Covalently Linked Heme in Cytochrome P450A4 Fatty Acid Hydroxylases*

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Three independent experimental methods, liquid chromatography, denaturing gel electrophoresis with heme staining, and mass spectrometry, establish that the CYP4A class of enzymes has a covalently bound heme group even though the heme is not cross-linked to the protein in other P450 enzymes. Covalent binding has been demonstrated for CYP4A1, -A2, -A3, -A8, and -A11 heterologously expressed in Escherichia coli. However, the covalent link is also present in CYP4A1 isolated from rat liver and is not an artifact of heterologous expression. The extent of heme covalent binding in the proteins as isolated varies and is substoichiometric. In CYP4A3, the heme is attached to the protein via an ester link to glutamic acid residue 318, which is on the I-helix, and is predicted to be within the active site. This is the first demonstration that a class of cytochrome P450 enzymes covalently binds their prosthetic heme group.

Heme1 is the essential prosthetic group of many important proteins, including the hemoglobins, catalases, peroxidases, and cytochromes. In cytochrome P450 enzymes, the heme sits deep inside the protein with its iron ligated to a cysteine thiolate group and the sixth iron coordination site occupied by a water molecule in the substrate-free state (1). This coordination arrangement gives cytochrome P450 enzymes their unique spectral and catalytic properties. These enzymes are involved in many vital processes, including the biosynthesis of steroids and other lipophilic physiological effectors, the metabolism of drugs, and the degradation of xenobiotics. A unique feature of the cytochrome P450 enzymes is their ability to catalyze the hydroxylation of unsaturated hydrocarbons under physiological conditions rather than the extreme conditions required for the reaction to occur under uncatalyzed conditions. Sequence homologies within the heme-binding domain of the protein suggest that, despite the existence of numerous P450 forms with different substrate specificities, individual P450 enzymes share a common catalytic mechanism of oxygen activation.

Work in this laboratory has focused on the P450 enzymes of family 4 (CYP4), especially the four known rat isoforms (CYP4A1, -A2, -A3, and -A8) and the single characterized human isoform (CYP4A11) (2–5). The first three rat enzymes are present in liver and are induced by clofibrate, whereas all four enzymes are constitutively expressed in the rat kidney. The enzymes of the CYP4 family are distinguished by their unique ability to hydroxylate preferentially the thermodynamically disfavored terminal methyl group of medium and long chain fatty acids, prostaglandins, and other eicosanoids. Although other P450 enzymes hydroxylate fatty acids at internal positions of the hydrocarbon chain (6–8), only the CYP4 enzymes preferentially hydroxylate the energetically disfavored terminal methyl group. Site-directed mutagenesis studies have begun to shed some light on the mechanism by which these enzymes achieve this specificity (4, 5, 9), but the detailed mechanism remains obscure.

In this paper we report another unique and completely unexpected feature of the CYP4A class of enzymes. Liquid chromatography, SDS-gel electrophoresis with heme staining, and LC/ESI-MS provide unambiguous evidence that the prosthetic group is at least partially covalently bound to the protein in the CYP4A enzymes. Release of the modified heme by proteolysis indicates the presence of an additional hydroxyl group on the heme, suggesting cross-linking to the protein through an ester function involving an acidic residue. Indeed, analysis of heme peptides indicates that a glutamic acid residue on the I-helix is involved in heme binding. Analysis of CYP4A1 isolated from rat liver demonstrates that the modified heme is not an artifact of the heterologous E. coli expression system. Finally, experimental data obtained with the CYP4A3 Tyr-117 and Leu-129 active site mutants suggest that covalent cross-linking of the heme is required for catalytic activity. This is the first time that covalent cross-linking of the heme to the protein is observed in native or wild-type P450 enzymes and that evidence is found that this cross-linking is a prerequisite for catalytic activity.

EXPERIMENTAL PROCEDURES

Materials—All primers were ordered from Life Technologies, Inc. Restriction endonucleases and ligases were purchased from New England Biolabs. Pronase, ampicillin, &-aminovulinic acid, glycerol, lipoxygenase, dilauroylphosphatidylcholine, glutathione, catalase, NADPH, and lauric acid were obtained from Sigma. Tris-EDTA and NaCl were purchased from Fisher. Emulgen 913 was a gift from Kao Chemicals. Reagents for “enhanced” CL were obtained from Amersham Pharmacia Biotech. [14C]Lauric acid (55 mCi/mmol) was from American Radiolabeled Chemicals, Inc. The human NADPH-cytochrome P450 450 reductase cDNA was provided by Stephen M. Black (University of California, San Francisco), and the protein was expressed and purified according to the protocol outlined previously (10). Purified rat liver cytochrome b5 was a gift from Lester Bornheim, and the pCWori vector was a gift from Robert Flodverick, both of whom are from the University of California, San Francisco. Rat liver CYP4A1 was isolated from clofibrate-pre-treated rats by Claire Cadac (11). CYP2E1 was kindly provided by F. P. Guengerich (Vanderbilt University) and CYP3A4 by M. A. Correia (University of California, San Francisco).

Mutations in CYP4A3—For mutagenesis the CYP4A3 DNA containing the 5’ modifications described by Barnes et al. (12) was subcloned...
into the pUC19 vector using the NdeI and SaI restriction sites. All mutants were generated using overlapping forward and reverse primers containing the desired residue changes in conjunction with the high-fidelity *Pfu* polymerase (Stratagene). The primers used for mutagenesis are summarized in Table I. The PCR consisted of 1 cycle of 95 °C for 20 s and then 16 cycles of the following: 95 °C for 30 s, 55 °C for 1 min, 68 °C for 8 min. This protocol resulted in the full-length constructs with changes at the desired residues. The PCR products were then digested with *DpnI*, transformed into DH15-competent cells, and DNA was isolated and analyzed for the desired mutations. Following PCR, the modified CYP4A3 were digested with *NdeI* and *SalI* and subcloned into the pCWori expression plasmid. All mutations were verified by double-stranded sequencing.

**Expression and Purification of CYP4A Proteins**—The previously described protocol for expression and purification of CYP4A2 and CYP4A3 was used (4).

**Spectroscopic Methods**—Reduced CO difference and substrate perturbation spectroscopic analyses were obtained on a Varian Cary 1E UV/Visible dual-beam spectrophotometer. Absolute spectra were recorded on a Hewlett-Packard 8452 diode array spectrophotometer. Both instruments were equipped with a temperature control accessory. The P450 content was determined using the method of Omura and Sato (13). Both turbidity spectroscopic analyses were obtained on a Varian Cary 1E spectrophotometer. Absorbance in dilauroylphosphatidylcholine, 0.1 mg of sodium cholate, 25 pmol of CYP4A, 250 pmol of cytochrome P450 reductase, 25 pmol of cytochrome *b*5, 1.5 pmol of glutathione, and 5 μg of catalase. This mixture was incubated for 10 min at room temperature before 50 μM Tris, pH 7.4, containing 0.02% sodium cholate, 250 mM NaCl, and 10% glycerol was added to a final volume of 490 μl. Finally, 5 μl of a 10 mM Hamilton syringe, resulting in a final sample volume change of less than 1% with less than 1% of MeSO present. All spectra were obtained at 25 °C. The spectral binding constant *Kb* was determined from the hyperbolic plot of the respective differences in the 420- to 390-nm peak to trough absorbance versus ligand concentration (14).

**Measurement of CYP4A Hydroxylation Activity**—CYP4A ω-hydroxylation activity was measured by mixing together 10 μg of dilauroylphosphatidylcholine, 0.1 mg of sodium cholate, 25 pmol of CYP4A, 250 pmol of cytochrome P450 reductase, 25 pmol of cytochrome *b*5, 1.5 pmol of glutathione, and 5 μg of catalase. This mixture was incubated for 10 min at room temperature before 50 μM Tris, pH 7.4, containing 0.02% sodium cholate, 250 mM NaCl, and 10% glycerol was added to a final volume of 490 μl. Finally, 5 μl of a 10 mM Hamilton syringe, resulting in a final sample volume change of less than 1% with less than 1% of MeSO present. All spectra were obtained at 25 °C. The spectral binding constant *Kb* was determined from the hyperbolic plot of the respective differences in the 420- to 390-nm peak to trough absorbance versus ligand concentration (14).

**Purification**—Covalently linked heme in cytochrome P4504A was isolated and characterized by HPLC, ESI-MS, and UV/Visible spectroscopy. The CYP4A proteins were subjected to LC analysis as described above, except that up to 1.5 μmol of protein was injected per run. The protein peaks were collected and concentrated under a stream of nitrogen. 20–50 μl of the concentrated protein solution was analyzed by LC/ESI-MS on a narrow bore Poros R2 column (0.254 × 100 mm) packed with the Capillary Perfusion Tool Kit from Applied Biosystems, connected to a Sciex API 300 quadrupole mass spectrometer (Applied Biosystems). The column was run isocratically with 70% buffer B (see below) at 500–1800 Da in 4.0 s, and the scans across the HPLC peak were summed to give an ESI mass spectrum that was subsequently deconvoluted using the Biomultiview computer program. These experiments were performed on at least 3 different days and were found to be highly reproducible. The intra-day variability in the determination of the molecular masses of the CYP4A proteins was found to range from 0.005 to 0.03%.

**Prosthetic Group Isolation and Characterization**—Desalted *E. coli*-expressed CYP4A1 and CYP4A3 (10–15 nmol) were digested with Pronase (1 mg/mg of enzyme) in 50 mM Tris-HCl buffer, pH 7.8, by overnight incubation at 37 °C. The samples were chromatographed by direct injection on a 150 × 4.6-mm C4 reverse phase column (Vydac). The prosthetic group was eluted with a linear gradient of 15–50% solvent B over 30 min after washing the column with 15% B for 5 min at a flow rate of 1.5 ml/min. The column was coupled to an HP-diode array detector with the effluent monitored at 280 and 400 nm. The solvent gradient consisted of buffer A (0.1% trifluoroacetic acid) and buffer B (0.1% trifluoroacetic acid in acetonitrile). Heme and protein were separated using a stepwise gradient elution of 30–50% buffer B from 0 to 5 min, hold at 50% buffer B from 5 to 6.5 min, and 50–95% buffer B from 6.5 to 9 min. Under these conditions heme eluted at 2.4 min and the CYP4A protein eluted at 9 min. This gradient was later changed to 30% buffer B for 3 min, 30–50% buffer B from 3 to 8 min, hold at 50% buffer B from 8 to 9.5 min, and 50–95% buffer B from 9.5 to 12 min. Under these conditions heme eluted at 6 min and the CYP4A protein eluted at 11 min.

**Protein Analysis by LC/ESI-MS**—For LC/ESI-MS analysis, the CYP4A proteins were subjected to LC analysis as described above, except that up to 1.5 μmol of protein was injected per run. The protein peaks were collected and concentrated under a stream of nitrogen. 20–50 μl of the concentrated protein solution was analyzed by LC/ESI-MS on a narrow bore Poros R2 column (0.254 × 100 mm) packed with the Capillary Perfusion Tool Kit from Applied Biosystems, connected to a Sciex API 300 quadrupole mass spectrometer (Applied Biosystems). The column was run isocratically with 70% buffer B (see above) at 50 μl per min. P450A4A eluted at 4.5 min. About 10% of the flow was diverted to the ESI mass spectrometer. Data acquisition was carried out from m/z 500–1800 Da in 4.0 s, and the scans across the HPLC peak were summed to give an ESI mass spectrum that was subsequently deconvoluted using the Biomultiview computer program. These experiments were performed on at least 3 different days and were found to be highly reproducible. The intra-day variability in the determination of the molecular masses of the CYP4A proteins was found to range from 0.005 to 0.03%.

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**RESULTS**

**Expression, Spectroscopic Characterization, and Activity of CYP4A3 Mutants**—The active site mutants L129F and Y117A, Y117S, Y117F, and Y117L were constructed and expressed as described under “Experimental Procedures.” The Tyr-117 and Tyr-129 mutants were prepared in a continuation of a study of controlling the substrate hydroxylation regiospecificity of the enzyme (cf. Ref. 5). All the mutants were expressed at levels reflecting the substrate hydroxylation regiospecificity of the enzyme (Fig. 1).

The spectroscopic constants ($K_s$, $k_{cat}$) for the binding of lauric acid, the catalytic activity, and the regiospecificity with which the mutants hydroxylate lauric acid are summarized in Table II. Surprisingly, all the mutations increased the spectroscopic dissociation constant for lauric acid by at least 40-fold. In the case of the L129F, Y117F, and Y117L mutants even titration with high levels of lauric acid no longer led to measurable saturation of the protein active site. Although we cannot rule out the possibility that the fatty acid binding channel is obstructed by a single point mutation, the absence of a spectroscopically observable binding constant is most likely due to an inability of the lauric acid to displace the water from the sixth coordination site, resulting in a failure to alter the spin state and therefore the absorption spectrum.

The increased binding constants (decreased binding) are reflected in the catalytic activities. Incubations were carried out at 37 °C with 25–100 pmol of purified enzyme and a 1:10:1 ratio of CYP4A/cytochrome P450 reductase/cytochrome b$_5$ in the presence of 100–500 μM lauric acid. Product formation was measured between 1 and 30 min, and the $k_{cat}$ was calculated from these plots. Low catalytic activities were observed for the Y117A and Y117S mutants, but the other mutants failed to detectably hydroxylate lauric acid. The hydroxylation regiospecificity in the Y117A and Y117S mutants was comparable to that of the parent enzyme, which gives a 3:1 ratio of ω- to α- hydroxylation products. A 1:1 ratio of ω- to α- hydroxylation products was observed in the L129F, Y117F, and Y117L mutants, which confirms the previous observation that hydroxylation at the ω-position is preferred in the absence of Tyr-117.

### Table II

| Enzyme          | $K_s$ (μM) | $k_{cat}$ (μM·min$^{-1}$) | ω/α-1 ratio |
|-----------------|------------|--------------------------|-------------|
| CYP4A3          | 3          | 73                       | 2.7         |
| CYP4A3 L129F    | Not saturated | 0$^d$                  |             |
| CYP4A3 Y117A    | 129        | 4                        | 3.0         |
| CYP4A3 Y117S    | 217        | 6                        | 2.6         |
| CYP4A3 Y117F    | Not saturated | 0                      |             |
| CYP4A3 Y117L    | Not saturated | 0                      |             |

*a* Spectroscopic binding constants were measured as reported under “Experimental Procedures” with a 1 μM enzyme solution and a 0.05–1 mM concentration of fatty acid.

*b* Turnover numbers were measured as reported under “Experimental Procedures” using 50 μM enzyme solutions. Hydroxylated lauric acid was separated by reverse phase-HPLC as described previously (5). The values are the average of two independent measurements that differed by no more than 10%.

*c* Binding constants could not be calculated because no saturation was obtained upon titration with up to 1 mM lauric acid.

*d* No hydroxylated products were observed with fatty acid concentrations ranging from 100 to 500 μM.

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**Fig. 2.** LC profiles of E. coli-expressed CYP4A1 (A), CYP4A3 (B), and the CYP4A3 Y117S mutant (C). Traces were recorded at two wavelengths, 280 nm, indicating protein absorbance (bold line), and 400 nm, indicating the heme absorbance (thin line). The insets show the UV-visible spectra at the maximum of the protein peak at 9 min of retention time. Proteins were analyzed on an analytical Poros R2 column using a water/acetonitrile (both containing 0.1% trifluoroacetic acid) gradient. Amounts of protein injected ranged between 100 and 200 pmol.
(os-1)-hydroxylauric acids.

HPLC Analysis of CYP4A Proteins—The iron protoporphyrin IX prosthetic group in the P450 enzymes is held in the active site by the pincer action of two protein helices, polar interactions of the heme propionic acid substituents with protein residues, and coordination of the iron to a cysteine residue. Hence, under denaturing conditions, the heme is separated from the protein during LC analysis. Wild-type CYP4A enzymes and the mutants of CYP4A3 were subjected to reverse phase-HPLC analysis on an analytical Poros R2 column using an acetonitrile/water gradient under acidic conditions. Representative LC traces obtained for CYP4A1 and CYP4A3 are shown in Fig. 2, A and B, respectively. Surprisingly, both enzymes yield a heme species that coelutes with the 280 nm absorbing protein peak! Whereas CYP4A1 was characterized by the presence of both free and protein-associated heme, the heme absorbance was almost exclusively associated with the protein peak in CYP4A3. In the case of CYP4A3, even the presence of 6 m urea or guanidinium HCl in conjunction with boiling of the sample for 10 min did not dissociate the heme absorbance from the protein absorbance. The association of different amounts of heme with the protein is also evidenced by the UV-visible spectra of the peak eluting at 9 min, as can be seen from the insets in Fig. 2. The RZ value, which is the ratio of the absorbance of 400 to 280 nm, depends on the ratio of free to protein-associated heme. Hence, for CYP4A3, this value is highest, whereas for CYP4A3 Y117S, the RZ is lowest. Every wild-type CYP4A enzyme was characterized by an individual ratio of free to protein-bound heme. Assuming that the extinction coefficients for free heme and the protein-bound heme are the same, this ratio, calculated from the area under the peak at 400 nm, was 70:30 for CYP4A1, 65:35 for CYP4A2, 3:97 for CYP4A3, 94:6 for CYP4A8, and 74:26 for CYP4A11.

In addition to wild-type CYP4A enzymes, CYP4A3 mutant proteins were subjected to LC analysis. The LC analysis revealed that, in contrast to their parent enzyme, none had more than 3% heme associated with the protein peak. Instead, we observed a strong heme peak with the retention time expected for free hemin. The difference in the LC analysis between the calculated molecular masses based on the primary structures of the heme-containing proteins was affected by the presence of reducing agents (15). Therefore SDS samples were prepared with and without 2-mercaptoethanol in the sample buffer. An SDS gel with the protein bands visualized by both Coomassie and CL staining is shown in Fig. 3. Protein molecular weight markers indicate that the protein band stained with CL corresponds to the CYP4A protein band. The intensity of the CL-heme stain was different for the various enzymes, and was closely correlated with the ratio of free to protein-bound heme calculated from the LC plots. Thus, CYP4A3, with more than 98% of the heme attached to the protein, was stained most intensively. The CL stain shown in Fig. 3 was developed after a 45-min exposure of the film to the gel. This long exposure time was necessary to visualize the stain for the weaker bands of lanes 1, 2, 5, and 6. For CYP4A3, an exposure time of 10 min was sufficient to show clearly the presence of heme (results not shown).

Most interestingly, in lane 1, CYP4A1 isolated from clofibrate-induced rat liver (2 pmol) was applied. The CYP4A1 isolated from this natural source clearly shows the presence of heme in the CL stain. By taking the amount of protein applied onto the gel into consideration, the intensity of the heme stain for the isolated protein exceeds that of the E. coli expressed isofrom in lane 2, indicating that a higher percentage of heme is bound to the protein isolated from rat liver than the recombinant protein. This result was confirmed by LC analysis; for CYP4A1 isolated from rat liver, only 20% of free heme was detected in the LC chromatogram. In contrast to the wild-type enzyme, none of the CYP4A3 mutants showed any heme stain when 10 pmol of protein (same amount as for wild-type CYP4A3 in lane 3) was applied to the gel. Besides confirming the LC experiments, these results also show that the conditions used for SDS electrophoretic analysis ensure a separation of the protein and heme, thus reinforcing the conclusion that the protein-heme stain, when observed, reflects the presence of a modified, cross-linked heme.

Protein Analysis by LC/ESI-MS—As a final test for covalent binding, the molecular masses of the CYP4A proteins were determined by LC/ESI mass spectrometry. So far, only a few P450 enzymes have been subjected to mass spectrometric analysis (19–21). In all cases the molecular masses corresponded to the calculated molecular masses based on the primary structure without the attachment of a heme or other secondary modifications. This result was confirmed by us for P450 cam using the experimental conditions described herein.

Due to the hydrophobic nature of CYP4A proteins, detergent
had to be present throughout the purification procedures. To obtain ESI-mass spectroscopic data, ideally all of the detergent should be removed. Therefore, we further purified the CYP4A proteins by reverse phase HPLC, using an analytical Poros R2 column with a water/acetonitrile/trifluoroacetic acid gradient. The protein peak eluting at around 9 min (see Fig. 2) was collected for ESI/MS analysis. A 50-μl aliquot of the protein fraction (corresponding to 10–20 pmol of CYP4A) was subsequently analyzed on a narrow bore column packed with Poros 10 R2 material connected to a Sciex API 300 mass spectrometer. The combination of analytical and narrow bore column allowed separation of the protein from the detergent that was present and facilitated the recording of ESI mass spectra. Fig. 4 shows representative ESI/MS spectra for CYP4A1, CYP4A3, and the CYP4A3 Y117S mutant. For CYP4A1, deconvolution of the ESI mass spectrum indicated the presence of two major CYP4A species differing by a mean of 618 atomic mass units. This difference in mass corresponds within experimental error to the presence of a heme (616 atomic mass units) on one of the two proteins. On the other hand, analysis of CYP4A3 and the CYP4A3 Y117S mutant provided evidence for only one major protein species in each instance, that for CYP4A3 with the molecular mass expected for the heme-bound protein and that for the mutant with the mass expected for the heme-free protein. Table III summarizes the measured and calculated molecular masses for all the proteins subjected to ESI/MS analysis. The numbers present the averaged results from three separate experiments. Consistent with the LC chromatograms, two major CYP4A species, one heme-bound and one heme-free, were detected for CYP4A1, CYP4A2, CYP4A3, and CYP4A11, whereas the CYP4A3 L129F and Y117S mutants and CYP4A8 were expressed as one major form with no detectable heme covalently attached.

**Prosthetic Group Isolation and Characterization**—Isolation of the prosthetic group was achieved by Pronase digestion of the heme-linked proteins. Overnight digestion of CYP4A1 and CYP4A3 released one polar heme species. A representative HPLC for CYP4A1 before and after Pronase digestion is shown in Fig. 5, A and B, respectively. The CYP4A1 preparation used for the digestion contained 70% free and 30% protein-bound heme. ESI/MS analysis confirmed that all the noncovalently bound heme was unmodified iron protoporphyrin IX. After digestion of the protein, one new polar heme species was obtained. The UV-visible spectrum of the new heme species is indistinguishable from that of iron protoporphyrin IX, as can be seen from the inset in Fig. 5B. Subsequent ESI/MS analysis established a molecular weight of 632 atomic mass units for the released heme, a molecular mass consistent with addition of one hydroxyl group to the porphyrin. Similar results were obtained for CYP4A3, whereas the noncovalently bound heme in the CYP4A3 Y117S mutant was, again, shown to simply be iron protoporphyrin IX.

**Isolation and Characterization of Heme-containing Peptides**—Heme-containing peptides were obtained by partial Pronase digestion of heme-linked CYP4A3. A representative chromatogram obtained after partial digestion is shown in Fig. 5C. In addition to the noncovalently bound heme and the hydroxyl-modified heme, several heme-containing peptides were detected at 400 nm. The peptides isolated in fraction a were further analyzed by matrix-assisted laser desorption ionization/time of flight spectrometry. The results obtained are summarized in Table IV. Fraction a consisted of four different peptides that differ by 1–3 amino acids and are all derived from the same parent peptide. In addition to the heme peptide masses, the corresponding non-heme peptide masses, as well as free heme, were detected. The contribution of the heme to the total mass of the peptides was 614.1 Da. The expected heme contribution to the total mass in a heme peptide with one ester bond is 2.01 mass units lower than with noncovalently bound heme (616.51 atomic mass units), i.e. 614.49, in perfect agreement with the observed masses. Hence, the results suggest that the heme of P4504A enzymes is attached via an ester link. Peptide 1 (Table IV) contains two acidic residues that could function as the site of attachment of the heme to the protein, namely Glu-318 and Asp-321. However, the heme is still covalently attached to peptide 4, which lacks the aspartic acid.
residue. Therefore, the glutamic acid residue at position 318 is likely to be involved in the ester bond to the prosthetic group.

**DISCUSSION**

In this paper we demonstrate that the prosthetic heme group of the CYP4A family of proteins is covalently cross-linked to different degrees to the protein. The evidence for this cross-linking is provided by three independent methods as follows: reverse phase LC, SDS-polyacrylamide gel electrophoresis with staining for the heme group, and ESI-MS. Furthermore, release of the covalently bound heme by Pronase digestion yields a mono-hydroxy derivative of iron protoporphyrin IX, and analysis of heme-bound peptides suggests that the heme is linked via a glutamic acid residue.

Analysis of CYP4A1 isolated from rat liver establishes that the enzyme species with the cross-linked heme is not an artifact of the heterologous expression of the protein in *E. coli*. Indeed, the protein isolated from rat liver contained 80% of the heme-linked species in which the attached heme with absorbance at 400 nm coelutes with the protein absorbance peak at 280 nm. This is to be compared with 30% covalent binding of the heme to the protein in the *E. coli* expressed protein. Furthermore, on the basis of protein and heme quantitation, the CYP4A1 isolated from rat liver had the same specific activity as the recombinant protein. Despite covalent binding, all the wild-type and mutant CYP4A proteins examined in this study have the characteristic spectroscopic properties typical of P450 enzymes (Fig. 1). Covalent attachment of the heme therefore does not influence the UV-visible spectroscopic properties of the enzymes. The precise site on the heme to which the extra hydroxyl group has been added remains unknown but probably involves one of the four methyl groups, as the heme absorption spectrum is not altered in the protein bound species.

The glutamic acid residue involved in heme binding is part of the highly conserved I-helix that also forms the substrate recognition site (SRS-4) in CYP4A enzymes. This glutamic acid, which is highly conserved in the CYP4A family, is glutamic acid 320, 315, 318, 319, and 321 in CYP4A1, -4A2, -4A3, -4A8, and -4A11, respectively. Fig. 6 shows the position of the glutamic acid with respect to the I-helix and the distal side active site residues in a CYP4A11 model. Mutations in the CYP4A3 active site Tyr-117 and Leu-129 residues, which correspond, respectively, to Tyr-120 and Leu-132 in CYP4A11 (Fig. 6), have already been shown to change the heme binding and catalytic properties of the enzyme. Ongoing investigations now focus on the role of the glutamic acid residue and its neighbors in the catalytic site.

We do not yet know how, and under what conditions, the heme is attached to the protein in the CYP4A proteins. There are precedents for two systems that result in covalent attachment of heme groups to protein frameworks under physiological (as opposed to pathological) conditions. One of these systems, typified by the assembly of the c-type cytochromes in which the heme is linked via its vinyl groups to cysteine residues (22–25), requires the intervention of proteins that catalyze the formation of the covalent cross-links. However, in these reactions the heme is bound to the two cysteines within a CXXCH heme-binding motif, a motif that is not present in the

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**TABLE III**

| Protein   | Free/bound heme<sup>a</sup> | % | Measured molecular masses | Δ<sup>b</sup> | Calculated mass<sup>c</sup> | Difference |
|-----------|----------------------------|---|---------------------------|-----------|----------------------------|------------|
| CYP4A1    | 70/30                      | 649          | 57,405                    | 623       | 57,425                     | 0.03       |
| CYP4A2    | 65/35                      | 35           | 57,543                    | 618       | 57,510                     | 0.06       |
| CYP4A3    | 32/68                      | 73           | 57,772                    | 625       | 57,760                     | 0.02       |
| CYP4A3 L129F | 95/5                   | 6            | 57,825                    | 618       | 57,794                     | 0.05       |
| CYP4A3 Y117S | 95/5                   | 6            | 57,707                    | 625       | 57,683                     | 0.03       |
| CYP4A8    | 94/6                       | 230          | 58,156                    | 625       | 58,143                     | 0.02       |
| CYP4A11   | 74/26                      | 42           | 58,519                    | 625       | 58,514                     | 0.01       |

<sup>a</sup> Calculated from the area under the peak corresponding to free heme and protein-bound heme at 400 nm.

<sup>b</sup> Difference obtained by subtracting the lower molecular mass from the higher molecular mass obtained by measurement of the same sample.

<sup>c</sup> Theoretical molecular mass calculated based on the primary structure of the proteins.
CYP4A enzymes (26). An even more serious shortcoming of this type of mechanism is that our observation of heme-protein cross-linking in bacterial cells requires that the entire machinery for this process be present in E. coli despite the fact that bacteria do not express CYP4A enzymes of their own.

The second paradigm for the formation of covalently bound heme groups is provided by the mammalian peroxidases. The heme group has been shown to be cross-linked to the protein in lactoperoxidase (27, 28), myeloperoxidase (29–31), eosinophil peroxidase (32), and thyroid peroxidase (33). The heme prosthetic group in lactoperoxidase, the only one that has been fully characterized, is iron 1,5-bis(hydroxymethyl)-3,8-dimethyl-2,4-divinylporphyrin-6,7-dipropionic acid. The prosthetic group is covalently attached to the protein via ester bonds between the 1- and 5-hydroxymethylene groups and the carboxylic acid side chains of Glu-275 and Asp-125, respectively (34). Proteolytic release of the heme from lactoperoxidase yields a heme species 32 atomic mass units heavier than heme itself, as expected from the presence of two additional hydroxy groups at the 1- and 5-methyl positions. In earlier work, we demonstrated that the heme is cross-linked to the protein in lactoperoxidase through an autocatalytic process in which the initial reaction with H₂O₂ is used to form the mature, catalytically active, cross-linked protein (6). It is likely that the heme is cross-linked to the protein in the other mammalian peroxidases by a similar mechanism. This precedent suggests that cross-linking of the heme to the protein in CYP4A proteins may occur through an autocatalytic process that utilizes the oxidizing species produced in the initial turnover of the protein to forge the covalent link. As reported here, proteolysis of the CYP4A enzymes releases a mono- rather than dihydroxy heme species, so that the autocatalytic process is only required to make one cross-link. Cross-linking of the heme via one of its methyl groups, as in lactoperoxidase (34), is a strong possibility in view of the observation that the hydroxyl group is added without significantly perturbing the spectrum of the heme in the bound or unbound state.

A critical question is whether covalent attachment of the heme to the protein enhances, or enables, the catalytic function of the enzyme or is the initial step in an aging process that results in an inactive or more rapidly degraded protein. It is not yet possible to answer this question with certainty. The fact that CYP4A1 isolated from rat liver with 80% of the heme bound to the protein has similar activity to heterologously expressed CYP4A1, which only has 30% covalently bound heme, clearly shows that covalent binding does not impair activity. On the other hand, it is less clear whether the protein with the noncovalently bound heme is catalytically active. With the exception of CYP4A8, all the wild-type proteins are expressed with at least 25% of their heme already covalently bound to the protein. If heme cross-linking is an autocatalytic event, one would expect an increase in the extent of heme covalent binding during catalytic turnover. Thus, it is possible that all the measured catalytic activity is due to the covalently linked protein. It is also interesting that the CYP4A3 L129F,
Y117A, Y117S, Y117F, and Y117L active site mutants, all of which are inactive, fail to bind covalently the heme even though their spectroscopic properties are those of normal P450 enzymes. However, these results do not establish that covalent binding enhances or is required for catalytic activity. A definitive answer to this question will only be possible after further experiments to determine the extent to which covalent binding increases during catalytic turnover and the effects of this binding on activity.

The failure to covalently bind the heme may be associated with the high $K_e$ value for lauric acid in the case of the CYP4A3 Y117A and Y117S mutants and the lack of spectroscopically measurable binding constants for the fatty acid in the other mutants. The weak or undetectable spectroscopic change that is used to determine the $K_e$ value can be explained either by inability of the fatty acid to bind in the active site or, if bound, an inability to displace the distal water ligand from the iron. Displacement of the water ligand normally causes the spin state change that gives rise to the observed spectroscopic change. In the absence of displacement of the water molecule, catalysis is not possible, and the heme cannot be processed into a covalently bound form. In an earlier paper (4) we reported that the CYP4A3_2 chimera, which derives its first 119 amino acids from CYP4A2 and the remainder from CYP4A3, retained a hydroxylation activity similar to that of the CYP4A2 parent, whereas the reciprocal CYP4A2_3 chimera was inactive even though its spectroscopic properties were those of a normal P450 enzyme. As found here for the active site mutations, the CYP4A2_3 chimera did not detectably bind lauric acid, failed to catalyze fatty acid hydroxylation, and as shown here, had less than 5% of its heme covalently attached to the protein. Thus, the evidence suggests that mutations that alter the ability of lauric acid to bind and initiate turnover also suppress covalent binding of the heme to the protein.

It is unclear what advantage is provided by covalent binding of the heme in the CYP4A proteins, given that the heme is fully functional in the unbound state in other P450 enzymes. This is a similar question to that posed by covalent linking of the heme to the protein in the mammalian peroxidases, given again that the plant and fungal peroxidases function perfectly well without the covalently bound heme. In the mammalian peroxidases, cross-linking of the heme may be related to the ability of some of its members, notably myeloperoxidase and eosinophil peroxidase, to oxidize high potential substrates such as chloride ion. In a similar vein, cross-linking of the heme in the CYP4A enzymes may be related to their oxidation of the thermodynamically disfavored terminal methyl group, although alternative roles, such as stabilization of the heme-protein complex, are also possible.

In summary, we have shown for the first time that CYP4A proteins have a modified, covalently attached heme prosthetic group and have identified Glu-318 as the specific site of attachment of the heme in CYP4A3. Our continuing investigation now focuses on the mechanism of its attachment and the resulting catalytic consequences.

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