Autocatalytic Mechanism and Consequences of Covalent Heme Attachment in the Cytochrome P4504A Family*

Laurie A. LeBrun, Ute Hoch, and Paul R. Ortiz de Montellano‡

From the Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94143-0446

The prosthetic heme group in the CYP4A family of cytochrome P450 enzymes is covalently attached to an I-helix glutamic acid residue. This glutamic acid is conserved in the CYP4 family but is absent in other P450 families. As shown here, the glutamic acid is linked, presumably via an ester bond, to a hydroxyl group on the heme 5-methyl group. Mutation of the glutamic acid to an alanine in CYP4A1, CYP4A3, and CYP4A11 suppresses covalent heme binding. In wild-type CYP4A3 68% of the heme is covalently bound to the heterologously expressed protein, but in the CYP4A3/E318D mutant, 47% of the heme is unchanged, 47% is present as noncovalently bound 5-hydroxymethylheme, and only 6% is covalently bound to the protein. In the CYP4A3/E318Q mutant, the majority of the heme is unaltered, and <2% is covalently linked. The proportion of covalently bound heme in the recombinant CYP4A proteins increases with time under turnover conditions. The catalytic activity is sensitive in some, but not all, CYP4A enzymes to the extent of covalent heme binding. Mutations of Glu318 in CYP4A enzymes increase with time under turnover conditions. The catalytic activity is sensitive in some, but not all, CYP4A enzymes to the extent of covalent heme binding.

It has been known for some time that the prosthetic heme group is covalently bound to the protein in both the cytochromes c and the mammalian peroxidases. In the cytochromes c, the heme is attached to the protein via thioether bonds between the original heme vinyl groups and two of the cysteine residues (1). In the mammalian peroxidases, the heme is linked to the protein via two ester bonds involving aspartate or glutamic acid residues and hydroxyl groups on two of the original heme methyl groups (2). In addition, in myeloperoxidase a third covalent link is present between a methionine sulfur atom and one of the heme vinyl groups (2, 3). Formation of the covalent bonds between the heme and the protein in the cytochromes c requires the intervention of several proteins specifically devoted to that task (4, 5). In contrast, recent work from this laboratory has demonstrated that the covalent bonds in lactoperoxidase are formed via an autocatalytic process when the noncovalent heme-protein complex reacts with H2O2 (6, 7). Evidence that similar autocatalytic processes mediate covalent attachment of the heme groups in the eosinophil and thyroid peroxidases has subsequently been reported (8, 9).

The role of covalent heme attachment in cytochrome c remains elusive (10), as removal of one or both of the covalent links to the heme by site-specific mutagenesis has little apparent effect on the properties of the protein (11). In contrast, recent work has shown that one covalent link is expendable in lactoperoxidase (7), but activity is lost when both covalent links are suppressed by site-specific mutations in lactoperoxidase and myeloperoxidase (7, 12–14).

The CYP4 family of cytochrome P450 enzymes catalyzes the ω and ω-1 hydroxylation of fatty acids (15). We recently established that the heme is covalently bound to the protein in the CYP4A family of proteins (16), and subsequent work has extended the evidence for heme covalent binding to members of the CYP4B and CYP4F families (17). The heme is bound to the CYP4A proteins via a conserved glutamic acid residue on the I-helix of the protein (16). BLAST searches of protein data banks show that the amino acid context of this glutamic acid, EGHDTT, is highly conserved in the CYP4A family (Table I). This conserved sequence is present in species that range from Drosophila to humans. The CYP4 family is one of the most ancient in the P450 superfamily, having originated over 1 billion years ago before the divergence of invertebrates and vertebrates (15). None of the known P450 families outside of the CYP4 family has a glutamic acid residue at the indicated position. In this paper, we identify the site on the heme that is involved in covalent bond formation, demonstrate that heme binding is the result of an autocatalytic process, and explore the consequences of covalent heme attachment to the protein in the P450 system.

MATERIALS AND METHODS

Materials—Primer sets were ordered from Invitrogen. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). [14C]Lauric acid (55 mCi/mmol) was from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Purified rat liver cytochrome b5 was a gift from Lester Bornheim (University of California, San Francisco). Cytochrome P450 reductase cDNA was expressed and purified as reported previously (18). Amicillin, δ-aminolevulinic acid, glycerol, lysoyzime, diuluroylphosphatidylcholine, glutathione, catalase, heme, NADPH, trifluoroacetic acid, and lauric acid were obtained from Sigma-Aldrich. Emulgen 913 was a gift from KAO Chemicals (Tokyo, Japan). Acetonitrile (HPLC-grade) was purchased from Fisher Scientific. The 1-, 5-, and 8-hydroxymethylhemes were obtained from Sigma-Aldrich. Emulgen 913 was purchased from KAO Chemicals (Tokyo, Japan). Acetonitrile (HPLC-grade) was purchased from Fisher Scientific.

Received for publication, December 19, 2001, and in revised form, January 29, 2002. Published, JBC Papers in Press, January 30, 2002, DOI 10.1074/jbc.M112155200

© 2002 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.
TABLE I

The EGHDTT sequence in the I-helix is highly conserved in the CYP4 family

| Protein                        | Sequence              |
|--------------------------------|-----------------------|
| CYP4A1 (rat)                   | FMEEGHDTTASG          |
| CYP4A2 (rat)                   | FMEEGHDTTASG          |
| CYP4A3 (rat)                   | FMEEGHDTTASG          |
| CYP4A4 (rabbit)                | FMEEGHDTTASG          |
| CYP4A5 (rabbit)                | FMEEGHDTTASG          |
| CYP4A6 (rabbit)                | FMEEGHDTTASG          |
| CYP4A7 (rabbit)                | FMEEGHDTTASG          |
| CYP4A8 (rat)                   | FMEEGHDTTASG          |
| CYP4A11 (human)                | FMEEGHDTTASG          |
| CYP4A1E1 (drosophila)          | FIFEGHDTTSSG          |
| CYP4B1 (human)                 | FMEEGHDTTSSG          |
| CYP4B1 (mouse)                 | FMEEGHDTTSSG          |
| CYP4B1 (rabbit)                | FMEEGHDTTSSG          |
| CYP4B1 (rat)                   | FMEEGHDTTSSG          |
| CYP4C1 (cockroach)             | FMEEGHDTTSSG          |
| CYP4D1 (drosophila)            | FMEEGHDTTSSA          |
| CYP4D2 (drosophila)            | FMEEGHDTTSSA          |
| CYP4D3 (drosophila)            | FMEEGHDTTSSA          |
| CYP4D14 (drosophila)           | FMEEGHDTTSSS          |
| CYP4E1 (drosophila)            | FMEEGHDTTSSG          |
| CYP4E2 (drosophila)            | FMEEGHDTTSSG          |
| CYP4E3 (drosophila)            | FMEEGHDTTSSG          |
| CYP4F1 (rat)                   | FMEEGHDTTASG          |
| CYP4F2 (human)                 | FMEEGHDTTASG          |
| CYP4F3 (human)                 | FMEEGHDTTASG          |
| CYP4F1 (rat)                   | FMEEGHDTTASG          |
| CYP4F1 (ram)                   | FMEEGHDTTASG          |
| CYP4G1 (drosophila)            | IMFEGHDTSSG           |
| CYP4G15 (drosophila)           | IMFEGHDTSSG           |
| CYP4S3 (drosophila)            | FMEEGHDTTSSA          |

Preparation of the CYP4A3/E318A, E318Q, and E318D Mutants—The CYP4A genes were excised from the pcWori/4A plasmid by digestion with NdeI and SacII restriction enzymes. The CYP4A genes were then subcloned into the pUC19 vector, and this plasmid was used for mutagenesis. The QuickChange (Stratagene, La Jolla, CA) method was used to prepare the mutants. Underlined codons represent the base pair changes that were made to effect the amino acid point mutations. The QuikChange (Stratagene, La Jolla, CA) method was then subcloned into the pUC19 vector, and this plasmid was used for mutagenesis. The QuikChange (Stratagene, La Jolla, CA) method was then subcloned into the pUC19 vector, and this plasmid was used for mutagenesis. The QuikChange (Stratagene, La Jolla, CA) method was then subcloned into the pUC19 vector, and this plasmid was used for mutagenesis.

Protein Purification—As described previously, the hexahistidine-tagged proteins were expressed in DH5-α cells, purified on a Ni²⁺-NTA columns eluted at 4.6 min, the 5-monohydroxylated heme at 8.2 min, the monohydroxylated heme from the CYP4A8 digest at 8.2 min, and free heme at ~19.6 min.

RESULTS

Spectroscopic Characterization of the Mutant Proteins—The CYP4A proteins with mutations at the site to which the heme is covalently attached were expressed in DH5-α cells and purified on a Ni²⁺-nitrilotriacetic acid-agarose column. In the CYP4A1/E320A, CYP4A3/E318A, and CYP4A11/E321A mutants, the Soret bands are blue shifted relative to those of the corresponding wild-type proteins (Table I). The absorption maximum of the CYP4A1/E320A mutant at 390 nm indicates that the protein is in a high rather than low spin state. The Soret bands of the CYP4A1/E320A, CYP4A3/E318A, and CYP4A11/E321A mutants at 406 nm are also lower than those of the corresponding wild-type proteins at 420 nm, respectively.
the wild-type proteins consist entirely of the 450 nm Soret band. The reduced CO difference spectra for the mutants and the wild-type proteins are contaminated by the presence of denatured cytochrome P450 species absorbing at 420 nm. The reduced CO difference spectra for the other mutants and the wild-type proteins consist entirely of the 450 nm species.

Wild-type CYP4A1, CYP4A3, and CYP4A11 have Soret maxima at 450 nm in the ferrous, CO-bound state. However, the reduced CO difference spectra indicate that the CYP4A3/E318A, CYP4A3/E318D, and CYP4A11/E321A mutants were contaminated by the presence of denatured cytochrome P450 species absorbing at 420 nm (Table II). The CYP4A3/E318A mutant contains ~75% of the 420 nm species, the CYP4A3/E318D mutant ~50%, and the CYP4A11/E321A mutant ~30%. The reduced CO difference spectra of the wild-type proteins and of the other mutants only contained the species absorbing at 450 nm. In all instances, the quantitative data reported in this manuscript are based exclusively on the protein with 450 nm absorption maximum.

**Analysis of Covalent Heme Binding in the CYP4A Mutants—**

Wild-type CYP4A1, CYP4A3, and CYP4A11 have ~36%, 68%, and 26%, respectively, of their heme covalently bound to the protein (16). When the indicated glutamic acid in these proteins is mutated to an alanine, the heme group is no longer covalently bound. The percent of covalently bound heme was calculated from the percentage of covalently bound heme was calculated from the mass spectrometric evidence that the heme is covalently bound, except that the protein need not be digested to release the derivative. The noncovalently bound monohydroxylated heme species obtained with the CYP4A3/E318D mutant also elutes at 8.2 min under the chromatographic conditions (Fig. 3). The 1-hydroxymethyl derivative eluted at a quite different position. The monohydroxylated heme species released by digestion of CYP4A8 elutes at 8.2 min, exactly where the 5-hydroxymethyl derivative elutes and at a position quite distinct from that of the 8- or 1-hydroxymethyl derivatives. These results, in conjunction with the mass spectrometric evidence that the heme has one extra oxygen atom and the fact that the absorption spectrum of the heme chromophore is unaltered (16), strongly suggest that the oxygen is attached to the 5-methyl of the heme as a hydroxyl moiety.

The noncovalently bound monohydroxylated heme species obtained with the CYP4A3/E318D mutant also elutes at 8.2 min under the chromatographic conditions (Fig. 3). The noncovalently bound species is thus the same as that obtained when the heme is covalently bound, except that the protein need not be digested to release the derivative.

**Auto- and Heme Site Involved in Binding to Protein—**

Earlier mass spectrometric analyses showed that the covalently bound heme released by Pronase from the CYP4A proteins is monohydroxylated, but the location of the hydroxyl group could not be determined. A CYP4A8 sample with a high percentage of covalently bound heme was selected to determine the location of the hydroxyl group on the heme. The CYP4A8 protein was digested with Pronase for 20 h to release the covalently attached heme (Fig. 2). The sample was then subjected to HPLC chromatography on a POROS column to identify the hydroxylated heme group by direct comparison with authentic samples. Authentic 8-hydroxymethylheme was obtained as reported previously from phenyldiazene-treated horseradish peroxidase (19). The 1- and 5-hydroxymethylhemes were obtained from lactoperoxidase mutants unable to form one of the two covalent bonds to the heme (7). The 8-hydroxymethylheme eluted at 4.6 min, the 5-hydroxymethylheme at 8.2 min, and unmodified heme at ~19.6 min in our HPLC system (Fig. 3). The 1-hydroxymethyl derivative eluted at a quite different position. The monohydroxylated heme species released by digestion of CYP4A8 elutes at 8.2 min, exactly where the 5-hydroxymethyl derivative elutes and at a position quite distinct from that of the 8- or 1-hydroxymethyl derivatives. These results, in conjunction with the mass spectrometric evidence that the heme has one extra oxygen atom and the fact that the absorption spectrum of the heme chromophore is unaltered (16), strongly suggest that the oxygen is attached to the 5-methyl of the heme as a hydroxyl moiety.

The noncovalently bound monohydroxylated heme species obtained with the CYP4A3/E318D mutant also elutes at 8.2 min under the chromatographic conditions (Fig. 3). The noncovalently bound species is thus the same as that obtained when the heme is covalently bound, except that the protein need not be digested to release the derivative.

**Auto- and Heme Site Involved in Binding to Protein—**

The 1-hydroxymethyl derivative eluted at a quite different position. The monohydroxylated heme species released by digestion of CYP4A8 elutes at 8.2 min, exactly where the 5-hydroxymethyl derivative elutes and at a position quite distinct from that of the 8- or 1-hydroxymethyl derivatives. These results, in conjunction with the mass spectrometric evidence that the heme has one extra oxygen atom and the fact that the absorption spectrum of the heme chromophore is unaltered (16), strongly suggest that the oxygen is attached to the 5-methyl of the heme as a hydroxyl moiety.

The noncovalently bound monohydroxylated heme species obtained with the CYP4A3/E318D mutant also elutes at 8.2 min under the chromatographic conditions (Fig. 3). The noncovalently bound species is thus the same as that obtained when the heme is covalently bound, except that the protein need not be digested to release the derivative.

**Auto- and Heme Site Involved in Binding to Protein—**

The 1-hydroxymethyl derivative eluted at a quite different position. The monohydroxylated heme species released by digestion of CYP4A8 elutes at 8.2 min, exactly where the 5-hydroxymethyl derivative elutes and at a position quite distinct from that of the 8- or 1-hydroxymethyl derivatives. These results, in conjunction with the mass spectrometric evidence that the heme has one extra oxygen atom and the fact that the absorption spectrum of the heme chromophore is unaltered (16), strongly suggest that the oxygen is attached to the 5-methyl of the heme as a hydroxyl moiety.

The noncovalently bound monohydroxylated heme species obtained with the CYP4A3/E318D mutant also elutes at 8.2 min under the chromatographic conditions (Fig. 3). The noncovalently bound species is thus the same as that obtained when the heme is covalently bound, except that the protein need not be digested to release the derivative.

**Auto- and Heme Site Involved in Binding to Protein—**

The 1-hydroxymethyl derivative eluted at a quite different position. The monohydroxylated heme species released by digestion of CYP4A8 elutes at 8.2 min, exactly where the 5-hydroxymethyl derivative elutes and at a position quite distinct from that of the 8- or 1-hydroxymethyl derivatives. These results, in conjunction with the mass spectrometric evidence that the heme has one extra oxygen atom and the fact that the absorption spectrum of the heme chromophore is unaltered (16), strongly suggest that the oxygen is attached to the 5-methyl of the heme as a hydroxyl moiety.

The noncovalently bound monohydroxylated heme species obtained with the CYP4A3/E318D mutant also elutes at 8.2 min under the chromatographic conditions (Fig. 3). The noncovalently bound species is thus the same as that obtained when the heme is covalently bound, except that the protein need not be digested to release the derivative.

**Auto- and Heme Site Involved in Binding to Protein—**

The 1-hydroxymethyl derivative eluted at a quite different position. The monohydroxylated heme species released by digestion of CYP4A8 elutes at 8.2 min, exactly where the 5-hydroxymethyl derivative elutes and at a position quite distinct from that of the 8- or 1-hydroxymethyl derivatives. These results, in conjunction with the mass spectrometric evidence that the heme has one extra oxygen atom and the fact that the absorption spectrum of the heme chromophore is unaltered (16), strongly suggest that the oxygen is attached to the 5-methyl of the heme as a hydroxyl moiety.

The noncovalently bound monohydroxylated heme species obtained with the CYP4A3/E318D mutant also elutes at 8.2 min under the chromatographic conditions (Fig. 3). The noncovalently bound species is thus the same as that obtained when the heme is covalently bound, except that the protein need not be digested to release the derivative.

**Auto- and Heme Site Involved in Binding to Protein—**

The 1-hydroxymethyl derivative eluted at a quite different position. The monohydroxylated heme species released by digestion of CYP4A8 elutes at 8.2 min, exactly where the 5-hydroxymethyl derivative elutes and at a position quite distinct from that of the 8- or 1-hydroxymethyl derivatives. These results, in conjunction with the mass spectrometric evidence that the heme has one extra oxygen atom and the fact that the absorption spectrum of the heme chromophore is unaltered (16), strongly suggest that the oxygen is attached to the 5-methyl of the heme as a hydroxyl moiety.

The noncovalently bound monohydroxylated heme species obtained with the CYP4A3/E318D mutant also elutes at 8.2 min under the chromatographic conditions (Fig. 3). The noncovalently bound species is thus the same as that obtained when the heme is covalently bound, except that the protein need not be digested to release the derivative.
The apparent $k_{cat}$ of the CYP4A1/E320A mutant was 3-fold lower than that of wild-type CYP4A1. For the CYP4A3E318D, -E318Q, and -E318A mutants the apparent lauric acid $k_{cat}$ values were 6-, 8-, and 20-fold lower, respectively, than that of wild-type CYP4A3. However, the apparent $k_{cat}$ value of the CYP4A11/E321A mutant was the same as that of the wild-type protein. It is perhaps significant that the extent of the changes in the apparent $k_{cat}$ values roughly parallel the corresponding changes in the lauric acid binding constants.

**DISCUSSION**

Previous investigations have demonstrated that the prosthetic heme group of several proteins of the CYP4 family is covalently bound to the protein. Although the studies were carried out primarily with proteins heterologously expressed either in *Escherichia coli* (16) or a baculovirus system (17), the extent of covalent binding was found to be as high or higher in (Table III). The apparent $k_{cat}$ of the CYP4A1/E320A mutant was 3-fold lower than that of wild-type CYP4A1. For the CYP4A3E318D, -E318Q, and -E318A mutants the apparent lauric acid $k_{cat}$ values were 6-, 8-, and 20-fold lower, respectively, than that of wild-type CYP4A3. However, the apparent $k_{cat}$ value of the CYP4A11/E321A mutant was the same as that of the wild-type protein. It is perhaps significant that the extent of the changes in the apparent $k_{cat}$ values roughly parallel the corresponding changes in the lauric acid binding constants.

**FIG. 1.** HPLC analysis of covalent heme attachment in CYP4A3 mutants. Desalted protein samples were injected onto a POROS R2 perfusive column, and the heme chromophore was monitored at 400 nm: A, heme standard; B, monohydroxylated heme; C, wild-type CYP4A3; D, CYP4A3/E318A; E, CYP4A3/E318D; and F, CYP4A3/E318Q. Unmodified heme elutes at 6 min, monohydroxylated heme at 3.3 min, and protein-bound heme around 12.5 min.

**FIG. 2.** HPLC analysis of the CYP4A8 protein before and after Pronase digestion. A desalted CYP4A8 sample was injected onto the HPLC and monitored at 400 nm (A) and 280 nm (B). The unmodified heme elutes at 6 min and the protein-bound heme at 12.5 min. In the CYP4A8 protein the majority of the heme is covalently bound. The CYP4A8 sample after overnight digestion with Pronase was similarly subjected to HPLC analysis with monitoring at 400 nm (C) and 280 nm (D). All of the covalently bound heme was released from the protein. The polar heme that is released elutes at 3.3 min.

**FIG. 3.** HPLC analysis of the polar heme species with the detector set at 400 nm. Samples were injected onto the POROS column and separated as indicated under "Materials and Methods." The reduced CO spectra of the CYP4A8 sample used for this experiment contained over 85% of the protein species absorbing at 450 nm: A, 8-hydroxymethylheme standard; B, unmodified heme standard; C, 5-hydroxymethylheme standard; D, digested CYP4A8 sample; and E, desalted CYP4A3/E318D protein. 8-Hydroxymethylheme elutes at 4.6 min, 5-hydroxymethylheme at 8.2 min, and unmodified heme around 19.6 min.

CYP4A1 isolated from rat liver (16). Covalent binding is thus not an artifact of heterologous expression. The site of covalent attachment was shown by proteolytic experiments to be a con-
served glutamic acid residue in the I-helix (16). However, the nature of the covalent link and the site on the heme involved in covalent attachment were not determined.

The present studies confirm the role of the glutamic acid, establish that it is the unique site of attachment of the heme to the protein, and demonstrate that the link to the heme involves a hydroxyl group on the 5-methyl of the heme (Fig. 5). Thus, mutation of the glutamic acid to an alanine in CYP4A1, CYP4A3, and CYP4A11 yields proteins with normal Fe$^{II}$−CO absorption spectra at 450 nm but in which covalent binding of the heme is completely suppressed (Fig. 1). Furthermore, replacement of the glutamic acid by a glutamine, a substitution that preserves the bulk of the side chain but eliminates the carboxylic acid terminus, yields a protein that again absorbs at 450 nm in the Fe$^{II}$ state but in which no more than a trace of the heme is covalently bound (Fig. 1). It is clear from these experiments that the carboxylic acid moiety of the glutamic acid is critical for the covalent attachment process.

Mutation of the indicated glutamic acid to an alanine in the CYP4A proteins results in a blue shift of the Soret band (Table II). A blue shift is also observed when the carboxylic acid residues that covalently bind to the heme are mutated in myeloperoxidase (Glu$^{318}$) (12). These mutations clearly affect the spectral properties of the protein. However, when the mutants retain a carboxylic acid at the site of attachment, as in CYP4A3/E318D (Table II) or a lactoperoxidase mutant (7), the Soret band is not shifted. The differences observed with the neutral substitutions could reflect the presence versus absence of the covalent bond, but could simply be due to differences in the active site polarity or water occupancy caused by the mutations.

Pronase digestion of CYP4A8, using a sample with a high degree of covalent heme binding, led to isolation of the modified prosthetic group. Previous mass spectrometric analysis of this prosthetic group established that its mass corresponded to that of heme plus an oxygen atom (16). As the absorption spectrum of the isolated heme was essentially the same as that of heme itself, the modified heme group was tentatively identified as a monohydroxylated heme. The site of hydroxylation could not be determined from the mass spectrometric data, but has been identified in the present experiments by comparison of the prosthetic group released proteolytically from the protein with authentic samples of the 1-hydroxymethyl-, 5-hydroxymethyl-, and 8-hydroxymethylheme derivatives (Fig. 3). The isolated prosthetic group has exactly the same retention time as 5-hydroxymethyl and a retention time well resolved from those of the 1- and 8-hydroxymethyl standards. In view of this chromatographic comparison and the earlier mass spectrometric and spectroscopic evidence (16), it is clear that the heme is linked to the protein via a hydroxyl group on the 5-methyl of the heme. It is virtually certain, therefore, that the heme-protein link is an ester bond involving the carboxylic mien of the glutamic acid and the hydroxyl on the heme (Fig. 5).

When Glu$^{318}$ in CYP4A3 is replaced by an aspartic acid, the side chain is shortened by one methylene group. This mutation greatly decreases covalent binding of the heme but, unlike the alanine mutation, does not completely suppress it. Only 6% of the heme is covalently bound, in contrast to the 68% observed with the wild-type protein. As a consequence, 47% of the prosthetic group in the CYP4A3/E318D mutant is unmodified heme. The most interesting finding, however, is that 47% of the prosthetic group is present as 5-hydroxymethylheme that is not covalently bound (Fig. 3)! HPLC analysis shows that the site of the hydroxyl moiety is the same in the covalently bound and noncovalently bound but modified heme group. The decrease in covalent binding due to the aspartate substitution reinforces the conclusion that the glutamic acid carboxylic acid group is required for covalent bond formation. More importantly, the finding that the substitution produces a substantial yield of modified but noncovalently bound prosthetic group considerably clarifies the mechanism of covalent bond formation (see below).

A major finding is that the extent of covalent binding of the heme to the protein increases with time when the recombinant enzyme is incubated under catalytic turnover conditions. Increases of 12%, 49%, and 10% were observed with CYP4A2, CYP4A8, and CYP4A11, respectively. These increases were absolutely dependent on catalytic turnover, as they did not occur when NADPH was omitted from the incubation system. Larger increases in covalent binding were difficult to observe, because the heme was already partially covalently bound, limiting the possible increase, and because incubation under turnover conditions also leads with time to inactivation of the protein. Nevertheless, the results clearly indicate that the heme is covalently bound to the protein through an autocatalytic process. Autocatalytic formation of an ester bond between the 5-methyl of the heme and the glutamic acid carboxyl moiety is likely to involve trapping of a heme 5-methyl carbocation by the carboxylic acid group. Support for this mechanism is provided by the finding that substantial amounts of uncovalently bound 5-hydroxymethylheme are formed when the glutamic acid is replaced by an aspartate group (Fig. 3). Shortening the chain that tethers the carboxylic acid group to the protein

### Table IV

Spectroscopic binding constants ($K_s$) for wild-type and mutant CYP4A proteins

| Enzyme          | Imidazole   | Lauric acid |
|-----------------|-------------|-------------|
| CYP4A1          | 1400$^{a}$  | 30$^{b}$    |
| CYP4A1/E320A    | 3300 (4000)$^{a}$ | 80          |
| CYP4A3          | 47          | 3           |
| CYP4A3/E318A    | 68          | ND$^{c}$    |
| CYP4A3/E318Q    | 379         | 23          |
| CYP4A3/E318D    | 1300        | 40          |
| CYP4A11         | 400$^{d}$   | 22$^{e}$    |
| CYP4A11/E321A   | 2400        | 27          |

$^{a}$Ref. 18.  
$^{b}$Ref. 20.  
$^{c}$Not determined.  
$^{d}$Ref. 21.  

For Fig. 4. Autocatalytic heme attachment in CYP4A proteins. The percentage of heme bound to the protein increases under catalytic conditions in a time-dependent manner with CYP4A2 (○), CYP4A8 (△), and CYP4A11 (□). In the absence of NADPH the percentage of protein-bound heme remains unchanged. The reduced CO spectra for the CYP4A8 sample used for this experiment contained a 60:40 mixture of 420 and 450 nm species. The change in the percent of covalently bound heme was calculated for the active 450 nm-absorbing species only.
backbone apparently decreases its ability to compete with water for trapping of the carboxylation, resulting in covalent binding of the prosthetic group when the carboxylic acid group is successful, but formation of noncovalently bound 5-hydroxymethylheme when the carboxylation is trapped by a water molecule in the enlarged active site. Radical and acid-base mechanisms can be written for formation of the 5-methyl carboxylation, but the available evidence does not allow a distinction to be made between the possible routes to this critical intermediate.

The autocatalytic mechanism demonstrated here for covalent attachment of the heme in CYP4 enzymes resembles that for covalent linking of the prosthetic group to the protein in the mammalian peroxidases (6, 7). There are two obvious differences, however. Covalent binding of the heme to the protein in lactoperoxidase involves two rather than one ester bond, one between Asp and the 5-methyl group and the other between Glu and the 1-methyl group (25). A second significant difference is that autocatalytic cross-linking of the heme in the mammalian peroxidases is promoted by reaction with H2O2, whereas the reaction in the CYP4A enzymes involves reaction with oxygen and NADPH-cytochrome P450 reductase. It is nevertheless likely that the basic mechanism will be the same in both systems, i.e., activation via a ferryl species of the methyl group to a carbocationic intermediate that is subsequently trapped by the appropriate carboxylic acid side chain (Fig. 5).

No crystal structure is available of a carboxylic protein, but a state of the art CYP4A11 homology model has been constructed by Chang and Loew (26). Although it is necessary to keep in mind the shortcomings of P450 homology models, the carboxylic acid side chain of Glu in the CYP4A11 model is closer by 1–2 Å to the 5- and 8-methyl groups of the heme than the 1- and 3-methyls. In the model, the 5- and 8-methyl groups are both ~7.7 Å from the critical glutamic acid residue. The model is thus consistent with formation of a covalent link to either the 5- or 8-methyl group, but is not sufficiently accurate to distinguish between these two positions.

Does covalent heme binding influence the catalytic function of CYP4 enzymes? The apparent k cat for lauric acid hydroxylation decreases from 150 to 54 min−1 when Glu is mutated to an alanine in CYP4A1, and from 77 to 3.4 min−1 when Glu is similarly mutated in CYP4A3 (Table III). However, mutating Glu to an alanine in CYP4A11 does not alter the lauric acid hydroxylation activity. These results clearly demonstrate that covalent heme binding is important, but not absolutely required, for the catalytic activity of CYP4A1 and particularly CYP4A3. In the case of CYP4A11, covalent heme binding is not a detectable determinant of the rate of lauric acid hydroxylation. Analysis of the binding of imidazole and lauric acid to the mutant and wild-type proteins reinforces these conclusions. Thus, the binding of imidazole is significantly decreased in all the mutants relative to the wild-type proteins (Table IV). More interestingly, the binding of lauric acid is also decreased in the CYP4A1 and CYP4A3, but not CYP4A11 mutants. The fact that both the wild-type and mutant proteins consist of mixtures of covalently and noncovalently bound heme complicates the quantitative interpretation of these ligand binding alterations, but the qualitative conclusion that the CYP4A1 and CYP4A3 protein active sites are more sensitive to covalent binding of the heme than the CYP4A11 protein appears warranted.

It must be kept in mind, of course, that covalent binding of the heme group may influence other properties of the CYP4A enzyme than their catalytic activity with lauric acid. A BLAST search of the protein data bases for the glutamic acid residue and its conserved context (EEDT) indicates that it is highly (but not absolutely) conserved in the CYP4 family (Table I). This high degree of conservation of the glutamic group suggests that covalent heme binding may be important in other aspects of CYP4A function, particularly those that are relevant to its in vivo function. However, no conclusions can yet be made relative to alternative mechanisms of covalent binding of the heme in the CYP4A family.

In summary, we have provided evidence that the covalent link to the heme in the CYP4A proteins is an ester bond between a glutamic acid residue and a hydroxyl group on the 5-methyl of the heme, that the covalent bond is formed by an autocatalytic process probably involving trapping of a carbocationic intermediate by the glutamic acid, and that covalent binding is important for lauric acid oxidation in some but not all CYP4A enzymes.

Acknowledgment—We thank Christophe Colas for the authentic 1-, 5-, and 8-hydroxymethylheme samples.

REFERENCES
1. Moore, G. R., and Pettigrew, G. W. (1990) in Cytochromes c: Evolutionary, Structural, and Physicochemical Aspects (Rich, A., ed) Springer-Verlag, Berlin
2. Fiedler, T. J., Davey, C. A., and Fenna, R. E. (2000) J. Biol. Chem. 275, 11964–11971
3. Fenna, R., Zeng, J., and Davey, C. (1995) Arch. Biochem. Biophys. 316, 653–658
4. Page, M. D., Sambongi, Y., and Ferguson, S. J. (1998) Trends Biochem. Sci. 23, 103–108
5. Thony-Meyer, L. (2000) Biochim. Biophys. Acta 1459, 316–324
6. DePillis, G. D., Ozaki, S., Kuo, J. M., Matthey, D. A., and Ortiz de Montellano, P. R. (1997) J. Biol. Chem. 272, 8857–8860
7. Colas, C., Kuo, J. M., and Ortiz de Montellano, P. R. (2002) J. Biol. Chem. 277, 7191–7200
8. Fayadat, L., Nicolet-Sire, P., Lanet, J., and Franc, J. L. (1999) J. Biol. Chem. 274, 10533–10538
9. Ovig, C., Thomsen, A. R., Overgaard, M. T., Sorensen, E. S., Hojrup, P., Bjerrum, M. J., Gleich, G. J., and Settrup-Jensen, L. (1999) J. Biol. Chem. 274, 16953–16958
10. Barker, P. D., and Ferguson, S. J. (1999) Structure (Lond.) 7, R281–R290
11. Tomlinson, E. J., and Ferguson, S. J. (2000) J. Biol. Chem. 275, 32530–32534
12. Suriano, G., Watanabe, S., Ghibaudi, E. M., Bollen, A., Ferrari, R. P., and Moguilevsky, N. (2001) _Bioorg. Med. Chem. Lett._ **11**, 2827–2831
13. Kooter, I. M., Néguilevsky, N., Bollen, A., Sijtsema, N. M., Otto, S., Dekker, H. L., and Wever, R. (1999) _Eur. J. Biochem._ **264**, 211–217
14. Kooter, I. M., Moguilevsky, N., Bollen, A., van der Veen, L. A., Otto, C. Dekker, H. L., and Wever, R. (1999) _J. Biol. Chem._ **274**, 26794–26802
15. Simpson, A. E. C. M. (1997) _Gen. Pharmacol._ **28**, 351–359
16. Hoch, U., and Ortiz de Montellano, P. R. (2001) _J. Biol. Chem._ **276**, 11339–11346
17. Henne, K. R., Kunze, K. L., Zheng, Y. M., Christmas, P., Soberman, R. J., and Rettie, A. E. (2001) _Biochemistry_ **40**, 12925–12931
18. Dierks, E. A., Davis, S. C., and Ortiz de Montellano, P. R. (1998) _Biochemistry_ **37**, 1839–1847
19. Ator, M., and Ortiz de Montellano, P. R. (1987) _J. Biol. Chem._ **262**, 1542–1551
20. Hoch, U., Zhang, Z. P., Kroetz, D. L., and Ortiz de Montellano, P. R. (2000) _Arch. Biochem. Biophys._ **373**, 63–71
21. Dierks, E. A., Zhang, A., Johnson, E. F., and Ortiz de Montellano, P. R. (1998) _J. Biol. Chem._ **273**, 23055–23061
22. Omura, T., and Sato, R. (1964) _J. Biol. Chem._ **239**, 2370–2374
23. Orrenius, S., Wilson, B. J., von Bahr, C., and Schenkman, J. B. (1972) in _Biological Hydroxylation Mechanisms: Biochemical Society Symposium_ (Boyd, G. S., and Smellie, R. M. S., eds) Vol. 34, pp. 55–77, Academic Press, New York.
24. Jacquet, A., Garcia-Quintana, L., Deleersnyder, V., Fenna, R., Bollen, A., and Moguilevsky, N. (1994) _Biochem. Biophys. Res. Commun._ **202**, 73–81
25. Rae, T. D., and Goff, H. M. (1998) _J. Biol. Chem._ **273**, 27968–27977
26. Chang, Y. T., and Loew, G. H. (1999) _Protein_ **34**, 403–415