-nonanol (14.38 min, m/z 117), 3-nonanol (14.09 min, m/z 131), 1-decanol (18.82 min, m/z 215), 2-decanol (16.99 min, m/z 117), and 3-decanol (16.67 min, m/z 131). 1-Octanol was used as an internal standard for heptane and decane assays, 1-nonanol was used for the octane assay, and 1-decanol was used for nonane assay. Standard deviations were determined from triplicate incubations for each enzyme assayed.

Spectral Binding with Octane.

Purified CYP4B1 (0.5 μ M) was first reconstituted with 50 μ M extruded liposomes of dilauryl phosphatidylcholine (DLPC). CYP4B1, or the E310A mutant, were then placed into two separate cuvettes containing 50 mM potassium phosphate at pH 7.4 in a final volume of 0.9 ml. After allowing the sample and reference cuvettes to reach room temperature, octane was titrated in from 0 to 5 μ M. Difference spectra were obtained over the range 350 to 500 nm using an Aminco DW2 double beam spectrometer. Data was fit with SigmaPlot 8.02 using the following function for the enzyme-substrate complex ($\Delta\text{Abs}_{(390-422)}$) versus octane concentration, as described by Segel (16):

$$[ES] = \frac{([E]_t + [S]_t + K_s) - \sqrt{([E]_t + [S]_t + K_s)^2 - 4[E]_t[S]_t}}{2}$$

4-Ipomeanol (IPO) bioactivation activity.

Purified P450 (25 pmol) was reconstituted with P450 reductase (50 pmol), DLPC (20 µg), and cytochrome b₅ (25 pmol) in 100 mM potassium phosphate buffer (pH 7.4). *N*-acetyl cysteine and *N*-acetyl lysine were then introduced in 100 mM potassium phosphate buffer to a final concentration of 10 mM each in order to trap the reactive enedial intermediate (17). IPO was then added to a final concentration of 1 mM (using a 10 mM stock solution made in 10% methanol). The total volume of the reconstituted mixture was 0.5 mL. Metabolic activity was initiated by the addition of NADPH (1 mM final concentration), and incubations allowed to proceed at 37 °C for 30 min. Reactions were terminated by the addition of 50 µl of a 15% zinc sulfate. The samples were then spun in a centrifuge (12,000 g) for 2 min to pellet the precipitated protein. The supernatant was then transferred to a 1.5 mL vial and aliquots (100 µL) were analyzed by HPLC. HPLC-UV analysis was performed on a Shimadzu instrument consisting of two LC-10ADvp pumps, an SPD-M10Avp UV-Vis detector, an SCL-10Avp controller, and an SIL-10ADvp autosampler (Shimadzu Scientific Instruments, Inc., Columbia, MD). During analysis, data were collected using EZSTART chromatography software (v 7.2, Shimadzu Scientific Instruments, Inc.) running on Windows 2000. A 4.0 × 125 mm Nucleosil 5 µm C-18 column (Agilent Technologies, Palo Alto, CA) served as the stationary phase. Initial conditions were 98% Solution A (0.05% TFA in H₂O) and 2% Solution B (0.05% TFA in acetonitrile) at a flow rate of 1 mL/min. The linear gradient increased B from 2-42% between 0 and 20 min. A second gradient was then initiated that increased B to 90% over 3 min, followed by an isocratic period for an additional 4 min. The total run time was 30 min. UV analysis was performed at a wavelength of 254 nm.

MS and NMR characterization of the NAC/NAL adduct.

LC/ESI-MS and LC/ESI-MS/MS analysis was performed using a Micromass Quattro II tandem quadrupole mass spectrometer (Micromass, Ltd., Manchester, U.K.) coupled to a Shimadzu LC instrument similar to that described above. The mass spectrometer was run in electrospray ionization (ESI) mode at a cone voltage of 25 V, source block temperature of 100 °C, and a desolvation temperature of 350 °C. In MS/MS mode, the collision energy was set to -30 V. Data analysis was carried out using Windows NT based Micromass MassLynxNT[®] 3.2 software.

In order to generate substantial quantities of the major NAC/NAL-IPO metabolite conjugate for NMR, high-resolution MS analysis and the quantitative assay, 20 CYP4B1 reactions were prepared as described above with the exception that each incubation contained 1 nmol of enzyme in a total incubation volume of 1 mL. Incubations were allowed to proceed for 2 hrs at 37 °C. The peak of interest, as determined by LC-MS/MS experiments, was collected over the course of 20 separate injections on a semi-preparative Beckman C-18 HPLC column (10 mm X 25 cm). Pooled fractions were frozen at -70 °C and lyophilized to remove the HPLC solvent. The resulting solid material was then dissolved in D₂O and re-lyophilized to help ensure removal of adventitious water and TFA. The NAC/NAL-IPO metabolite adduct was dissolved in 300 µl of *d*₆-DMSO, and TMS was added as an internal chemical shift reference. An aliquot of this sample was also used to generate high-resolution MS data to verify the elemental composition of the trapped metabolite. Following NMR analyses, the sample was re-purified by HPLC to remove the DMSO and recapture the metabolite.

High resolution MS experiments were carried out using a PE Biosystems Mariner ESI-TOF mass spectrometer (PE Biosystems, San Jose, CA). Sample was introduced in a methanol solvent delivery system with an integrated syringe infusion pump and a Rheodyne 7125i injector (Rheodyne, Inc., Cotati, CA). Data were collected and analyzed by Windows NT based PE Biosystem Mariner Control[®] software and Biospectrometry Data Explorer[®] 3.0 software.

NMR data were acquired at 500 MHz on a Varian Inova 500 spectrometer equipped with an actively shielded z-axis gradient and a triple resonance probe. All experiments were conducted at 25°C. 1D ^1H -NMR spectra were acquired with a spectral width of 8000 Hz using 4096 acquired points. ^1H - ^{13}C HSQC spectra of carbon-proton pairs were acquired using a spectral width of 8000 Hz and 128 points in F_1 and a spectral width of 8000 Hz and 512 points in F_2 . The ^1H ROESY data set included an 8000 Hz spectral width, 32 transients, 256 increments, and 4096 points along F_2 . ROESY experiments were acquired with a mixing time of 300 ms. 2D data sets were processed using MestRe-C software (version 3.5.9, <http://www.mestrec.com>).

IPO trapped metabolite standard curve.

For preparation of the quantitative assay standard curve, 2.70 mg of the lyophilized IPO metabolite adduct was weighed on an MT5 microbalance (Mettler Toledo, Switzerland) and dissolved in H_2O (1.034 mL) to make a 5 mM stock solution. The standard curve was subsequently prepared from diluted solutions ranging from 2.5 μM to 30 μM .

Pyrogallol oxidation.

CYP4B1 (500 pmol), cytochrome P450 reductase (1 nmol), 20 μg of DLPC, and 0.5% pyrogallol were combined in 500 μL of 100 mM KPi buffer. The absorbance at 420 nm relative to 700 nm was monitored until a background reaction rate could be measured. Then 0.025% H_2O_2 was added at 5 min and the rate of P450 dependent pyrogallol oxidation was measured by the increase in absorbance at 420 nm. Reactions were also attempted with cytochrome P450 reductase and NADPH substituting for H_2O_2 . The extinction coefficient for purpurogallin is $3.2 \text{ mM}^{-1} \text{ cm}^{-1}$.

1,2,4,5-Tetramethoxybenzene synthesis.

1,2,4,5-Tetramethoxybenzene was synthesized as previously described by Sato *et al.* (18). 2,5-Dihydroxy-1,4-benzoquinone (1.0 g) was stirred in CH_3OH (85 mL) with 3 mL

of conc. HCl at room temp overnight and the resulting yellow-brown solid was collected. The crude 2,5-dimethoxy-1,4-benzoquinone (1.0g) and $\text{Na}_2\text{S}_2\text{O}_4$ (2.0g) were stirred vigorously in boiling H_2O (10 mL) for 1 min. After cooling of the mixture to 4 °C a solid was formed, and was washed with H_2O . The crude 2, 5-dimethoxy-1,4-hydroquinone (0.5 g) was stirred in $\text{C}_2\text{H}_5\text{OH}$ (1.2 mL), with dimethyl sulfate (1.75 mL), $\text{Na}_2\text{S}_2\text{O}_4$ (50 mg), and NaOH (1.6 mL of a 12 N solution) for 1.5 h on ice. The mixture was warmed to 70-80 °C for 20 min, diluted with H_2O (5 mL), and cooled to 4 °C. The resulting 1,2,4,5-tetramethoxybenzene was collected and recrystallized from $\text{C}_2\text{H}_5\text{OH}$. The structure was confirmed by $^1\text{H-NMR}$: (CDCl_3) δ 3.87 (s, 12H), δ 6.63 (s, 2H) (Figure 5.2).

1,2,4,5-Tetramethoxybenzene oxidation.

CYP4B1 (1 nmol), cytochrome P450 reductase (2 nmol), 20 μg of DLPC, and 1 mM NADPH were combined in 500 μL of 100 mM KPi buffer. The reaction was initiated with the addition of 0.5 mM 1,2,4,5-tetramethoxybenzene. The absorbance from 400 to 500 nm was recorded every min and the reaction rate can be determined by the increase of absorbance at 450 nm using $\epsilon_{450} = 9800 \text{ M}^{-1}\text{cm}^{-1}$. Reactions were also attempted with 0.025% H_2O_2 substituting for cytochrome P450 reductase and NADPH.

5.3 Results

CYP4B1 Heme Content.

To determine if the covalent linkage affects the extent of heme incorporation into CYP4B1, the amount of protein as determined by the Lowry Assay, was compared to the concentration of heme as determined by the haemochromogen assay, for the wild type enzyme as well as the E310 mutants (Table 5.1). CYP4B1 wild type and the E310A and E310Q mutants were found to have similar levels of holoprotein (80-86%). Interestingly, the E310D mutant, which contains unbound 5-hydroxymethylheme, consisted of only 39% holoprotein. CYP4B1 wild type, E310A, and E310Q has a ratio of P450:P420 of 95:5 and the E310D mutant has a ratio of approximately 50:50 (data not shown). The

P450 concentration, based on the reduced CO-bound spectra, was similar to the calculated heme content for both CYP4B1 wild type and E310A (Table 5.1).

Thermostability.

The CYP4B1 ferrous CO-bound complex was incubated at 37 °C for 40 min and the half life was determined from the first order rate decay of the absorbance at 450 nm. The spectra for CYP4B1 wild type over the 40 min period is shown in Figure 5.3 and the decrease in spectral P450 for CYP4B1 wild type, as well as E310 mutants, is shown graphically in Figure 5.4. CYP4B1 E310A was determined to have a half life of 18 min, slightly longer than the 12 min half life observed for the wild-type enzyme (Table 5.2). In contrast, the absorbance at 450 nm decayed much more rapidly for the E310D (0.7 min) and E310Q (1.5 min) mutants. The increase in the 424 nm peak, for CYP4B1 wild type and E310 mutants, did not correlate well with the decrease in 450 nm absorbance, appearing to have an initial rapid rate followed by a second rate that is much slower than the decrease in absorbance at 450 nm. If the short half life for the decrease in 450 nm absorbance correlates with a decrease in activity, estimation of steady-state product formation would be greatly underestimated during typical 30 min metabolic assays. Therefore, reconstitution components were added sequentially to determine their effect on CYP4B1 thermostability. The addition of DLPC increased the half-life of CYP4B1 to 55 min, and the further addition of cytochrome P450 reductase reduced the half life back to 23 min. The most interesting finding was that the addition of substrate drastically stabilized CYP4B1 wild type, as well as all E310 mutants, with no observable loss of 450 nm absorbance over the duration of the experiment. The ferric form of CYP2C9 has previously been reported to be much more thermostable (10) with a half life of 16 min at 48 °C, even in the absence of ligand. This temperature completely abrogated CYP4B1 absorbance at 450 nm before the first time point. Therefore for comparison, CYP2C9 as well as CYP3A4 were subjected to this thermostability assay, which examines the ferrous CO complex. The absorbance at 450 nm for CYP2C9 did not decrease during the course of the experiment, indicating that this isoform is exceptionally thermostable as compared to CYP4B1. CYP3A4 was determined to have a half life of 21 min.

Metabolic Profiles for Hydrocarbon Metabolism.

The metabolism of heptane, octane, nonane, and decane by CYP4B1 and E310 mutants was analyzed to compare substrate chain length selectivity, as well as product regioselectivity. The results of this study are displayed numerically in Table 5.3 and graphically in Figure 5.5. CYP4B1 wild type activity and regioselectivity were similar to results previously reported (19). The total hydroxylation rates for CYP4B1 wild type and E310A were similar for octane (21 nmol/ nmol P450/ min versus 20 nmol/ nmol P450/ min), nonane (13 nmol/ nmol P450/ min versus 14 nmol/ nmol P450/ min), and decane (7 nmol/ nmol P450/ min versus 10 nmol/ nmol P450/ min), although the wild type enzyme showed a slightly higher rate for hydroxylation of heptane (26 nmol/ nmol P450/ min versus 16 nmol/ nmol P450/ min). The activities for E310D and E310Q were significantly less than the wild type enzyme for all hydrocarbon substrates analyzed (Table 5.3). The E310A mutant had less stringency for ω -hydroxylation for all substrates compared to wild type enzyme, whereas the E310D and E310Q mutants appear to be even more selective for terminal hydroxylation. As for chain length selectivity, the wild type enzyme preferred the short chain substrate, heptane, while E310A metabolized octane to a greater extent. Both E310D and E310Q preferred the medium chain hydrocarbons, octane and nonane. To address concerns about thermal inactivation, octane hydroxylation was measured every 5 min for 30 min for CYP4B1 and E310 mutants. Turnover was linear with respect to time for all enzymes, indicating that thermostability was not an issue during the 30 min incubation and at saturating substrate concentrations.

Binding Spectra with Octane.

When CYP4B1 wild type was titrated with octane, a characteristic type I spectra was observed with a λ_{max} at 390 nm and λ_{min} at 422 nm (Figure 5.6A). Saturation was reached after adding approximately one equivalent of octane, indicating that this hydrocarbon is a tight binding ligand (Figure 5.7). Typical hyperbolic binding kinetics could not be applied because that analysis relies on the assumption that the concentration

of free ligand is much greater than the concentration of ligand-protein complex. Instead, an equation was used that could more accurately determine the binding constants at high enzyme concentrations relative to the K_S . The E310A mutant was also titrated with octane to give an identical spectrum, although the absorbance intensity was slightly higher (Figure 5.6B). The binding constant for wild type and mutant were almost identical, with a K_S of 126 nM for CYP4B1 wild type and 125 nM for the E310A mutant. The E310D and E310Q mutants were not subjected to spectral binding studies because of the rapid depletion of active P450 observed at sub-saturating ligand concentrations.

Assay Development for 4-Ipomeanol Bioactivation. An IPO bioactivation assay was developed to quantify a reactive ene-dial intermediate, by trapping it with NAC and NAL. A major NADPH- and CYP4B1-dependent adduct was detected by LC-UV analysis that eluted at 14 min, while a second, minor adduct (t_r = 13 min) was also observed (Figure 5.8, upper panel). MS data from the major adduct (Figure 5.9A) showed ions of m/z 538 (pseudo-molecular ion), 520, 482 (base-peak), and 353. MS/MS evaluation of the base-peak ion at m/z 482 (Figure 5.9B) produced fragment ions at m/z 353, 271, 229, 210, 166, and 130 amu. Identical MS data was generated for the minor adduct, suggesting that the two adducts were isomers. The major reaction product accounted for 87% of total NAC/NAL-dependent peak area, and the minor product accounted for 13% (based on integration of m/z 482 ion current common to both products, shown in Figure 5.8, lower panel). Based on our findings and other work from the Peterson laboratory (20, 21), a NAC/NAL-IPO adduct consistent with the available data was proposed (Figure 5.9). Although a peak at m/z 500 representing $[M+H]^+$ was not observed in the Q1 mass spectrum (Figure 5.9A), the masses at m/z 538, 520, and 482 were assigned to $[M+K]^+$, $[M+K-H_2O]^+$, and $[MH-H_2O]^+$ ions, respectively. MS/MS analysis of the m/z 482 base-peak showed that the loss of 129 amu was a prominent peak in the spectrum (m/z 353), indicating cleavage adjacent to the cysteine sulfur atom. The peak at m/z 166 likely represented loss of the *N*-acetyl cysteine and *N*-acetyl lysine groups from the pyrrole structure. Formation of the m/z 271 ion could be attributed to loss of the IPO aliphatic side chain and the NAC moiety.

Additional evidence for the proposed pyrrole structure was obtained by high-resolution mass spectral analysis using a TOF/MS instrument, and by 2D-NMR. Firstly, the major trapped NAC/NAL-IPO conjugate gave three molecular species at m/z 522.1902, 538.1613, and 544.1694 that corresponded to $[M+Na]^+$, $[M+K]^+$, and $[M+2Na]^+$ ions, respectively. In each case, the experimentally determined mass differed from the theoretical mass for the proposed elemental composition by no more than 3.1 ppm. These data demonstrate that the identified adduct and the proposed structure possess the same molecular formula. Secondly, 1H -NMR analysis of the major putative NAC/NAL-IPO adduct (Figure 5.10) showed two resonances (δ 7.79 and 6.73 ppm) indicative of two pyrrole hydrogen atoms (Table 5.4). Also present in the spectrum were two singlets (δ 1.87 and 1.84 ppm) representing the two acetyl methyl groups ($-COCH_3$) expected from the NAC and NAL moieties. Resonances from the IPO side-chain were also present in the spectrum. The terminal methyl group hydrogen atoms adjacent to the hydroxyl group ($-CHOHCH_3$) appeared at δ 1.07 ppm as a doublet—almost identical to that in the unaltered IPO spectrum (Table 5.4). Protons α to the carbonyl functionality (δ 2.73 ppm, triplet) were slightly shifted relative to the IPO substrate, presumably due to the transformation of the furan functionality to a cysteinyl-substituted pyrrole. Other resonances in the spectrum appeared at chemical shifts consistent with those reported previously for NAC/NAL trapping of a reactive intermediate of furan itself (20, 21). Therefore, all signals observed in the NMR spectrum of the isolated adduct support the structure proposed in Figure 5.10. 2D NMR ROESY experiments allowed for confirmation of the previous assignments and identification of the position of adduct attachment to the pyrrole ring (Figure 5.10). The proton on the C3-pyrrole (6H) showed proximity to both protons on the ipomeanol side chain (4H) and protons on the cysteine chain (7_aH, 7_bH, 8H), but there were no interactions with protons on the lysine chain. The proton on the C5-pyrrole (5H) showed proximity to both protons on the ipomeanol side chain (4H) and protons on the lysine chain (10H, 11H, 12H). These interactions indicated that the two pyrrole protons were

arranged on either side of the ipomeanol side chain, one located near the cysteine chain and one located near the lysine chain as shown in Figure 5.10. The chemical shifts for the carbons attached to the proposed pyrrole protons (129 and 117 ppm), obtained from the HSQC spectra, agreed with predicted carbon chemical shifts for the substituted pyrrole.

Effect of covalent link on bioactivation.

The assay was then utilized to compare the activity of CYP4B1 wild type to that of the E310 mutants to evaluate the contribution of the covalent link to IPO bioactivation. The E310A mutant showed a similar rate of bioactivation (646 ± 24 nmol/ nmol P450/ 30 min) as the wild type enzyme (696 ± 41 nmol/ nmol P450/ 30 min). In contrast, the E310D (21 ± 0.2 nmol/ nmol P450/ 30 min) and E310Q (77 ± 1 nmol/ nmol P450/ 30 min) mutants showed drastically reduced levels of the reactive intermediate trapped by NAC and NAL.

Peroxidase Activity.

Pyrogallol and 1,2,4,5-tetramethoxybenzene were used as substrates to examine the ability of CYP4B1 and E310 mutants to perform single electron oxidations. Pyrogallol conversion to purpurogallin has been previously observed with CYP2B4 and is a typical substrate used to assay peroxidases (22, 23). The oxidation of pyrogallol by CYP2B4 was determined to be 23 nmol/nmol P450 /min (calculated from linear region of the curve between 12 and 17 min) above the background oxidation rate of 3.5 nmol/nmol P450/min (calculated from linear region of the curve between 1 and 4 min, prior to the addition of H_2O_2). Unfortunately, no activity was observed above the background rate for CYP4B1 wild-type or E310A under identical conditions (Figure 5.11). The assay was attempted with both H_2O_2 and reductase / NADPH, but to no avail. Oxidation of 1,2,4,5-tetramethoxybenzene to a radical cation has been demonstrated with a variety of P450s including CYP1A2 (18), which was used as a control in the current experiments. CYP1A2 showed an increase in absorbance at 450 nm, indicative of the 1,2,4,5-tetramethoxybenzene radical cation, but neither CYP4B1 wild type nor the E310A

mutants showed any activity. Figure 5.12 shows the absorbance spectra acquired every min over 3 min for CYP1A2, CYP4B1 wild type and CYP4B1 E310A.

5.4 Discussion

The conserved nature of the glutamic acid on the I-helix of many CYP4 family P450s suggests that the ester linkage formed between this residue and the heme could have an important functional role. The experiments described in this chapter were designed to identify structural and functional advantages that might be conferred by this unique structural feature. The CYP4B1 wild type enzyme, which essentially covalently binds 100% of the heme to protein, was compared to E310 mutants that lack any covalent link. Mutagenesis is a better method for evaluation of the functional effects of covalent linkage than comparing CYP4 isoforms with and without the linkage because the latter introduces problems associated with inherent differences in substrate selectivity and physical properties. The CYP4B1 E310A mutant is a good choice for comparison because almost all other families of P450s have a conserved alanine residue in place of the glutamic acid residue in the CYP4 enzymes. The main concerns with this substitution are that it is much smaller in size ($22.7 \text{ \AA}^3$ for alanine versus $63.5 \text{ \AA}^3$ for glutamic acid) and it lacks any hetero atoms that might participate in hydrogen bonding interactions that are possible with the oxygen atoms of the ester linkage of the wild type enzyme. Therefore, the E310D ($46.5 \text{ \AA}^3$) and E310Q ($71 \text{ \AA}^3$) mutants were also generated to more closely mimic the properties of the ester linkage involving the carboxylate of Glu310.

Typical P450s bind heme with a coordinate covalent bond between the sulfur of a conserved distal cysteine residue and the heme iron. In addition, there are ionic interactions between the propionic groups and basic residues, and hydrophobic interactions between the heme periphery and hydrophobic active site (24). To determine if the covalently bound heme provided additional heme retention capabilities, the amount of total heme content was compared to total protein content for CYP4B1 and the E310 mutants. The preparations of wild type enzyme, E310A, and E310Q all exhibited 80-

86% holoprotein, indicating that the covalent link did not offer any advantage in this respect.

The additional intramolecular linkage in CYP4B1 could conceivably increase the thermostability of the enzyme, and therefore, decrease the conversion of P450 to P420 at elevated temperatures. Any differences in the thermostability between the forms of CYP4B1 wild type, with and without bound heme, would be difficult to detect unless we had a preparation of the completely unbound form to analyze. However, when the thermostability of CYP4B1 and E310 mutants was analyzed, we found that the half life for the E310A mutant was even slightly prolonged compared to the wild type enzyme, indicating that the ester linkage does not offer any additional structural support. It is interesting that the more thermostable mutant more closely represents other families of P450s that have a conserved alanine at this position on the I-helix (25). The E310D and E310Q mutations resulted in rapid conversion to P420, suggesting that a polar substitution is not tolerated. Based on this result, we can speculate that the glutamic acid of the wild-type enzyme would not be tolerated either if it were not incorporated into the ester bond.

The fact that the half life for CYP4B1-CO complex at 37 °C is so short was concerning because thermostability studies with CYP2C enzymes indicated that these P450s were much more resistant to heat inactivation. CYP2C2 and CYP2C9 were reported to have half lives of 20 min and 16 min, respectively, at 48 °C (10, 26). When CYP2C9 was subjected to the current assay at 37 °C, there was no observable conversion from P450 to P420 during the 40 min incubation, in agreement with previous observations. The half life for CYP3A4 was found to be 21 min, more closely resembling the heat stability of CYP4B1. These findings suggest that the CYP2C family may be the exception, but analysis of more P450s is required to make the general statement. The short thermostability half life for CYP4B1 led us to question if enzyme inactivation was a problem during metabolic assays, so we determined the effect of reconstitution components on thermostability. Lipid stabilized the enzyme 4.6-fold but

the addition of reductase lowered the half life back to 23 min. This means that over half of the enzyme would be inactivated during the typical 30 min incubation. Yet, when octane or lauric acid were added to the enzyme solution, complete protection against heat inactivation was afforded during the course of the experiment. Therefore, steady-state kinetics determined at saturating substrate concentrations will not be significantly affected, but thermal inactivation at sub-saturating substrate concentrations may result in apparent non-linear kinetics.

Functional consequences of covalently bound heme have previously been evaluated by comparing the lauric acid hydroxylation activity of CYP4B1 wild type to that of the E310A and E310D mutants (12). The E310A mutant was found to preferentially metabolize at the ω -1 carbon, indicating that the covalent linkage in the wild type enzyme may be important for maintaining terminal carbon regioselectivity. Because the E310D mutant was inactive toward lauric acid, evaluation of the more conserved mutation on regioselectivity could not be evaluated (12). Therefore, to more fully investigate the effect of the covalent link on metabolism we examined the activity of CYP4B1 wild type, E310A, E310D, as well as E310Q toward hydrocarbons of various chain lengths. Total turnover by CYP4B1 E310A was similar to that of CYP4B1 wild type, although the mutant preferred internal, rather than terminal carbon oxidation for all hydrocarbons. These results support the measurements carried out previously with lauric acid, and demonstrate that the linkage is not required for the hydroxylation activity. Levels of activity for the E310D and E310Q mutants were easily detectable in all hydrocarbon hydroxylation assays, allowing us to compare the effect of more conservative mutations on regioselectivity. We found that both mutants demonstrated a higher degree of ω regioselectivity than did the wild type enzyme. This finding disproves the initial suggestion that the covalently attached heme is responsible for the preferential hydroxylation at the terminal carbon. By examining the turnover of hydrocarbons with varying lengths, the effect of the covalent heme linkage on chain length preference could be determined. Maximal CYP4B1 wild type activity was observed with heptane, whereas the E310 mutants preferred the slightly longer chain lengths of octane and nonane.

Although this shift in chain length preference appears significant, what advantage this chain-length preference of CYP4B1 might confer is unclear from evolutionary perspective.

The binding spectra acquired for CYP4B1 wild type and the E310A mutant demonstrate that octane binds tightly with an affinity of about 125 nM for both enzymes. The covalent linkage does not appear to affect octane binding, but the fact that the affinity for octane is so high is interesting in itself. Generally, ionic interactions ($\Delta G \approx -5$ kcal/mol) and hydrogen bonds ($\Delta G \approx -3$ kcal/mol) are considered responsible for the high affinity of substrates, whereas octane must bind strictly *via* hydrophobic interactions ($\Delta G \approx -0.7$ kcal/mol for each methylene unit). The bacterial fatty acid hydroxylase, P450_{BM3}, has been reported to have a K_m of 20 μ M for octane, 160-fold less than the K_S for CYP4B1 (27). Even after directed evolution was utilized to maximize octane turnover (from 80 to 3020 mol/min/mol enzyme), the K_S was still determined to be only 10 μ M (28). The ability of octane to bind so tightly may help explain why this ligand offers such a protective effect against thermal inactivation.

The bioactivation of 4-ipomeanol by CYP4B1 and E310 mutants was analyzed in order to compare activity toward a completely different substrate. This reaction is believed to occur with an initial epoxidation of the furan ring of 4-ipomeanol, a much more facile oxidation relative to hydrocarbon hydroxylation (17). A quantitative assay was first developed by trapping the reactive intermediate with NAC and NAL and analyzing the products by HPLC-UV. The major product was unambiguously identified as a *N*-substituted cysteinyl pyrrole derivative of IPO by high resolution MS, MS/MS, and 2D NMR. Additional information regarding the mechanism of bioactivation and trapping by NAC/NAL, as well as the toxicological implications are reported in Baer *et al* (17). The activities of CYP4B1 wild type and the E310A mutant are similar, indicating that the covalently bound heme does not affect the epoxidation reaction either. The E310D and E310Q mutants had lower activities, as observed in the hydroxylation

reaction. For the final attempt at differentiating activity between the CYP4B1 wild type and E310A, these enzymes were subjected to two peroxidase activity assays. Unfortunately, neither enzyme showed any oxidation of pyrogallol nor 1,2,4,5-tetramethoxybenzene.

In conclusion, the covalent linkage in CYP4B1 does not appear to offer an obvious advantage over the E310A mutant. The thermostability for both enzymes is similar and both retain heme to the same extent. The hydrocarbon metabolic studies with the wild type enzyme and the E310A mutant indicate a slight change in chain length preference and regioselectivity of hydroxylation, but these effects may simply be due to the gap that is left by elimination of the side chain. More importantly, the E310D and E310Q mutants show a greater preference for terminal carbon hydroxylation than wild type, indicating that the covalent link to the heme is not responsible for this regioselectivity. The comparison of 4-ipomeanol bioactivation, pyrogallol and 1,2,4,5-tetramethoxybenzene oxidation by these enzymes did not offer any other notable changes in activity. In sum, we can only conclude that a random mutation at position 310 resulted in a fully functional enzyme, and therefore was not eliminated by natural selection. The fact that this mutation is conserved in the CYP4 family may be because many other mutations, such as E310D or E310Q, caused drastic changes in metabolism that resulted in an evolutionary disadvantage.

Table 5.1. Heme and protein concentrations for CYP4B1 wild-type and E310 mutants. Holoprotein (%) was calculated by (heme / protein) x 100.

	CYP4B1 WT	E310A	E310D	E310Q
Heme (uM)	17.7	15.0	14.3	25.6
Protein (uM)	22.1	17.5	36.6	31.2
P450 (uM)	17.2	11.5	1.9	14.5
Holoprotein (%)	80.2	85.5	39.0	82.0

Table 5.2. Half-lives for CYP4B1 and E310 mutants. The half-lives were calculated from the first order decay of absorbance at 450 nm. For enzymes for which no significant change was observed over the experiment, a value of > 250 was assigned.

	Half life (min)
CYP4B1 WT	12
E310A	18
E310D	0.7
E310Q	1.5
CYP4B1 WT + octane	> 250
E310A + octane	> 250
E310D + octane	> 250
E310Q + octane	> 250
CYP2C9	> 250
CYP3A4	21
CYP4B1 WT + DLPC	55
CYP4B1 WT + DLPC + Reductase	23

Table 5.3. CYP4B1 and E310 mutant metabolic profiles for hydroxylation of heptane, octane, nonane, and decane. Rates are reported as nmol / nmol P450/ min. Standard deviations are calculated from triplicate measurements.

Heptane								
	ω		$\omega-1$		$\omega-2$		$\omega/\omega-1$	Total Rate
	AVG	STDEV	AVG	STDEV	AVG	STDEV		
Wild-type	24.68	2.08	1.13	0.17	0.02	0.01	21.89	25.83
E310A	14.07	0.49	2.00	0.07	0.14	0.01	7.02	16.21
E310D	2.63	0.23	0.03	0.02	0.02	0.03	99.79	2.67
E310Q	1.79	0.21	0.03	0.01	0.00	0.00	53.60	1.82
Octane								
	ω		$\omega-1$		$\omega-2$		$\omega/\omega-1$	Total Rate
	AVG	STDEV	AVG	STDEV	AVG	STDEV		
Wild-type	18.43	2.35	2.56	0.39	0.02	0.01	7.19	21.01
E310A	12.72	0.63	7.26	1.20	0.15	0.01	1.75	20.13
E310D	7.03	0.57	0.26	0.13	0.01	0.01	26.91	7.30
E310Q	4.16	0.21	0.24	0.02	0.01	0.01	17.16	4.41
Nonane								
	ω		$\omega-1$		$\omega-2$		$\omega/\omega-1$	Total Rate
	AVG	STDEV	AVG	STDEV	AVG	STDEV		
Wild-type	8.93	0.69	3.68	0.20	0.05	0.01	2.43	12.65
E310A	5.27	0.11	8.74	0.27	0.35	0.02	0.60	14.36
E310D	8.06	0.12	1.62	0.10	0.01	0.00	4.96	9.69
E310Q	3.44	0.08	0.83	0.01	0.02	0.00	4.15	4.28
Decane								
	ω		$\omega-1$		$\omega-2$		$\omega/\omega-1$	Total Rate
	AVG	STDEV	AVG	STDEV	AVG	STDEV		
Wild-type	4.21	0.27	2.90	0.18	0.22	0.02	1.45	7.32
E310A	2.16	0.32	5.61	0.81	1.91	0.36	0.39	9.68
E310D	3.93	0.08	1.26	0.04	0.06	0.00	3.12	5.25
E310Q	1.90	0.10	0.71	0.01	0.13	0.01	2.68	2.73

Table 5.4. Chemical shifts and coupling constants for protons and corresponding carbon chemical shifts of the NAC/NAL-IPO adduct and IPO.

	Position	Proton Chemical Shift (ppm)	Splitting Pattern	Coupling Constants (Hz)	Carbon Chemical Shift (ppm)	Structure
NAC/NAL-IPO Adduct						
IPO Chain	1H	1.07	d	6.3	23.28	
	2H	3.61	m	--	65.06	
	3H	1.60	m	--	30.22	
Pyrrole Ring	4H	2.73	t	8.0	34.69	
	5H	7.79	d	1.7	128.89	
	6H	6.73	d	1.7	117.14	
NAC	7 _a H	2.84	dd	9.5, 13.7	37.82	
	7 _b H	2.99	dd	4.5, 13.7	37.85	
	8H	4.23	m	--	51.20	
NAL	9H	1.87	s	--	22.02	
	N _a H	8.33	d	8.3	--	
	10H	3.99	t	7.8	46.11	
	11H	1.69	m	--	30.28	
	12H	1.28	m	--	22.33	
	13H	1.60	m	--	33.40	
	14H	4.15	m	--	51.31	
IPO	15H	1.84	s	--	22.02	
	N _b H	8.10	d	8.3	--	
IPO						
IPO Chain	1H	1.07	d	6.2	--	
	2H	3.61	m	--	--	
	3H	1.62	m	--	--	
Furan Ring	4H	2.83	t	7.3	--	
	5H	8.56	m	1.0	--	
	6H	6.67	m	1.0	--	
	7H	7.78	m	1.6	--	

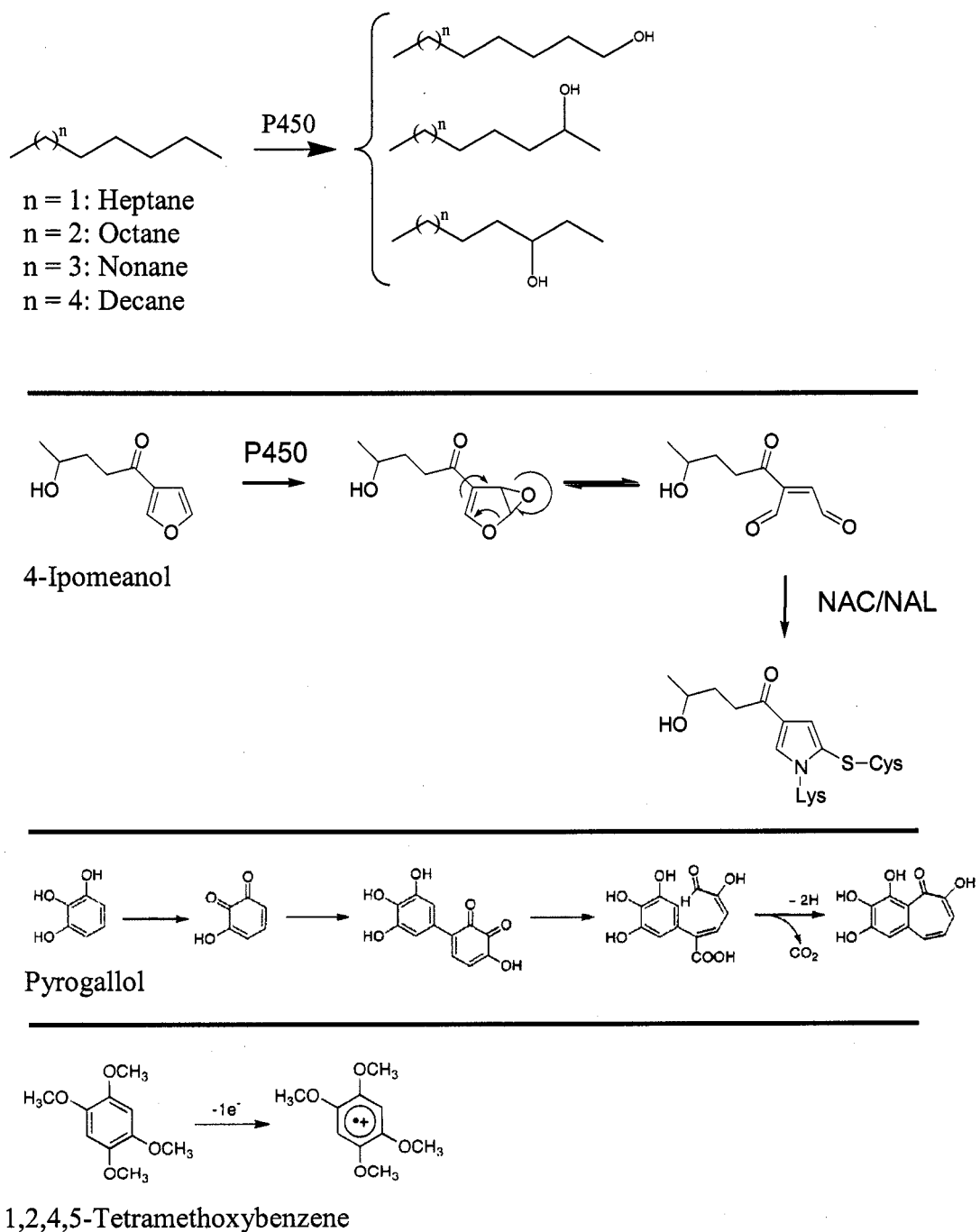


Figure 5.1. Structures of substrates used to probe the functional consequences of the covalently attached heme CYP4B1, as well as predicted metabolites.

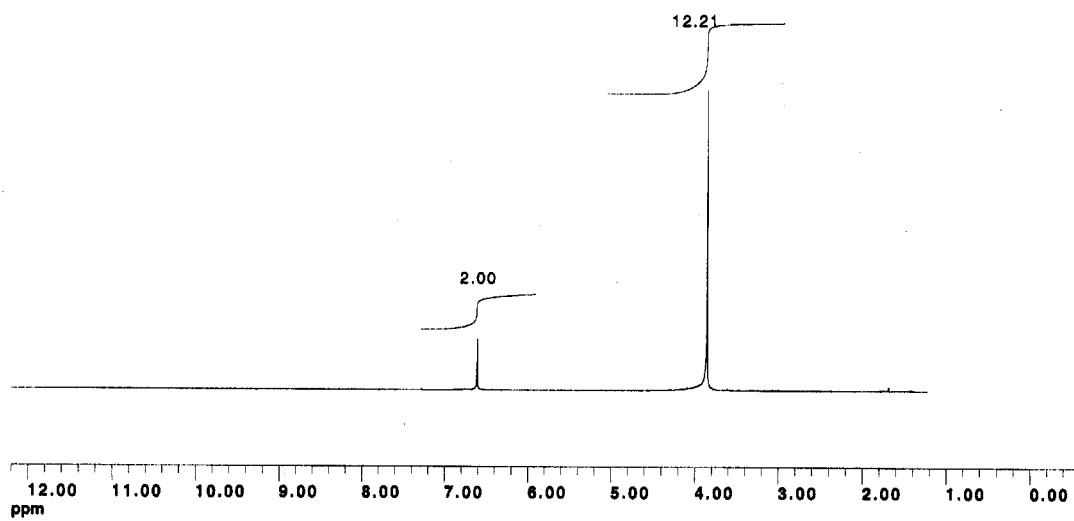


Figure 5.2. ^1H -NMR spectrum of synthesized 1,2,4,5-tetramethoxybenzene in CDCl_3 .

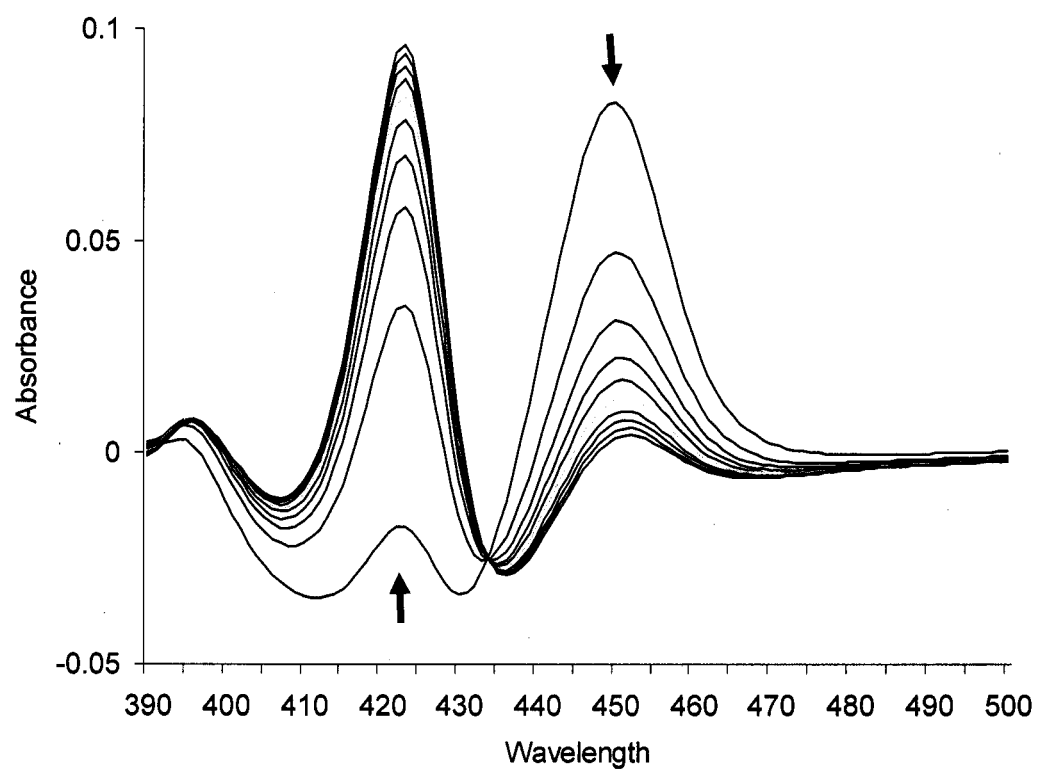


Figure 5.3. Ferrous CO-bound P450 spectra of CYP4B1. Scans are shown for CYP4B1 in 4 min intervals during incubation at 37 °C.

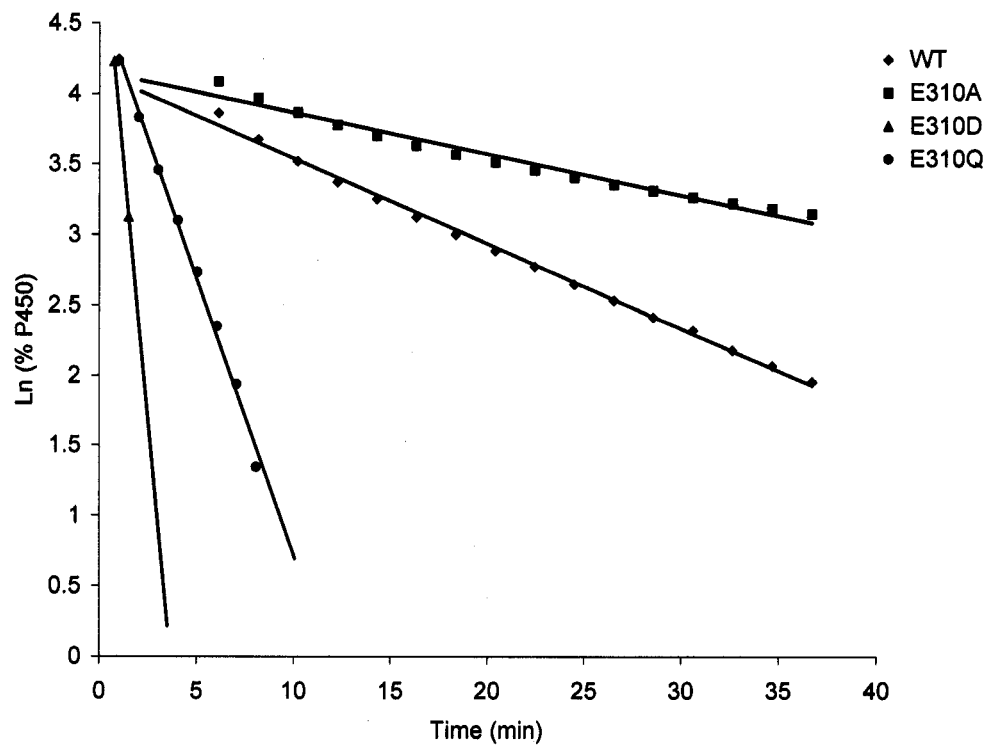


Figure 5.4. Thermostability of CYP4B1 and E310 mutants at 37 °C. The amount of spectral P450 remaining at the indicated time point is shown relative to the zero time point.

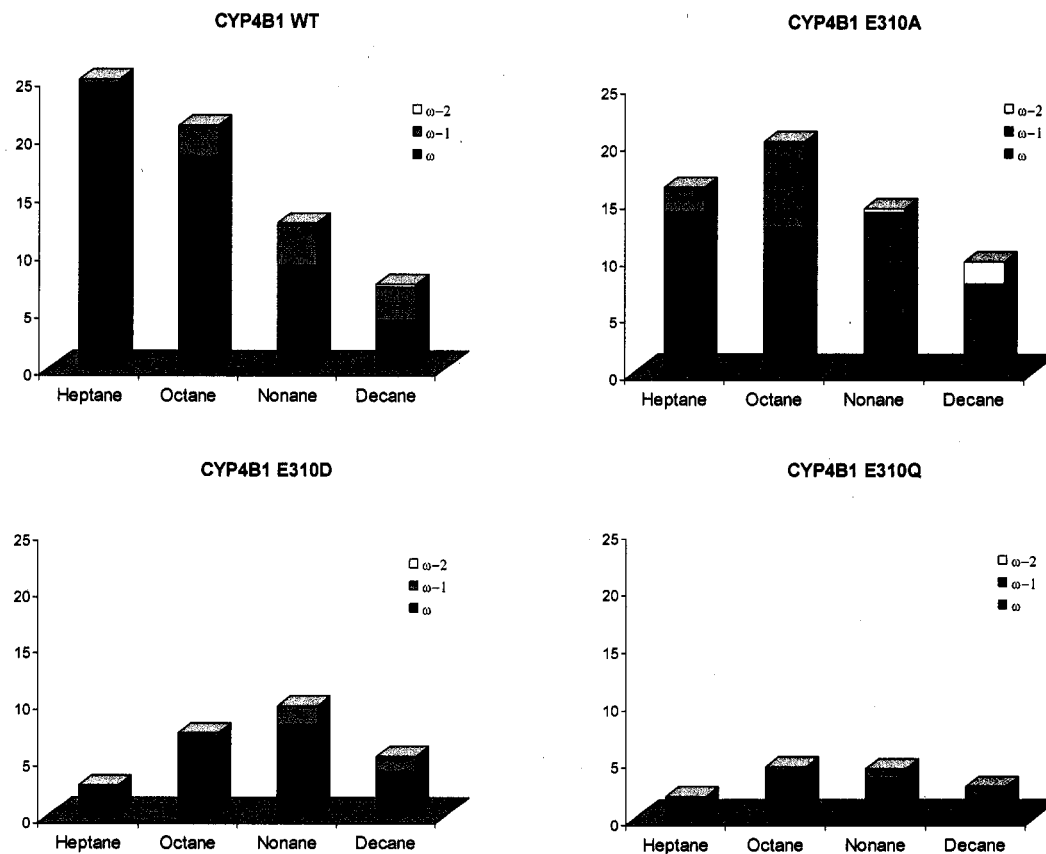


Figure 5.5. CYP4B1 and E310 mutant metabolic profiles for hydroxylation of heptane, octane, nonane, and decane. Rates are reported as nmol / nmol P450/ min.

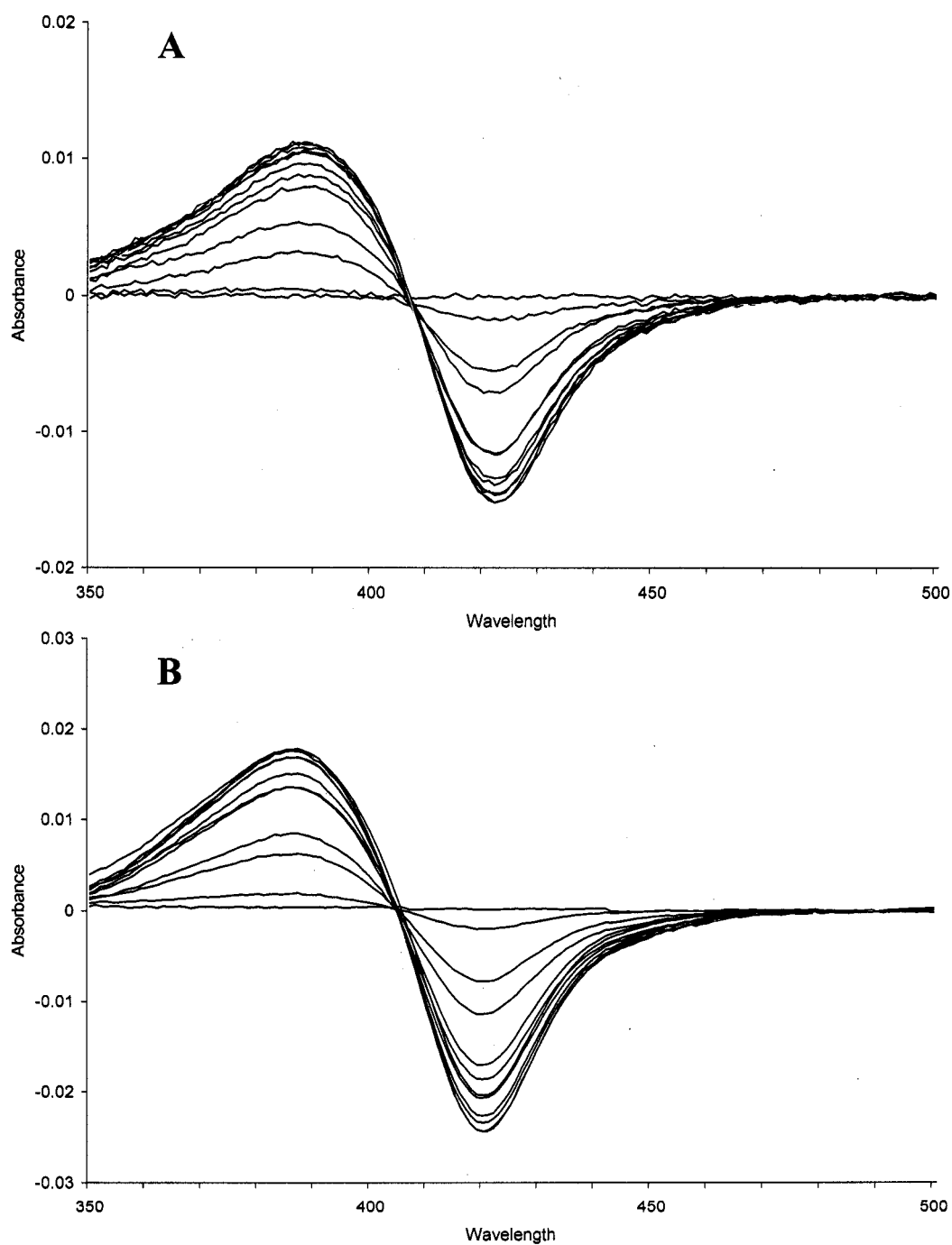


Figure 5.6. Binding spectra for CYP4B1 wild type (A) and E310A (B) titrated with octane.

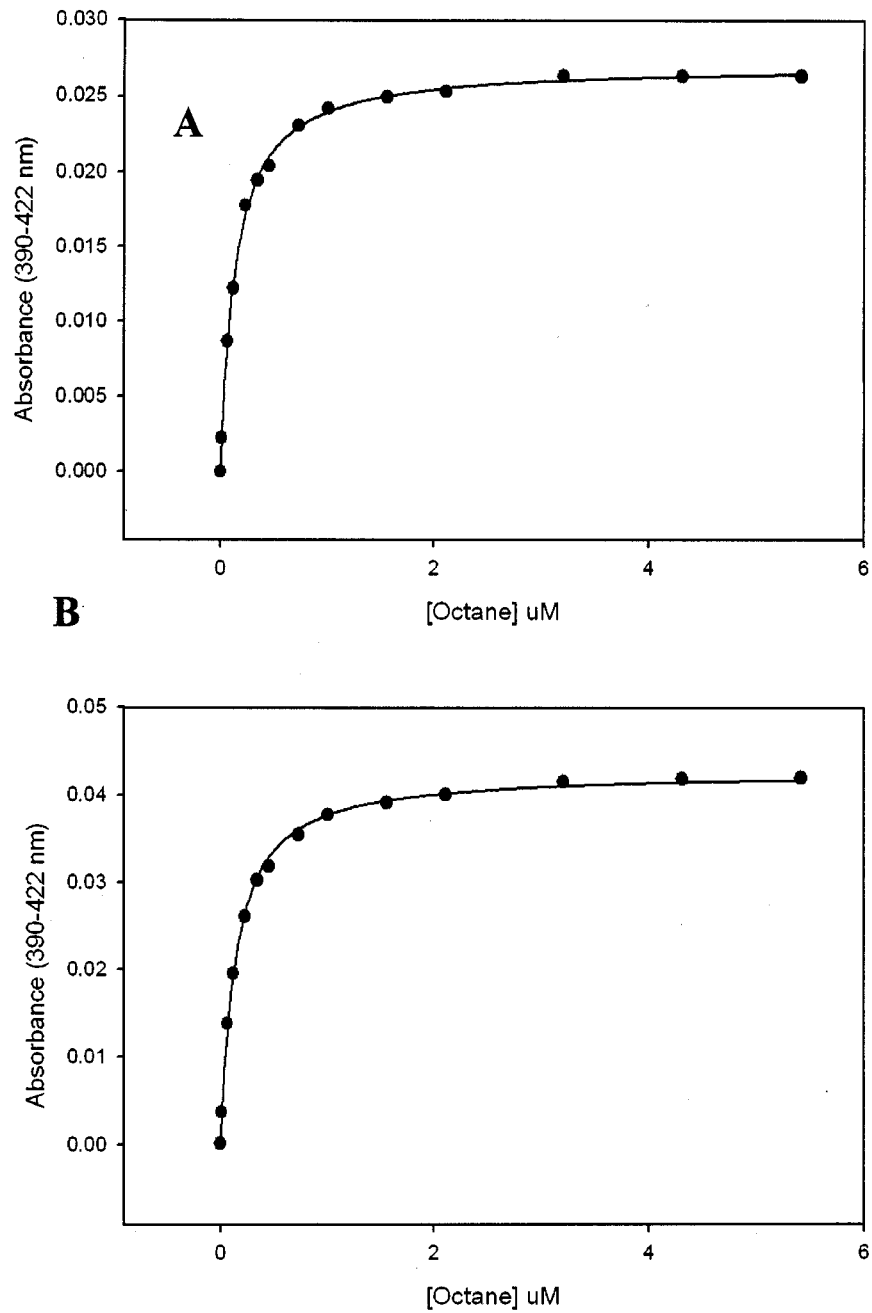


Figure 5.7. Absorbance change (390 nm – 422 nm) versus octane concentration curve for CYP4B1 WT (A) and the E310A mutant (B).

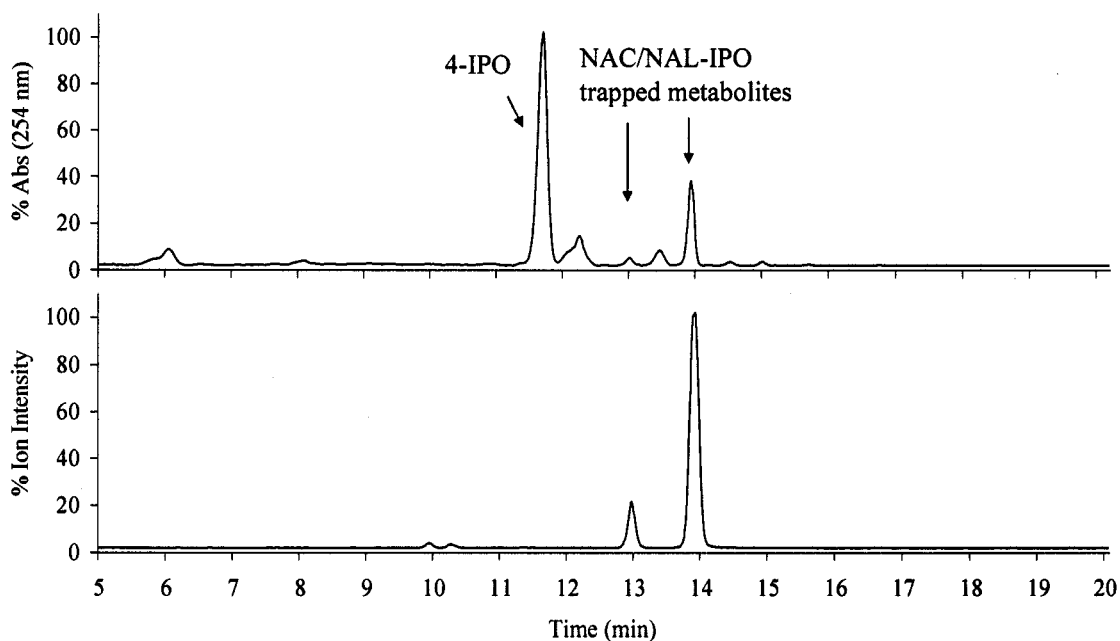


Figure 5.8. UV (upper panel) and reconstructed m/z 482 ion current (lower panel) chromatograms from CYP4B1 incubations containing IPO, NADPH, and equimolar NAC/NAL. 4-IPO elutes at 11.7 min and the minor and major NAC/NAL-IPO adducts at 13.0 and 14.0 min, respectively, under the HPLC conditions described in Methods. Unlabeled peaks at 12.1, 12.2, and 13.5 min in the UV chromatogram are present in control samples, or are attributable to impurities in the NAC material.

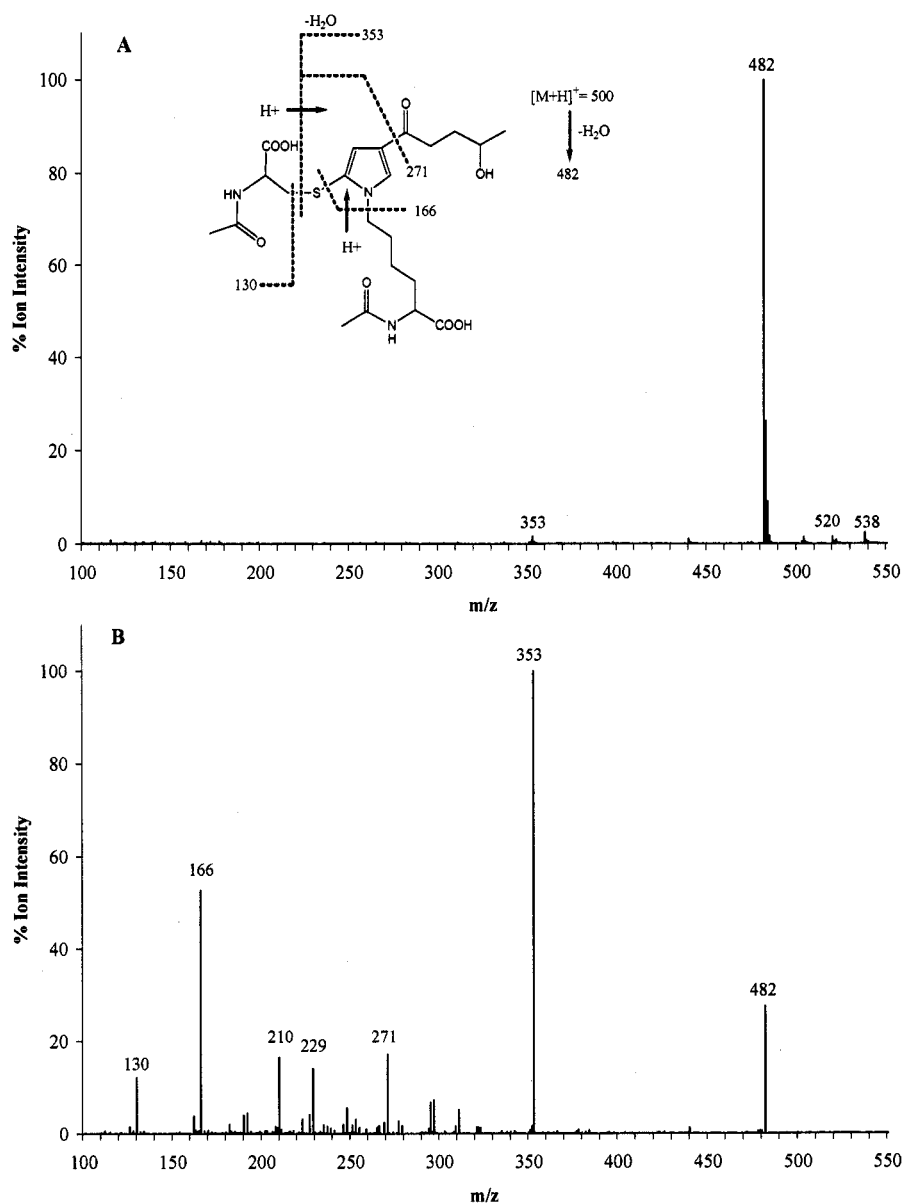


Figure 5.9. A representative Q1 mass spectrum of the major NAC/NAL-IPO trapped metabolite, RT=14.0 min (A), and a representative MS/MS spectrum showing the daughter ions of m/z 482 (B). Fragmentation of the trapped metabolite produces product ions consistent with the proposed structure. The MS/MS spectrum for the minor NAC/NAL-IPO trapped metabolite, RT=13.0 min, is identical.

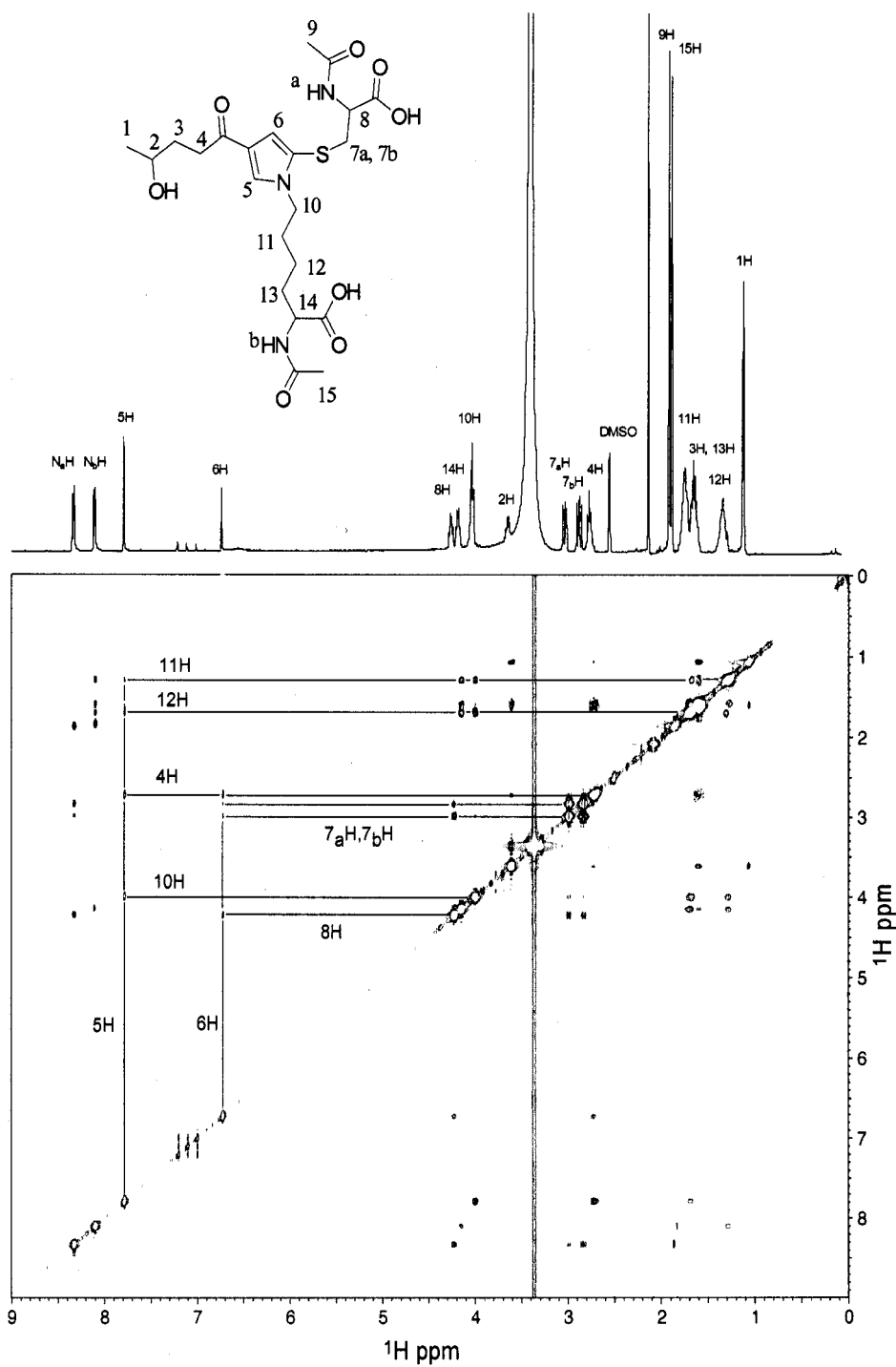


Figure 5.10. The ^1H -NMR and ROESY spectra of the NAC/NAL-IPO adduct containing a labeled structure showing the position of each H-atom.

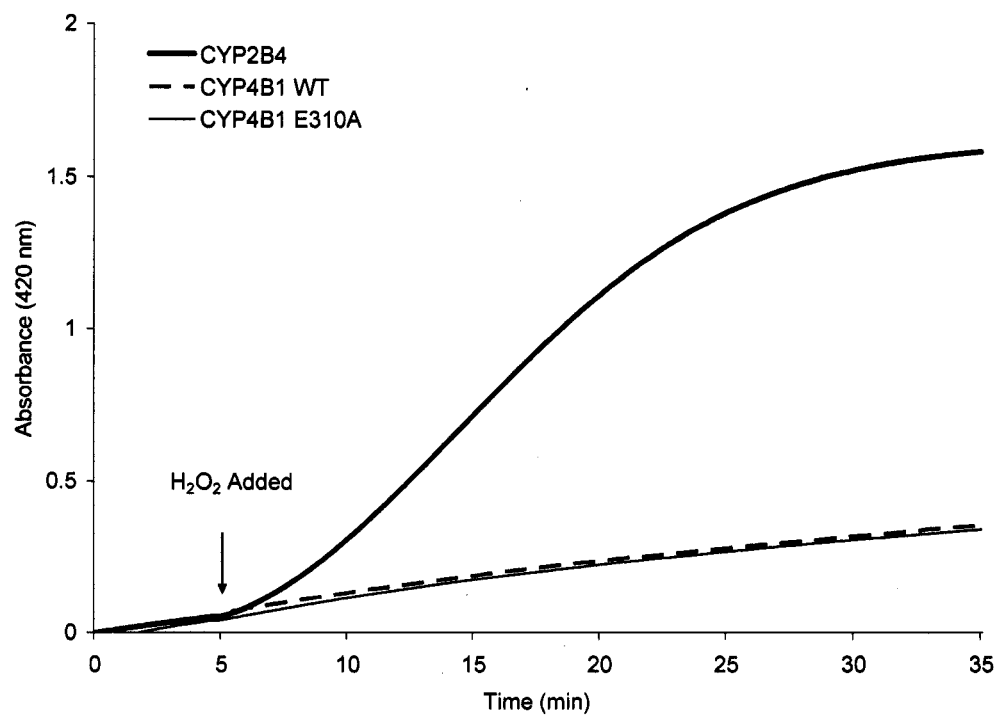


Figure 5.11. Absorbance change at 420 nm relative to 700 nm, representing the formation of purpurogallin by CYP2B4, CYP4B1 wild type, and CYP4B1 E310A.

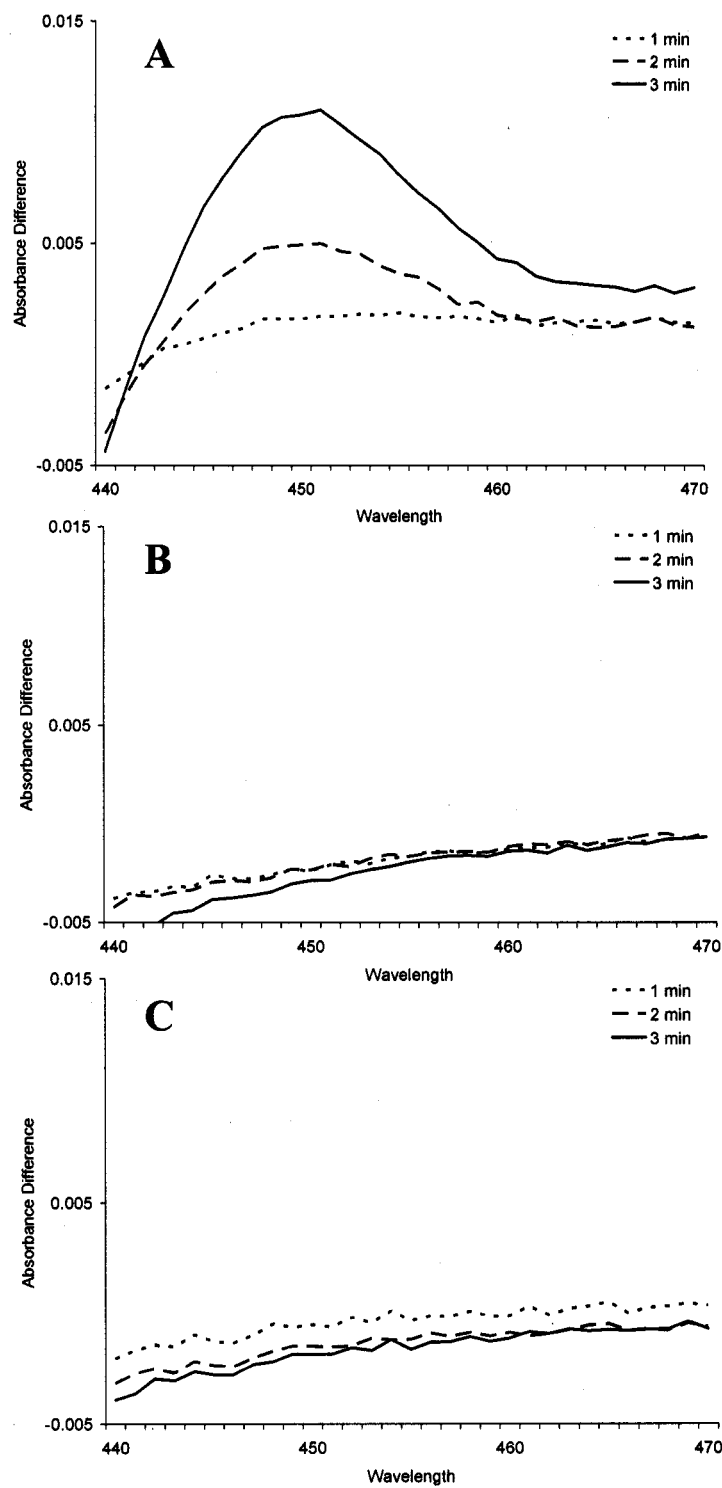


Figure 5.12. Absorbance spectra at 1 min intervals showing the oxidation of 1,2,4,5-tetramethoxybenzene by CYP1A2 (A), CYP4B1 wild type (B), and CYP4B1 E310A (C).

5.5 Notes to Chapter 5

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Chapter 6

General Conclusions and Future Directions

6.1 General Conclusions

The discovery of the covalently attached heme group in CYP4B1 raises many intriguing questions about the structural, mechanistic, and functional aspects of this phenomenon. In the search for the sites of the ester bond attachment, we found that the protein was bound at Glu310, and more interestingly, that the heme was bound at both the C-5 and C-8 methyl groups in recombinantly expressed CYP4B1 and CYP4A3 (1). From these observations we realized that we had stumbled upon a unique method for evaluating the microheterogeneity of heme orientation in P450s, which is otherwise difficult to address. Because these results suggest that the heme may be inserted in two distinct orientations in all recombinant P450s it prompted us to consider the effects that this could have in extrapolation from *in vitro* metabolic studies with the major drug metabolizing P450s to predictions of *in vivo* clearance. Further studies demonstrated that the dual heme orientations in CYP4 proteins are an artifact of recombinant expression systems. There is no evidence to support that this structural alteration significantly alters *in vitro* drug metabolism, at least with CYP4B1.

Mechanisms were then proposed that could explain the formation of an ester bond with the carboxylate of Glu310 and a heme methyl group, through an oxidative process involving known P450 oxidants. Mechanisms evolved from the facts 1) the process was autocatalytic, as demonstrated clearly with CYP4A8, (ii) the evidence that a carboxylate group was required for heme activation, and (iii) the assumption that there was a carbocation intermediate, suggested by the hydroxymethylheme formed in studies with the CYP4B1 E310D mutant (2). The overall mechanism, which parallels that proposed for covalent heme attachment in the mammalian peroxidases, involves initial oxidation of the carboxylate of Glu310 to form an acyloxyl radical, followed by hydrogen abstraction from the heme methyl, intramolecular electron transfer to generate a heme methyl

carbocation, and final quenching of the carbocation by the same carboxylate of Glu310. We proposed that the acyloxyl radical could be generated by one of two ways, either by direct hydrogen abstraction from the carboxylate by the iron-oxo P450 oxidant, or by initial attack of the nucleophilic iron-peroxo oxidant on the carboxylate with subsequent homolytic scission of the O-O bond. Studies with ^{18}O -labeled glutamate revealed that the former mechanism was responsible, or at least the dominant pathway (3). This detailed mechanism gives us further insight into the wide range of oxidations carried out by P450s. Only one other case of direct carboxylate oxidation by P450s has been reported in the literature, and this reaction terminated with decarboxylation (4). By experimentally dissecting the mechanism of covalent heme attachment, we provide the first evidence for the formation of an ester bond *via* an oxidative event.

Discovery of the covalently attached heme group in CYP4 enzymes specifically, led to the hypothesis that this structural peculiarity was related to substrate catalysis that is unique to this family of enzymes, notably fatty acid hydroxylation with a high degree of ω -specificity. Yet when CYP4B1 wild-type and the E310 mutants were subjected to metabolic assays with lauric acid and various hydrocarbons, no obvious distinction was apparent. In addition we found that CYP4B1 was comparable to the E310A mutant in the retention of heme and thermostability. Based on the accumulated data, we can only deduce that a random mutation at the 310-position from alanine to glutamic acid did not perturb the selective pressures imposed on the P450 and therefore was not eliminated during the evolution of the gene.

6.2 Future Directions

Crystallography studies with CYP4B1 would provide valuable information regarding the structural effects of the covalently attached heme group. First of all, a solved crystal structure could be used to confirm the site of the ester linkage on the protein and, if high resolution was achieved, may verify that the heme is bound at the C-5 methyl position (and the C-8 methyl position since recombinantly expressed enzyme would be analyzed).

It would be interesting to observe the conformational distortions of the porphyrin structure caused by the covalent linkage. The crystal structure of myeloperoxidase revealed that the heme plane was distorted by covalent bonds to the heme, so that the A and C rings were bowed toward the distal side (5). There may also be interesting conformational changes in the active site, as compared to the solved structures of CYP2B4, CYP2C9, and CYP3A4. Possibly, the covalent linkage can compact the active site of CYP4B1 by pulling the I-helix closer to the heme, or may alter the relative distance between the iron of the heme and the cysteine residue to which it is ligated. A solved crystal structure could also provide clues to how the active site can restrict access to the heme so that fatty acids and hydrocarbons are preferentially metabolized at the ω -position, yet still allow bioactivation of larger substrates such as 2-aminofluorene and 4-ipomeanol.

From an experimental standpoint, CYP4B1 is an excellent target for crystallography, at least when compared to other CYP4 enzymes. First of all, high levels of expression are achieved in the *E. coli*. expression system (660 nmol/L), compared to 80 – 120 nmol/L for CYP4As and CYP4Fs. Second, the purified enzyme is homogenous in the sense that 100% of the heme is covalently bound, whereas all other CYP4 enzymes are reported to have 6% - 68% bound heme. Third, we have identified octane as a tight binding ligand that provides protection against thermal denaturation, which inevitably occurs during the purification and crystallization process.

Unfortunately, to date, we have not been able to adequately truncate the hydrophobic N-terminus, which has been a requirement for the solubilization of CYP2B4, CYP2C9, and CYP3A4 in ionic detergents, and the eventual generation of crystals. We have made several attempts in our laboratory at deleting portions of the N-terminus of CYP4B1, but poor expression of the modified proteins has limited progress (Figure 6.1). There are two constructs that could potentially lead to CYP4B1 crystallization, designated MC-7 and YM-1 in figure 1. CYP4B1 MC-7 lacks one of the major hydrophobic regions in the N-terminus and still expresses at high levels. CYP4B1 YM-1 is a few residues shorter, but

still contains a similar number of hydrophobic residues due to the addition of the Barnes sequence, and expresses at much lower levels. Future studies may include modifying CYP4B1 MC-7 so that there are fewer hydrophobic residues, and attempting to form crystals from protein purified solely with ionic detergents.

Comparisons between CYP4B1 wild-type and E310 mutants in the oxidation of xenobiotics such as lauric acid, hydrocarbons, and 4-ipomeanol have not revealed an obvious benefit of the covalently linked heme in CYP4B1, but the functional consequences of this unique structural feature in the metabolism of endogenous substrates has not been evaluated. To date, the only work on the physiological contribution of CYP4B1 comes from the laboratory of Lanaiido-Schwartzman where the production of 12-HETE in response to hypoxia and inflammation in the rabbit cornea, has been associated with CYP4B1 (6-8). In future experiments it would be interesting to evaluate the effects of the covalent linkage on the conversion of arachidonic acid to 12-HETE. Studies in our laboratory are underway to generate a CYP4B1 knockout mouse. Evaluation of resulting phenotype may lead to the identification of additional endogenous substrates for CYP4B1 that could be used to further probe the *in vivo* functional effect of covalently bound heme.

Covalent binding of heme in the human form of CYP4B1 has not been characterized because the holoenzyme has not been successfully expressed and purified in our laboratory. When human CYP4B1 is expressed in the baculovirus expression system, immunoreactive protein is observed, but membrane preparations lack the typical ferric CO-bound P450 spectra (9). This human CYP4B1 apo-protein does not show any activity toward lauric acid. Only when the S427P mutation is made, can holoenzyme be produced and purified, resulting in active P450 in the metabolism of lauric acid. Imaoka *et al.* expressed a human CYP4B1-P450 reductase fusion protein in yeast that gave a ferric CO-bound P450 spectra, catalyzed ω -hydroxylation of lauric acid, and activated 2-aminofluorene (10). Since this is the most convincing piece of evidence for human CYP4B1 activity, this fusion protein should be recreated in our laboratory and evaluated

using our standard metabolic assays. The extent of covalent binding could be easily determined from the purified fusion protein, and may provide an additional avenue for evaluation of its functional role.

The investigation into the structural alterations, mechanism of formation, and functional consequences of the covalent bond between heme and protein has been an academically oriented project, with little relation to humans thus far. A clear path to follow with the CYP4B1 story is to address the *in vivo* importance of the human form in endogenous metabolism and physiological function, as well as the bioactivation of protoxins. To this extent, studies are underway in our laboratory to generate a human CYP4B1 transgenic mouse, starting with the CYP4B1 knockout mouse described previously. Results from these studies may provide us with a better understanding of the role human CYP4B1 plays *in vivo* and could potentially lead to treatments for inflammation, by modulation of arachidonic acid metabolism, or the design of safer drugs, by minimizing bioactivation.

CYP4B1 may have multiple functional roles, acting on endogenous substrates to produce inflammatory mediators, as well as metabolizing xenobiotics. Typically, P450s that have a physiological function, such as P450s in families 5, 7, 11, 17, 19 and 21, have active sites which are customized for their substrates, limiting their participation in the oxidation of exogenous compounds. On the other hand, the common drug metabolizing enzymes in the CYP1, CYP2, and CYP3 families have much less stringent active sites allowing the metabolism of a variety of foreign compounds. Yet, CYP4B1 appears to be at an interface between these two distinct roles for P450s. The only other P450s identified to date that may play an important physiological role as well metabolize xenobiotics, are CYP4F12 and CYP2J2 (11, 12). This phenomenon leads us to question if there could be significant drug – endogenous substrate interactions. Possibly, an elevated immune response that triggers elevated levels of CYP4B1 or increased levels of a substrate, such as arachidonic acid, could increase or decrease the metabolism of an administered drug. Conversely, drug administration could affect the inflammatory

response, which may or may not be a desired effect. If human CYP4B1 is found to possess activity, it would be interesting to investigate the potential for these types of drug interactions.

In conclusion, studies presented in this thesis have established the precise structure and sites of attachment of the covalent linkage that binds heme to CYP4B1, revealed a viable autocatalytic mechanism for its formation, and probed the functional consequences with known substrates for this P450. As final efforts to correlate this structural peculiarity with function, future work may entail determining the active site structural distortion caused by the link and identifying CYP4B1-dependent metabolic pathways that necessitate covalently attached heme. Lastly, research may be directed at establishing the significance of CYP4B1 in humans and exploring the similarities to the rabbit form, which has been so well characterized. Identifying the role of human CYP4B1 may pave the way for many more investigative projects and could potentially lead to the design of new drug therapies.

	CYP4B1	M L G F L S R L G L W A S	S	G L I L I L G F L K L L R L L R R Q R L A R A M D S F P G P P	nmo/L
MC-1		M A L L L A V F G L W A S		G L I L I L G F L K L L R L L R R Q R L A R A M D S F P G P P	660
MC-2				M A L L L A V F L K L L R L L R R Q R L A R A M D S F P G P P	-
MC-7				M A G F L K L L R L L R R Q R L A R A M D S F P G P P	450
YM				M A R R Q R L A R A M D S F P G P P	-
YM-1				M A L L L A V L R R Q R L A R A M D S F P G P P	110
YM-2				M A G F L S R R R Q R L A R A M D S F P G P P	-
BB				M A K K T S S K G K L P G P P	-
BB-1				M A K K T S S K R R Q R L A R A M D S F P G P P	-
BB-2				M A L L L R R Q R L A R A M D S F P G P P	-

Figure 6.1. Modifications made to the N-terminus of CYP4B1 and expression levels of each construct in *E. coli*. Constructs were generated by Matt Cheesman (MC), Yi-Min Zheng (YM), and Brian Baer (BB). Hydrophobic residues are in bold print.

6.3 Notes to Chapter 6

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