Published in final edited form as:

Biochemistry. 2007 October 16; 46(41): 11598–11605. doi:10.1021/bi701064b.

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

Brian R. Baer, Kent L. Kunze, and Allan E. Rettie*

Department of Medicinal Chemistry, University of Washington, Seattle, Washington 98195

Abstract

Cytochrome P450s in the CYP4 family covalently bind their heme prosthetic group to a conserved acidic I-helix residue *via* an autocatalytic oxidation. The present study was designed to evaluate the source of oxygen atoms in the covalent ester link in CYP4B1 enzymes labeled with ¹⁸O-glutamate and ¹⁸O-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% ¹⁸O. Base hydrolysis of labeled protein released 5-hydroxyheme which contained $12.8 \pm 1.9\%$ ¹⁸O. Aspartate-containing peptides of the E310D mutant were labeled with 6.0 – 6.5% ¹⁸O 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 perferferryl-initiated carbocation mechanism for covalent heme formation in CYP4B1.

Members of the CYP4 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 hemelinked 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,

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

*To whom correspondence should be addressed. Telephone: (206) 685-0615. Fax (206) 685-3252. rettie@u.washington.edu.

in which an endogenous or exogenous compound is converted to a more lipophilic metabolite *via* hydroxylation, epoxidation, or heteroatom oxidation (8). Yet, 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 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 (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 solved 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 heme attachment 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 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 the present study was to globally label glutamic acid residues of wild-type CYP4B1 with ¹⁸O 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 ¹⁸O-aspartic acid. These studies were designed to 1) determine if a pre-formed 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 media, L- α -dilauroylphosphatidylcholine (DLPC), and TFA were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Trypsin was purchased from Promega (Madison WI). H₂ ¹⁸O (98 atom mol%) 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 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; by dissolving 600 mg of the amino acid in 3.6 ml of H_2^{18}O . The pH of the aspartate solution had to be adjusted to pH 1 by addition of 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 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 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 $\mu\text{l}/\text{min}$. 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 ^{18}O -Labeled CYP4B1 Enzymes

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.* (19). In addition, the media was supplemented with 250 μL of trace elements solution (20). For the wild-type enzyme, 200 μl 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 (200 mg) was added. For the E310D mutant, 300 μL of culture was used to inoculate 300 ml of media and 2 ml of the labeled aspartate solution (150 mg) 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 -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 hr 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., Manchester, U.K.) coupled to a Shimadzu LC instrument 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 gradient increased B from 20–95% 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.

LCMS 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 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 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 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.

Calculations to Determine ^{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 4 ^{18}O atoms) and non-labeled glutamic acid, aspartic acid, peptides, and 5-hydroxymethylheme were recorded by LCMS in the selected ion monitoring mode. The ion envelope abundances for each run were normalized to the ion count observed for the mass of the base peak of the non-labeled 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 % of ^{18}O label incorporation was calculated individually for masses M , $M+2$, $M+4$, $M+6$ and $M+8$ that correspond to 0, 1, 2, 3 or 4 ^{18}O atoms, respectively. Total mole% ^{18}O incorporation was then obtained by summation of the contributions of species containing 0–2 labels (hydroxyheme), 0–3 labels (tryptic peptides) or 0–4 labels (^{18}O labeled glutamate and aspartate) by an extension of the method reported by Racha et al. (21) to analyze ^{18}O content. In order to minimize the contribution of adventitious labeling of amino acids, estimates of the mol % incorporation of specifically three ^{18}O labels into tryptic peptides containing Glu or Asp was accomplished using a correction factor of 89.0/63.0 or 83.4/48.2, respectively, based on 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 LCMS for ^{18}O incorporation. 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 1A). After accounting for the natural abundance of isotopes, which were based on ion intensities measured for unlabeled glutamate, the mole % of each isotopic species was calculated (Table 1). Overall, $89.0 \pm 0.23\%$ of the glutamate oxygen atoms were labeled with ^{18}O . Similarly, unlabeled aspartate had a molecular ion of m/z 132, whereas the labeled aspartate sample showed masses at m/z 136, m/z 138, and m/z 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 *E. 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 media 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 LCMS. Fourteen peptides in the control protein digest were identified based on 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 LCMS 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 unlabeled protein, the mole % of each species was calculated (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 % 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. Considerable incorporation of 1 label or 2 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 % 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 figures 2A and 2B.

The most reliable method for determining the mole % of labeled oxygen atoms in CYP4B1 is to assume that the label distribution in the glutamic acid in the protein does not differ from the original amino acid that was added to the expression system, and to base the calculation on the mole % of the triple-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* inter-conversion of amino acids by biosynthetic enzymes. Consequently, we also summed the contribution from 1, 2, and 3 labels to calculate the total mole % of label in the peptide. This method produces slightly higher values for the T17 (16.0 ± 0.2 mole %) and T18 (14.3 ± 1.7 mole %) peptides, and reflects the maximum possible levels of label incorporation.

^{18}O -Labeling of the CYP4B1 E310D Mutant

The mole % of label incorporation 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 for incorporation of labeled aspartate can be seen in the triple-labeled species for each of these peptides, values which range from 2.1 to 3.9 mole %. Again it is apparent that label has been transferred to other amino acids, notably asparagine, glutamate, and glutamine, as evidenced by the increase in 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 % 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 figures 2C and 2D. 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 (Table 3). When the 1-, 2-, and 3-labeled species for these peptides are included in the calculation, maximal label incorporation of the T8 peptide is 7.6 ± 0.6 mole % and of the T13 peptide, 8.2 ± 0.3 mole %.

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 intensities of the labeled hydroxymethylheme-acetonitrile complex and the mole % 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 mole % 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 mole %.

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 ($0.09 \pm 0.7\%$) (Figure 3B), a base-line level close to that obtained for unmodified heme from the mutant protein ($-0.1 \pm 0.4\%$). This result is in agreement with findings from an earlier study that indicated that the oxygen in non-covalently bound 5-hydroxyheme from the E310D mutant is derived from water (5).

Discussion

Covalent heme attachment to the protein backbone of mammalian peroxidases was established 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 di-ester 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. Based on a homology model of LPO, it has been proposed that the carboxylate groups that eventually form the ester linkages are themselves initially oxidized, and are responsible for subsequent hydrogen atom abstraction 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 to form aryl radicals (11).

Further evidence for 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, evidence for the single-electron oxidation of carboxylic acids has recently been demonstrated with HRP in the presence of acetate and H₂O₂ (15). 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.* showed that HRP can be engineered to covalently bind heme (16). These investigators inserted a glutamic acid into the active site of HRP, *via* mutagenesis at Phe41. When HRP F41E was reacted with H₂O₂, 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 mono-ester 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, non-covalently bound 5-hydroxymethylheme is recovered (3, 5). Moreover, when the CYP4B1 E310D mutant was expressed in media containing H₂ ¹⁸O, 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 heme covalent attachment to the P450s and peroxidases, surprisingly no data have been presented regarding 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 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 by LCMS. Based on the T17 peptide, the carboxylates were labeled to an extent of 13.4 ± 0.12 %, and based on the T18 peptide this value was 10.6 ± 0.6 %. Incorporation of the heavy isotope into the ester bond was evaluated by releasing 5-hydroxymethylheme from the enzyme by base hydrolysis and analyzing the heme for ¹⁸O content. We found that the modified heme was labeled with 12.8 ± 1.9 % ¹⁸O, representing the mole % 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 in order 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 mole % of the carboxylates of aspartic acid residues in CYP4B1 E310D were globally labeled with ¹⁸O. 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 H₂ ¹⁸O reveals quantitative incorporation of the heavy isotope. As suspected previously, a heme methyl carbocation is formed similarly in the E310D mutant as the wildtype enzyme, but is then quenched by water instead of the side chain carboxylate.

Quantitation of ^{18}O incorporation 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 artificial 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 oxygen from Glu310 carboxylates is transferred to the P450 mono-ester linkage. These data support a mechanism in which the 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 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 enzyme.

Abbreviations

CYP	cytochrome P450
LPO	lactoperoxidase
HRP	horseradish peroxidase
TFA	trifluoroacetic acid
LCMS	liquid chromatography mass spectrometry
SIM	single ion monitoring.

REFERENCES

1. Henne KR, Kunze KL, Zheng YM, Christmas P, Soberman RJ, Rettie AE. Covalent linkage of prosthetic heme to CYP4 family P450 enzymes. *Biochemistry*. 2001; 40:12925–12931. [PubMed: 11669629]
2. Hoch U, Ortiz De Montellano PR. Covalently linked heme in cytochrome p4504a fatty acid hydroxylases. *J Biol Chem*. 2001; 276:11339–11346. [PubMed: 11139583]
3. LeBrun LA, Hoch U, Ortiz de Montellano PR. Autocatalytic mechanism and consequences of covalent heme attachment in the cytochrome P4504A family. *J Biol Chem*. 2002; 277:12755–12761. [PubMed: 11821421]
4. LeBrun LA, Xu F, Kroetz DL, Ortiz de Montellano PR. Covalent attachment of the heme prosthetic group in the CYP4F cytochrome P450 family. *Biochemistry*. 2002; 41:5931–5937. [PubMed: 11980497]

5. Zheng YM, Baer BR, Kneller MB, Henne KR, Kunze KL, Rettie AE. Covalent heme binding to CYP4B1 via Glu310 and a carbocation porphyrin intermediate. *Biochemistry*. 2003; 42:4601–4606. [PubMed: 12693958]
6. Baer BR, Schuman JT, Campbell AP, Cheesman MJ, Nakano M, Moguilevsky N, Kunze KL, Rettie AE. Sites of covalent attachment of CYP4 enzymes to heme: evidence for microheterogeneity of P450 heme orientation. *Biochemistry*. 2005; 44:13914–13920. [PubMed: 16229480]
7. Osawa Y, Pohl LR. Covalent bonding of the prosthetic heme to protein: a potential mechanism for the suicide inactivation or activation of hemoproteins. *Chem Res Toxicol*. 1989; 2:131–141. [PubMed: 2519716]
8. Sono M, Roach MP, Coulter ED, Dawson JH. Heme-Containing Oxygenases. *Chem Rev*. 1996; 96:2841–2888. [PubMed: 11848843]
9. Rae TD, Goff HM. The heme prosthetic group of lactoperoxidase. Structural characteristics of heme l and heme l-peptides. *J Biol Chem*. 1998; 273:27968–27977. [PubMed: 9774411]
10. Colas C, Kuo JM, Ortiz de Montellano PR. Asp-225 and glu-375 in autocatalytic attachment of the prosthetic heme group of lactoperoxidase. *J Biol Chem*. 2002; 277:7191–7200. [PubMed: 11756449]
11. Colas C, Ortiz de Montellano PR. Autocatalytic radical reactions in physiological prosthetic heme modification. *Chem Rev*. 2003; 103:2305–2332. [PubMed: 12797831]
12. DePillis GD, Ozaki S, Kuo JM, Maltby DA, Ortiz de Montellano PR. Autocatalytic processing of heme by lactoperoxidase produces the native protein-bound prosthetic group. *J Biol Chem*. 1997; 272:8857–8860. [PubMed: 9083001]
13. Fiedler TJ, Davey CA, Fenna RE. X-ray crystal structure and characterization of halide-binding sites of human myeloperoxidase at 1.8 Å resolution. *J Biol Chem*. 2000; 275:11964–11971. [PubMed: 10766826]
14. Ator MA, Ortiz de Montellano PR. Protein control of prosthetic heme reactivity. Reaction of substrates with the heme edge of horseradish peroxidase. *J Biol Chem*. 1987; 262:1542–1551. [PubMed: 3805041]
15. Huang L, Colas C, Ortiz de Montellano PR. Oxidation of carboxylic acids by horseradish peroxidase results in prosthetic heme modification and inactivation. *J Am Chem Soc*. 2004; 126:12865–12873. [PubMed: 15469283]
16. Colas C, De Montellano PR. Horseradish peroxidase mutants that autocatalytically modify their prosthetic heme group: insights into mammalian peroxidase heme-protein covalent bonds. *J Biol Chem*. 2004; 279:24131–24140. [PubMed: 15039425]
17. Cheesman MJ, Baer BR, Zheng YM, Gillam EM, Rettie AE. Rabbit CYP4B1 engineered for high-level expression in *Escherichia coli*: ligand stabilization and processing of the N-terminus and heme prosthetic group. *Arch Biochem Biophys*. 2003; 416:17–24. [PubMed: 12859977]
18. Gallo KA, Tanner ME, Knowles JR. Mechanism of the reaction catalyzed by glutamate racemase. *Biochemistry*. 1993; 32:3991–3997. [PubMed: 8097109]
19. Muchmore DC, McIntosh LP, Russell CB, Anderson DE, Dahlquist FW. Expression and nitrogen-15 labeling of proteins for proton and nitrogen-15 nuclear magnetic resonance. *Methods Enzymol*. 1989; 177:44–73. [PubMed: 2691846]
20. Bauer S, Shiloach J. Maximal exponential growth rate and yield of *E. coli* obtainable in a bench-scale fermentor. *Biotechnol Bioeng*. 1974; 16:933–941. [PubMed: 4606791]
21. Racha JK, Rettie AE, Kunze KL. Mechanism-based inactivation of human cytochrome P450 1A2 by furafylline: detection of a 1:1 adduct to protein and evidence for the formation of a novel imidazomethide intermediate. *Biochemistry*. 1998; 37:7407–7419. [PubMed: 9585555]
22. Taylor, JR. *An Introduction to Error Analysis: The Study of Uncertainties in Physical Measurements*. 2nd ed.. Sausalito: University Science Books; 1997. Propagation of Uncertainties; p. 45-92.
23. Morell DB. The prosthetic group of lactoperoxidase. *Aust J Exp Biol Med Sci*. 1953; 31:567–571. [PubMed: 13149557]
24. Akhtar M, Calder MR, Corina DL, Wright JN. Mechanistic studies on C-19 demethylation in oestrogen biosynthesis. *Biochem J*. 1982; 201:569–580. [PubMed: 7092812]

25. Kuo CL, Raner GM, Vaz AD, Coon MJ. Discrete species of activated oxygen yield different cytochrome P450 heme adducts from aldehydes. *Biochemistry*. 1999; 38:10511–10518. [PubMed: 10441147]

Figure 1.

Mass spectra of ^{18}O -labeled glutamic 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 shaded grey and the spectrum for the labeled amino acid is shaded black.

Figure 2.

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 3.

Mass spectra of 5-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 5-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. Mechanism for covalent heme attachment involving hydrogen abstraction by the iron-oxo species. The labeled oxygens (bold face print) are traced throughout the reactions. This mechanism results in 100% ^{18}O incorporation of glutamate oxygen into the ester linkage.

Figure 5.

Alternative mechanism for covalent heme attachment involving homolytic scission of a perester oxygen-oxygen bond. The labeled oxygens (bold face print) are traced throughout the reactions. This mechanism would result in 50% ¹⁸O incorporation of glutamate oxygen into the ester linkage.

Table 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 % 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
Aspartate	0.40	5.72	29.07	48.18
				88.98
				83.38

Table 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		

CYP4B1	Peptide	Sequence	Mole % labeled				
			1 label	2 label	3 label	4 label	5 label
	T56	LPQLVLR	8.4	1.1	-0.2		

Table 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 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	