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Structural Design of the Active Site for Covalent Attachment of the Heme to the Protein Matrix: Studies on a Thermostable Cytochrome P450[†]

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ABSTRACT: The molecular basis of the post-translational modification involving covalent attachment of the heme with a glutamic acid observed in some enzymes of the CYP4 family of heme monooxygenases has been investigated using site-directed mutagenesis of CYP175A1 from *Thermus thermophilus*. Earlier studies of CYP4 as well as the G248E mutant of CYP101A1 showed covalent linkage of the heme to a conserved glutamic acid of helix I. We have introduced Glu/Asp at the Leu80 position in the β-turn of CYP175A1, on the basis of molecular modeling studies, to assess whether formation of such a covalent linkage is limited only to helix I or whether such modification may also take place with the residue that is spatially located at a position appropriate for activation by the heme peroxidase reaction. Tandem mass spectrometry analyses of the tryptic digest of the wild type and mutants of CYP175A1 were conducted to identify any heme-bound peptide. Tryptic digestion of the L80E mutant of CYP175A1 preincubated with H_2O_2 showed formation of GLE(−heme)TDWGESWKEARK supporting covalent linkage of Glu80 with the heme in the mutant enzyme. No such heme-bound peptides were found if the sample was not preincubated in H_2O_2, indicating no activation of the Glu by the heme peroxidase reaction, as proposed earlier. The wild type or L80D mutant of the enzyme did not give any heme-bound peptide. Thus, the results support the idea that covalent attachment of the heme to an amino acid in the protein matrix depends on the structural design of the active site.

The cytochrome P450 enzymes form a ubiquitous superfamily of heme *b*-containing monooxygenases, which are responsible for a large number of physiologically and biotechnologically important transformations of numerous endogenous and exogenous compounds (*1*−*3*). The crystal structure of most of the P450 enzymes shows that the active site of the enzyme consists of the heme[1] anchored to the protein matrix through a coordinate bond with a highly conserved cysteine (thiolate ligand) residue on its proximal site, which probably serves as the most important interaction between the heme and protein matrix. Also, heme binding in P450 enzymes is facilitated via other noncovalent interactions like ionic, hydrophobic, and van der Waals interactions between active site amino acid residues of the protein and functional groups of the porphyrin ring (*2*, *4*). However, exceptions are members of the CYP4 family of cytochrome P450 enzymes in which post-translational modification involving covalent attachment of the heme to the protein matrix has been observed in CYP4A, CYP4B, and CYP4F (not in CYP4F5 and -6) (*1*, *2*, *5*−*8*). In CYP4A3, the heme is attached to the protein via an ester link to Glu318, which is in helix I, and is predicted to be within the active site (*9*). The percentage of covalent linkage in CYP4A1, CYP4A3, CYP4A11, and CYP4B1 was found to decrease by more than 90% when the glutamic acid residue of helix I was mutated to alanine and other amino acid residues (*1*, *5*). No covalent attachment, however, was found in CYP4F5, which has glycine at the respective position, but the G330E mutant of CYP4F5 shows the partial covalent attachment of the heme (*6*).

In case of myeloperoxidase, two types of linkages are found, one involving an ester link between the Glu242 and Asp94 residues and the 1- and 5-methyl groups of heme, respectively, and the other a sulfonium link between the sulfur of Met243 and the β-carbon of the 2-vinyl group of heme (*10*, *11*). Similarly, the lactoperoxidases are well-known to contain ester links of the heme 1- and 5-methyl groups with Glu and Asp residues (*12*−*15*). In eosinophil peroxidase, Glu241 and Asp93 have been shown to be involved in the formation of covalent links with the 1- and 5-methyl groups of heme, respectively (*16*). Not only the information described above but also numerous examples of the modifications of heme group are well-known and may or may not be involved in the same type of covalent linkage. Proteins that contains a covalent linkage between the heme prosthetic group and the protein matrix include cytochrome *c*, ascorbate peroxidase (APX), cytochrome *c* peroxidase (*CcP*), and thyroid peroxidase (*11*, *17*−*20*). In cytochrome *c*, heme is covalently attached to the protein matrix through thioether bonds between two vinyl groups of the heme and the cysteine residues (*17*). In ascorbate peroxidase and cytochrome *c* peroxidase, covalent links between Trp41 and Trp51, respectively, and one of vinyl groups of heme were reported (*18*, *19*).

The presence of covalently bound heme in some enzymes of the CYP4 family and its absence in a vast majority of other P450

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[1]Abbreviations: heme, iron protoporphyrin IX regardless of oxidation and ligation state; WT, wild type; L80D and L80E, leucine 80 to aspartic acid and glutamic acid mutants of CYP175A1, respectively; CD, circular dichroism; T_m, melting temperature; PDB, Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank.

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enzymes indicate that the covalent link satisfies a requirement that is specific for some of those CYP4 enzymes (*8*). Removal of the covalent linkage in CYP4A1 and CYPA3 was shown to cause either a decrease or a loss of the catalytic activity of the enzyme. However, such linkage did not have any effect on the enzymatic activity of CYP4A11. The role of covalent linkages in the catalytic activity of lactoperoxidase and myeloperoxidase has been established in detail, but in the class I *c*-type cytochrome, loss of either of the two heme-binding cysteines has a small effect on the physiological properties (*21*). In contrast, when both the covalent links are suppressed by site-specific mutations in lactoperoxidase and myeloperoxidase, they lose their catalytic activity (*5*).

The specific conditions under which the covalent link between the heme and protein matrix can be formed have been explored less in P450 enzymes. The results from earlier studies (*2*, *5*) have shown the importance of a conserved glutamic acid residue of helix I in covalent linkage. Surprisingly, in CYP4 or in some other families of P450 enzymes (e.g., cytochrome $P450_{cam}$), only the position that corresponds to the conserved glutamic acid residue of helix I has been explored in detail (*2*) for possible covalent linkage between the heme and the protein. To the best of our knowledge, no report of formation of a covalent link between heme and protein via the placement of an appropriate amino acid in P450 enzymes at a position other than that corresponding to the conserved glutamic acid residue of helix I of members of the CYP4 family is available (*22*). In view of this, we set out to investigate whether the covalent linkage of the heme in a P450 enzyme requires the glutamic acid to be located at the same position as in the case of CYP4 or whether such a covalent link can form if the glutamic acid is situated at some other position near the heme center.

The aim of this work is to test the innate distinctiveness of the conserved glutamic acid residue in helix I of members of the CYP4 family in the formation of the covalent link with the heme. The crystal structure of thermostable CYP175A1 (*23*) shows the absence of any suitable amino acid residue in the distal heme pocket that could act as a potential candidate for the formation of a covalent link as in members of the CYP4 family of cytochromes P450 enzymes. It is, however, not known whether a linkage can be formed between the heme and a carboxylate-containing residue residing an appropriate distance from the heme, though the position of the residue is not equivalent to that of the glutamic acid of helix I observed in members of the CYP4 family.

To understand the molecular basis of formation of the covalent linkage between the heme and the protein matrix, we have conducted detailed molecular modeling and rational design of the active site of the thermostable cytochrome P450, CYP-175A1 (*23*). The Ala221 residue of CYP175A1 is sequentially equivalent to the glutamic acid in helix I of members of the CYP4 family. Considering the distance from the heme 5-methyl to the C_α of the amino acid residue along with the orientation of its side chain, the crystal structure of wild-type (WT) CYP175A1 suggests that apart from Ala221, the Leu80 site could also be a candidate for covalent linkage with the heme, if replaced with an Asp/Glu acid residue. In view of testing this hypothesis, we have introduced two mutations, L80D and L80E, into WT CYP175A1 for which the Leu80 position close to heme belongs to a β-turn (type I, Gly78−Thr81) and not to helix I. Detailed UV−vis and circular dichroism (CD) spectroscopic studies were conducted to characterize WT CYP175A1 and its mutants. In this report, we demonstrate that covalent linkage can occur between heme and Glu80 of the L80E mutant of CYP175A1 upon exposure of the enzyme to H_2O_2, supporting the involvement of high-valent heme redox intermediates in such post-translational modification. The tandem mass spectrometry (MS/MS) studies with the tryptic peptides revealed that the heme moiety was indeed linked to Glu80 in the L80E mutant. However, covalent linkage was not observed in the L80E mutant in the absence of H_2O_2. Similar studies with WT and the L80D mutant did not show any covalent link between the heme and the protein.

EXPERIMENTAL PROCEDURES

Materials. The salt ammonium sulfate [$(NH_4)_2SO_4$], dipotassium hydrogen orthophosphate and potassium dihydrogen orthophosphate, Tris-HCl [2-amino-2-(hydroxymethyl)propane-1,3-diol hydrochloric acid], and Tris base were purchased from USB Chemicals, and components of bacteriological culture media were purchased from Himedia. Ampicillin sodium salt, chloramphenicol, DNaseI (from bovine pancrease), phenylmethanesulfonyl fluoride (PMSF), δ-aminolevulinic acid (δ-ALA), isopropyl β-D-1-thiogalactopyranoside (IPTG), sodium cholate, and oligonucleotide primers were purchased from Sigma Aldrich. The lysozyme (from hen egg white) was purchased from Fluka. The column material Phenyl Sepharose was purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Hydroxyapatite material was purchased from Bio-Rad Laboratories Inc. (Hercules, CA). The Quik-Change site-directed mutagenesis kit was purchased from Stratagene. The restriction enzymes were purchased from New England Biolabs. Dithiothreitol (DTT) and trypsin gold were purchased from Promega. HPLC grade water, acetonitrile, methanol, trifluoroacetic acid (TFA), ammonium bicarbonate, and urea were obtained from Merck Chemicals. Ethylenediaminetetraacetic acid dipotassium salt (EDTA) and sodium dithionite was purchased from s. d. Fine Chemicals. Hydrogen peroxide was purchased from Qualigens. All other buffer components were from Qualigen/s. d. Fine Chemicals or of analytical grade. Millipore water was used for buffer preparation and for all other experimental work.

Mutation of Recombinant WT CYP175A1. The *pKK-223* plasmid, encoding the WT CYP175A1 enzyme, was a kind gift from V. B. Urlacher (University of Stuttgart, Stuttgart, Germany). Site-directed mutagenesis was conducted using the Quik-Change site-directed mutagenesis kit (Stratagene). The forward and reverse oligonucleotide primers for the L80D mutant were GGGAGGGG***t***CTC***ga***CACCGACTGGGG and CCCCAGTCGGTG***tc***GAG***a***CCCCTCCC, respectively, and for L80E mutant were GGGAGGGGCCTC***gaa***ACCGACTGGGG and CCCCAGTCGGT***ttc***GAGGCCCCTCCC, respectively. The respective primers introduced a restriction site for *Bsa*I at position 239 in the structural gene along with the desired mutation in L80D but not in L80E. The *Bsa*I site was introduced for screening of the mutations.

Expression and Purification of WT CYP175A1 and Its Mutant. WT CYP175A1 and its mutants (L80D and L80E) were expressed by taking a single colony of the BL21-DE3 codon plus RP cells harboring the plasmid encoding the gene for the WT or mutant and inoculating them into the 2× YT medium containing ampicillin (100 μg/mL) and chloramphenicol (50 μg/mL). For the mutant proteins, the heme precursor δ-aminolevulinic acid (50 μM) was added to the culture 30 min before the induction of protein expression with IPTG. The protein was precipitated with $(NH_4)_2SO_4$ (35−50% saturation) and purified with a hydro-

phobic column [HIC, Phenyl Sepharose (high sub) fast flow], and fractions having an R_z (Reinheits Zahl, A_{417}/A_{280}) value of ≥1.0 were collected. The protein was further purified on a hydroxyapatite column, and fractions having an R_z of ≥1.5 were collected. The purified fractions were concentrated using centriprep concentrators (10 kDa cutoff membrane, Millipore), dialyzed in 50 mM KP_i buffer (pH 7.5), and stored at −25 °C in 50% glycerol.

UV−Visible Absorption Study. The UV−visible absorption spectra of WT CYP175A1, its mutants in 50 mM KP_i (pH 7.5), and various other species were recorded from 220 to 700 nm (as needed) in a quartz cuvette with a path length of 1 cm on a double-beam Perkin-Elmer (Lamda750-UV/vis/NIR) spectrophotometer. The wavelength accuracy for the spectrophotometer was ±0.15 nm, and it was equipped with a Peltier cell temperature controller with an accuracy of ±0.3−0.5 °C.

Carbon Monoxide (CO) Difference Spectra. The carbon monoxide difference spectra were recorded on the same UV−vis spectrophotometer. To record carbon monoxide difference spectra, we first reduced WT CYP175A1 and its mutant proteins with sodium dithionite and then recorded the spectra. Afterward, CO complexes of these reduced proteins were prepared by passing CO gas through the protein solutions, and spectra were again recorded. Final difference spectra were obtained by subtracting the spectrum of reduced P450 from the spectrum corresponding to the CO-bound species.

Circular Dichroism (CD) Study. The CD absorption spectra of WT CYP175A1 and its mutants [in 50 mM KP_i (pH 7.5)] were recorded on a JASCO J-810 spectropolarimeter that was equipped with a Peltier cell temperature controller. The spectral range of 190−250 nm in far-UV region was used for probing the secondary structure of the protein, and the spectral range of 250−450 nm in the near-UV and visible region was used for probing the tertiary structure of the proteins. Quartz cuvettes with path lengths of 1 and 10 mm were used for the determination of secondary and tertiary structures of proteins, respectively. Protein concentrations of 2 μM for secondary and 20 μM for tertiary structure were used. The temperature-dependent CD experiments were performed by monitoring the heme CD signal at a fixed wavelength, in the temperature range of 25−90 °C.

Resonance Raman (RR) Study. Resonance Raman spectra were recorded using a Horiba Yvon Raman spectrometer (T64000), with an inverted optical microscope adapted to a triple grating equipped with (1024 × 256) liquid N_2-cooled CCD. A Spectra-Physics model with an argon ion laser provided the excitation at 488 nm. The laser power at the sample was 5−6 mW and was focused using a 10× objective lens. Wavenumber calibration was conducted using the 521 cm^{-1} line of the silicon wafer. Spectra were recorded by scanning the 1300−1700 cm^{-1} region with a total acquisition time of 400 s, and data were averaged over five scans. The concentrations of the proteins (wild type and mutants of CYP175A1) were 600−800 μM in 50 mM KP_i buffer (pH 7.5). All measurements were taken at room temperature.

Stopped-Flow Experiment. The rapid mixing stopped-flow experiments (single wavelength and multiple wavelengths) for WT CYP175A1 and its mutants (L80D and L80E) were conducted using a Hi-Tech SF61MX stopped-flow spectrometer equipped with a xenon lamp. The dead time of the instrument was 3−4 ms. For diode array experiments, an integration time of 1 ms was used. The sample handling unit was mounted inside a thermostated bath compartment, and the temperature of the compartment was maintained (within ±0.3 °C) using a circulating water bath. The final concentration of various proteins was 10 μM in 50 mM KP_i buffer (pH 7.5). All measurements were taken at room temperature.

Electrospray Ionization Liquid Chromatography and Mass Spectrometry (ESI-LCMS) Studies. ESI-LCMS was performed on a Thermo Finnigan LCQ Deca Electrospray quadrupole ion trap mass spectrometer (ThermoElectron Co., Hemel Hempstead, Herts, U.K.) with a Thermo Surveyor high-performance liquid chromatography (HPLC) pump and microelectrospray source operated with Thermo Xcalibur, version 1.4. Peptides were resolved with an 11 cm, 100 μm inside diameter, Monitor C18 (Column Engineering) microcapillary column with a 5−15 μm tip opening. The flow rate from the HPLC pump was adjusted to 1 mL/min. Solvents were as follows: A, 0.1% (w/v) TFA in water; B, 0.1% (w/v) TFA in acetonitrile. The gradient of mixing of the two solvent was appropriately programmed for the best resolution of the peptide peaks. The total run time for the method was 70 min. All MS analyses were performed in positive ion mode. The MS/MS results for each peptide were subjected to a database search using SEQUEST (ThermoElectron Co.). In the peptide identification process, searches were performed for the routine tryptic peptides; usually only those peptides that follow the trypsin cleavage rules, i.e., cleavages after a basic amino acid residue (arginine or lysine) except when followed by a proline, are considered. The search parameters also included a differential modification of 616.5 Da on aspartic/glutamic acid for data analysis using the software to identify the covalent attachment. The MS/MS spectrum was recorded at 35% normalized collision energy to ensure efficient fragmentation. The principal fragmentation pathway was typically the loss of water followed by the loss of ammonia or, in some cases, vice versa. The spectra contain most of the expected series of b-, y-, a-, and x-type fragment ions.

Enzymatic Digestion of WT CYP175A1 and Its Mutants Using Trypsin. WT CYP175A1 and its mutant protein solutions were desalted using a PD-10 column (Amersham Biosciences), and solutions were concentrated using a centriprep tube (10 kDa cutoff, Millipore). This concentrated solution (~180−200 μL of a 100 μM solution) was used for the enzymatic digestion with 50 equiv of H_2O_2 (overnight incubation) or in the absence of H_2O_2. The H_2O_2 stock solution was freshly prepared every time, and its concentration was quantified (*24*) spectrophotometrically (ε_{240} = 39.4 M^{-1} cm^{-1}). The enzyme solutions were heated at 95 °C for 3 h in the presence of dithiothreitol [DTT, 200 mM, freshly prepared in 100 mM ammonium bicarbonate buffer (pH 8.5)], urea (100 μL, 4 M), and 50% methanol to denature the protein to allow full access by the proteolytic enzyme for improved sequence coverage. Vials containing the enzyme samples were incubated with trypsin (1 mg of trypsin for every 25 mg of protein to be digested) at 37 °C for 16 h to ensure complete digestion. To inactivate the trypsin, 0.5 μL of acetic acid was added to bring the pH at 3. The sample was cooled, and the supernatant was transferred to a fresh vial and dried. To a 50 μL portion of the digested protein solution was added 100 μL of 50% acetonitrile in water to dissolve the peptides for mass spectrometric analysis. Tryptic digests were stored at −20 °C. The shelf life of such frozen solution is 3−4 months.

RESULTS AND DISCUSSION

Molecular Modeling and Design of the Active Site. There are different types of covalent linkages between the heme and the protein (*2*, *5*, *6*, *12*, *13*, *16*, *18*−*20*, *25*−*27*). Ester linkage between

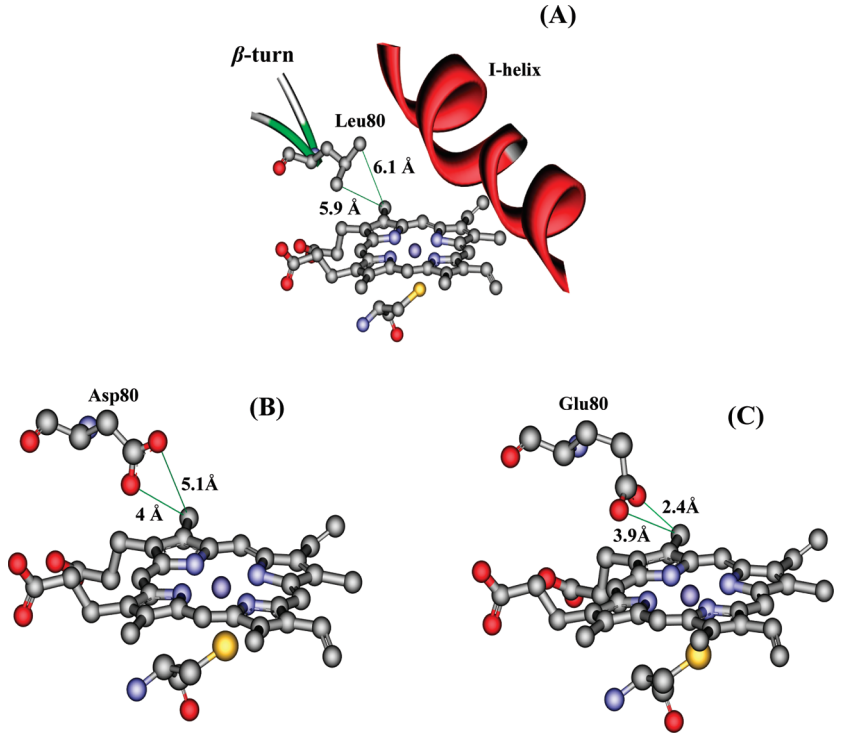

FIGURE 1: Crystal structures of the active sites of (A) WT CYP175A1 (PDB entry 1N97) and the (B) L80D and (C) L80E mutants of CYP175A1 based on a molecular modeling study. This figure shows the various distances from CD1, CD2, OE1, OE2, OD1, and OD2 of the respective amino acids to the 5-methyl group of heme.

the 5-CH_3 group and an acidic amino acid (glutamate) has been proposed in the CYP4 family of enzymes (*5*, *6*). Similar linkage has also been found in the case of lactoperoxidase (*13*) and myeloperoxidase (*27*). Analysis of the crystal structure of myeloperoxidase [PDB entry 1MHL (*27*)] shows that Asp94 is linked to the 5-CH_3 group of the heme through an ester linkage. The distance between the C_α of Asp94 and the 5-CH_3 group of heme in the enzyme is 4.9 Å. Ester linkage between the 1-CH_3 group (of heme) and Glu242 was also found in myeloperoxidase where the distance between the methyl group and the C_α of Glu242 is 5.7 Å. Similarly, lactoperoxidase [PDB entry 3KRQ (*13*)] consists of the 5-CH_3 group linked to Asp108 with the C_α of the residue 4.8 Å from the methyl group. Lactoperoxidase also has a linkage of the 1-CH_3 group of heme with Glu258 with the C_α of Glu258 5.7 Å from the methyl group of the heme. These results indicate that to form an ester linkage between a methyl group of the heme and an aspartic acid, the distance from the backbone residue should be 4.8−4.9 Å. Analogously, a similar linkage with a glutamic acid residue would require the backbone residue to be ~5.7 Å from the methyl group.

The crystal structure of WT CYP175A1 (PDB entry 1N97) shows that the active site of the enzyme consists of the heme anchored to the protein matrix through coordination with Cys336 (*23*). The distal pocket of the enzyme, analogous to those of all P450 systems, consists of a cavity that could accommodate the substrate of the enzyme. Earlier studies of members of the CYP4 family of enzymes (*5*, *6*) as well as of the G248E mutant of CYP101A1 (*2*) suggested formation of an ester linkage between the 5-CH_3 group of the heme and a glutamic acid residue in helix I of the enzyme. The crystal structure analysis of the active sites and helix I of WT CYP175A1 showed that there is no suitable amino acid residue (aspartate/glutamate) at distances appropriate for formation of a covalent linkage with a methyl group of the heme.

To explore whether there were any residues in the enzyme at a distance that may allow formation of the covalent linkage with the heme upon mutation of that residue to an appropriate acidic amino acid, we determined the backbone distances of residues that are <6 Å from the 5-CH_3 group of the heme. The results indicate that Leu80, Leu79, and Leu218 are the only three residues in the distal region of the heme that have their C_α atoms <6 Å from the 5-CH_3 group of the heme. The sequence alignment showed that Ala221 is at a position equivalent to that of the glutamic acid in helix I of members of the CYP4 family of enzymes (Table S1 of the Supporting Information), and C_α of Ala221 was found to be 6.1 Å from the 5-CH_3 group of the heme in CYP175A1, a distance slightly longer than that required (~5.7 Å) for formation of a covalent linkage. These analyses thus indicate that apart from position 221, there could be other sites in the protein structure, which if modified with glutamic acid or aspartic acid, may form an ester linkage with the 5-CH_3 group of the heme in CYP175A1. Leu80 resides in a β-turn (type I, Gly78−Thr81, Figure S1 of the Supporting Information) in the distal region of the heme and was chosen as a candidate to test this hypothesis. The C_α of Leu80 is located 5.8 Å from the 5-CH_3 group of the heme, which matches the distance of the Glu residue involved in linkage formation in the case of lactoperoxidase (LPO) and myeloperoxidase (MPO). NCBI BLAST analysis (http://blast.ncbi.nlm.nih.gov/) of the sequence of CYP175A1 showed that the Leu80 is highly conserved in the P450 superfamily. Thus, the absence of the natural occurrence of any acidic residue (Glu/Asp) at this position indicates that such residues could have different functional and structural implications that are absent in the P450 superfamily of enzymes. The possible structures of the enzyme upon mutation of the Leu80 residue were constructed on the basis of the wild-type structure (PDB entry 1N97), and both L80D and L80E mutants were considered. The shortest distance from the acidic oxygen of aspartate in the

Table 1: Distances between Atoms of an Amino Acid and 5-Methyl of Heme in WT CYP175A1 and Its Mutants (model structures)

protein	atom	distance (Å)
L80 (CYP175A1)	C_α	5.8
D94 and E242 (MPO)	C_α (Asp and Glu)	4.9 and 5.7
D108 and E258 (LPO)	C_α (Asp and Glu)	4.8 and 5.7
L80 (CYP175A1)	CD1	6.1
	CD2	5.9
D80 (L80D CYP175A1)	OD1	4.0
	OD2	5.1
E80 (L80E CYP175A1)	OE1	3.9
	OE2	2.4

L80D mutant to the 5-CH_3 group of the heme was found to be ~4.0 Å, while that of the acidic oxygen of glutamate in the L80E mutant was much shorter (~2.4 Å) (Figure 1 and Table 1). The exact conformation of the residue at position 80, however, cannot be predicted from this modeling study, and it represents the most probable rotamer with the shortest distance between aspartate/glutamate and the 5-CH_3 group of the heme. The molecular modeling study thus indicates that the mutation of the Leu80 to a glutamate may provide a structure of the enzyme similar to that in case of myeloperoxidase, lactoperoxidase, or members of the CYP4 family and may favor formation of an ester linkage between the acidic residue and the 5-CH_3 group of the heme. In the case of the L80D mutant of CYP175A1, the side chain oxygen atoms are slightly farther from the heme than those in the L80E mutant, indicating that formation of the covalent linkage may be more favorable in the case of the latter mutant. It is important to note that mutations to glutamic acid near the heme residue do not always promote formation of a covalent linkage between the heme and the protein matrix, and recent studies (*22*) showed that I401E, F261E, and L86E mutants of CYP102A1 did not form heme ester bonds with the glutamate residue in the mutant enzyme. However, such mutations were shown to affect the reactivity of the enzyme (*22*). Thus, one needs to consider both the structural constraints and the mechanistic requirements (see later) to understand the formation of covalent modification of the enzyme.

Site-Specific Mutation of WT CYP175A1. The mutations of Leu80 giving the L80D and L80E mutants were confirmed by restriction enzyme digestion and by sequencing, respectively. Our initial attempts to obtain the folded and purified proteins by using the same procedure that was employed for WT were unsuccessful, indicating that introduction of a charged residue into the hydrophobic cavity of the protein affects the folding pathway of the protein. Addition of δ-ALA to the culture medium 0.5 h before the addition of IPTG was found to help in folding of the protein, though the overall yield of the folded holoprotein was much smaller than that of the wild-type enzyme (*28*−*30*). The exogenous addition of δ-ALA has previously been shown to promote the folding of protein and increase the yield of heme-containing enzymes such as cystathionine β-synthase (*31*). Addition of δ-ALA has also been shown to enhance the level of expression of other P450 enzymes (*32*−*34*) analogous to that observed in this case.

UV−*Visible Absorption Spectroscopy*. The UV−visible absorption spectra of WT CYP175A1 and its mutants L80D and L80E are shown in Figure 2. The substrate-free ferric form of WT CYP175A1 exhibited a Soret band at 417 nm along with two visible bands at 534 nm (β-band) and 568 nm (α-band) (*35*). These spectral features are characteristic of typical low-spin

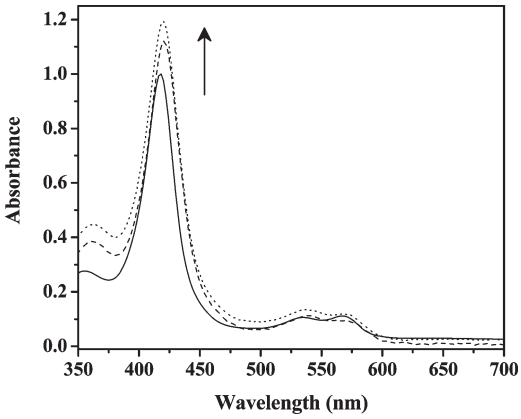

FIGURE 2: UV−visible absorption spectra of WT CYP175A1 (—) and the L80D (---) and L80E (· · ·) mutants of CYP175A1 in 50 mM potassium phosphate buffer (pH 7.5).

substrate-free ferric P450 enzymes (*36*, *37*). In contrast, the L80D and L80E mutants exhibit a red shift in the Soret absorption maxima at ~420 nm. The visible α-band exhibited a red shift of 2−3 nm for L80D but not for L80E. The visible β-band also exhibited a red shift of 4−5 nm for both L80D and L80E. Unlike the WT enzyme, the intensity of the β-band was found to be greater than that of the α-band for both L80D and L80E. The red shift in the Soret absorption maxima in the mutants of CYP175A1 may be due to the increase in the polarity of the heme environment (*5*). Earlier studies of the A264E mutant of P450BM3 and the G248E mutant of $P450_{cam}$ demonstrated that axial coordination of the glutamate causes a red shift of the Soret absorption peak in the P450 enzyme (*2*, *38*, *39*). Analogously, weak interaction of aspartate in L80D and glutamate in L80E with the ferric heme might also be responsible for the red shift of the Soret absorption maxima. An increase in the pH of the solution was shown to cause a further red shift (~422 nm), while lowering of the pH causes a blue shift (~416 nm) (Figure S2 of the Supporting Information) in the Soret band maxima of L80D and L80E mutants. These results support the possibility that the L80D and L80E mutants may weakly interact with the ferric heme that becomes weaker with a decrease in pH and vice versa. A red shift in the absorption bands and a change in the α/β-band intensity indicate that the electronic environment of the heme has been slightly changed via introduction of the mutations into CYP175A1. The reduced CO complex of the mutants of the enzyme shows the band at 450 nm that is characteristic of the P450 enzymes (Figure 3) along with a weaker band at 420 nm indicative of formation of a small amount of inactive P420 species (*35*, *40*). This result possibly indicates that the heme cavity of the mutants is less stable than that of the wild-type enzyme because of the introduction of the acidic residues into the hydrophobic pocket, and small perturbations may affect the folding and change in the coordination state of the metal ion leading to the formation of a P420-type species.

Near-UV and Heme Tertiary Circular Dichroism Spectroscopy. The heme CD provides information about the integrity of the heme active site of the protein. The CD in this region originates in the coupling of heme π−π* electric dipole transition moments with those of nearby aromatic residues and with the π−π* transition of the protein backbone (*41*). The near-UV and heme tertiary CD spectra of the WT and its mutants are shown in Figure 4. The CD spectra of WT CYP175A1 and its mutants are well-structured, with the Soret CD band at ~400 nm and the

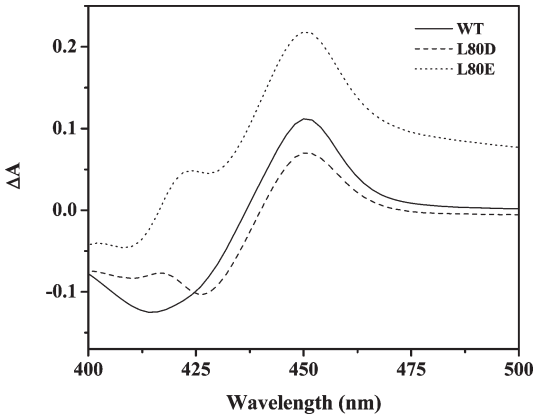

FIGURE 3: CO difference spectra of WT CYP175A1 (—) and the L80D (---) and L80E (···) mutants of CYP175A1 in 50 mM potassium phosphate buffer (pH 7.5).

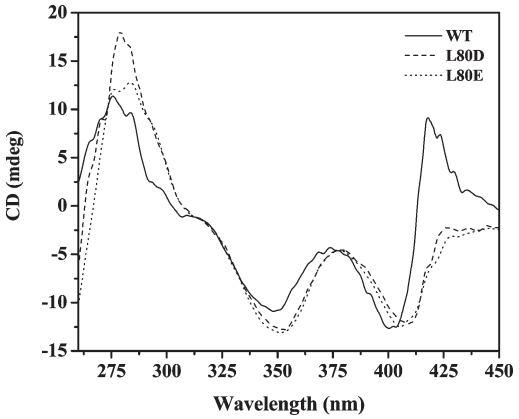

FIGURE 4: Near-UV and heme CD spectra of WT CYP175A1 (—) and the L80D (---) and L80E (···) mutants of CYP175A1 in 50 mM potassium phosphate buffer (pH 7.5).

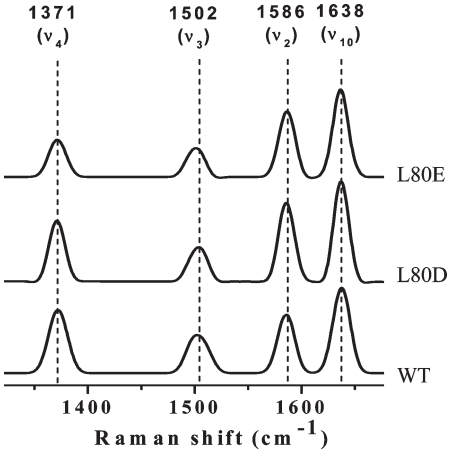

FIGURE 5: Resonance Raman spectra of the ferric wild type (WT), L80D, and L80E (600−800 μM) in 50 mM potassium phosphate buffer (pH 7.4). All measurements were taken using excitation at 488 nm and incident powers of 5−6 mW at room temperature. All spectra were recorded with data accumulated over 400 s averaged over five scans. The positions of the marker bands (ν_2−ν_4 and ν_{10}) are shown as vertical lines.

ΔCD band at ∼350 nm as observed in substrate-free P450 enzymes (*35*). The heme tertiary CD spectra of the L80D and L80E mutants exhibit a red shift in both the CD bands in the UV−visible region of ∼5−8 nm compared to the respective bands of WT CYP175A1. A small increase in the intensity of ΔCD bands in the mutants was also observed compared to that of the WT enzyme, though the intensities of the Soret CD bands were almost unaffected. The tertiary CD spectra described above suggest that the heme environment is slightly perturbed in the L80D and L80E mutants compared to that in WT CYP175A1. However, the secondary structure of the CYP175A1 enzyme was found to remain almost identical to that of WT CYP175A1 as indicated by the far-UV CD spectra of the enzymes (Figure S3 of the Supporting Information) (*42*). To check the thermal and conformational stabilities, temperature-dependent heme tertiary CD studies were conducted for WT CYP175A1 as well as its L80D and L80E mutants. Temperature variation of the heme tertiary CD bands of L80D and L80E mutants and the WT enzyme monitored at 409, 405, and 400 nm, respectively, gave the melting temperatures (T_m) of the enzymes. A melting temperature of 84 °C was observed for WT CYP175A1 and agrees with earlier reports (*35*). The melting temperatures for the tertiary structure of L80D and L80E mutants were drastically decreased to 70 and 71 °C, respectively, a fact that supports the idea that the heme cavity is indeed less stable in the mutants compared than in the WT enzyme (Figure S4 of the Supporting Information). This may be due to the introduction of a charged residue (D or E) inside the protein cavity that decreases the stability of the predominantly hydrophobic environment in the distal pocket of the heme active site. The thermostability of CYP175A1 (*42*) has been proposed to arise from the extended hydrogen bonding network in the enzyme. Introduction of a charged residue into the active site may also affect the water structure and thus alter the hydrogen bonding network, causing a decrease in the thermostability of the mutant as observed here.

Resonance Raman Spectroscopy. The heme CD studies indicate small changes in the tertiary structure of the mutant enzymes. To determine whether these changes could arise because of any alteration in the coordination geometry and/or oxidation state of the iron or the environment of the heme center, we conducted resonance Raman studies with the wild type and mutants of CYP175A1 (*43*). Figure 5 shows the RR spectra of WT CYP175A1 and its mutants obtained by excitation of a heme electronic transition (at 488 nm). The heme proteins exclusively exhibit bands originating from the vibrations of the heme group when they are excited at heme transitions, which is caused by the resonance enhancement mechanism. An extensive amount of experimental data for heme model compounds (*44*) has been generated, assigning the RR spectra of CYP175A1 to the corresponding vibrational modes. Some of these bands are regarded as the marker bands for the metal oxidation, spin, and coordination states. In particular, the RR bands between 1340 and 1375 cm^{-1} are marker bands for the oxidation state of the metal ion, while the marker bands for the coordination and spin states are above 1450 cm^{-1}. The oxidation state marker band ν_4 of the wild-type enzyme at 1371 cm^{-1} indicates a ferric state, and the heme core size marker bands ν_3, ν_2, and ν_{10} at 1502, 1586, and 1638 cm^{-1}, respectively, are characteristic of a hexacoordinate low-spin heme (6cLS). The frequencies of the Raman lines of WT CYP175A1 are very similar to the values reported for the other P450 enzymes (*43*−*45*). Comparing the resonance Raman spectra of the wild type (ν_4 at 1371 cm^{-1}, ν_3 at 1502 cm^{-1}, ν_2 at 1586 cm^{-1}, and ν_{10} at 1638 cm^{-1}) and its mutants (ν_4 at 1370−1371 cm^{-1}, ν_3 at 1500−1502 cm^{-1}, ν_2 at 1584−1585 cm^{-1}, and ν_{10} at 1637−1638 cm^{-1}) showed that the structure of the heme active site was almost unchanged after the mutation

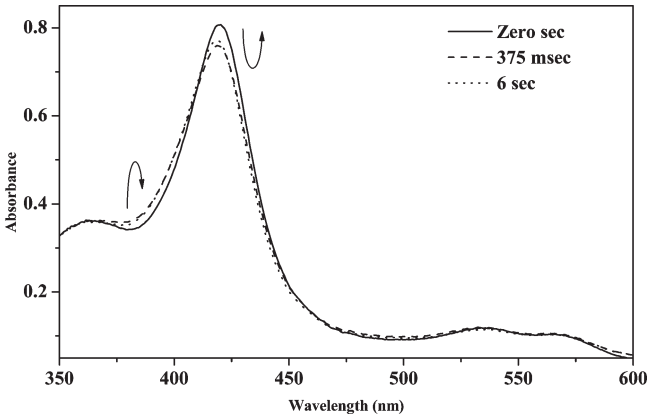

FIGURE 6: Stopped-flow rapid scan spectra using a diode array detector for WT CYP175A1 at $t = 0$, 0.375, and 6 s after it had been mixed with H_2O_2.

of Leu80. Hence, the coordination geometry, spin state, and oxidation state of iron in the heme of the L80D and L80E mutants of CYP175A1 are similar to those of the wild-type enzyme. The resonance Raman results thus support the idea that the ferric ion in the heme of the L80D and L80E mutants is in a six-coordinate low-spin state analogous to that of the wild-type enzyme as discussed above, though the exact nature of the sixth axial ligand could not be confirmed by these studies (*38*).

Reaction of WT CYP175A1 and Its Mutants with Hydrogen Peroxide. Among all the families of P450 enzymes, the CYP4 family is the only one that has been found to undergo self-catalyzed covalent attachment of the prosthetic group to the protein. An autocatalytic mechanism involving formation of a high-valent heme redox intermediate has been proposed to forge the formation of a covalent link between the heme and glutamate of the protein (*2*, *5*). Compound I, a ferryl-(π) porphyrin cation radical [($P^{+\bullet}$)Fe(IV)=O], formed during the catalytic cycle of the P450 enzyme, was proposed to oxidize the glutamate to a carboxylic radical forming a Compound II [(P)Fe(IV)=O]-type intermediate species. Subsequently, the glutamate carboxyl radical was thought to abstract a hydrogen atom from the 5-methyl of the heme that is structurally close to the carboxylic radical, yielding a methylene radical, which then is converted to a carbocation intermediate through an internal electron transfer. In many P450 enzymes, it is possible to form the high-valent redox intermediates by the peroxide shunt mechanism with H_2O_2 or analogues (*m*-CPBA, cumene hydroperoxide, and peroxy acid) (*46*, *47*). Evidence of the formation of short-lived Compound I in P450 enzymes upon reaction with peroxides was reported in the case of CYP101A1, CYP102A1, CYP119A1, etc. (*43*, *48*−*51*). Fast stopped-flow mixing kinetic studies were conducted with WT CYP175A1 and its L80E mutant to determine the formation of the redox intermediate that was proposed to be essential for the covalent linkage. The reaction of WT CYP175A1 with H_2O_2 confirmed the rapid formation of a species analogous to Compound I as reported previously (*43*, *50*, *51*) (Figure 6). The rapid wavelength scan data in Figure 7 show that there is an initial increase in absorbance at ~370 nm with a simultaneous decrease in absorbance at the Soret band, indicating formation of the high-valent redox intermediate upon reaction of H_2O_2 with the substrate-free WT, L80D, and L80E of CYP175A1. The absorbance at the Soret band slowly recovers (30% recovery in 20 s for the mutant proteins), indicating conversion of the intermediate back to the resting state of the

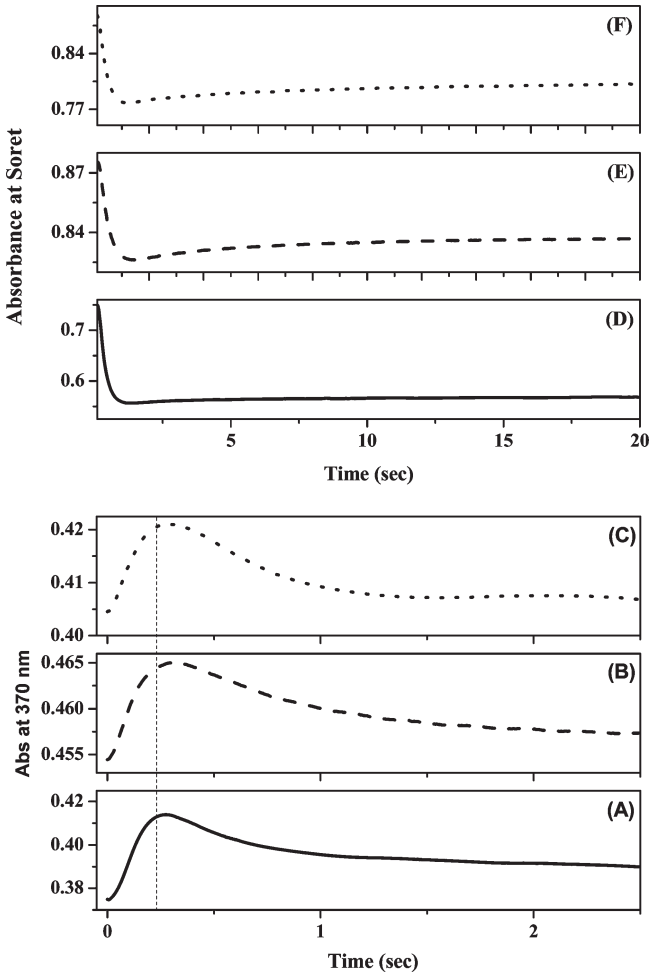

FIGURE 7: Time-dependent formation and decay of the intermediate detected at 370 nm for (A) WT, (B) L80D, and (C) L80E. Time-dependent decay and regeneration observed at the Soret band for (D) WT, (E) L80D, and (F) L80E. The vertical line in panels A−C designates the maximum absorbance at 370 nm for the WT (A).

enzyme in the absence of any reducing substrate (*49*, *51*, *52*). These results show that the high-valent redox intermediate of CYP175A1 was indeed formed upon treatment of hydrogen peroxide with WT CYP175A1, as well as its L80D and L80E mutants.

ESI-LCMS Analysis. The kinetic studies, however, cannot determine whether the reactions of this intermediate lead to formation of a covalent linkage of the heme with an amino acid residue, as proposed previously (*2*, *5*). To assess any such covalent linkage formation, we conducted tandem mass spectrometric identification of the peptides produced upon tryptic digestion of the proteins. Peptide sequencing of the tryptic digests of the enzyme sample before the treatment of H_2O_2 was conducted as the control. To identify any covalent attachment between the heme and the protein matrix, we have analyzed mass spectrometric data of tryptically digested protein solutions that were incubated with H_2O_2 (1:50 protein:H_2O_2 ratio). WT CYP175A1 and its mutants L80D and L80E were enzymatically digested with trypsin (Experimental Procedures) and subsequently separated by high-pressure liquid chromatography, and peptide sequences were analyzed by MS/MS using the reported procedures (*53*−*55*). For all the proteins, good sequence coverage of the overall sequence was observed, with most of the expected peptide fragments detected. The quadruple ion trap MS/MS

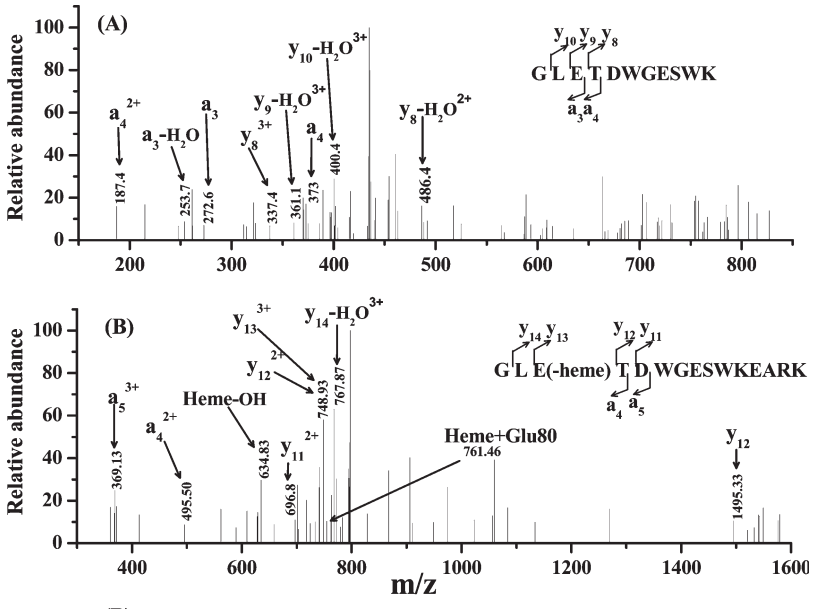

FIGURE 8: MS/MS spectra for the tryptic peptides of interest with a +3 charge state: (A) peptide sequence GLETDWGESWK (78−88) observed in the L80E mutant without H_2O_2 treatment and (B) heme-bound peptide GLE(−heme)TDWGESWKEARK (78−92) observed in the L80E mutant after treatment with H_2O_2 (only a- and y-type ions indicated for the sake of simplicity). Of particular interest are the peaks indicating positions for the covalent attachment of the heme.

strategy with differential modification of 616.5 Da (unmodified heme) on Asp80/Glu80 in the mutant enzymes was used to detect formation of any heme-bound peptide that could indicate the covalent linkage with the heme. Analyses of the mass spectrometric data of the control samples of WT, L80D, and L80E CYP175A1 without the treatment of H_2O_2 did not show any peptide covalently attached to heme (Tables S2, S4, and S6, respectively, of the Supporting Information). Moreover, the WT enzyme also did not show any covalently linked heme peptide upon tryptic digestion of the sample incubated with H_2O_2 (Table S3 of the Supporting Information), supporting the possibility that the heme in the WT enzyme does not form any covalent linkage with any amino acids in the heme cavity. It was interesting to note that the tryptic digest of the L80D mutant of CYP175A1 with or without incubation with H_2O_2 also did not show any heme-bound peptide (Tables S4 and S5 of the Supporting Information). Thus, analogous to the WT enzyme, the L80D mutant also does not favor covalent linkage of the heme. Molecular modeling analysis proposed that the L80E mutant of the enzyme may be a candidate for formation of a covalent linkage between the heme and the glutamic acid residue. Tryptic digestion of the L80E mutant without incubation with H_2O_2 was used as the control sample, and ~50% sequence coverage of the overall sequence was observed with most of the expected peptide fragments detected (Table S6 of the Supporting Information). The tryptic peptide containing the mutated residue in the L80E mutant of CYP175A1 was identified as GLE*TDWGESWK (residues 78−88, E*-mutated residue) in the control sample by tandem mass analyses. The ion at m/z 437.13 corresponding to the +3 charge state of the peptide (1308 Da) was obtained with a retention time (t_R) of 53.55 min. The tandem MS/MS spectrum of this peptide is shown in Figure 8A. Analyses of the MS/MS data by SEQUEST (ThermoElectron Co.) using a nonredundant (NR) protein database assigned the b-, y-, a-, and x-type fragment ions (Figure 8A and Table S7 of the Supporting Information) for the peptide sequence of residues 78−88. Several fragment ions of the type $b_n(m+)$, $y_n(m+)$, $a_n(m+)$, and $x_n(m+)$ (where n = 1−10 and m = 1−3) were detected (Table S7 of the Supporting Information), which allowed us to identify the sequence of the GLETDWGESWK peptide. The control sample of the L80E mutant enzyme that was not incubated with H_2O_2 did not exhibit any covalent modification with heme. The L80E mutant of CYP175A1 that was incubated in H_2O_2 was digested with trypsin, and tandem mass spectrometry of the peptide mixture showed ~63% sequence coverage of the overall sequence as given in Table S6 of the Supporting Information. Analyses of the data (Table S6 and Figure S5 of the Supporting Information) showed that the peptide without covalently attached heme (amino acid positions 78−91) having a mass of 1665 Da (m/z 555.83) and the peptide with covalently attached heme (Figure 8B and Table S8 of the Supporting Information, amino acid positions 78−92) having a mass of 2409 Da (m/z 803.74) are present in the solution. The presence of both unbound peptide and heme-bound peptide sequences revealed the partial formation of a covalent linkage of heme in the L80E mutant when the enzyme was preincubated with H_2O_2. Unbound peptide GLETDWGESWKEAR (78−91) in L80E, with a +3 charge state (m/z 555.83) and a t_R of 55.98 min, was identified from the analysis of the MS/MS spectrum shown in Figure S5 of the Supporting Information, and observed fragment ions are listed in Table S9 of the Supporting Information. Peptide GLETDWGESWKEARK (78−92) bound to heme in L80E obtained at a t_R of 53.45 with +3 charge state (m/z 803.74) was analogously analyzed from the MS/MS spectrum shown in Figure 8B. The calculated mass for the peptide (residues 78−92) without heme is 1790 Da. The average mass of 2409 Da observed for the GLETDWGESWKEARK (78−92) fragment is greater than the mass calculated from the sequence (1790 Da) by the mass of heme (616.5 Da), which is expected for the peptide fragment containing covalently bound heme. The spectrum contains most of the expected collision-induced dissociation (CID) ions of the $b_n(m+)$, $y_n(m+)$, and $a_n(m+)$ series (where n = 1−10 and m = 1−3) (Table S8 of the Supporting Information). In addition, we have observed a singly charged heme fragment ion at m/z 634.8, which indicates

formation of hydroxylated heme in the case of the L80E mutant incubated with H_2O_2. This peak was not observed in the MS (MS1) spectrum of the digestion mixture; hence, this species arises because of the CID of the heme ester bond of the heme-bound Glu80-containing peptide. This further indicated that hydroxylated heme was possibly not formed from the ferryl heme intermediate of the mutant enzyme (*5*). The MS/MS spectrum of the covalently linked heme peptide given in Figure 8B also shows a peak at *m*/*z* 761.4, which corresponds to a heme bound to a glutamic acid fragment [i.e., GLE-(−heme)TDWGESWKEARK]. These observations strongly suggest that the heme moiety undergoes covalent attachment with the glutamic acid (Glu80).

The fraction of covalently attached heme-containing enzyme in the solution could not be directly determined by FPLC of the mixture, possibly because the overall structure and surface charge of the enzyme remained unchanged in the folded form even after the formation of a heme ester linkage with the glutamic acid residue in the mutant. However, mass spectrometric analyses of the Glu80-containing tryptic peptides of the L80E mutant of CYP175A1 helped us to estimate the ratio of heme-bound to heme-free peptide fragment. The liquid chromatography−mass spectrometry results showed that the H_2O_2-treated solution of the L80E mutant had ∼30% of the Glu80-containing tryptic peptides with heme covalently linked to the peptide (Figure S6 of the Supporting Information). This indicates that ∼30% heme-linked L80E enzyme was formed upon treatment with H_2O_2 in this case. The H_2O_2-treated L80E mutant was, however, found to have no significant difference in the UV−visible absorption or CD spectral features (data not shown), suggesting that the observed covalent attachment possibly had no major effect on the heme cavity of the mutant enzyme.

As mentioned above, a covalent link was not observed in the L80D mutant even after incubation of the enzyme with H_2O_2 for an extended period of time. There was no peak corresponding to the formation of hydroxylated heme (*m*/*z* 634.8) observed in the case of the L80D mutant incubated with H_2O_2. This further supported the possibility that hydroxylated heme was possibly not formed because of water interception of the ferryl heme intermediate in this case. The analyses of the possible structures of the two mutants (L80D and L80E) indeed show that the side chain of aspartate being smaller than that of glutamate probably precludes formation of a covalent linkage in the former and indicates that either the aspartate carboxyl group is unable to abstract the hydrogen atom from the heme methyl group or it cannot be oxidized to the radical for the water molecule to quench the methyl carbocation (*5*) of the heme moiety.

Our studies thus demonstrate that post-translational modification, such as covalent attachment of the heme to a glutamate residue, does not need to take place only at a position equivalent to the conserved glutamic acid site of helix I as observed in members of the CYP4 family. Such covalent linkage may also be introduced into a P450 by rational design of the active site, where the carboxylate residue is present at a site structurally favorable for such attachment. Our results showed that the L80E mutant of CYP175A1 forms a covalent linkage between Glu80 and the heme upon incubation with H_2O_2, although position 80 in the enzyme is not equivalent to the conserved glutamic acid residue (Glu318) in helix I of members of the CYP4 family but belongs to one of the β-turns. These results indicate that there is no intrinsic impediment for the formation of a covalent link if a suitable amino acid is structurally well placed within the appropriate

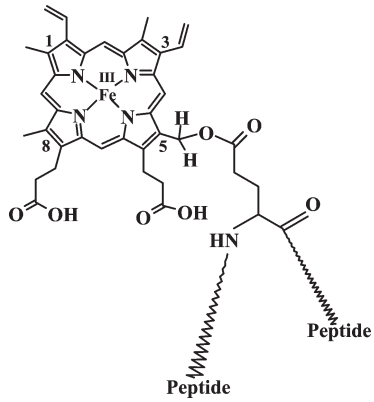

FIGURE 9: Schematic diagram showing the ester linkage between the 5-methyl group of heme and the side chain carboxylic group of Glu80 in the L80E mutant of CYP175A1.

distance as well as an orientation toward the desired site of the heme where covalent linkage is to take place. It is, however, important to note that these studies do not provide any clear evidence of the exact position of covalent attachment in the heme with Glu80 in the L80E mutant of CYP175A1. However, molecular modeling studies and structural considerations as well as earlier reports about the G248E mutant of CYP101A1 indeed indicate that the 5-CH_3 group of the heme is the possible site for formation of the ester linkage with glutamic acid in the L80E mutant of CYP175A1 (Figure 9).

CONCLUSIONS

Covalent attachment of the heme to a glutamic acid residue from helix I has previously been observed as a post-translational modification in several CYP4 families of enzymes. Recent studies have also shown that a glutamic acid residue introduced by mutation of Gly248 in CYP101A1 that is sequentially equivalent to Glu318 of CYP4 can also form such a covalent linkage. We investigated whether there could be any site in the heme cavity of the cytochrome P450 enzyme other than the one in helix I that can be structurally designed to form such linkages. Molecular modeling studies of CYP175A1 from *Thermus thermophilus* suggested that site-specific mutation of Leu80 to Glu may indeed provide appropriate structural disposition to the glutamate residue in the mutant enzyme that can be activated by the heme peroxidase-type reaction to form a covalent linkage between the glutamate and the heme. ESI-LCMS studies of the tryptic peptides were used to identify the modified peptides formed because of the covalent linkage between the heme and the protein matrix. The wild-type and L80D enzymes did not exhibit any evidence of covalent modification of the heme in the protein. The L80E mutant of CYP175A1 without incubation with H_2O_2 also did not exhibit any tryptic peptide associated with the heme. However, tryptic digestion of the L80E mutant of CYP175A1 that was preincubated with H_2O_2 showed formation of the peptide GLE(−heme)TDWGESWKEARK as well as its fragments (such as heme-OH and heme-Glu). These results support the possibility that covalent attachment of the heme to an amino acid in the protein matrix depends on the structural design of the active site and not only on the sequential equivalence of the residue with those commonly found in nature. Moreover, the formation of a covalent linkage required preincubation of the mutant enzyme with H_2O_2, supporting the earlier proposition that activation of the glutamate residue by the heme peroxidase

reaction is possibly essential for formation of the covalent linkage between the residue and the heme prosthetic group of the enzyme. These results thus add a new dimension to our understanding of the post-translational covalent linkage of the heme in these enzymes and highlight the importance of the carboxylic group of the amino acid at an appropriate spatial disposition for the formation of the bond with the heme. Such structural design of the active site may be important for stabilization of the heme inside the enzyme.

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SUPPORTING INFORMATION AVAILABLE

Sequence alignment by the ClustalW server with different CYP4 isoenzymes and CYP101 and CYP175A1 showing the helix I region (Table S1), peptide sequences observed by tryptic digestion of WT CYP175A1 without incubation in H_2O_2 (Table S2), peptide sequences observed by tryptic digestion of WT CYP-175A1 preincubated in H_2O_2 (Table S3), peptide sequences observed by tryptic digestion of L80D without incubation in H_2O_2 (Table S4), peptide sequences observed by tryptic digestion of L80D preincubated in H_2O_2 (Table S5), peptide sequences observed by tryptic digestion of L80E with and without incubation in H_2O_2 (Table S6), fragment ions observed for tryptic peptide 78−88 (GLETDWGESWK) for the +3 charge state (t_R = 53.55) for L80E without incubation in H_2O_2 (Table S7), fragment ions observed for tryptic peptide 78−92 (GLETDW-GESWKEARK) for the +3 charge state (t_R = 53.45) for L80E preincubated in H_2O_2 (Table S8), fragment ions observed for tryptic peptide 78−91 (GLETDWGESWKEAR) without heme for the +3 charge state (t_R = 55.98) for L80E preincubated in H_2O_2 (Table S9), location of Leu80 in one of the β-turns of CYP175A1 (Figure S1), pH titration of mutants of CYP175A1 (Figure S2), far-UV CD spectra of all the enzymes (Figure S3), thermal unfolding study of all the enzymes (Figure S4), MS/MS spectrum of peptide GLETDWGESWKEAR (78−91) for the +3 charge state (t_R = 55.98, without heme) of the L80E mutant of CYP175A1 preincubated in H_2O_2 (Figure S5), and MS spectra of the trypsin-digested L80E sample (preincubated in H_2O_2) (A) with heme (t_R = 53.42, m/z 803.74), (B) without heme (t_R = 55.95, m/z 555.83), and (C) with a percentage of bound and unbound heme (Figure S6). This material is available free of charge via the Internet at http://pubs.acs.org.

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