Molecular Engineering of Horseradish Peroxidase: Thioether Sulfoxidation and Styrene Epoxidation by Phe-41 Leucine and Threonine Mutants

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Received March 17, 1995[⊗]

Abstract: Although a high-resolution crystal structure is not available for horseradish peroxidase isozyme c (HRP), sequence alignments with peroxidases for which crystal structures are available indicate that Phe-41 is adjacent to the catalytic histidine in the active site of HRP. The replacement of Phe-41 by smaller amino acids has been investigated to determine if limited access to the ferryl species is responsible for the poor peroxygenase activity of HRP. The leucine (F41L) and threonine (F41T) mutants have been expressed in a baculovirus/insect cell system and have been purified and characterized. The spectroscopic properties of the two mutants are nearly identical to those of native HRP, and their kinetic constants for the oxidation of guaiacol, ABTS, and iodide differ by no more than a factor of 3 from those of the native enzyme. The F41L and F41T mutants, as expected from earlier work, have higher V_{max} values for the sulfoxidation of phenyl alkyl thioethers. Furthermore, F41L yields sulfoxides with higher enantioselectivity than native HRP. More significantly, they catalyze the epoxidation of styrene and *cis*-β-methylstyrene, reactions not catalyzed by native HRP. These reactions proceed with low to high enantioselectivity and, in the case of *cis*-β-methylstyrene, yield both the *cis*- and *trans*-epoxides. Studies with ^{18}O-labeled H_2O_2 and O_2 indicate that the oxygen incorporated into the *cis*-β-methylstyrene epoxide derives exclusively from the peroxide, whereas a substantial fraction of the oxygen in the styrene and *trans*-β-methylstyrene products derives from O_2. Surprisingly, *trans*-β-methylstyrene is oxidized by both the native and mutant enzymes, and a large fraction of the oxygen incorporated into the epoxide derives from O_2. The results indicate that the F41L and F41T mutations greatly enhance the peroxygenase activity of HRP and show that the epoxidation reactions catalyzed by the mutants occur in part by mechanisms other than ferryl oxygen transfer.

The catalytic turnover of HRP involves reaction of the enzyme with H_2O_2 to give a ferryl (Fe^{IV}=O) porphyrin radical cation two oxidation equivalents above the resting ferric state.[1,2] This intermediate, known as compound I, is normally reduced by sequential one-electron transfers from different substrate molecules. The first of these electrons quenches the porphyrin radical cation and gives compound II, an intermediate that is one oxidation equivalent above the ferric state.[2] The second electron reduces compound II to the ferric state. The substrates that provide the two electrons are normally released as free radical products. The reduction of compound II is the rate-limiting step of the process, so that the absorption spectrum of compound II is normally observed during steady-state catalytic turnover of the enzyme.[2a,b] In contrast, the oxidation of thioanisoles to sulfoxides occurs without the accumulation of compound II, although a compound II species can be detected by stopped flow methods.[3,4] This led to the suggestion that thioanisoles undergo a net two-electron oxidation, possibly via stepwise commitment of two electrons from the same substrate molecule. Evidence that thioanisole sulfoxidation is indeed mediated by a peroxygenase rather than peroxidase mechanism is provided by the demonstration that the sulfoxide oxygen derives from the peroxide.[5−7] Iodide is also oxidized without the detectable formation of compound II,[8] but there is no independent evidence that HRP oxidizes iodide or any other class of substrates via a peroxygenase rather than peroxidase mechanism. HRP has specifically been shown to be ineffective as a catalyst for the relatively facile epoxidation of styrene[9] or butadiene.[10]

Catalytic turnover of cytochrome P450 monooxygenases requires two-electron reduction of molecular oxygen to a species that is thought to resemble compound I of HRP, although it is not known if the second oxidation equivalent resides on the porphyrin or the protein.[11] The electrons required for the reductive activation of molecular oxygen are provided by NADPH via cytochrome P450 reductase. The oxygen activation process can be circumvented, however, because H_2O_2 supports what appears to be normal catalytic turnover of some (but not

[⊗] Abstract published in *Advance ACS Abstracts*, July 1, 1995.

(1) The abbreviations used are as follows: HRP, horseradish peroxidase isozyme c; heme, iron protoporphyrin IX regardless of the oxidation or ligation state; ABTS, 2,2'-aminobis(3-ethylbenzothiazoline-6-sulfonic acid), diammonium salt; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ee, enantiomeric excess.

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all) cytochrome P450 enzymes.[12,13] Despite the probable similarities in catalytic species, cytochrome P450 differs from HRP in that it routinely catalyzes the two-electron insertion of an oxygen atom into its substrates rather than the removal of a single electron. Two factors that presumably contribute to this fundamental catalytic divergence are the difference in the axial iron ligand, a cysteine thiolate in P450 and a histidine imidazole in HRP, and the difference between the essentially hydrophobic active site of P450 and the polar active site of HRP.[14,15] Furthermore, we have provided evidence that peroxygenase activity is suppressed by the active site architecture of HRP and other peroxidases by restricting access of the substrate to the ferryl oxygen.[16-20] This inference is based on evidence that (a) the iron of peroxidases is much less accessible to arylhydrazines than that of hemoproteins in which the iron is known to be accessible,[21] (b) several catalytically generated radicals react exclusively with the δ-meso carbon or the adjacent 8-methyl group of the heme in peroxidases,[16-20] (c) δ-meso alkyl substituents block peroxidase activity,[17,22] and (d) NMR NOE studies indicate that aromatic substrates bind near the 1- and 8-methyl groups flanking the δ-meso carbon.[23] Furthermore, the active sites in the crystal structures of cytochrome *c* peroxidase,[15] lignin peroxidase,[24] manganese peroxidase,[25] and *Arthromyces rhamosus* peroxidase[26] are sterically encumbered and are only accessible via a channel over the δ-meso edge.

A histidine and an arginine function as catalytic residues in plant and fungal peroxidases, and a phenylalanine or a tryptophan is always found adjacent to the catalytic histidine.[25-28] In HRP, the catalytic histidine is His-42, the arginine is Arg-38, and the aromatic residue is Phe-41. Although a high-resolution crystal structure of HRP is not available, a comparison of the four known peroxidase X-ray structures indicates that the histidine, arginine, and aromatic residues occupy essentially identical positions in the distal heme pockets of all four enzymes (Figure 1).[15,24-26] In each instance, the aromatic residue sits to the left of the catalytic histidine as one looks down the access channel into the active site. In light of the proposal that the inaccessibility of the ferryl species limits the peroxygenase activity of peroxidases, site specific replacement of Phe-41 is a potentially promising approach to expanding the peroxygenase activity of HRP.

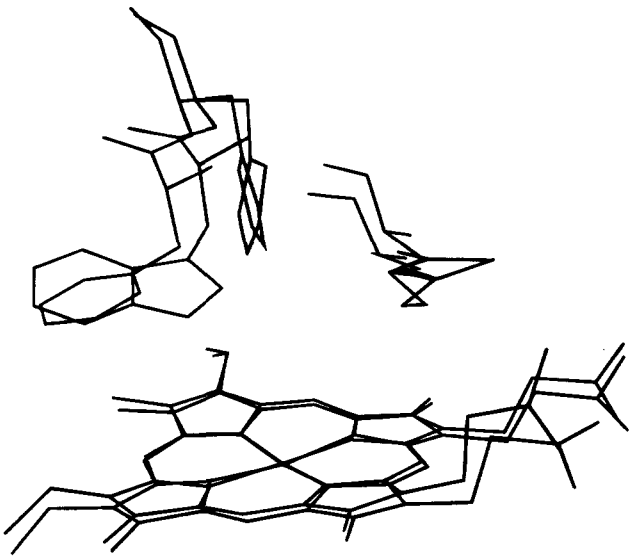

Figure 1. Superposition of the heme and three distal residues in the crystal structures of cytochrome *c* peroxidase (CcP) and lignin peroxidase (LiP). The viewer faces the δ-meso edge of the heme from the access channel that leads into the active sites of these proteins. The residues that are shown are the catalytic histidine (His-52 in CcP, His-47 in LiP), the catalytic arginine (Arg-48 in CcP, Arg-43 in LiP), and the aromatic residue (Trp-51 in CcP, Phe-46 in LiP). These are the residues equivalent to Phe-41, His-42, and Arg-38 in HRP.

As part of a project to elucidate the relationship between protein structure and the control of peroxidase vs peroxygenase function, we have expressed HRP in a baculovirus/insect cell system[29] and recently reported in a preliminary communication that the F41L and F41T mutants of HRP catalyze the sulfoxidation of thioanisoles at higher rates and, in the case of F41L, with higher stereospecificity than the native protein.[30] Sulfoxidation, however, is not a strong indicator of peroxygenase activity because the reaction is catalyzed by all known peroxidases. We provide here additional information on the sulfoxidation of thioethers by the F41L and F41T mutants and, more importantly, report that the same two mutations endow HRP with the ability to epoxidize styrene, a reaction that is not generally catalyzed by classical peroxidases.

Experimental Section

Materials. Native HRP was purchased from Boehringer-Mannheim. Styrene, (±)-styrene oxide, (*R*)-styrene oxide, *cis*- and *trans*-β-methylstyrene, (1*R*,2*R*)-(+)-1-phenylpropylene oxide, (1*S*,2*S*)-(−)-1-phenylpropylene oxide, ABTS, and H_2O_2 (30% in water) were from Aldrich. Guaiacol was obtained from Sigma. $[^{18}O]O_2$ (98% ^{18}O) was from Isotec (Miamisburg, OH), and $[^{18}O]H_2O_2$ (90% ^{18}O) from ICON (Summit, NJ). General molecular biology supplies were from BRL.

Gas−liquid chromatography was carried out on a Hewlett-Packard model 5890A instrument equipped with an HP3365 data system. Mass spectrometry was done on a VG Fisons 70S mass spectrometer equipped with an HP 5890A gas chromatography system. Electronic absorption spectra were recorded on an Aminco DW-2000 instrument. Processing of kinetic data to obtain K_m and V_{max} values was done by fitting the data to the Lineweaver−Burke equation with the KinetAsyst program.[31]

Construction of the HRP Mutants. Mutants were constructed by cassette mutagenesis. The *Sac I//Nhe I* fragment of pVLHRP2, a plasmid containing a synthetic HRP gene,[29] was replaced by a cassette coding for a change of residue 41 from a phenylalanine to a leucine (TTC → TTA) or threonine (TTC → ACC). Approximately 0.1 pmol

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of the annealed synthetic oligonucleotides were used for plasmid construction. *E. coli* strain DH5α was used for transformation and the codon change was confirmed by sequencing.

Production of the HRP Mutant Virus. Transfection of insect cells was done using the BaculoGold transfection kit (Pharmingen). Plaque purification was performed by following the instructions provided with the kit, and the first passage viral stock (pass I stock) was amplified from a single plaque. In order to confirm the production of HRP mutant virus, the viral DNA was isolated from pass I stock and was used as a PCR template using the primers provided by Invitrogen. The size of the PCR product confirmed that the HRP gene was inserted into the viral DNA. The second passage viral stock (pass II stock) was produced from pass I stock, and the titer was determined by plaque assay. Sf9 cells grown at 27 °C in TNM-FH medium containing 10% heat inactivated fetal bovine serum were used for all of these procedures.

Expression of the HRP Mutants. Sufficient pass II virus stock was added to approximately 250 mL of mid-log *Trichoplusia ni* (HighFive) cells grown at 27 °C in the Sf-900 II serum free medium (BRL) to give a multiplicity of infection of 100. The cells were then incubated at 27 °C for 2−4 h. The culture was diluted to 1000 mL with the medium before streptomycin and penicillin (100 μg/mL of streptomycin sulfate and 100 units/mL of penicillin G) and 10 mL of filter sterilized 0.5 mM hemin solution were added. The hemin was prepared by dissolving it in 0.01 M aqueous NaOH prepared with sterile water followed by adjustment of the pH nearly to neutrality by dropwise addition of 1 M HCl. The titration was stopped just before a precipitate was observed. The *T. ni* cells were then allowed to grow for 3−4 days at 27 °C in a shaker oscillating at 150 rpm.

Purification of HRP and HRP Mutants. The insect cell culture (2−3 L) was centrifuged at 5000 rpm on a GS-3 rotor (4200*g*) for 10 min. The supernatant was then concentrated to approximately 150 mL and diafiltrated with 25 mM Tris-HCl (pH 8.0) on a CH2 Hollow Fiber Concentrator (Amicon). The concentrated medium containing protein was loaded on a mono-Q anion exchange column and was washed with 25 mM Tris-HCl (pH 8.0). The fractions which exhibited guaiacol oxidizing activity were combined, concentrated to approximately 20 mL, and dialyzed against 50 mM (pH 7.0) sodium phosphate buffer containing 1.5 M ammonium sulfate. A 7 × 1 cm column packed with Chelating Sepharose Fast Flow (Pharmacia) and a similar column packed with thiophilic gel (T-gel, Sigma) were washed with 2 vol of distilled water.[32] At least 2 column vol of 0.1 M $CuSO_4$ solution were applied to the Chelating Sepharose, and the column was washed with 5 vol of distilled water. The two columns were then connected in tandem, with the Chelating Sepharose column first, and were equilibrated with 50 mM (pH 7.0) sodium phosphate buffer containing 1.5 M ammonium sulfate. The protein sample was then loaded onto the tandem column system. The two-column system was washed with 100 mL of the same buffer. The protein bound to the T-gel was then eluted with 50 mM (pH 7.0) sodium phosphate buffer. The fractions with R_z values (A_{404}/A_{280}) greater than 3 were combined and dialyzed against 50 mM sodium phosphate buffer (pH 7.0) that had been pretreated with Chelex (5 g/L stirred overnight). The final samples were stored at −80 °C for periods of up to 4 months without detectable loss of activity.

Absorption Spectra. Spectra were taken at 25 °C in 50 mM sodium phosphate buffer at pH 7. Compounds I and III were formed by adding respectively 1 and 100 equiv of H_2O_2. Compound II was formed both by adding 1 equiv of potassium ferrocyanide to compound I or by simply allowing compound I to decay with time. The same spectra were obtained by both methods. The cyanide complex was obtained by adding a 2000-fold excess of KCN. The reduced forms of HRP and its mutants were formed by adding a 1000-fold excess of sodium dithionite, after which the solutions were saturated with CO to form the CO complexes.

Guaiacol Peroxidase Assay. The reaction mixture contained 2 nM HRP, 0.6 mM H_2O_2, and variable amounts of guaiacol in 50 mM sodium phosphate buffer (pH 7.0). For routine assays the guaiacol concentration was 5 mM. The rate of formation of the guaiacol oxidation product was determined from the increase in the absorbance at 470 nm during the first 30 s, using a molar absorbance coefficient of $\epsilon = 2.6 \times 10^4$ M^{-1} cm^{-1}.

ABTS Peroxidase Assay. The reaction mixture contained 2 nM HRP, 2.5 mM H_2O_2, and variable amounts of ABTS in 50 mM sodium phosphate buffer (pH 5.0) containing 25 mM citric acid. For routine assays the ABTS concentration was 1.7 mM. The initial rate of the reaction was determined by measuring the increase in absorbance at 415 nm using a molar absorbance coefficient of $\epsilon = 3.6 \times 10^4$ M^{-1} cm^{-1}.

Iodide Oxidation Assay. The reaction mixture contained 1 nM HRP and 0.1 mM H_2O_2 in 50 mM sodium acetate buffer (pH 5.3). The concentration of potassium iodide was varied (10, 15, 20, 40, 80, and 100 mM), and potassium nitrate was added (90, 85, 80, 60, 20, and 0 mM, respectively) to keep the ionic strength constant. The rate of triiodide formation was determined for the first 20 s of the reaction from the increase in the absorbance at 353 nm using a molar absorption coefficient of 2.62×10^4 M^{-1} cm^{-1}.

Thioether Sulfoxidation. To 1 mL of a solution at 25 °C of native or mutant HRP (25 μM) and thioether substrate (10 mM, added in 10 μL of methanol) in sodium phosphate buffer (pH 7.0) was added H_2O_2 in 10 equal aliquots (final nominal concentration of 4 mM). After a total incubation time of 2 h, acetophenone was added as an internal standard, the solution was extracted with CH_2Cl_2, and the extracts were concentrated nearly to dryness under a stream of argon. The residue, taken up in the HPLC solvent, was analyzed by isocratic HPLC on a Daicel chiral column installed on a Hewlett Packard model 1040A system equipped with a diode array detector and a Varian 1090 solvent pump system. The HPLC effluent was monitored at 254 nm. The column was eluted with 80% hexane:20% isopropyl alcohol at a flow rate of 0.5 mL/min for methyl phenyl sulfoxide and with 90% hexane: 10% isopropyl alcohol at the same flow rate for all the other sulfoxides. The retention times for the *S* and *R* isomers were methyl phenyl sulfoxide (26 and 44 min, respectively), ethyl phenyl sulfoxide (30.8 and 57.2 min, respectively), cyclopropyl phenyl sulfoxide (32.6 and 58.3 min, respectively), and *n*-propyl phenyl sulfoxide (30.8 and 60.2 min, respectively). By analogy with the simple aryl methyl sulfoxides, the *S* isomer was assumed to be the first to elute from the column. A sulfoxide standard curve was constructed from the peak ratios obtained after analogous workup of known sulfoxide−acetophenone mixtures. The amount of racemic sulfoxide from control incubations without the enzyme was subtracted from the experimental values to obtain the true enantiomeric excess.

To test the linearity of the relationship between time and product formation, 1 mL incubations were carried out containing 12.5 μM native HRP, 1 mM H_2O_2, and 2 mM thioanisole in 50 mM sodium phosphate buffer (pH 7.0). Analogous incubations with the mutant contained 5 μM enzyme, 0.5 mM H_2O_2, and 1 mM thioanisole. Incubations were stopped at various time points and were worked up and analyzed as described above.

For the determination of K_m and V_{max} values, the reaction mixture contained 5 μM enzyme, the indicated amounts of thioether, and 0.5 mM H_2O_2 in 50 mM sodium phosphate buffer (pH 7.0). The incubations were carried out for 2 min at 25 °C, and the products were analyzed as already described. When it was necessary to monitor product formation by HPLC, the incubation mixture was scaled up 2−10-fold. A linear relationship between time and product formation was observed for at least 5 min. H_2O_2 was shown not to be the limiting factor in the oxidation reaction. Kinetic constants were determined from the data for a minimum of six substrate concentrations, including concentrations smaller and larger than the K_m.

Styrene Epoxidation. Native or mutant HRP (0.25 μM) in 0.5 mL of sodium phosphate buffer (50 mM, pH 7.0) was incubated with styrene, *cis*-β-methylstyrene, or *trans*-β-methylstyrene (10 mM, delivered neat). The olefins were prepurified by silica gel flash chromatography with hexane. An aliquot of H_2O_2 (5 μL of 100 mM solution) was added five times at 10 min intervals, giving a final nominal 10 mM peroxide concentration, and the incubation was continued for 1 h at 25 °C. The mixture was then extracted twice with 1 mL of CH_2Cl_2 and the combined extracts, after concentration nearly to dryness under a stream of argon, were analyzed by gas−liquid chromatography on a Hewlett Packard 5890A instrument equipped with a 0.25 mm × 30 m

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Chiraldex G-TA capillary column (Advanced Separation Technologies, Whippany, NJ). The column temperature was 90 °C for the analysis of styrene oxide and 100 °C for the analyses of both *cis*- and *trans*-β-methylstyrene oxides. The retention times for (*S*)- and (*R*)-styrene oxide were 31.9 and 38.4 min, respectively, for (1*S*,2*R*)- and (1*R*,2*S*)-*cis*-β-methylstyrene oxide 23.4 and 28.2 min, respectively, and for (1*R*,2*R*)- and (1*S*,2*S*)-*trans*-β-methylstyrene oxide 22.4 and 25.1 min, respectively. The retention times for the side products were benzaldehyde (13.9 min at 100 °C, 20 min at 90 °C), phenylacetaldehyde (32 min at 90 °C), and phenylacetone (33.7 min at 100 °C). The styrene oxide enantiomers were identified by chromatographic comparisons with authentic (*R*)-styrene oxide and racemic styrene oxide and the *trans*-β-methylstyrene oxide enantiomers by comparison with the authentic isomers. The *cis*-β-methylstyrene oxide enantiomers were assigned as previously described by comparison to an unequal synthetic mixture.[33,34]

Experiments to determine whether a linear relationship exists between time and product formation were carried out at 0 °C (ice bath) with reaction mixtures containing native or mutant HRP (25 μM), H_2O_2 (15 mM), and olefin (10 mM, delivered neat). The incubations were stopped at increasing time points and the appropriate internal standard (10 μL of a 1 mM methanol solution of α-methylstyrene for styrene and *trans*-β-methylstyrene and of vinylcyclohexane for *cis*-β-methylstyrene) was added. The incubations were then extracted with 1 mL of CH_2Cl_2, and the extract, after concentration nearly to dryness under a stream of argon, was analyzed by gas–liquid chromatography on a 0.5 mm × 30 m DB-5 column. The column was programmed to run at 80 °C for 3 min, then to rise at 2.5 °C/min to 105 °C, and finally to rise at 70 °C/min to 250 °C, where it remained for 10 min to clear the column. The retention times of styrene oxide, *cis*-β-methylstyrene oxide, and *trans*-β-methylstyrene oxide were 10.4, 11.4, and 12.8 min, respectively. Control experiments indicated that a linear relationship existed between product formation and time for at least the initial 30 min.

In order to confirm that H_2O_2 is not a limiting factor in the epoxidation reaction, 0.5 mL incubations were carried out with HRP (25 μM), 10 mM *trans*-β-methylstyrene, and varying concentrations of H_2O_2 in 0.5 mL of 50 mM sodium phosphate buffer (pH 7.0). The incubations were worked up and analyzed as described above.

Reaction mixtures (0.5 mL final volume) containing native or mutant HRP (25 μM), the indicated amount of olefin, and 15 mM H_2O_2 in 50 mM phosphate buffer (pH 7.0) were used to determine the K_m and V_{max} values. Styrene and *cis*- and *trans*-β-methylstyrene were added as methanol solutions, and incubations were carried out for 30 min at 0 °C (ice bath). Extraction and quantitation of the products was performed as described above.

Determination of the Source of the Epoxide Oxygen. To quantitate the incorporation of oxygen from H_2O_2 into the epoxides, incubations (0.5 mL volume) were carried out with native or mutant HRP (25 μM) and the olefin (10 mM, delivered neat) in 50 mM sodium phosphate buffer (pH 7.0). Ten equal aliquots of [^{18}O]H_2O_2 (90% ^{18}O) were added at 10 min intervals to give a final nominal peroxide concentration of 5 mM. The incubations were continued for a further 20 min (2 h total) at 25 °C, after which they were extracted with CH_2Cl_2 (2 × 1 mL). The extracts were concentrated nearly to dryness under a stream of argon and were then analyzed by tandem gas–liquid chromatography–mass spectrometry to determine the extent of ^{18}O enrichment. Similar control experiments were carried out using H_2O_2, and the M:M - 2 peak ratio in the mass spectrum was used to calculate ^{18}O incorporation.

To quantitate the incorporation of molecular oxygen into the epoxide, native HRP (25 μM) in 1 mL of 50 mM sodium phosphate buffer (pH 7.0) was frozen by immersion in dry ice–acetone and the mixture was degassed three times with a water aspirator. The first two times the sealed flask was filled with argon, but the third time it was filled with $^{18}O_2$ (98% atom % ^{18}O). Styrene (10 mM) was then added to the mixture, followed by 10 aliquots of H_2O_2 at 10 min intervals (final H_2O_2 concentration of 5 mM). The mixture was incubated at 25 °C for a total of 2 h, including the peroxide addition, and was then extracted with CH_2Cl_2, and the extracts were analyzed to determine the extent of ^{18}O incorporation as described above.

Results

Plasmid Construction and Protein Expression. HRP mutants were constructed by cassette mutagenesis to take advantage of the restriction sites incorporated into the synthetic HRP gene.[29] The presence of appropriately located single *Sac I* and *Nhe I* sites in the pVLHRP2 construct made possible direct replacement of a fragment including the codons for residue Phe-41. Genes were thus prepared in which Phe-41 was replaced by either a smaller, nonpolar (leucine) or smaller, polar (threonine) residue. The pVLHRP2 construct employed in this work contained a 5′-leader sequence identical to that in the plant gene that codes for excretion of the protein into the medium.[29] The efficiencies of the transfections of insect cells with the mutant constructs were approximately 100%, and the titers of the pass II stocks were normally in the order of 10^{10}. Initial expression trials were carried out in *Spodoptera frugiperda* Sf9 cells grown at 27 °C in TNM-FH (JRH Biosciences) medium containing 10% heat-inactivated fetal bovine serum. The cell line and culture system were subsequently changed to *T. ni* egg cells (HighFive cells, Invitrogen) grown at 27 °C in Sf-900 medium without the bovine serum. The *T. ni* cells gave higher protein yields, and the bovine serum was deleted because purification of the proteins was simplified in its absence. A variety of expression conditions were examined in efforts to optimize the expression system. Little difference was observed in protein yields when the infection of *T. ni* cells was undertaken with the equivalent excess of viral stock at cell densities ranging from 0.5 × 10^6 to 2 × 10^6 cells/mL. Culturing the cells for 7 rather than 3–4 days did not improve yields because cell growth normally reaches a plateau after 3–4 days. Heme was normally added on the first day of culture. Addition of a second aliquot of heme on the third day did not reproducibly result in increased protein yields.

Purification of Wild-Type and F41L and F41T HRP. MonoQ anion exchange chromatography was used as the first step in the purification procedure to remove nutrients and secreted proteins other than HRP from the medium. Neither wild-type, F41L, nor F41T HRP was retained by the column. The R_z value (A_{404}/A_{280}) for F41L was typically improved from 0.3 to 1.1 for F41L by this purification step. The next step in purification of the recombinant wild-type protein is binding of the protein to a cation exchange column at pH 4.3.[29] The same procedure could not be used for the F41L or F41T mutants because they did not have a strong affinity for the cation exchange resin at pH 4.5 and lowering the pH to 4.3 resulted in accelerated enzyme degradation. This problem was overcome by chromatography on a tandem set of columns, the first one an immobilized metal ion affinity column loaded with Cu^{2+} and the second a thiophilic gel (divinyl sulphone mercaptoethanol Sepharose 4B). The thiophilic column bound the recombinant HRP proteins in 50 mM sodium phosphate buffer containing 1.5 M ammonium sulfate (pH 7.0). The proteins were eluted from the column by decreasing the ammonium sulfate concentration. The metal ion column did not bind the HRP proteins but removed protein impurities bearing ligating residues such as His or Cys on their surfaces. In combination, the two columns proved to be an efficient purification system. The R_z value after this step for F41L HRP was >3.0. Furthermore, single major bands of slightly under 43 kDa molecular mass were observed on SDS-PAGE for the purified F41L (Figure 2) and F41T (Figure 3) proteins. The small difference in molecular mass between the recombinant and native proteins can be attributed to differences in the extent of glycosylation of the

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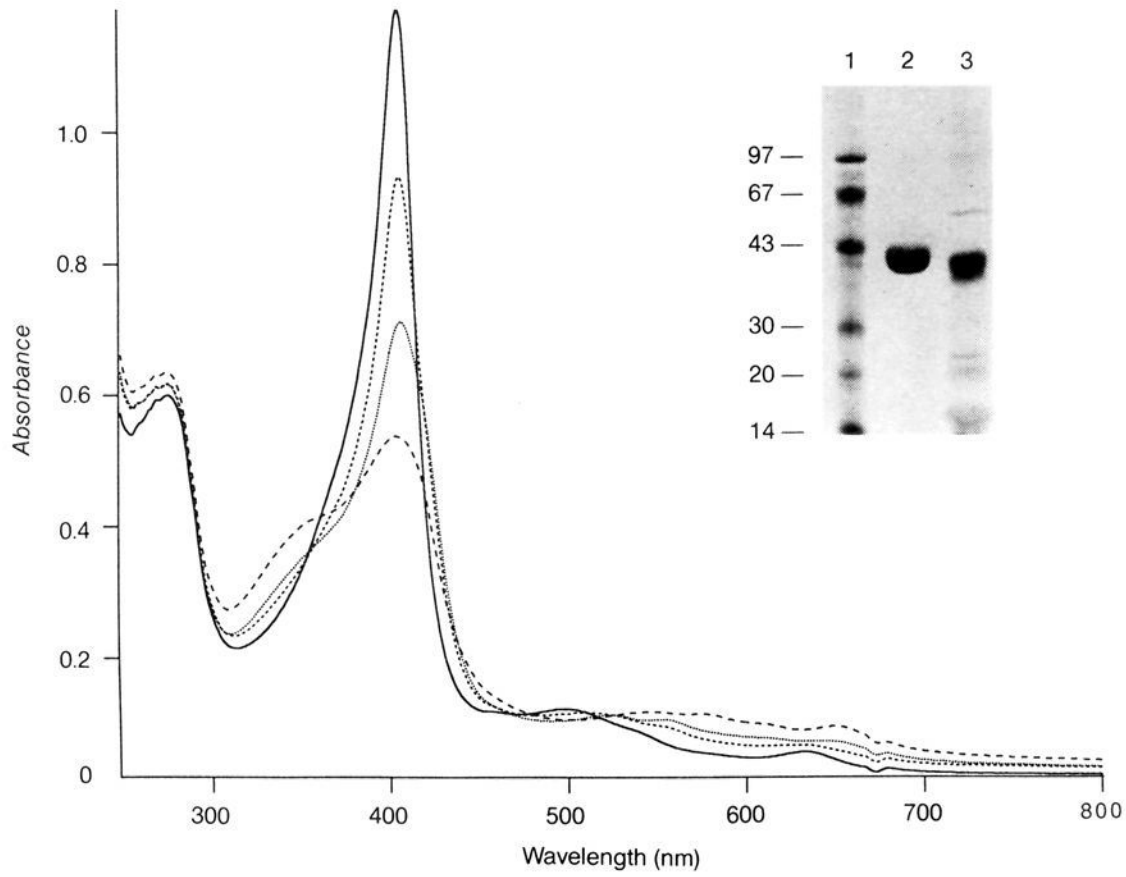


Figure 2. Spectra of F41L HRP in the ferric (—), compound I (•••), and compound III (— — —) states. The result of adding 1 equiv of ferrocyanide to the compound I intermediate is also shown (- - -). An SDS-PAGE gel of the F41L mutant of HRP is presented in the inset: lane 1, molecular weight standards; lane 2, native HRP; lane 3, F41L HRP. Approximately 5 μg of protein was loaded onto each lane.

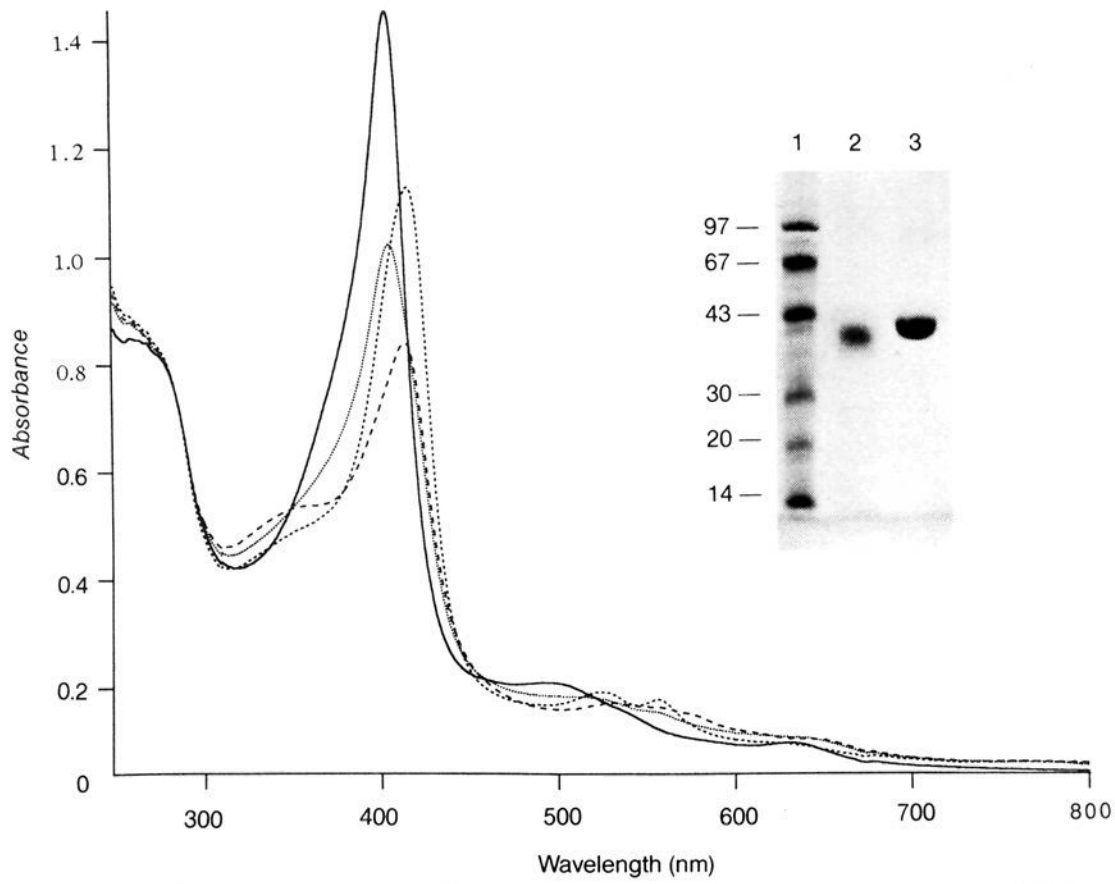


Figure 3. Spectra of F41T HRP in the ferric (—), compound I (•••), and compound II (- - -), and compound III (— — —) states. An SDS-PAGE gel of the F41T mutant of HRP is presented in the inset: lane 1, molecular weight standards; lane 2, F41T HRP; lane 3, native HRP. Approximately 5 μg of protein was loaded onto each lane.

proteins in insect cells and plants. The yield of purified F41L and F41T by this procedure was 2−5 mg/L of cell culture.

Characterization of the F41L and F41T HRP Mutants. The spectra of the ferric, ferric-CN, and ferrous-CO states of the F41L and F41T mutants are very similar to the corresponding states of the native enzyme (Figures 1 and 2, Table 1). Oxidation of the mutant enzymes with 1 equiv of H_2O_2 produces compound I chromophores that differ little from those of compound I of the native or wild-type recombinant enzymes (Figures 2 and 3, Table 1). Addition of 100 equiv of H_2O_2 to the ferric F41T mutant produces a compound III ($Fe^{+2}O_2$) species with the same spectroscopic properties as compound III of native HRP (Figure 3), although similar treatment of the F41L mutant gives a species with a shifted Soret band (Figure 2). Reduction of compound I of the F41T mutant with 1 equiv of ferrocyanide produces a compound II chromophore that is essentially identical to that of native compound II (Figure 3). The same spectrum is obtained by simply allowing compound I to decay for 20 min. Compound II of the F41L mutant, however, appears to be relatively unstable. Addition of 1 equiv of ferrocyanide to compound I of the F41L mutant produces a spectrum similar to that of the ferric state (Figure 2). Allowing the compound I species to decay with time simply results in a decrease in the intensity of the absorption without the shift characteristic of compound II. The similarities in the spectroscopic properties of various states of the two mutants and the

Table 1. Electronic Absorption Maxima for HRP and Its F41L and F41T Mutants[a]

	Soret (nm)	visible (nm)
	Native HRP[b]	
ferric	403	499, 638
ferric-CN	422	539, 580
ferrous-CO	423	542, 572
compound I	404	526, 578, 654
compound II	413	527, 555
compound III	415	546, 568, 665
	F41L HRP	
ferric	405	500, 634
ferric-CN	421	540, 575
ferrous-CO	424	540, 577
compound I	406	523, 554, 658
compound II	nd[c]	nd[c]
compound III	405	548, 581, 658
	F41T HRP	
ferric	404	499, 629
ferric-CN	423	541, 580
ferrous-CO	424	541, 570
compound I	405	527, 560, 648
compound II	416	527, 557
compound III	414	547, 575, 648

[a] The spectra were recorded in 50 mM phosphate buffer (pH 7.0) at 25 °C. [b] The absorption spectra of native and wild-type recombinant HRP are essentially identical.[29] [c] nd = not detected.

Table 2. Kinetic Constants for the Oxidations of Guaiacol, ABTS, and Iodide by HRP, F41L-HRP, and F41T-HRP

	guaiacol		ABTS		iodide	
enzyme	K_m (mM)	V_{max}[a]	K_m (mM)	V_{max}[a]	K_m (mM)	V_{max}[a]
native HRP	1.4	1.1×10^7	0.22	4.6×10^7	50	3.7×10^7
F41L-HRP	1.1	2.2×10^7	0.41	8.4×10^7	25	2.3×10^7
F41T-HRP	1.8	1.8×10^7	0.12	1.4×10^7	27	2.8×10^7

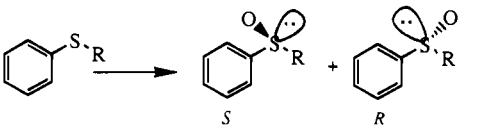
[a] V_{max} values refer to product formation and are reported as pmol/min/nmol of enzyme.

native enzyme indicate that the active sites of the mutants are structurally intact. Apart from the instability of compound II of the F41L mutant, the similarities in the three oxygenated states of the native and mutant proteins support this inference.

The K_m and V_{max} values for the oxidation of guaiacol, ABTS, and iodide by the F41L and F41T mutant enzymes differ by little from the corresponding values for the native enzyme (Table 2). The K_m values for oxidation of the three substrates by the mutants differ by less than a factor of 2 from the values for the native enzyme. The V_{max} values for guaiacol oxidation are slightly higher (<2-fold) and for iodide oxidation slightly lower (~70%) for the two mutants compared to the native enzyme. For ABTS, the V_{max} of F41L is approximately twice, and that of F41T approximately half, that of the native protein. There is no clear or marked trend in the values for the mutants. The minor changes in K_m and V_{max} confirm that the active site structure is not significantly reorganized in the mutants.

Oxidation of Thioanisoles and Related Compounds. Native HRP catalyzes the sulfoxidation of thioethers with the incorporation of an oxygen from the peroxide, presumably via the ferryl species, into the sulfoxide.[6,7] The F41L and F41T mutants of HRP retain this sulfoxidation activity but differ from the native protein in terms of K_m, V_{max}, and enantiomeric specificity (Table 3). The K_m values, except for those of the F41L and F41T HRP-catalyzed oxidations of thioanisole and F41L-catalyzed oxidation of ethyl phenyl thioether, are lower than the corresponding values for the native enzyme. No trend is seen in the K_m values for oxidation of the four substrates by native HRP, but for the two mutants, a correlation exists between

Table 3. Kinetic Parameters and Stereochemistry for the Sulfoxidation of Phenyl Alkyl Thioethers by HRP



S *R*

	native[a]			F41L[a]			F41T[a]		
R	K_m	V_{max}	%ee	K_m	V_{max}	%ee	K_m	V_{max}	%ee
methyl	0.17	3500	77	4.5	19000	97	1.1	8100	10
ethyl	0.90	3600	35	2.0	20000	94	0.6	15000	10
n-propyl	0.50	700	12	0.2	4600	94	0.4	7500	44
cyclopropyl	0.88	600	7	0.2	5900	70	0.3	11000	5(*R*)

[a] The units are as follows: V_{max}, pmol/min/nmol; K_m, mM. The absolute stereochemistry of the dominant isomer is *S* except where indicated.

Table 4. Kinetic Parameters for the Epoxidations of Styrene, *cis*-β-Methylstyrene, and *trans*-β-Methylstyrene by HRP, F41L-HRP, and F41T-HRP

	native[a]		F41L[a]		F41T[a]	
substrate	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
styrene	*b*		2.0	1.4	3.0	2.3
cis-β-methylstyrene	*b*		*c*		12	2.0
trans-β-methylstyrene	12	1.5	8.4	2.4	9.8	1.2

[a] V_{max} refers to product formation and is given in pmol/min/nmol; K_m is in mM units. [b] A trace of product detected. [c] No product detected.

the K_m value and the size or lipophilicity of the thioether alkyl group. The V_{max} values for the oxidation of all four substrates are much higher in the F41L and F41T mutants than they are for the native protein (Table 3). The largest increases in V_{max} versus native HRP are the 18.3- and 10.7-fold increases observed for the F41T-catalyzed oxidations of, respectively, the phenyl cyclopropyl and phenyl propyl thioethers. In addition, the enantiomeric excesses of the sulfoxides obtained in the reaction are considerably higher for all the substrates in the F41L- than native HRP-catalyzed reactions (Table 3). In contrast, except for phenyl propyl thioether, the enantiomeric excess is decreased in the oxidations catalyzed by F41T HRP. In fact, the favored absolute stereochemistry for the phenyl cyclopropyl sulfoxide produced by F41T is opposite to that observed with native HRP or the F41L mutant. The greatest improvement in enantiomeric excess with respect to native HRP is the 10-fold enhancement seen for the oxidation of phenyl cyclopropyl sulfide by the F41L mutant.

Oxidation of Styrene and *cis*-β-Methylstyrene. Styrene, as reported earlier,[9] undergoes no more than trace oxidation by native HRP (Table 4). In agreement with this result and the finding that butadiene is not a substrate,[10] *cis*-β-methylstyrene is also not oxidized by native HRP (Table 4). Decreasing the volume of the residue at position 41 by replacing the phenylalanine with a leucine conveys on HRP the ability to epoxidize styrene but not *cis*-β-methylstyrene, whereas replacement of the phenylalanine by a threonine allows the mutant enzyme to oxidize both olefins. Styrene is oxidized to styrene oxide, benzaldehyde, and phenylacetaldehyde (Table 5). The ratios of the rates for the formation of these three products by F41L and F41T HRP are approximately 28:29:1 and 27:32:1, respectively. Benzaldehyde is formed in the H_2O_2-supported oxidation of styrene by most hemoproteins by an unknown mechanism, but phenylacetaldehyde is formed by a hydrogen rearrangement reaction.[9,35,36] The oxidation of *cis*-β-methylstyrene by the F41T

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Table 5. Products Formed in the Reactions of Styrene, *cis*-β-Methylstyrene, and *trans*-β-Methylstyrene with F41L and F41T HRP

		F41L[a]		F41T[a]	
substrate	product	rate	relative rate[b]	rate	relative rate[b]
styrene	styrene oxide	8.3	28	8.0	27
	benzaldehyde	8.6	29	9.5	32
	phenylacetaldehyde	0.3	1	0.3	1
cis-β-methylstyrene	*cis*-β-methylstyrene oxide	nd		9.4	5.9
	trans-β-methylstyrene oxide	nd		2.0	1.3
	1-phenyl-2-propanone	nd		1.6	1.0
	benzaldehyde	nd		trace	trace
trans-β-methylstyrene	*trans*-β-methylstyrene oxide	5.2	1.9	37	7.0
	benzaldehyde	2.7	1.0	5.3	1.0

[a] Rates are in pmol/min/nmol protein. The absence of detectable product is indicated by nd. [b] Relative rates compare the formation of products from a single substrate by a single enzyme.

mutant yields *cis*-β-methylstyrene oxide, *trans*-β-methylstyrene oxide, 1-phenyl-2-propanone, and a trace of benzaldehyde (Table 5). The epoxidation of *cis*-β-methylstyrene thus proceeds with both partial loss of the olefin stereochemistry and the formation of a rearranged ketone product.

The epoxidation of styrene proceeds with negligible to modest stereospecificity. The styrene epoxides produced by the F41L and F41T mutants are 27:73 (46% ee) and 51:49 (2% ee) mixtures of the 1*S* and 1*R* enantiomers, respectively (Table 6). The oxidation of *cis*-β-methylstyrene by F41T HRP provides the (1*S*,2*R*)-*cis*-epoxide with greater than 99% ee and the (1*S*,2*S*)-*trans*-β-methylstyrene epoxide with 10% ee. Thus, epoxidation of *cis*-β-methylstyrene with retention of the olefin stereochemistry proceeds with high absolute stereospecificity whereas epoxidation with loss of the olefin stereochemistry proceeds with little absolute stereospecificity.

The source of epoxide oxygen has been examined by ^{18}O-labeling studies to determine whether the epoxides arise by ferryl oxygen transfer to the olefins (Table 7). Oxidation of styrene by F41T HRP and $[^{18}O]H_2O_2$ produces styrene oxide with 79% ^{18}O-labeled oxygen. Under the same conditions, the *cis*-β-methylstyrene epoxide produced by F41T HRP from *cis*-β-methylstyrene was 99% ^{18}O-labeled. In contrast, the *trans*-β-methylstyrene oxide produced from *cis*-β-methylstyrene was only 63% ^{18}O-labeled. The results suggest that epoxidation with retention of stereochemistry proceeds exclusively by a ferryl oxygen transfer mechanism. Epoxidations that result in inversion of stereochemistry, however, involve two reaction mechanisms, one that results in incorporation of the ferryl oxygen and another that does not. The epoxidation of styrene presumably involves both mechanisms although the products obtained with retention and loss of stereochemistry cannot be distinguished.

Oxidation of *trans*-β-Methylstyrene. The failure of native HRP to oxidize styrene or *cis*-β-methylstyrene suggests that the enzyme should not oxidize the more sterically hindered *trans*-β-methylstyrene. Surprisingly, however, *trans*-β-methylstyrene is oxidized not only by F41T HRP but also by native and F41L HRP (Table 4). The two products of the reaction are *trans*-β-methylstyrene oxide and benzaldehyde (Table 5). No *cis*-β-methylstyrene epoxide or 1-phenyl-2-propanone was detected. In contrast to the oxidations of styrene and *cis*-β-methylstyrene, the V_{max} values for the epoxidation of *trans*-β-methylstyrene are essentially the same for all three enzymes. The K_m values are the same for the native and F41T HRP, but the K_m is higher by a factor of 2 for the F41L mutant.

The enantiomeric excesses of the *trans*-β-methylstyrene epoxide produced by native, F41L, and F41T HRP are 6%, 36%, and 64%, respectively (Table 6). There is a clear increase in the stereospecificity of the reaction in traversing the above series of proteins, but the increase in stereospecificity does not correlate with changes in either the K_m or V_{max} of the reaction.

Studies of the source of the oxygen in the epoxide produced from *trans*-β-methylstyrene have been carried out with both $[^{18}O]H_2O_2$ and $[^{18}O]O_2$. Incubations of *trans*-β-methylstyrene and $[^{18}O]H_2O_2$ with native, F41L, and F41T HRP in an open vessel resulted in incorporation of 65, 68, and 91%, respectively, of the labeled oxygen into the epoxide (not shown). It appears that the F41T mutation significantly increases the fraction of the reaction that incorporates peroxide oxygen into the epoxide. More detailed studies have been carried out with the native enzyme. Incubation of the olefin with native HRP and $[^{18}O]H_2O_2$ under an atmosphere of $[^{16}O]O_2$ yielded epoxide that was 37% ^{18}O-labeled (Table 7). If the reaction was carried out with unlabeled H_2O_2 under an atmosphere of $[^{18}O]O_2$, the oxygen was 44% ^{18}O-labeled (Table 7). Oxygen can thus be incorporated into the epoxide from both the peroxide and molecular oxygen, and the proportion of the oxygen incorporated from these two sources depends on the concentration of molecular oxygen. In accord with this conclusion, if the reaction is run with $[^{18}O]H_2O_2$ under an atmosphere of argon gas to suppress the incorporation of molecular oxygen, the oxygen in the epoxide is found to be 85% ^{18}O-labeled.

Discussion

Site-specific replacement of Phe-41 by either a leucine or a threonine yields proteins that differ that in spectroscopic terms from native or wild-type recombinant HRP. Only minor differences are observed in the spectra of the ferric, ferric-CN, ferrous-CO, and compound I states of the proteins (Table 1). These results indicate that the mutations have not greatly altered the electronic structure of the heme chromophore or the physical properties of its environment. The only anomalies appear to be the instability of compound II of the F41L mutant and the shift in the Soret band of its compound III species. The basis for the instability of compound II is not clear but is reminiscent of the instability of the compound II species of *A. rhamosus* peroxidase at neutral pH.[37] The instability of the F41L compound II, however, does not have important catalytic repercussion because the K_m and V_{max} values for the oxidations of guaiacol, ABTS, and iodide by the two mutants differ by no more than a factor of 2 from those for native HRP (Table 2). The only exception is the oxidation of ABTS by F41T HRP, for which the V_{max} is slightly less than one-third that of the native enzyme. These results agree with previous spectroscopic and kinetic results on the deglycosylated Phe-41 → Val and Phe-41 → Trp HRP mutants expressed in *E. coli*.[38,39]

Mutation of Phe-41 to either a leucine or a threonine increases the ability of HRP to catalyze the sulfoxidation of thioethers (Table 3). In a preliminary communication we reported that the relative rates for the sulfoxidation of *p*-substituted thioanisoles and aryl alkyl thioethers are increased by both mutations and demonstrated a marked increase in the stereospecificity of

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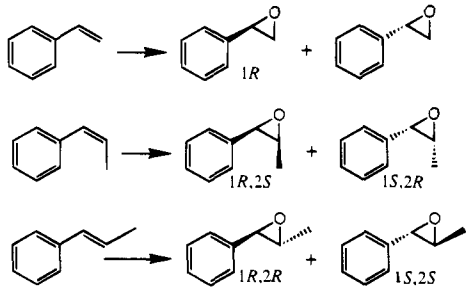
Table 6. Stereochemistries of the Epoxides Formed in the Reactions of Styrene, *cis*-β-Methylstyrene, and *trans*-β-Methylstyrene with native, F41L, and F41T HRP



substrate	epoxide	% ee (major isomer) native	F41L	F41T
styrene	styrene oxide		46 (*R*)	2 (*S*)
cis-β-methyl-styrene	*cis*-β-methyl-styrene oxide			>99 (1*S*,2*R*)
	trans-β-methyl-styrene oxide			10 (1*S*,2*S*)
trans-β-methyl-styrene	*trans*-β-methyl-styrene oxide	6T(1*R*,2*R*)	36 (1*S*,2*S*)	64 (1*S*,2*S*)

the reaction catalyzed by F41L but not F41T HRP.[30] The sulfoxidation of *para*-substituted thioanisoles by the F41L mutant was shown to increase as a function of the electron-donating strength of the *para*-substituent. These trends are confirmed by the present studies, in which we have determined the kinetic constants for the oxidation of phenyl alkyl thioethers by HRP and its mutants as a function of the size of the alkyl group. The thioanisole K_m values are larger for the F41L and F41T mutants than for native HRP, but the mutant K_m values decrease relative to the wild-type enzyme as the size of the alkyl group increases (Table 3). In contrast, the V_{max} values for the mutants are consistently higher than those for the native enzyme. This is clearly shown by the 18-fold difference in V_{max} for the oxidation of *n*-propyl phenyl thioether by F41T and native HRP. These results suggest that thioanisole binds in a restricted site that poorly accommodates larger alkyl groups but that it accommodates them more readily when Phe-41 is replaced by smaller residues.

As found before, the absolute stereospecificity of the sulfoxidation reaction is much higher with F41L than native or F41T HRP (Table 3). For example, the (*S*)-thioanisole sulfoxide is formed by F41L HRP with 97% ee, compared to 77% ee with native HRP and only 10% ee with the F41T mutant. The results indicate that the polarity as well as the size of the amino acid residue at position 41 contributes to the stereospecificity of the reaction.

Sulfoxidation is the only confirmed peroxygenase reaction that has been shown to be catalyzed by native HRP or any other peroxidase with a histidine iron ligand.[2c,5-7] The one exception is cytochrome *c* peroxidase and its W51A mutant, which have been shown to oxidize styrene and *cis*- and *trans*-β-methylstyrene.[35] Cytochrome *c* peroxidase is a special case, however, because its normal substrate is a protein. Less evolutionary pressure may therefore have existed to sequester the ferryl species. We have therefore chosen the epoxidation of styrene and its derivatives as more definitive probes of the peroxygenase activity of HRP and its mutants. The epoxidation of styrene is catalyzed by the F41L and F41T mutants of HRP even though the reaction is essentially not catalyzed by native HRP (Table 4). The similarities in the styrene K_m and V_{max} values for the two mutants suggest that both open the active site to similar extents, but this is contradicted by the lower stereospecificity of the F41T mutant and the observation that *cis*-β-methylstyrene is only oxidized by the F41T mutant. The inference that the cavity adjacent to the ferryl species is effectively less restricted in the F41T than F41L mutant is supported by the finding that F41T HRP is less stereoselective in the sulfoxidation of thioethers than F41L HRP or, except for one substrate, than native HRP. The increase in the effective binding site thus appears to be greater when Phe-41 is replaced by a threonine than a leucine despite the similarities in the sizes of the side chains of these two amino acids. It is possible, for example, that hydrogen bonding of the hydroxyl group displaces the side chain with respect to the position of the non-hydrogen bonding leucine side chain.

In view of the results with styrene and *cis*-β-methylstyrene, it is surprising that *trans*-β-methylstyrene is a substrate not only for F41L and F41T HRP but also for native HRP (Table 4). The susceptibility of *trans*-β-methylstyrene but not styrene or *cis*-β-methylstyrene to HRP-catalyzed epoxidation must stem from interactions of the substrate with the protein because the intrinsic reactivity of the π-bond in *cis*- and *trans*-β-methylstyrene toward the electron-deficient ferryl species should be approximately the same. The 4-fold higher V_{max} for epoxidation of the β-methylstyrenes over styrene could simply reflect the higher reactivity of a di- rather than monosubstituted π-bond. A steric argument without recourse to protein interactions is untenable because the *cis*-isomer is intrinsically less sterically hindered than the *trans*-isomer. It is possible that binding of *trans*-β-methylstyrene perturbs the active site residues and exposes the ferryl oxygen to interaction with the olefin π-bond, whereas binding of *cis*-β-methylstyrene does not cause a similar perturbation. This would provide an additional rationale for the more difficult question of why styrene, which is less sterically hindered than either of the β-methyl styrenes, is not oxidized.

If the epoxide is formed in these epoxidation reactions by a ferryl oxygen transfer mechanism, the oxygen incorporated into the epoxide should derive from H_2O_2. This is not always the case, however (Table 7). Thus, the oxygen in the *cis*-epoxide produced from *cis*-β-methylstyrene by F41T HRP derives exclusively from the peroxide, but only 63% of the oxygen in the *trans*-epoxide derived from the *cis*-olefin and 79% of the epoxide derived from styrene has its origins in H_2O_2. Similar studies of the epoxidation of *trans*-β-methylstyrene by native HRP indicate that comparable amounts of the oxygen derive from H_2O_2 (37%) and O_2 (44%), although the exact percent of each is influenced by the conditions of the experiment. Differences are also found in the extent of incorporation of oxygen from H_2O_2 into the *trans*-β-methylstyrene epoxide produced under a standard set of conditions by native HRP (65%), F41L HRP (68%), and F41T HRP (91%). These results clearly establish that the epoxidation of all three substrates, but more so the epoxidation of *trans*-β-methylstyrene, is mediated by at least two mechanisms, one of which involves a "P450-like" ferryl oxygen transfer. The mechanism that incorporates an atom of molecular oxygen into the epoxide, on the other hand, is obscure.

We have previously demonstrated that the epoxidation of styrene and *cis*- and *trans*-β-methylstyrene by myoglobin and H_2O_2 proceeds by two mechanisms, one that incorporates an oxygen from the peroxide with retention of the olefin stereochemistry and another that incorporates an atom of molecular oxygen with scrambling of the olefin stereochemistry.[36,40] The first of these mechanisms involves a ferryl oxygen transfer to the π-bond, whereas the second involves the formation of a

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Table 7. Origins of the Oxygen in the Epoxidations of Styrene, *cis*-β-Methylstyrene, and *trans*-β-Methylstyrene

substrate	enzyme	oxidizing agent	atmosphere	product	%^{18}O incorporation
styrene	F41T	[^{18}O]H_2O_2	air	epoxide	79
cis-β-methylstyrene	F41T	[^{18}O]H_2O_2	air	*cis*-epoxide	99
				trans-epoxide	63
trans-β-methylstyrene	native	[^{18}O]H_2O_2	[^{16}O]O_2	*trans*-epoxide	37
	native	[^{16}O]H_2O_2	[^{18}O]O_2	*trans*-epoxide	44
	native	[^{18}O]H_2O_2	argon	*trans*-epoxide	85

protein radical, binding of oxygen to the radical, and cooxidation of the olefin by the resulting protein peroxy radical. The properties of these two myoglobin mechanisms resemble those observed here for the epoxidation of styrene and *cis*- and *trans*-β-methylstyrene. There is no evidence, however, for the formation of a protein radical during the catalytic turnover of HRP. If a cooxidation mechanism is responsible for the incorporation of oxygen from molecular oxygen into the product, the protein radical involved in the reaction must be a very minor species or the cooxidizing agent is something other than a protein radical (e.g., an impurity in the enzyme preparation).

Replacement of Phe-41 with either of two smaller residues, in accord with the hypothesis that inaccessibility of the ferryl oxygen suppresses direct interaction with substrates,[2c,16] greatly enhances the peroxygenase activity of HRP. Not only is thioether sulfoxidation accelerated but the enzyme acquires the ability to epoxidize styrene and *cis*-β-methylstyrene. Although the picture is complicated by the unexpected findings that *trans*-β-methylstyrene is oxidized by native HRP and that part of the epoxidation reaction proceeds via a mechanism other than ferryl oxygen transfer, the results obtained with the F41L and F41T mutants support the view that limited access to the ferryl oxygen steers HRP turnover toward peroxidative rather than peroxygenative catalysis. The enhancement of peroxygenase activity as the ferryl group is made more accessible suggests that practical peroxygenase catalysts may be constructed by appropriate mutation of peroxidase structures.

Acknowledgment. We thank Sherri L. Newmyer and Vaughn P. Miller for assistance in the initial baculovirus expression of HRP. This work was supported by Grant GM32488 from the National Institutes of Health. Mass spectrometry was carried out in the University of California, San Francisco, Biomedical Bioorganic Mass Spectrometry Facility (A. Burlingame, Director) supported by NIH Grant RR 01614 and Liver Core Center Grant 5 P30 DK26743.

JA9508939