Mechanism of Formation of the Ester Linkage between Heme and Glu310 of CYP4B1: ^{18}O Protein Labeling Studies†

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Received May 31, 2007; Revised Manuscript Received July 26, 2007

ABSTRACT: Cytochrome P450s in the CYP4 family covalently bind their heme prosthetic group to a conserved acidic I-helix residue via an autocatalytic oxidation. This study was designed to evaluate the source of oxygen atoms in the covalent ester link in CYP4B1 enzymes labeled with [^{18}O]glutamate and [^{18}O]aspartate. The fate of the heavy isotope was then traced into wild-type CYP4B1 or the E310D mutant-derived 5-hydroxyhemes. Glutamate-containing tryptic peptides of wild-type CYP4B1 were found labeled to a level of 11−13% ^{18}O. Base hydrolysis of labeled protein released 5-hydroxyheme which contained 12.8 ± 1.9% ^{18}O. Aspartate-containing peptides of the E310D mutant were labeled with 6.0−6.5% ^{18}O, but as expected, no label was transmitted to recovered 5-hydroxyheme. These data demonstrate that the oxygen atom in 5-hydroxyheme derived from wild-type CYP4B1 originates in Glu310. Stoichiometric incorporation of the heavy isotope from the wild-type enzyme supports a perferryl-initiated carbocation mechanism for covalent heme formation in CYP4B1.

Members of the CYP4[1] family can forge an ester linkage between the protein and heme group under normal turnover conditions (*1*−*6*). Specifically, native rabbit CYP4B1 forms a cross-link between Glu310, a glutamic acid residue on the I-helix that is present in all heme-bound CYP4 isoforms (*1*, *5*), and the C-5 methyl group of the heme (*6*). Preparations of CYP4B1 in which the heme is completely bound to the protein are capable of oxidizing substrates such as lauric acid and 4-ipomeanol (*6*), thereby demonstrating that the modified enzyme is catalytically competent. Furthermore, elimination of covalent heme binding via mutagenesis of the critical glutamate to alanine in CYP4B1 (*5*) or CYP4A11 (*3*) does not significantly affect activity toward lauric acid.

Retention of enzymatic activity by CYP4 enzymes following cross-link formation contrasts with the suicide inactivation and enhanced proteolytic degradation observed with previously characterized heme-linked P450s that have been generated in reactions with suicide substrates, such as carbon tetrachloride or 2-isopropyl-4-pentenamide (*7*). These substrate-dependent reactions are believed to be initiated by the oxidation of a substrate to a radical species that, in turn, can oxidize either the heme periphery or protein residues, followed by cross-link formation (*7*). In the case of the CYP4 enzymes, a substrate may be required to initiate the autocatalytic event, but there is no evidence to suggest that the substrate itself is involved in the chemistry of the cross-linking reaction. Thus, the reaction leading to covalent heme attachment in the CYP4s appears to proceed through a novel mechanism and warrants detailed investigation.

The oxidative formation of the ester bond in the CYP4 enzymes cannot be simply rationalized using the oxygen transfer 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 (*8*). However, 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 (*9*−*11*). Mutagenesis studies with lactoperoxidase (LPO) indicate that a properly positioned carboxylic acid group is strictly required for ester bond formation (*10*). Furthermore, covalent link formation can be promoted by the addition of hydrogen peroxide, suggesting that the mechanism is autocatalytic (*12*). To accommodate these experimental observations, a mechanism 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, has been proposed (*11*). Subsequently, the heme methyl radical is converted to a carbocation by transfer of one electron to the Fe^{IV} of the ferryl species. The carbocation can then be quenched by the properly positioned carboxylate group of the glutamate or aspartate residue to form the ester linkage that has been observed directly in the determined X-ray crystal structure of myeloperoxidase (*13*).

While evidence in favor of the foregoing mechanism continues to accrue for the mammalian peroxidases (*14*−*16*), there is a relative paucity of experimental data in support of an analogous mechanism for covalent attachment of heme to the CYP4 enzymes. Mutagenesis studies with CYP4B1 (*5*) and CYP4A3 (*3*) have provided evidence that a properly positioned carboxylate is required for heme activation and that a heme carbocation is a likely intermediate in the

† This investigation was supported by NIH Grants GM49054 and GM07750.

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[1] Abbreviations: CYP, cytochrome P450; LPO, lactoperoxidase; HRP, horseradish peroxidase; TFA, trifluoroacetic acid; LC−MS, liquid chromatography−mass spectrometry; SIM, single-ion monitoring.

10.1021/bi701064b CCC: $37.00
Published on Web 09/19/2007

mechanism of covalent bond formation. However, central to mechanistic inferences for covalent heme linkage in the P450s is information about the origin of the oxygen atoms in the ester bond between the conserved I-helix glutamate and the C-5 methyl heme.

Therefore, the primary goal of this study was to globally label glutamic acid residues of wild-type CYP4B1 with ^{18}O and trace the source of oxygen in the CYP4B1 ester link. As a control measure, we also performed the identical experiment with the E310D mutant that had been labeled with [^{18}O]aspartic acid. These studies were designed to (1) determine if a preformed heme methyl carbocation is quenched by the carboxyl group of Glu310 of native CYP4B1, as opposed to quenching by water in the CYP4B1 E310D mutant, and (2) evaluate the stoichiometry of these processes to provide information regarding the nature of the P450 oxidant involved in the heme activation step.

MATERIALS AND METHODS

Materials. Unlabeled amino acids and nucleic acids used in the minimal medium, L-α-dilauroylphosphatidylcholine (DLPC), and TFA were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Trypsin was purchased from Promega (Madison, WI). $H_2{}^{18}O$ (98 at. %) was purchased from Cambridge Isotope Laboratories (Andover, MA).

Enzymes. Recombinant rabbit CYP4B1 was cloned, expressed, and purified as described previously by Cheesman et al. (*17*). 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*).

Preparation of ^{18}O-Labeled 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 $H_2{}^{18}O$, and the solution was heated at 90 °C for 4 days at pH 1 (*18*). 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, 600 mg of the amino acid being dissolved in 3.6 mL of $H_2{}^{18}O$. The pH of the aspartate solution had to be adjusted to 1 by addition of 440 μL of concentrated HCl. After being heated for 4 days, the aspartic acid solution was neutralized on ice with 540 μL of 15 M NaOH. The water was removed from the dicarboxylic acid solutions by rotary evaporation. 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. Each solution was 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 liquid chromatograph. Each amino acid was diluted 1:10000 for analysis. A volume of 5 μL was injected into a mobile phase consisting of 50% methanol and 50% water at 100 μL/min. The mass spectrometer was run in negative electrospray ionization (ESI) mode at a cone voltage of 40 V, a source block temperature of 150 °C, and a desolvation temperature of 380 °C. Data analysis was carried out using Windows NT-based Micromass MassLynxNT version 3.2. 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 ^{18}O-Labeled CYP4B1 Enzymes. Wild-type CYP4B1 and E310D CYP4B1 were expressed in growth medium containing defined amounts of salts, nucleic acids, and amino acids, as described previously by Muchmore et al. (*19*). In addition, the medium was supplemented with 250 μL of a trace elements solution (*20*). For the wild-type enzyme, 200 μL of an overnight culture in Luria-Bertani medium was used to inoculate 200 mL of the complete medium in a 2.8 L Fernbach flask, and 1 mL of the labeled glutamate solution (200 mg) was added. For the E310D mutant, 300 μL of culture was used to inoculate 300 mL of medium and 2 mL of the labeled aspartate solution (150 mg) was added. These cultures were allowed to shake at 180 rpm and 37 °C for approximately 4 h. At this time, IPTG (1 mM), δ-aminolevulinic acid (0.5 mM), and [^{18}O]glutamic acid (200 mg) or [^{18}O]aspartic acid (150 mg) were added to the cultures. The temperature was reduced to 27 °C, and the shaking speed was reduced to 140 rpm. The remaining glutamic acid (500 mg) or aspartic acid (300 mg) solutions were added to the appropriate culture at regular intervals over the 48 h growth period. Control cultures for each enzyme were prepared in the same manner except that unlabeled amino acid was added. All proteins were purified as previously described on a nickel column (*17*), except that the hydroxyapatite column step was omitted. The final yield of labeled wild-type CYP4B1 and E310D mutant proteins was 15−20 nmol.

Mass Analysis of 5-Hydroxymethylheme. Covalently bound heme was released from wild-type CYP4B1 by hydrolysis 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 because the modified heme is not covalently bound and is released under the acidic chromatographic conditions. 5-Hydroxymethylheme was analyzed on a Micromass Quattro II tandem quadrupole mass spectrometer (Micromass, Ltd.) coupled to a Shimadzu liquid chromatograph similar to that described above. Modified heme was separated from the protein on a POROS R2 column. Initial conditions were 80% solution A (0.05% TFA in H_2O) and 20% solution B (0.05% TFA in acetonitrile) at a flow rate of 1 mL/min. The linear B gradient increased from 20 to 95% between 0 and 15 min. 5-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 5-hydroxymethylheme−acetonitrile complex.

LC−MS Analysis of CYP4B1 Tryptic Digests. 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 h 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 B gradient increased from 5 to 65% between 0 and 20 min. The mass spectrometer was run in (ESI) mode at a cone voltage of 45 V, a 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 version 3.2. The tryptic peptides that were used for the calculation of label incorporation were analyzed in triplicate.

Calculations for Determining ^{18}O Abundances. The relative integrated ion abundances of the parent ion envelopes for ^{18}O-labeled (span included masses representing the incorporation of up to four ^{18}O atoms) and nonlabeled glutamic acid, aspartic acid, peptides, and 5-hydroxymethylheme were recorded by LC−MS in the SIM mode. The ion envelope abundances for each run were normalized to the ion count observed for the mass of the base peak of the nonlabeled species (MH^+). The normalized abundances were determined in triplicate runs, and the averages and variances for the normalized intensity at each mass were calculated. The observed normalized ion envelopes for each species were utilized to sequentially correct the abundances that were observed for the corresponding labeled species. From the corrected ion intensities, the relative mole percentage of ^{18}O label incorporation was calculated individually for masses M, M + 2, M + 4, M + 6, and M + 8 that correspond to zero, one, two, three, and four ^{18}O atoms, respectively. The total mole percentage of ^{18}O incorporation was then obtained by summation of the contributions of species containing zero to two labels (hydroxyheme), zero to three labels (tryptic peptides), or zero to four labels (^{18}O-labeled glutamate and aspartate) by an extension of the method reported by Racha et al. (*21*) for the analysis of ^{18}O content. To minimize the contribution of adventitious labeling of amino acids, estimates of the mole percentage of incorporation of specifically three ^{18}O labels into tryptic peptides containing Glu or Asp were obtained using a correction factor of 89.0/63.0 or 83.4/48.2, respectively, on the basis of the analysis of the labeled amino acids. These ratios reflect the extent to which each of these amino acids was labeled with four ^{18}O atoms. Errors were fully propagated through calculations based on the assumption of independent random error (*22*).

RESULTS

Preparation of ^{18}O-Labeled Glutamate and Aspartate. Labeled amino acids, generated by incubating glutamate or aspartate hydrochloride at 90 °C in $H_2{}^{18}O$ for 4 days, were analyzed by LC−MS for ^{18}O incorporation. Unlabeled glutamate had a molecular ion at *m*/*z* 146, whereas the labeled glutamate sample exhibited masses at *m*/*z* 150, 152, and 154, representing a mixture of species with multiple ^{18}O atoms (Figure 1A). After accounting for the natural abundance of isotopes, which were based on ion intensities measured for unlabeled glutamate, we calculated the mole percentage of each isotopic species (Table 1). Overall, 89.0 ± 0.23% of the glutamate oxygen atoms were labeled with ^{18}O. Similarly, unlabeled aspartate had a molecular ion at *m*/*z* 132, whereas the labeled aspartate sample exhibited masses at *m*/*z* 136, 138, and 140 (Figure 1B). Following correction for natural abundance, 83.4 ± 0.3% of the aspartate oxygen atoms were found to be labeled with the heavy isotope (Table 1).

^{18}O Labeling of Wild-Type CYP4B1. Recombinant CYP4B1 was expressed in *Escherichia coli*, in a defined medium of salts, nucleic acids, amino acids, vitamins, and minerals. In a parallel experiment, natural glutamate was omitted from the defined medium and replaced with ^{18}O-labeled glutamate. Following purification on a nickel column, both labeled and unlabeled proteins were digested with trypsin and analyzed by LC−MS. Fourteen peptides in the control protein digest were identified on the basis of predicted masses and isotopic distribution. Four of these peptides contained glutamic acid residues (Table 2). The tryptic peptide containing Glu310 could be observed but did not ionize well enough to be analyzed for heavy isotope incorporation. Chymotrypsin and AspN were also utilized in attempts to obtain a heme-bound peptide amenable to LC−MS analysis, but appropriate masses could not be detected. Thirteen ions, spanning the possible isotopic distribution patterns, were monitored with SIM for each of the 14 tryptic peptides. After subtracting the intensities due to the natural abundance of isotopes, which were based on ion intensities of the peptides from the

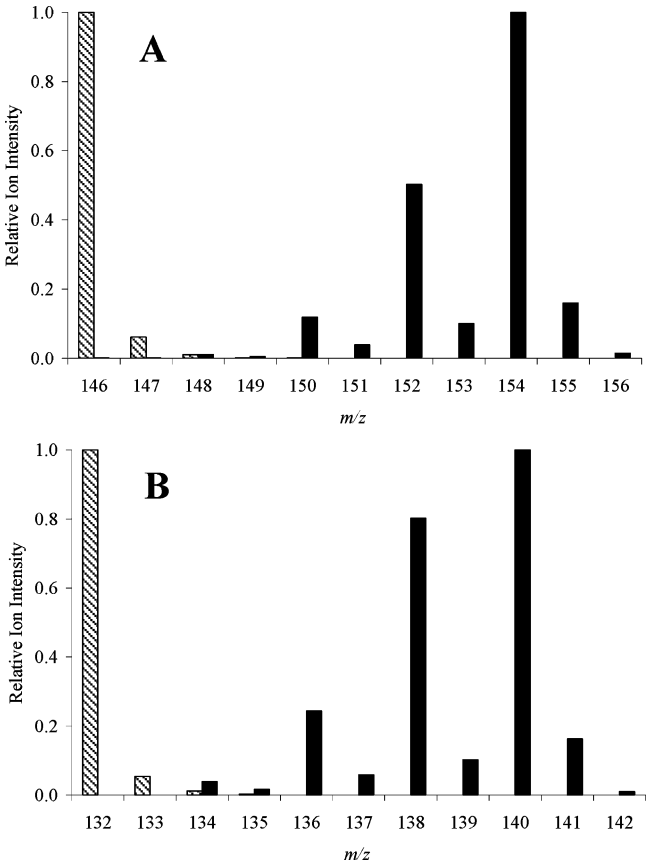

FIGURE 1: Mass spectra of ^{18}O-labeled glutamic acid and ^{18}O-labeled aspartic acid. Relative ion intensities for isotopic masses are shown for glutamic acid (A) and aspartic acid (B). The spectrum for the unlabeled amino acid is cross-hatched, and the spectrum for the labeled amino acid is black.

Table 1: Mole Percentage of Glutamate and Aspartate Labeled with ^{18}O[a]

	mol % labeled				
	one label	two labels	three labels	four labels	% total contribution
glutamate	0.11	2.79	23.11	62.98	88.98
aspartate	0.40	5.72	29.07	48.18	83.38

[a] A total of four labels can be incorporated into each dicarboxylic acid. The total contribution is calculated by assuming that the mole percentage of the fully labeled amino acid contributes 100%, the amino acid containing three labels contributes 75%, etc.

Table 2: Calculated Percentage of Label Incorporation for Each of 14 Identified CYP4B1 Wild-Type and E310D Tryptic Peptides

			mol % labeled				
CYP4B1	peptide	sequence	one label	two labels	three labels	four labels	five labels
wild-type [^{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	IML**E**K	5.7	7.0	**9.5**		
	T18	W**E**K	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	AALWD**E**K	12.9	14.5	**8.7**	2.8	1.9
	T36	IQNR	11.6	14.4	1.2	−0.1	
	T50	FSP**E**NSSGR	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	TGSL**D**K	6.7	2.7	**3.4**	0.9	
	T13	GLLVL**D**GPK	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	G**D**SGLNHR	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	AALW**D**EK	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		

unlabeled protein, we calculated the mole percentage of each species (Table 2). Tryptic peptides T17, T18, T33, and T50 all showed significant incorporation (up to 10%) of three ^{18}O atoms, which is expected for peptides that contain the labeled glutamic acid. By examining the mole percentage of one or two labels in the remaining peptides, it is clear that the ^{18}O label was transferred to other amino acids during biosynthesis. Considerable incorporation of one label or two labels was observed in peptides containing proline, glutamine, aspartic acid, and asparagine. This phenomenon is not surprising because glutamic acid is central to the production of several other amino acids. T17 and T18 peptides were then used to calculate the mole percentage of labeled oxygen atoms because these two peptides contained the fewest additional residues that were labeled with one or two ^{18}O atoms. Mass spectra for peptides T17 and T18 are shown in panels A and B of Figure 2.

The most reliable method for determining the mole percentage of labeled oxygen atoms in CYP4B1 is to assume that the label distribution in the glutamic acid in the protein does not differ from that in the original amino acid that was added to the expression system, and to base the calculation on the mole percentage of the triply labeled species. This method minimizes the contribution of label in other amino acids to the calculated value. When this method was applied to the T17 and T18 peptides, values of 13.4 ± 0.1 and 10.6 ± 0.6% ^{18}O incorporation, respectively, were obtained (Table 3). There is a possibility that the distribution of the label was not maintained during expression. This could occur via interconversion of amino acids by biosynthetic enzymes. Consequently, we also summed the contribution from one, two, and three labels to calculate the total mole percentage of label in the peptide. This method produces slightly higher values for the T17 (16.0 ± 0.2 mol %) and T18 (14.3 ± 1.7 mol %) peptides and reflects the maximum possible levels of label incorporation.

^{18}O Labeling of the CYP4B1 E310D Mutant. The mole percentage of incorporation of the label into CYP4B1 E310D was calculated from the ion intensities for the same 14 peptides analyzed for the wild-type enzyme (Table 2). Four of the observed peptides, T8, T13, T23, and T33, contained a single aspartic acid residue. Evidence of incorporation of labeled aspartate can be seen in the triply labeled species for each of these peptides, with values that range from 2.1 to 3.9 mol %. Again it is apparent that label has been transferred to other amino acids, notably asparagine, glutamate, and glutamine, as evidenced by the increase in the ion intensity of the M + 2 and M + 4 peaks of certain tryptic peptides. Therefore, tryptic peptides T8 and T13 were used to calculate the mole percentage 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. The mass spectra for these two peptides are shown in panels C and D of Figure 2. Solely on the basis of the heavy isotope levels in the triply labeled species, the T8 peptide is 6.0 ± 0.9 mol % labeled and the T13 peptide is 6.5 ± 0.5 mol % labeled (Table 3). When the singly, doubly, and triply labeled species for these peptides are included in the calculation, the maximal level of incorporation of the label into the T8 peptide is 7.6 ± 0.6 mol % and of the T13 peptide 8.2 ± 0.3 mol %.

Source of Oxygen in the Ester Linkage between Wild-Type CYP4B1 and Heme. 5-Hydroxymethylheme derived from base hydrolysis of the ester bond in wild-type CYP4B1 was analyzed for heavy isotope incorporation (Figure 3A). The natural isotope abundance was subtracted from the ion

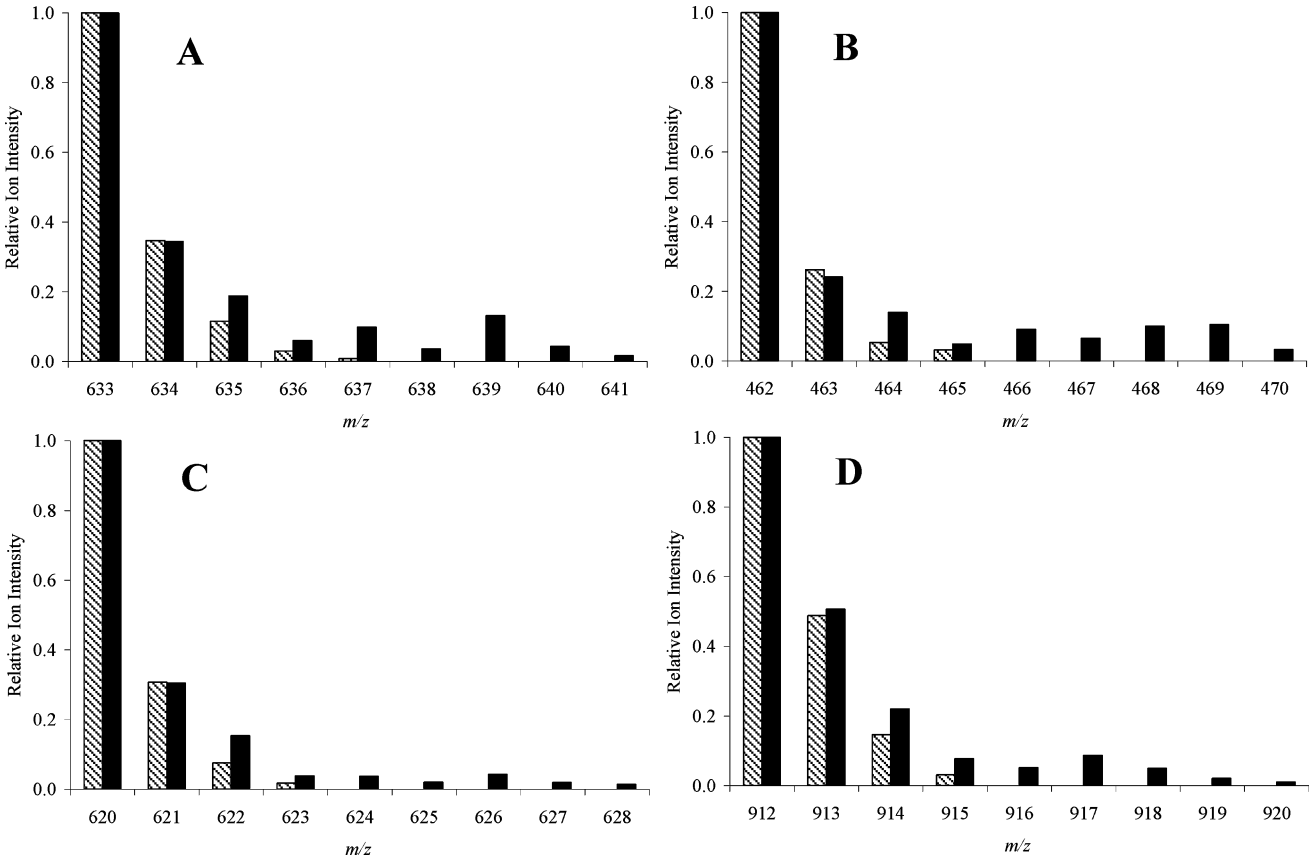

FIGURE 2: Mass spectra of tryptic peptides from wild-type CYP4B1 expressed with ^{18}O-labeled glutamic acid and E310D CYP4B1 expressed with ^{18}O-labeled aspartic acid. Relative ion intensities for isotopic masses are shown for peptides T17 (A) and T18 (B) from wild-type CYP4B1 and for peptides T8 (C) and T13 (D) from E310D CYP4B1. The spectrum for the unlabeled peptide is cross-hatched, and the spectrum for the labeled peptide is black.

Table 3: Calculated Percentage of ^{18}O-Labeled Atoms in Glutamic Acid Residues of Wild-Type CYP4B1, Aspartic Acid Residues of E310D CYP4B1, and Their Respective Modified Hemes[a]

CYP4Bl	peptide	sequence	total label based on three-label content (%)	label in hydroxy-methylheme (%)
wild-type [^{18}O]Glu	T17	IML**E**K	13.4 ± 0.1	12.8 ± 1.9
	T18	W**E**K	10.6 ± 0.6	
E310D [^{18}O]Asp	T8	TGSL**D**K	6.0 ± 0.9	0.1 ± 0.7
	T13	GLLVL**D**GPK	6.5 ± 0.5	

[a] The total calculated label content in glutamic acid and aspartic acid residues is based on the relative intensity of the triply labeled peptide containing these amino acids, assuming that the original glutamate or aspartate was not isotopically diluted.

intensities of the labeled hydroxymethylheme−acetonitrile complex and the mole percentage of label calculated from the additional M + 2 ion intensity at *m/z* 675. 5-Hydroxymethylheme derived from CYP4B1 labeled with [^{18}O]-glutamate contained 12.8 ± 1.9 mol % of the heavy isotope. To ensure that ^{18}O was not incorporated into the propionic acid carboxylates of heme, unmodified heme from CYP4B1 was analyzed by the same method. No label incorporation was observed, as evidenced by the calculated value of −0.7 ± 1.4 mol %.

Source of Oxygen in Monohydroxylated Heme Released from the E310D Mutant. In contrast to the data obtained for the wild-type enzyme, 5-hydroxymethylheme released from the [^{18}O]aspartate E310D mutant contained essentially no heavy label (0.1 ± 0.7%) (Figure 3B), a baseline level close to that obtained for unmodified heme from the mutant protein (−0.1 ± 0.4%). This result is in agreement with findings from an earlier study that indicated that the oxygen in noncovalently bound 5-hydroxyheme from the E310D mutant is derived from water (*5*).

DISCUSSION

Covalent attachment of heme to the protein backbone of mammalian peroxidases was elucidated more than 50 years ago (*23*), and a considerable amount of data since then has led to the development of a consensus mechanism for heme diester bond formation (*11*). Important features of this mechanism include formation of a Compound I-like intermediate followed by a process that involves removal of a hydrogen atom from each of the two affected heme methyl groups. Intramolecular transfer of one electron from Fe^{IV} generates a heme methyl carbocation that can be trapped by a carboxylic acid residue to form the observed ester linkage(s) between the heme and protein. A heme iron oxidant, such as the iron−oxo species, does not seem suitable for direct oxidation of the methyl group because of the large distance to the iron on the rigid heme plane. Therefore, it is more likely that a protein residue functions as a transient oxidized intermediate that can, in turn, oxidize the heme methyl group to a methyl radical. On the basis of a homology model of LPO, it has been proposed that the carboxylate groups that

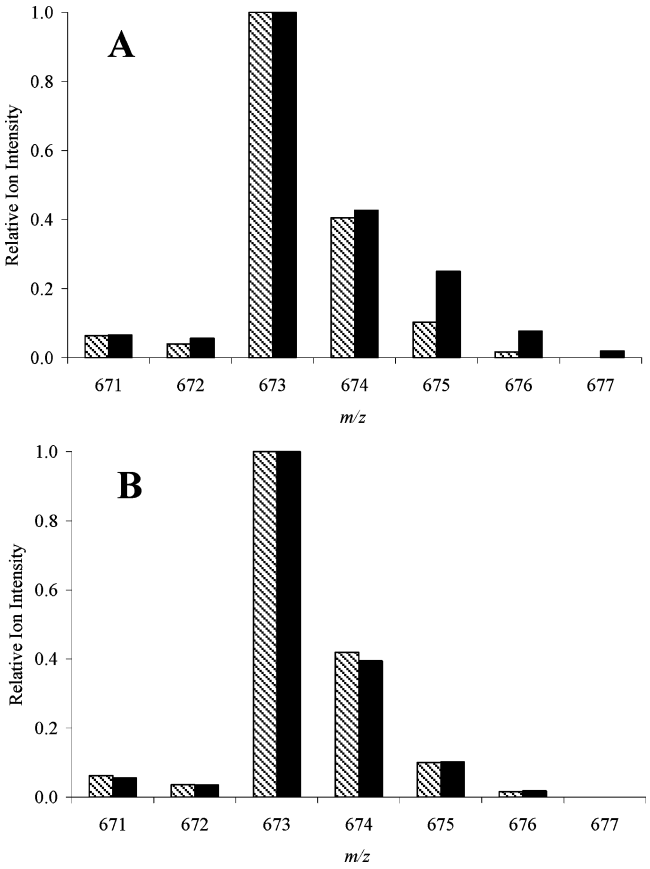

FIGURE 3: Mass spectra of the 5-hydroxymethylheme−acetonitrile complex from wild-type CYP4B1 expressed with ^{18}O-labeled glutamic acid and E310D CYP4B1 expressed with ^{18}O-labeled aspartic acid. Relative ion intensities for isotopic masses are shown for hydroxymethylheme following base hydrolysis of wild-type CYP4B1 (A) and unbound 5-hydroxymethylheme from E310D CYP4B1 (B). The spectrum for the unlabeled amino acid is cross-hatched, and the spectrum for the labeled amino acid is black.

eventually form the ester linkages are themselves initially oxidized and are responsible for the subsequent abstraction of a hydrogen atom from the heme methyl groups (*11*). In the mechanism considered for LPO, it is assumed that the carboxylic acid side chain is oxidized by Compound I to produce a carboxyl radical, a single-electron oxidation resembling the reaction with small aromatic substrates that yields aryl radicals (*11*).

Further evidence of a heme methyl carbocation has been gleaned from the reaction of phenylhydrazine and hydrogen peroxide with horseradish peroxidase (HRP) (*14*). In this reaction, a phenyl radical abstracts a hydrogen atom 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 $H_2{}^{18}O$ demonstrated that water attacks the carbocation to form 8-hydroxymethylheme (*14*). Even more relevant is evidence for the single-electron oxidation of carboxylic acids that has recently been demonstrated with HRP in the presence of acetate and H_2O_2 (*15*). The existence of a carboxyl radical intermediate was deduced from the formation of ester-linked heme adducts and the production of 8-hydroxymethylheme (*15*). To prove that a suitably positioned carboxylate is the only requirement for heme activation, Colas et al. (*16*) showed that HRP can be engineered to covalently bind heme. These investigators inserted a glutamic acid into the active site of HRP, via mutagenesis of Phe41. When F41E HRP was reacted with H_2O_2, nearly all of the heme became covalently linked to the protein, presumably via formation of an ester bond between Glu41 and the 3-methyl group.

Since the identification of the covalently attached heme in the CYP4 family of P450s, evidence has accrued to support a mechanism for monoester bond formation similar to that proposed for the mammalian peroxidases. Mutagenesis studies with CYP4B1 and CYP4A3 have uncovered key mechanistic features of the reaction, including a properly positioned carboxylate and heme activation to a putative carbocation intermediate. When the catalytically relevant glutamic acid is mutated in either enzyme to an aspartic acid residue, noncovalently bound 5-hydroxymethylheme is recovered (*3*, *5*). Moreover, when the E310D CYP4B1 mutant was expressed in media containing $H_2{}^{18}O$, the heavy isotope was found to be incorporated into 5-hydroxymethylheme, suggesting that water had attacked the preformed carbocation during the autocatalytic cycle (*5*).

While many parallels appear to exist between the mechanism of covalent attachment of heme to the P450s and peroxidases, surprisingly no data have been presented with regard to a key component of either mechanism, viz., the origin of the oxygen atoms in the ester linkage. Studies with the E310D mutant of CYP4B1 support a carbocation intermediate, but they do not directly address the origin of the ester oxygen atoms because no covalent link is formed with this mutant. To focus on this issue, an experiment was designed in which the carboxylates of glutamate residues in CYP4B1 were labeled globally with ^{18}O. Following expression and purification, the amount of label in the glutamate carboxylates of CYP4B1 was determined by the increase in the intensity of the [M + 6] ions of tryptic peptides by LC−MS. On the basis of the T17 peptide, the carboxylates were labeled to an extent of 13.4 ± 0.12%, and on the basis of the T18 peptide, this value was 10.6 ± 0.6%. Incorporation of the heavy isotope into the ester bond was evaluated by releasing 5-hydroxymethyheme from the enzyme by base hydrolysis and analyzing the heme for ^{18}O content. We found that the modified heme was labeled with 12.8 ± 1.9% ^{18}O, representing the mole percentage of label located in the ester bond. Therefore, incorporation of the label from the glutamic acid residue into the ester bond (96 ± 15% based on T17 and 121 ± 20% based on T18) was essentially stoichiometric. These data are consistent with the view that a preformed heme methyl carbocation is quenched by the carboxylate of Glu310 to generate the heme covalent link in native CYP4B1 (Figure 4).

The same experimental technique was applied to the E310D mutant of CYP4B1 to confirm previous results, which showed that the oxygen atom in hydroxymethylheme originates from water (*5*). Between 6.0 ± 0.9 and 6.5 ± 0.5 mol % of the carboxylates of aspartic acid residues in E310D CYP4B1 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 $H_2{}^{18}O$ revealed quantitative incorporation of the heavy isotope. As suspected previously, a heme methyl carbocation is formed similarly in the E310D mutant and the wild-type enzyme but is then quenched by water instead of the side chain carboxylate.

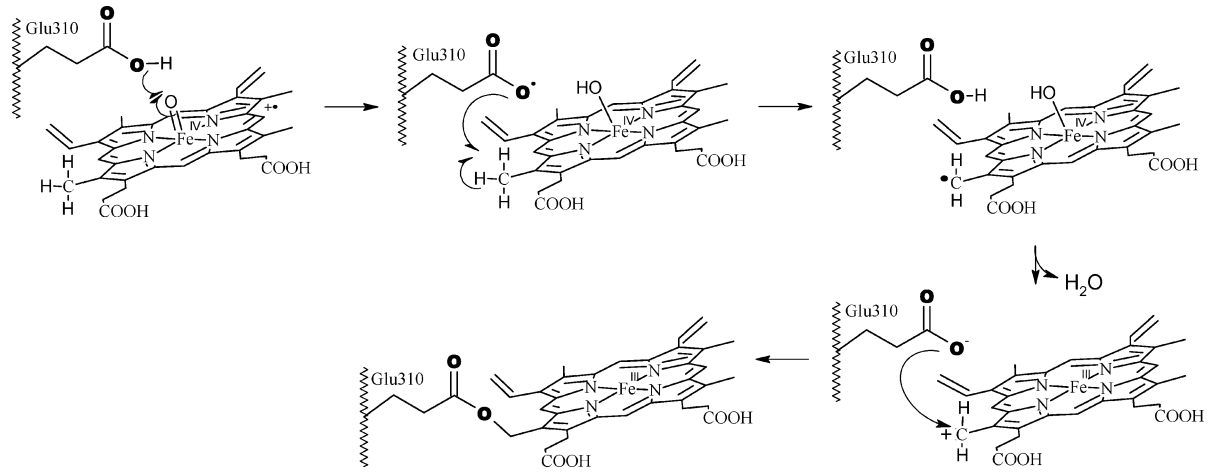

FIGURE 4: Mechanism for covalent heme attachment involving hydrogen abstraction by the iron−oxo species. The labeled oxygens (boldface) are traced throughout the reactions. This mechanism results in 100% incorporation of glutamate ^{18}O into the ester linkage.

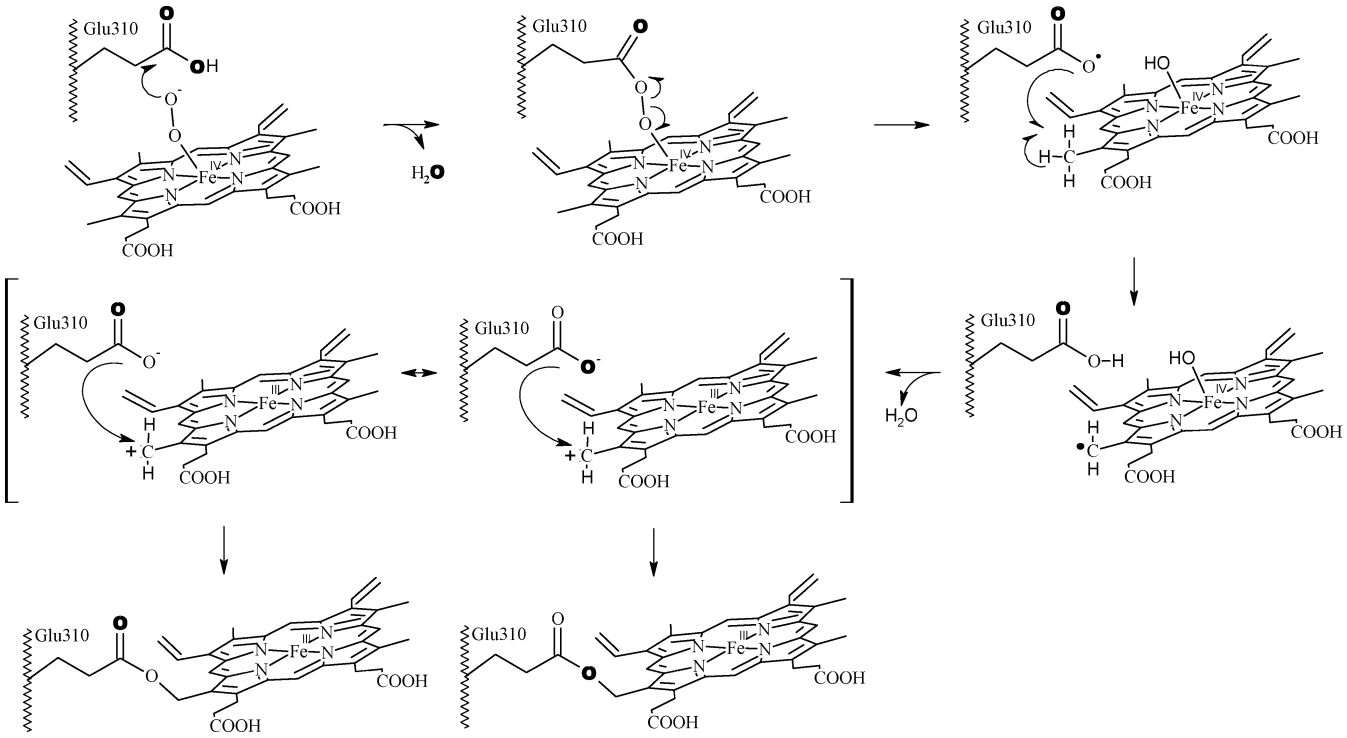

FIGURE 5: Alternative mechanism for covalent heme attachment involving homolytic scission of a perester oxygen−oxygen bond. The labeled oxygens (boldface) are traced throughout the reactions. This mechanism would result in 50% incorporation of glutamate ^{18}O into the ester linkage.

Quantitation of incorporation of ^{18}O from the carboxylates of Glu310 not only reinforces the proposed mechanism based on the mammalian peroxidases but also excludes other potential reactions that could lead to the observed heme−protein linkage. We can assuredly 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 liberated 5-hydroxymethylheme could potentially originate from water, as experimentally determined 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 into hydroxymethylheme, and so this mechanism is not valid. Another mechanism can be envisioned in which the P450 peroxo−iron oxidant reacts with the carboxylate to form a perester intermediate, similar to the reaction of aromatase with aldehydes (*24*). Homolytic scission of the oxygen−oxygen bond could then produce the proposed carboxyl radical (Figure 5). Indeed, homolytic scission of a perester intermediate between CYP2B4 and the artifical oxidant, *m*-CPBA, has previously been shown to result in a carboxyl radical that subsequently reacts with the heme periphery to form a relevant ester-bound adduct (*25*). In this alternate mechanism, one of the oxygen atoms in the carboxylate would derive from iron-bound dioxygen prior to quenching of the carbocation (Figure 5). This reaction pathway would result in the transfer of 50% of the heavy isotope into the heme, and therefore, these data are also inconsistent with a mechanism involving a perester intermediate.

In conclusion, studies with the labeled glutamate residues of CYP4B1 demonstrate that 100% of the oxygen from Glu310 carboxylates is transferred to the P450 monoester linkage. These data support a mechanism in which the iron−oxo P450 oxidant removes an electron from the glutamic acid carboxylate, followed by abstraction of hydrogen 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 generate the ester bond (Figure 4). These experiments provide the first experimental support for the mechanism of ester bond formation in a wild-type CYP4 enyzme.

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BI701064B