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

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ABSTRACT: Recently we found that CYP4B1, and several other members of the CYP4 family of enzymes, are covalently linked to their prosthetic heme group through an ester linkage. In the current study, we mutated a conserved CYP4 I-helix residue, E310 in rabbit CYP4B1, to glycine, alanine, and aspartate to examine the effect of these mutations on the extent of covalent heme binding and catalysis. All mutants expressed well in insect cells and were isolated as a mixture of monomeric and dimeric forms as determined by LC/ESI-MS of the intact proteins. Rates of metabolism decreased in the order E310 > A310 ≫ G310 > D310, with the A310 and G310 mutants exhibiting alterations in regioselectivity for ω-1 and ω-2 hydroxylation of lauric acid, respectively. In marked contrast to the wild-type E310 enzyme, the G310, A310, and D310 mutants did not bind heme covalently. Uniquely, the acid-dissociable heme obtained from the D310 mutant contained an additional 16 amu relative to heme and exhibited the same chromatographic behavior as the monohydroxyheme species released upon base treatment of the covalently linked wild-type enzyme. Expression studies with $H_2{}^{18}O$ demonstrated incorporation of the heavy isotope from the media into the monohydroxyheme isolated from the D310 mutant at a molar ratio of ∼0.8:1. These data show (i) that E310 serves as the site of covalent attachment of heme to the protein backbone of rabbit CYP4B1; (ii) this I-helix glutamate residue influences substrate orientation in the active site of CYP4B1; and (iii) the mechanism of covalent heme attachment most likely involves a carbocation species located on the porphyrin.

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,[1] 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*, *5*). 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.

Covalent attachment of prosthetic heme also occurs in the mammalian peroxidase and cytochrome *c* families of proteins (*7*−*11*). Whereas enzyme activity increases as a function of the heme diester link in lactoperoxidase (*12*), covalent attachment via thioether formation is not recognized to provide any functional advantage for the *c*-type cytochromes (*10*). The focus of our studies is CYP4B1, primarily because of its close association with the bioactivation of a diverse array of xenobiotics, including valproic acid (*13*), 4-ipomeanol (*14*), and 3-methylindole (*15*). The relatively broad substrate specificity of CYP4B1 may provide enhanced opportunities for evaluation of the functional consequences of heme covalent attachment to this isoform. However, many questions remain to be answered about the nature of the heme covalent link to CYP4B1.

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) (*16*), near the oxygen-binding pocket in the active site. Therefore, the primary goal of the present study was to determine the effect of the rabbit CYP4B1 I-helix mutants—E310G, E310A, and E310D—on covalent heme binding and catalytic activity

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[1] Abbreviations: CYP, cytochrome P450; HPLC, high-pressure liquid chromatography; LC/ESI-MS, liquid chromatography/electrospray ionization-mass spectrometry; amu, atomic mass units; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; GC/FID, gas chromatography-flame ionization detection; BSTFA, *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide.

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toward lauric acid, a general metabolic probe for the CYP4 enzymes. The studies provide valuable information about the site of the heme ester link in CYP4B1 and the functional consequences of these active site mutations. Unexpectedly, we also 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.

EXPERIMENTAL PROCEDURES

Materials. Restriction enzymes and T4 ligases were purchased from New England Biolabs, Inc. (Beverly, MA). PfuTurbo DNA polymerase was purchased from Stratagene (La Jolla, CA). The pFastBac vector and Bac-to-Bac expression system was purchased from Gibco-BRL (Grand Island, NY). Ex-Cell 405 media was from JRH Biosciences (Lenexa, KS). NADPH, lauric acid, and C-α-dilauroylphosphatidylcholine (DLPC) were purchased from Sigma-Aldrich (St. Louis, MO). $H_2{}^{18}O$ (95 atom %) and heme were obtained from Aldrich Chemical Co. (Milwaukee, WI). 8-Hydroxyheme was synthesized according to the method of Ator et al (*26*). Hydroxylated lauric acid metabolites were synthesized as described previously (*17*). 15-Hydroxypentadecanoic acid was purchased from Wiley Organics (Coshocton, Ohio). *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Supelco, Inc. (Bellefonte, PA). P450 reductase and cytochrome *b*5 were purified from *Escherichia coli* expression systems as previously described (*18*).

Site-Specific Mutagenesis. Site-specific mutations were introduced by overlap extension PCR, with the following mutagenic primers: E310A, forward primer, 5′ C ACG TTC ATG TTC **GCA** GGT CAT GAC ACC 3′, reverse primer, 5′ GGT GTC ATG ACC **TGC** GAA CAT GAA CGT G 3′; E310D, forward primer, 5′ C ACG TTC ATG TTC **GAT** GGT CAT CAC ACC 3′, reserve primer, 5′ GGT GTC ATG ACC **ATC** GAA CAT GAA CGT G 3′; E310G, forward primer, 5′ C ACG TTC ATG TTC **GGA** GGT CAT GAC ACC 3′, reverse primer, 5′ GGT GTC ATG ACC **TCC** GAA CAT GAA CGT G 3′. For overlap extension, the 5′ end primer (5′ CCGGAATTCCATGCTCGGCTTCCTCTCCC 3′, EcoR I underlined) was paired with a reverse mutagenic primer to generate a 5′ fragment. The 3′ end primer (5′ TGCTCTAGAGCTACTTCTCAGCCTTGGG 3′, Xba I underlined) was paired with a corresponding forward mutagenic primer to generate a 3′ fragment. The 5′ and 3′ fragments were then reannealed to amplify ∼1.5 kb fragments containing the mutations of interest. These PCR products were subsequently digested with EcoR I and Xba I and cloned into the pFastBac vector, in which all mutations were confirmed by DNA sequencing. The resulting pFastBac vectors incorporating the various E310 mutations were transfected into *Trichoplusia ni* cells (High Five) using cellfectin (Life Technology, Grand Island, NY) according to the manufacturer's instructions.

Protein Expression and Purification. Generation of recombinant baculoviruses and mutant proteins in suspension culture (100−250 mL) was performed according to the methods described previously (*17*, *19*). Each mutant was purified to near homogeneity following solubilization with sodium cholate and column chromatography on Octyl-Sepharose 4B and hydroxyapatite (*17*). The E310D mutant was also expressed on plates (10 mL) in the presence or absence of $H_2{}^{18}O$ that had been filtered and added to the culture to give final concentrations of 6.79 ± 0.06% $H_2{}^{18}O$ (1 g of 95% $H_2{}^{18}O$ added to ∼9 mL of media), or 14.0 ± 0.14% $H_2{}^{18}O$ (2 g of 95% $H_2{}^{18}O$ 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.

HPLC−UV 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 × 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-MS analysis was performed 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 100 °C, and a desolvation temperature of 350 °C. Data analysis was carried out using Windows NT based Micromass MassLynxNT 3.2 and MaxEnt software.

LC/ESI-MS Analysis of Monohydroxyheme from the E310D Mutant. The membrane fractions containing the E310D mutant expressed in $H_2{}^{18}O$ 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 the heme. The sample was then spun down in a centrifuge to remove insoluble components. LC separation of the supernatant was performed on a 2.1 × 250 mm C4 protein column (Vydac, Hesperia, CA) to eliminate other compounds that may have a similar molecular weight and to assist with ionization of the polar heme. 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 heme isotope ratios due to ^{18}O 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 × 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 °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 °C over 2 min, then from 60 to 120 °C in 1 min, and held at 120 °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 ± 0.06% $H_2{}^{18}O$ and 14.04 ± 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.

Assay for Lauric Acid Hydroxylation. Rabbit CYP4B1 (100 pmol of each purified mutant form) was preincubated at 37 °C with P450 reductase (200 pmol), cytochrome *b*5 (100 pmol), and 10 μg of dilaurylphosphatidylcholine. Incubations were carried out in 0.1 M potassium phosphate buffer, pH 7.4 containing 0.3 mM lauric acid and 1 mM NADPH. Reactions were terminated after 20 min by the addition of 1 mL of 10% HCl, spiked with 5 mg of the internal standard, 15-hydroxy pentadecanoic acid, and the rates of lauric acid ω, ω-1, and ω-2 metabolite formation were determined by GC/FID after derivatization with BSTFA as described previously (*17*).

Spectroscopic Characterization of CYP4B1 Proteins. P450 spectra (reduced-CO complex vs 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 mM cm^{-1} for ΔA (450−480) nm.

RESULTS

Characterization of Purified I-Helix Mutants of CYP4B1. Mutations were introduced into the CYP4B1 gene corresponding to the E310 position, and the expressed enzymes were purified from insect cell membranes as shown in Figure 1. Each of the E310G, E310A, and E310D mutants exhibited a Soret maximum near 418 nm, indicative of the low spin form of the oxidized enzyme and a typical reduced P450-CO spectrum (Table 1). ESI-MS analysis of the intact proteins provided molecular weight data in close agreement (0.0002−0.01% error) to the calculated molecular weights for holo-CYP4B1, apo-E310A, apo-E310G, and apo-E310D (Table 1).

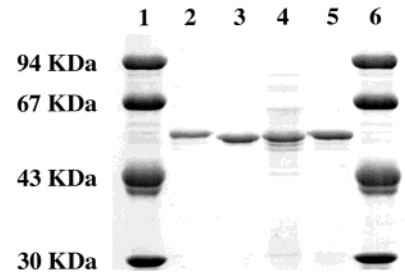

FIGURE 1: SDS−PAGE gel of purified rabbit CYP4B1 I-helix mutants. Lanes 1 and 6, molecular weight markers; lane 2, CYP4B1 wild-type (10 pmol); lane 3, E310G (10 pmol); lane 4, E310A (10 pmol); and lane 5, E310D (10 pmol).

Table 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

Rates and Regioselectivity of Lauric Acid Metabolism by CYP4B1 E310 Mutants. Native rabbit CYP4B1 catalyzed the formation of both 12-hydroxy and 11-hydroxy lauric acid in near equal amounts with a combined turnover number of approximately 13 min^{-1}. The E310A mutant metabolized laurate at ~60% of the rate of the wild-type enzyme and displayed a clear change in regioselectivity to predominantly ω-1 hydroxylation. The E310G mutant turned over laurate at very low but measurable rates (<3% of wild-type) and with a further shift in regioselectivity toward ω-2 hydroxylation. Turnover numbers for the E310D mutant were below the limit of detection (Table 2).

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 2A). 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 2B−D), and mass analysis of the earlier eluting peak (Table 3) confirmed its identity as either native heme (E310G and E310A) or monohydroxy heme (E310D, see below). When combined with the molecular weight data

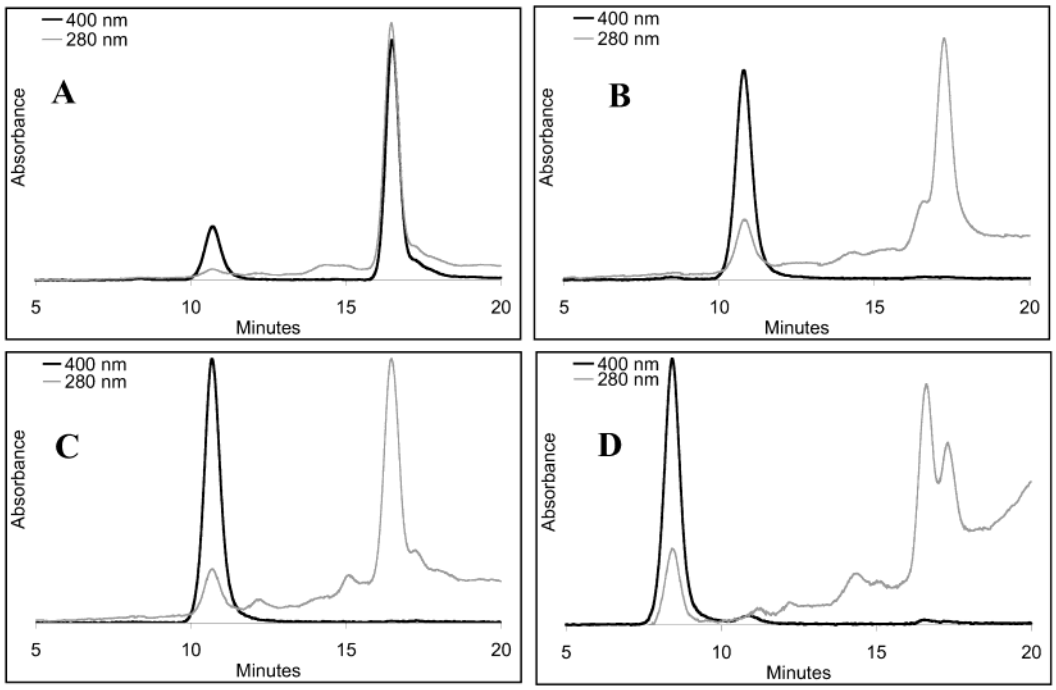

FIGURE 2: POROS R2 chromatography of rabbit CYP4B1 mutants with HPLC−UV detection at 400 nm (dark trace) and 280 nm (gray trace). Panel A: Wild-type CYP4B1; panel B: E310G mutant; panel C: E310A mutant; and panel D: E310D mutant. CYP4B1 protein elutes at 16.8 min, free heme elutes at 10.8 min, and monohydroxyheme 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.

Table 2: Lauric Acid Hydroxylase Activities of Rabbit CYP4B1 I-Helix Mutants

enzyme	total turnover[a]	12-OH	11-OH (nmol/nmol/min)	10-OH (nmol/nmol/min)	dominant pathway
wild-type	13.4	8.2 ± 0.2	5.2 ± 0.1	<0.1	ω-hydroxylation
E310A	8.2	1.6 ± 0.1	4.1 ± 0.3	2.5 ± 0.2	ω-1 hydroxylation
E310G	<0.5	<0.1	<0.1	0.33 ± 0.10	ω-2 hydroxylation
E310D	<0.3	<0.1	<0.1	<0.1	

[a] Min^{-1}.

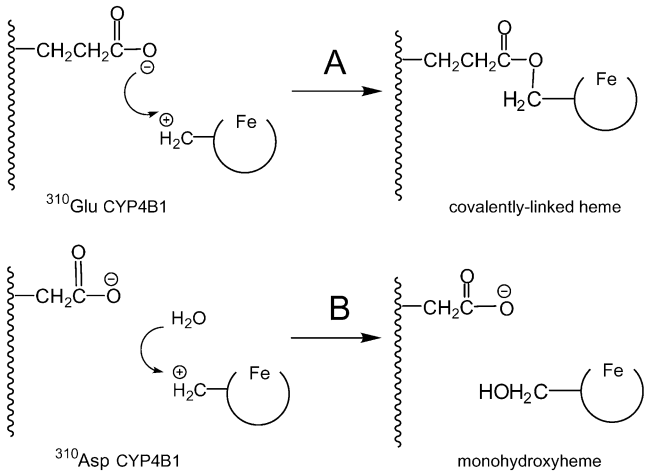

FIGURE 3: Alternative fates of a heme carbocation in CYP4B1 that partitions between (A) reaction with the appropriately positioned carboxylate of E310 to generate an ester bond and (B) quenching by an active site water molecule to generate monohydroxyheme in the E310D mutant.

in Table 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 2D). This heme species co-chromatographed with the polar heme released from wild-type CYP4B1 upon base treatment (data not shown) and exhibited the same 16 Da increase in mass observed previously (*3*) (Table 3). These data indicate that monohydroxylated heme is bound to the E310D mutant in a noncovalent manner.

Table 3: Covalent Heme Binding and Molecular Weight of the Acid-Dissociable Heme of Rabbit CYP4B1 I-Helix Mutants

enzyme	% covalently bound	heme (Da)
wild-type	75 ± 4[a]	616.2
E310G	1	616.2
E310A	0	616.2
E310D	2	632.3

[a] Mean ± standard deviation of three separate preparations.

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 ± 0.06% $H_2{}^{18}O$ and 14.0 ± 0.14% $H_2{}^{18}O$, and analyzed directly by LC/MS because of the limiting sample size. Fractional ion intensities for monohydroxyheme were

Table 4: Experimentally Determined Fractional Ion Intensities for the Polar Heme−Acetonitrile Adduct Obtained from CYP4B1 E310D Expressed in $H_2{}^{16}O$ or $H_2{}^{18}O$

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)

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

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 4). 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 ± 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 ± 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 2B,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 and 117 230 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, no dimer was apparent on SDS−PAGE performed under reducing conditions (Figure 1), and 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.

DISCUSSION

In addition to CYP4B1, covalent heme binding has been demonstrated for several members of the CYP4A and CYP4F subfamilies (*3*−*5*). Both CYP4A1 isolated from rat liver and CYP4A5/7 isolated from rabbit kidney 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. 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 (*8*, *12*, *20*, *21*).

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.

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 (*22*−*24*). However, in the present studies, dimer formation did not occur significantly with the wild-type enzyme and was not clearly associated with disruption or augmentation of the heme covalent link. Additional studies are needed to identify the cysteine residue(s) involved in the putative disulfide bond and to determine the effect of dimer formation on catalytic activity.

An important question concerns the effect of the heme covalent link on substrate and product specificity. For CYP4B1, elimination of the covalent link in the E310A, E310G, and E310D mutants resulted in a 40−100% loss of lauric acid hydroxylase activity. The low catalytic rates for the E310G and E310D mutants may reflect extensive dimerization of the enzyme preparation and/or monohydroxylation of the prosthetic group, respectively. However, interpretation of these data is complicated not only by the background of dimer and polar heme formation but also by the nonconservative nature of the mutations introduced, each of which could independently affect substrate binding and/or oxygen activation. What is clear is that the nature of the amino acid at position 310 in rabbit CYP4B1 is an important determinant of the regiospecificity of laurate hydroxylation because mutation of E301 → A310 → G310 shifted the dominant site of hydroxylation from $\omega \rightarrow \omega$-1 → ω-2 in concert with progressive reduction of amino acid side-chain volume.

Finally, 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 3A,B). Similar findings have been reported recently for the E318D mutant of CYP4A3 (*6*) and for the E375D and D225E mutants of lactoperoxidase (*25*). To test the hypothesis that a carbocation is featured on the reaction pathway, 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 (*12*).

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 and absence of detectable laurate turnover 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.

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 substitution of this I-helix glutamate influences fatty acid alkyl chain orientation in the active site of CYP4B1; and (iii) that the mechanism of formation of the heme−CYP4B1 covalent link most likely involves a carbocation intermediate located on the porphyrin.

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