Covalent Attachment of the Heme Prosthetic Group in the CYP4F Cytochrome P450 Family†

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ABSTRACT: We demonstrated earlier that the heme in cytochrome P450 enzymes of the CYP4A family is covalently attached to the protein through an I-helix glutamic acid residue [Hoch, U., and Ortiz de Montellano, P. R. (2001) *J. Biol. Chem. 276,* 11339−11346]. As the critical glutamic acid residue is conserved in many members of the CYP4F class of cytochrome P450 enzymes, we investigated covalent heme binding in this family of enzymes. Chromatographic analysis indicates that the heme is covalently bound in CYP4F1 and CYP4F4, which have the required glutamic acid residue, but not in CYP4F5 and CYP4F6, which do not. Catalytic turnover of CYP4F4 with NADPH−cytochrome P450 reductase shows that the heme is covalently bound through an autocatalytic process. Analysis of the prosthetic group in the CYP4F5 G330E mutant, into which the glutamic acid has been reintroduced, shows that the heme is partially covalently bound and partially converted to noncovalently bound 5-hydroxymethylheme. The modified heme presumably arises by trapping of a 5-methyl carbocation intermediate by a water molecule. CYP4F proteins thus autocatalytically bind their heme groups covalently in a process that requires a glutamic acid both to generate a reactive (cationic) form of the heme methyl and to trap it to give the ester bond.

The CYP4F family of cytochrome P450[1] enzymes consists of proteins that catalyze the ω-hydroxylation of fatty acids, eicosanoids, and steroids. The first member of this family to be discovered was CYP4F1, a protein that is constitutively expressed at relatively high levels in rat hepatoma (*1*). This protein catalyzes the ω-hydroxylation of leukotriene B_4, lipoxin A, prostaglandin A_1, and several hydroxyeicosatetraenoic acids (HETEs), but not of lauric, palmitic, or arachidonic acids (*2*). The human proteins CYP4F2 and CYP4F3 also catalyze the ω-hydroxylation of leukotriene B_4, but in contrast to CYP4F1, they also support the ω-hydroxylation of arachidonic acid (*3*, *4*). Rat CYP4F4, CYP4F5, and CYP4F6 have only been superficially characterized, but like other members of the CYP4F family are able to hydroxylate leukotriene B_4 (*5*). The CYP4F and CYP4A families, which share approximately 40% sequence identity, differ in their substrate specificities in that the latter universally catalyze the ω-hydroxylation of fatty acids but more rarely that of leukotriene B_4 or the prostaglandins (*6*).

We recently demonstrated that the prosthetic heme group in CYP4A1, -4A2, -4A3, -4A8, and -4A11 is covalently bound to the protein (*7*). Pronase digestion of CYP4A3 and mass spectrometric analysis of the peptides identified the site of attachment to the CYP4A3 protein as Glu318, a highly conserved glutamic acid residue in the I-helix. Mass spectrometric analysis of the modified prosthetic group released from the protein indicated that the heme had incorporated an additional oxygen atom. A subsequent independent study established that the heme is also covalently bound in rabbit CYP4A5/7 and reported that the same is true in CYP4B1 and CYP4F3B (*8*). In a very recent study, we have further demonstrated that the CYP4A protein and the prosthetic group are linked via an ester bond between the conserved glutamic acid and a hydroxyl group on the 5-methyl of the heme (*9*). We demonstrated, furthermore, that the covalent bond is formed by an autocatalytic process that is supported by the native electron donor partner NADPH−cytochrome P450 reductase and thus involved catalytic turnover of the protein.

The presence of ester links to the heme and the involvement of an autocatalytic mechanism recall the properties of lactoperoxidase, except that in lactoperoxidase and the other mammalian peroxidases there are two rather than one ester bond to the heme, and the catalytic turnover required for their formation is supported by H_2O_2 rather than NADPH−cytochrome P450 reductase and O_2 (*10*, *11*).

In the present study, we (a) establish that the heme is covalently bound to the protein in CYP4F1 and CYP4F4, proteins that have the glutamic acid residue in the I-helix (Table 1), (b) show that the heme is probably covalently

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[1] Abbreviations: heme, iron protoporphyrin IX regardless of oxidation and ligation state; 5-hydroxymethyl heme, the derivative of heme with a hydroxyl group on the 5-methyl group; P450, cytochrome P450; HPLC, high-pressure liquid chromatography; TFA, trifluoroacetic acid.

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Table 1: CYP4F Family Partial I-Helix Sequence Alignments

protein	I-helix sequence
CYP4F1	FMF**E**GHDTTASG
CYP4F2	FMF**E**GHDTTASG
CYP4F3	FMF**E**GHDTTASG
CYP4F4	FMF**E**GHDTTASG
CYP4F5	FMF**G**GHDTTASA
CYP4F6	FMF**G**GHDTTASA
CYP4F8	FMF**G**GHDTTASA
CYP4F21	FMF**E**GHDTTASA

linked through a hydroxyl group on the heme 5-methyl group, (c) establish that covalent bond formation, as in the CYP4A enzymes, is the result of an autocatalytic process, and (d) show that the heme is not covalently bound in CYP4F5, which has a glycine instead of the glutamic acid on the I-helix (Table 1), but the heme is modified and, in part, covalently bound when the glycine is mutated to a glutamic acid.

MATERIALS AND METHODS

Materials. Primers were ordered from Invitrogen Life Technologies (Carlsbad, CA). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). [^{14}C]Lauric acid (55 mCi/mmol) was from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Purified rat liver cytochrome b_5 was a gift from Lester Bornheim (UCSF). Cytochrome P450 reductase was expressed and purified as previously reported (*12*). Ampicilin, D-aminolevulinic acid, glycerol, lysozyme, DLPC, glutathione, catalase, heme, NADPH, lauric acid, and TFA were obtained from Sigma-Aldrich (St. Louis, MO). Emulgen 913 was a gift from KAO Chemicals (Tokyo, Japan). Acetonitrile (HPLC grade) was purchased from Fisher Scientific (Fair Lawn, NJ). The 1-, 5-, and 8-hydroxymethylheme standards were obtained and purified as reported elsewhere and were kindly provided by Christophe Colas (*11*, *13*). Leukotriene B_4 was purchased from Cayman Chemical Co. (Ann Arbor, MI).

Cloning. The CYP4F1 cDNA from Sprague−Dawley rat hepatic tumor was kindly provided by Dr. James P. Hardwick (Northeastern Ohio Universities College of Medicine). The full-length cDNAs of CYP4F4 (1608 bp), CYP4F5 (1660 bp), and CYP4F6 (1707 bp) were isolated from Sprague−Dawley rat liver or kidney by one-step reverse transcription−polymerase chain reaction (RT-PCR) (Life Technologies Inc., Rockville, MD) using the following primers based on the published sequences (*14*): CYP4F4, forward 5′-ttcagacaggggggtttcca-3′ and reverse 5′-cgtccagatgtacgtagtcactg-3′; CYP4F5, forward 5′-atgcgtttttgcaggagtgggg-3′ and reverse 5′-ggggagtttacacagggttactg-3′; and CYP4F6, forward 5′-acagcttccggcaagtcggaa-3′ and reverse 5′-gagatctcaagaggtaggagg-3′. The full-length cDNAs were ligated directly into the pCRII vector (Invitrogen, Carlsbad, CA). The identities of the CYP4F4, CYP4F5, and CYP4F6 clones isolated by RT-PCR were confirmed by DNA sequencing of the entire inserts. The sequences were in complete agreement with the published sequences (*14*). A sequence encoding a six-histidine tail at the carboxy terminus was added to all the CYP4F cDNAs, and the sequences coding for the first 23 amino acids of the CYP4F enzymes were replaced by the N-terminus of bovine sterol 17α-hydroxylase with the codon modifications introduced by Barnes et al. (*15*). This was done by PCR using the following primers: CYP4F1, forward 5′-ggaattccatatggctctgttattagcagtttttctgctgtttggagcctcctgg-3′ and reverse 5′-ggtcctttaaagcttttaatgatgatgatgatgctgcgcgcccgccgtcag-3′; CYP4F4, forward 5′-ggaattccatatggctctgttattagcagtttttctgcttctgatcggagcttcctgg-3′ and reverse 5′-ggaattccatatggctctgttattagcagtttttctgcttctgatcggagcttcctgg-3′ and reverse 5′-ggtcctttaaagctttaatgatgatgatgatgtccctgtgtgctcagtggctc-3′; and CYP4F5, forward 5′-ggaattccatatggctctgttattagcagtttttctgcttggagccgcttcttgg-3′ and reverse 5′-ggtcctttaaagctttaatgatgatgatgatgtccctgagtgtctgtgct-3′; CYP4F6, forward 5′- ggaattccatatggctctgttattagcagtttttctgctgggaggagcctcttgg-3′ and reverse 5′-ggtcctttaaagctttaatgatgatgatgatgatgagaggtaggagggtgggc-3′. The above primers also introduced *Nde*I and *Hin*dIII restriction sites. The PCR products were ligated into the pCRII-TOPO vector (Invitrogen, Carlsbad, CA) directly. DNA sequencing of the entire inserts confirmed the modifications made to the CYP4F genes. The modified CYP4F cDNAs were then digested with *Nde*I and *Hin*dIII enzymes and ligated into pCWori vector.

Preparation of the CYP4F5/G330E Mutant. Gly330 was mutated to a glutamic acid using the QuikChange (Stratagene, La Jolla, CA) method. The CYP4F5 gene was excised from the pCWori/4F5 plasmid by digesting with *Nde*I and *Hin*dIII restriction enzymes. The CYP4F5 gene was then subcloned into the pUC19 vector, and this plasmid was used for mutagenesis. The following primers were used to prepare the mutants, where underlined nucleotides represent the base pair changes that were made to prepare the amino acid point mutations. The forward primer used was 5′GAC ACC TTC ATG TTT GAA GGC CAT GAC ACC3′, and the reverse primer was 5′GGT GTC ATG GCC TTC AAA CAT GAA GGT GTC3′. The mutation was confirmed by sequence analysis.

Protein Expression and Purification. The proteins were expressed in *Escherichia coli* and purified as previously described for CYP4A proteins (*9*, *16*). The CYP4F1, CYP4F4, CYP4F5, CYP4F5/G330E, and CYP4F6 hexahistidine-tagged proteins were expressed in DH5-α cells and purified on a Ni^{2+}-NTA agarose column from Qiagen (Chatsworth, CA) and desalted on a P10 column from Amersham Pharmacia Biotech (Solna, Sweden) for analysis.

Spectroscopic Methods. Reduced CO-difference and absolute spectra were recorded on a Hewlett-Packard 8452 diode array spectrophotometer. The P450 content was determined using the method of Omura and Sato (*17*). Difference spectra were recorded on a Varian Cary 1E UV/visible dual-beam spectrophotometer.

HPLC−UV Analysis of CYP4F Proteins To Determine the Percent of Covalently Bound Heme. Purified and desalted CYP4F proteins were analyzed on a POROS R2 perfusive particle column (4.6 × 100 mm) from Perceptive Biosystems (Framingham, MA). The solvent gradient consisted of a mixture of buffer A (0.1% TFA in water) and buffer B (0.05% TFA in acetonitrile) with a flow rate of 1.5 mL/min. The bound and unbound heme species were separated with a linear stepwise gradient of 30% buffer B for 3 min, 30−50% buffer B from 3 to 8 min, held at 50% buffer B from 8 to 9.5 min, 50−95% buffer B from 9.5 to 13 min, 95% buffer B from 13 to 18 min, and then adjusted back to 30% buffer B for 2 min. The free heme eluted at 6 min, the polar heme at 3.3 min, and the bound heme around 12.5

min. The heme group and protein were monitored at 400 and 280 nm, respectively.

Identification of the Site of Hydroxylation on the Heme by HPLC Analysis. The samples were injected onto the reverse-phase POROS R2 perfusive particle column (4.6 × 100 mm) from Perceptive Biosystems (Framingham, MA). Buffer A (water with 0.1% TFA) and buffer B (acetonitrile with 0.05% TFA) were varied at a flow rate of 1 mL/min. The heme species were eluted with a linear gradient of 0.16% buffer B/min. Conditions of the column were 3 min at 27% buffer B, 3−40.5 min from 27 to 33% buffer B, then 40.5−43 min from 33 to 95% buffer B, 43−48 min with 95% buffer B, 48−52 min from 95 to 27% buffer B, and then 52−55 min at 27% buffer B. Under these conditions, the 8-hydroxymethylheme standard elutes at 4.6 min, the 5-monohydroxylated at 8.2 min, and the free heme at 20 min.

Measuring Autocatalytic Attachment. Autocatalytic binding of the heme group to the CYP4F4 protein was measured by mixing together 10 mg of dilauroylphosphatidylcholine, 800 pmol of cytochrome P450 reductase, 300 pmol of cytochrome b_5, 5 mg of catalase, and 28 pmol of CYP4F4. This mixture was incubated for 10 min at room temperature, and then 50 mM Tris, pH 7.5, buffer containing 250 mM NaCl and 10% glycerol was added to a final volume of 180 μL. Finally, 10 μL leukotriene B_4 was added, giving a final concentration of 30 μM. The samples were incubated for 2 min at 37 °C, and then the reaction was started by adding NADPH to a final concentration of 2 mM. The reaction was carried out for 2 h at 37 °C and then injected directly onto the POROS column to determine the percentage of bound and free heme (as described above). When the proteins were incubated in the presence of all of the reaction components except NADPH, the percentage of covalently bound heme did not increase.

RESULTS

CYP4F Protein Expression. The genes coding for CYP4F1, CYP4F4, CYP4F5, CYP4F5/G330E, and CYP4F6 were cloned into a pCWori vector and were expressed in *E. coli*. The proteins, each of which had a hexahistidine tag, were then purified by Ni^{2+}-NTA agarose affinity column chromatography. The proteins, after desalting, exhibited absorption spectra in the oxidized ferric state consistent with their identification as heme-containing cytochrome P450 enzymes (Figure 1, panels A−E). The four wild-type proteins exhibited Soret maxima at 418 or 422 nm (Table 2), as expected for low-spin proteins with a distal water ligand to the heme iron atom (*18*). The CYP4F5/G330E mutant had a somewhat lower absorption maximum at 412 nm and may be partially in the high-spin state. Reduction of the proteins with dithionite in the presence of CO yielded the Fe^{2+}−CO complexes. The absorption spectra of these proteins indicated that the proteins were present as mixtures of the intact, thiolate-ligated protein with an absorption maximum at 450 nm, and a denatured species in which the absorption maximum was at 420 nm (Figure 1, panels F−J). The proportion of the protein in the intact, active state ranged from 22 to 85%, CYP4F5 and CYP4F6 giving the highest, and CYP4F1 and CYP4F4 the lowest, proportion of intact protein (Table 2). All the experimental data cited in this study have been corrected for the presence of the denatured, inactive protein and relate only to the protein with an Fe^{2+}−CO absorbance maximum at 450 nm.

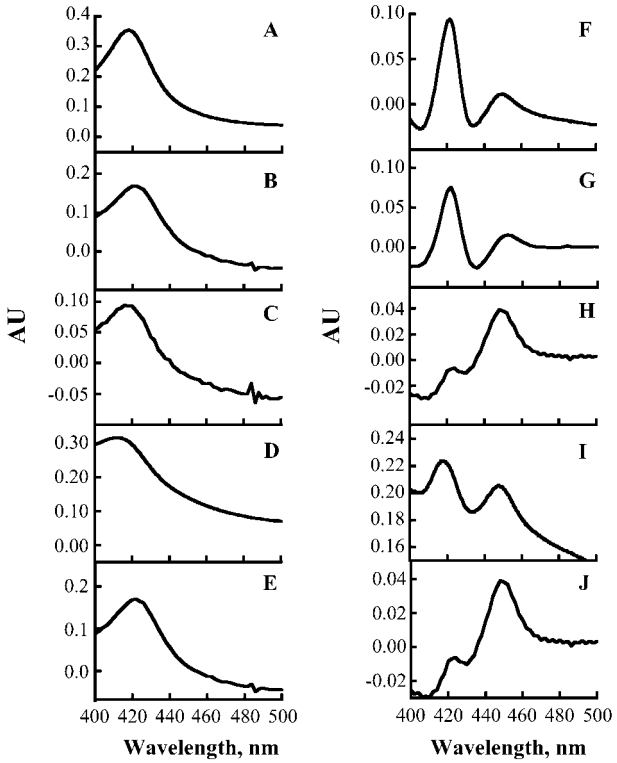

FIGURE 1: Spectroscopic properties of CYP4F proteins. The absolute absorbance spectra are shown in panels A−E for CYP4F1 (A), CYP4F4 (B), CYP4F5 (C), CYP4F5/G330E (D), and CYP4F6 (E). The reduced CO spectra are in panels E−J for CYP4F1 (F), CYP4F4 (G), CYP4F5 (H), CYP4F5/G330E (I), and CYP4F6 (J).

Table 2: Spectral Analysis of CYP4F Proteins

protein	Fe^{3+} (nm)	Fe^{2+}−CO[a] (nm)
CYP4F1	418	450 (22)
CYP4F4	422	450 (24)
CYP4F5	418	450 (82)
CYP4F5/G330E	412	450 (46)
CYP4F6	422	450 (85)

[a] The reduced CO spectra for the CYP4F proteins contained a mixture of 420 and 450 nm species. The percent of the intact, active species with the absorption maximum at 450 nm is indicated in parentheses. The percentage of 450−420 nm species is variable, and the values given here are for typical preparations.

Analysis of Covalent Heme Binding. HPLC analysis of the CYP4F proteins with dual detection at 280 (protein) and 400 nm (heme) shows that a fraction of the heme is covalently bound in the CYP4F1 and CYP4F4 proteins (Figure 2, panels A and B). The heme is not covalently bound in the CYP4F5 and CYP4F6 proteins (Figure 2, panels C and E). The extent of heme binding has been calculated by assuming that the heme is unmodified in the fraction of the protein with an Fe^{2+}−CO absorbance maximum at 420 nm. This assumption is justified by the fact that covalent binding of the heme is an autocatalytic process and requires a catalytically active protein (vide infra). The unmodified heme peak in the HPLC trace thus includes all the heme from the denatured protein plus the fraction of the heme not covalently bound in the intact enzyme. The fraction of the unmodified heme contributed by the intact protein can therefore be calculated from the ratio of the 450 to 420 nm absorbances

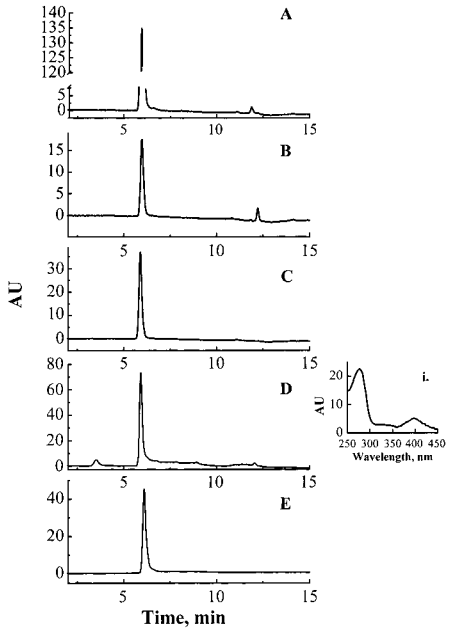

FIGURE 2: HPLC analysis for covalent heme attachment in CYP4F proteins. Desalted protein samples were injected onto a POROS R2 perfusive column, and the heme chromophore was monitored at 400 nm: (A) CYP4F1; (B) CYP4F4; (C) CYP4F5; (D) CYP4F5/G330E; (E) CYP4F6. Inset i: UV−visible spectra at the maximum of the protein peak at approximately 12 min. Unmodified heme elutes at 6 min, monohydroxylated heme at 3.3 min, and the protein-bound heme around 12.5 min.

Table 3: Percent of Covalently Bound Heme to Intact Cytochrome P450

protein	% of bound heme[a]
CYP4F1	10
CYP4F4	48
CYP4F5	0
CYP4F5/G330E	5
CYP4F6	0

[a] The percent of covalently bound heme was calculated for the percent of 450 nm species in the reduced CO spectra assuming that the 420 nm species only has the free heme species. The percent values are for one preparation, as the values vary from preparation to preparation.

in the Fe^{2+}−CO state. After this correction was made, it could be estimated that approximately 10% of the heme was covalently bound in CYP4F1, 48% in the CYP4F4, and none in the CYP4F5 and CYP4F6 (Table 3). The extent of covalent binding in these proteins presumably reflects the history of the proteins in the expression system and is not a measure of the maximum binding possible.

Analogous analysis of the prosthetic group in the CYP4F5/G330E mutant indicates that a small fraction of the heme (5%) was covalently bound (Figure 2, panel D, and Table 3). Although the covalently bound heme is just detectable above the baseline in the HPLC trace (Figure 2), the absorption spectrum of the protein peak (as detected at 280 nm) clearly shows that the protein has incorporated a heme chromophore (Figure 2, inset to trace D). In addition, a new polar peak with absorption at 400 nm is detected at approximately 3.3 min in the HPLC trace (Figure 2, trace D). Analysis of the heme species present in the CYP4F5/G330E mutant under different conditions shows that the polar peak has the same retention time (Figure 3, panel C) and

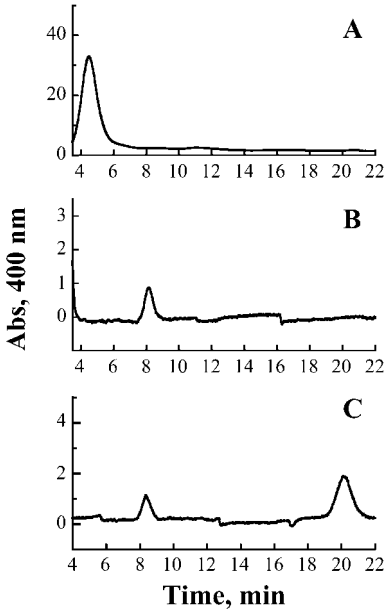

FIGURE 3: HPLC analysis of the polar heme species of the CYP4F5/G330E mutant with the detector set at 400 nm. Samples were injected onto the POROS column and separated by using a flat gradient of 0.16% buffer B/min (range 27−33% of buffer B): (A) 8-hydroxymethylheme standard; (B) 5-hydroxymethylheme standard; (C) desalted CYP4F5/G330E protein. The 8-hydroxymethylheme elutes at 4.6 min, the 5-hydroxymethylheme at 8.2 min, and unmodified heme around 20 min.

Table 4: Heme Species in the CYP4F5/G330E Mutant As Compared to Wild-Type CYP4F5

protein	% free heme	% bound heme	% monohydroxylated heme
CYP4F5	100	ND[a]	ND[a]
CYP4F5/G330E	77	5	18

[a] ND, not detectable.

spectrum (not shown) as authentic 5-hydroxymethylheme. Quantitation of the heme species (Table 4) indicates that in the isolated CYP4F5/G330E mutant 5% of the heme was covalently bound, 18% was converted to the 5-hydroxymethylheme derivative but was not covalently bound, and 77% was unmodified heme. In contrast, only unmodified heme was found in wild-type CYP4F5 (Table 4). Introduction of the glutamic acid at position 330 in CYP4F5 thus results in partial covalent binding of the heme and partial conversion of the heme to noncovalently bound 5-hydroxymethylheme (Figure 4).

Increase in Covalent Binding under Turnover Conditions. To determine if covalent binding of the heme to the protein is the result of an autocatalytic event, as previously found with the CYP4A proteins (*9*), CYP4F4 was incubated for 2 h under turnover conditions with NADPH−cytochrome P450 reductase and leukotriene B_4. The fraction of the heme that was covalently bound to catalytically active CYP4F4 increased from 39% to 90% as a result of this incubation (Figure 5, panel A). This increased extent of covalent heme binding is evident not only from the ratio of the 400 nm peak that migrates with the protein versus the free heme peak, but also from the absorption spectrum of the protein peak itself (Figure 5, panel B). The protein isolated from a similar incubation without the NADPH cofactor has exactly the same proportion of covalently and noncovalently bound heme (not

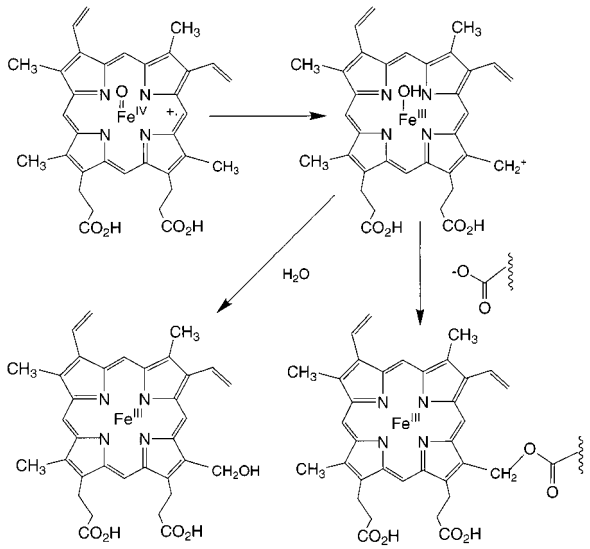

FIGURE 4: Structure of 5-hydroxymethylheme, and proposed mechanism for its formation involving competition for a carbocationic intermediate in the normal heme covalent binding process.

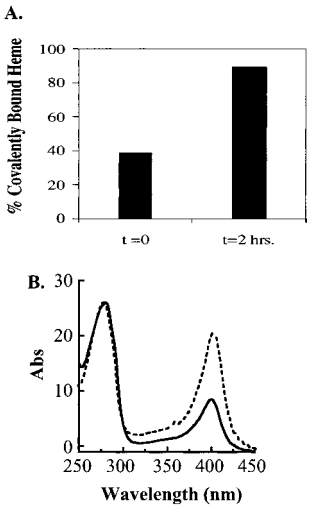

FIGURE 5: Percentage of covalently bound heme increases with catalysis in CYP4F4. The percentage of heme bound to the protein greatly increases when incubated under catalytic turnover conditions for 2 h. The percent of heme bound to the proteins is originally at 39% and increases to 90% heme bound after 2 h incubation at 37 °C. The spectrum of the HPLC protein peak before and after incubation under catalytic turnover conditions. When the protein is incubated under the same conditions but in the absence of NADPH, the percentage of covalently bound heme does not change.

shown) as the protein before the incubation. Catalytic turnover thus clearly increases the amount of the heme that is covalently attached to the protein.

DISCUSSION

Heterologous expression of CYP4F1 and CYP4F4 yields proteins in which a fraction of the prosthetic heme group is partially attached to the protein (Figure 2, Table 2). If the assumption is made (vide infra) that covalent binding only occurs with catalytically active proteins, the extent of covalent binding in the isolated CYP4F1 and CYP4F4, respectively, can be calculated to be 10% and 48% (Table 3). The prosthetic group that is not covalently bound to these two proteins is shown by HPLC analysis to be unmodified heme (Figure 2). These results agree with the finding that the heme in CYP4F3 is partially covalently bound to the protein (*8*), and with the demonstration that the same is true of the CYP4A family of proteins (*7*−*9*). Covalent attachment of the heme to the protein thus appears to be a widespread property of the CYP4 family of cytochrome P450 enzymes even though it has not been detected in the members of any other P450 family (*7*, *8*).

In contrast to the results with CYP4F1 and CYP4F4, the heme in the heterologously expressed CYP4F5 and CYP4F6 was not detectably cross-linked to the protein (Figure 2, traces C and E). These are the first naturally occurring members of the entire CYP4 family that have not been found to covalently bind their heme group. The earlier studies of the CYP4A family indicated that the covalent bond from the heme to the protein involves a conserved glutamic acid residue, specifically Glu318 in CYP4A3 (*7*). This glutamic acid residue is found in all of the CYP4 enzymes that have so far been found to bind the heme covalently (*7*−*9*), including the CYP4F1 and CYP4F4 proteins examined here (Table 1). As shown for the CYP4A enzymes (*9*), the ability to covalently bind the heme is suppressed when the glutamic acid is replaced through site-specific mutagenesis. The collective results unambiguously establish that the most critical feature for covalent binding of the heme in the CYP4 family is the presence of the conserved I-helix glutamic acid residue.

To further evaluate the relationship between the presence of the glutamic acid and covalent heme binding, the CYP4F5/G330E mutant was prepared. This mutation introduces the requisite glutamic acid into a protein that has a glycine rather than a glutamic acid at the critical site and that therefore does not bind its heme covalently. This is a more demanding and informative experiment than simply showing that mutation of the glutamic acid in a CYP4 protein results in loss of covalent heme binding, as the acquisition of a property through mutagenesis is more difficult than its loss. In the event, introduction of the glutamic acid into CYP4F5 restores the ability of the enzyme to covalently bind the heme (Figure 2, trace D). Thus, 5% of the heme in the CYP4F5/G330E mutant is covalently bound when the heterologously expressed protein is isolated (Table 3). Interestingly, a substantial fraction of the noncovalently bound prosthetic group in the remaining protein is not unmodified heme. Analysis of the noncovalently bound prosthetic group shows that 18% of it is identical by HPLC and absorption spectroscopy with 5-hydroxymethylheme, the rest being unmodified heme (Figure 3). The combined extent of heme modification in the mutant is therefore 23%, although only 5% of that was actually covalently bound heme.

To interpret the observation of noncovalently attached 5-hydroxymethylheme, it is necessary to first address the mechanism of covalent heme binding. As we show here, if a CYP4F4 sample with 39% of the heme covalently bound is incubated for 2 h under turnover conditions, the extent of covalent binding increases to 90% (Figure 5). These findings exactly parallel those obtained earlier with the CYP4A family of proteins, for which the extent of covalent binding also increases upon catalytic turnover of the protein (*9*). It is clear from these results that the covalent heme−protein link is produced by an autocatalytic process that requires catalytically active protein and electrons from NADPH−cytochrome P450 reductase. In this respect, covalent binding of the heme

in the CYP4F and CYP4A proteins resembles that observed in the mammalian peroxidases. The prosthetic group of lactoperoxidase is attached to the protein via ester bonds between an aspartate and a glutamic acid and hydroxyl groups on the 1- and 5-methyls of the heme (*10*, *19*−*21*). We have demonstrated that these bonds are forged during the H_2O_2-dependent catalytic turnover of the apoprotein−heme complex (*10*, *11*). Evidence is now available that eosinophil and possibly thyroid peroxidase also undergo autocatalytic covalent attachment of their prosthetic heme groups (*22*, *23*).

The formation of an ester link between a methyl group and a glutamic acid residue in a single catalytic step appears, at first glance, a daunting process. However, both radical- and base-catalyzed pathways can be formulated for conversion of one of the heme methyls to a methylene cation. The radical process requires one-electron oxidation of a protein residue by the activated "Compound I" ferryl form of the enzyme, followed by abstraction by the protein radical of a hydrogen from the methyl group. Intramolecular reduction of the still hypervalent iron by an electron from the resulting methylene radical produces the required methylene cation. Alternatively, acidification of the methyl by the "Compound I" porphyrin radical cation, followed by two electron transfers from the methylene anion to the hypervalent ferryl species, generates the same state. Although these two mechanisms cannot yet be differentiated, both convert the methyl group to a methylene carbocation. Formation of the ester bond then simply requires trapping of the cation by the glutamic acid carboxylate group.

The carbocation generated in the above mechanisms is highly reactive and would be expected to react with water if not immediately trapped by the glutamic acid carboxylic acid function. The actives sites of the CYP4A proteins and of CYP4F1 and CYP4F4 are presumably engineered so that water does not compete effectively in this reaction with the carboxylic acid group. In contrast, CYP4F5 and CYP4F6 have presumably evolved so as to optimize their active site with a glycine rather than glutamic acid at the position in question in the I-helix. Thus, it is not surprising that the glutamic acid introduced into CYP4F5 by mutagenesis competes less effectively with water for trapping of the carbocation intermediate (Figure 4). This scenario readily explains the fact that 5% of the heme is covalently bound and 18% is converted to the noncovalently bound 5-hydroxymethyl derivative in the G330E mutant (Figures 2 and 3). It is interesting, in this context, that noncovalently bound 5-hydroxymethylheme is found in the active site when the CYP4A3 glutamic acid is mutated to an aspartic acid but not to other residues (*9*). The smaller aspartate side chain in the CYP4A3 mutant apparently allows a water molecule to compete effectively for the carbocation intermediate.

The results with CYP4F5/G330E clarify one further important aspect of the covalent binding mechanism. The fact that the noncovalently bound hydroxyheme derivative is formed with the CYP4F5/G330E mutant, but not with the wild-type proteins, shows that the carboxylate side chain is required not only for trapping the cation *but also for its formation*. Support for this conclusion is provided by the earlier study in which the hydroxymethyl derivative was observed when the glutamic acid in CYP4A3 was mutated to an aspartate, but not when it was mutated to a glutamine or alanine (*9*). We did not realize the significance of this finding in the earlier paper, but in conjunction with the similar observation made here, it is clear that a carboxylic acid side chain, either that of an aspartic or that of a glutamic acid, is required to generate the cationic charge on the heme 5-methyl group. The results do not establish whether the carboxylate group is required as an acid−base catalyst, as a potential radical site, or simply as a center of negative charge, but it is clearly an intimate part of the heme modification mechanism. It is interesting, in this context, that the cross-links in lactoperoxidase also involve ester bonds between the heme methyl groups and aspartic or glutamic acid residues. The carboxylic acid side chains in lactoperoxidase may also participate in formation of the cationic center that they subsequently trap to give the ester bonds.

In summary, we have (a) confirmed that CYP4F family proteins covalently bind their heme group, (b) established that an I-helix glutamic acid residue is the key determinant of CYP4F covalent heme binding, (c) found that the heme, as in the CYP4A enzymes, is probably bound to the protein through its 5-methyl group, (d) demonstrated that an autocatalytic process results in covalent heme binding in CYP4F proteins, (e) found that small perturbations in the active site structure influence whether the heme is covalently bound or simply converted to its 5-hydroxymethyl derivative, and (f) provided evidence that the carboxylic acid side chain is required to form as well as to trap the methyl-derived cation.

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