Horseradish Peroxidase Mutants That Autocatalytically Modify Their Prosthetic Heme Group

INSIGHTS INTO MAMMALIAN PeroxidASE HEME-PROTEIN COVALENT BONDS

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The mammalian peroxidases, including myeloperoxidase and lactoperoxidase, bind their prosthetic heme covalently through ester bonds to two of the heme methyl groups. These bonds are autocatalytically formed. No other peroxidase is known to form such bonds. To determine whether features other than an appropriately placed carboxylic acid residue are important for covalent heme binding, we have introduced aspartate and/or glutamic acid residues into horseradish peroxidase, a plant enzyme that exhibits essentially no sequence identity with the mammalian peroxidases. Based on superposition of the horseradish peroxidase and myeloperoxidase structures, the mutated residues were Leu[57], Phe[41], Gl[69] and Ser[73]. The F41E mutant was isolated with no covalently bound heme, but the heme was completely covalently bound upon incubation with H2O2. As predicted, the modified heme released from the protein was 3-hydroxymethylheme. The S73E mutant did not covalently bind its heme but oxidized it to the 8-hydroxymethyl derivative. The hydroxyl group in this modified heme derived from the medium. The other mutations gave unstable proteins. The rate of compound I formation for the F41E mutant was 100 times faster after covalent bond formation, but the reduction of compound I to compound II was similar with and without the covalent bond. The results clearly establish that an appropriately situated carboxylic acid group is sufficient for covalent heme attachment, strengthen the proposed mechanism, and suggest that covalent heme attachment in the mammalian peroxidases relates to peroxidase biology or stability rather than to intrinsically catalytic properties.

Mammalian peroxidases share a unique feature, the formation of two or three covalent bonds to their prosthetic heme group, not found in the peroxidases of other organisms. The two common bonds in all mammalian peroxidases are ester links between a conserved glutamate and aspartate and the heme 1-methyl and 5-methyl substituents, respectively. In MPO a third bond is forged between a methionine and the β carbon of the 2-vinyl substituent (1). Mutagenesis studies with LPO have demonstrated that at least one of the two ester bonds is required for catalytic activity (2). Interestingly, a single covalent link between the heme and protein is also found in most members of the CYP4A, -4B, and -4F classes of cytochrome P450 enzymes (3–7). In contrast to LPO, however, mutagenesis studies show that the heme-protein covalent bond is not essential for the catalytic activity of at least some of these P450 enzymes (8, 6). A long known example of heme covalent binding is provided by cytochrome c, in which a cysteine residue is covalently bound to each of the two porphyrin vinyl groups. Although many hypotheses have been formulated concerning the functional advantages of the links in cytochrome c, including bending of the heme, increasing the heme affinity (9), and increasing the stability of the methionyl-Fe(II) coordination (10), the question has yet to be definitively answered.

Despite mutagenesis studies of MPO and LPO (for a review, see Ref. 11), both the mechanism by which the heme covalent bonds are formed in the mammalian peroxidases and their functional advantage remain unclear. One reason for the slow progress in this area is the relative complexity and relatively low yield of the expression systems required to obtain recombinant mammalian peroxidases, as well as the sensitivity and insensitivity of these peroxidases to mutations. In addition, the expression systems only yield partially or completely processed enzymes. It has therefore not been possible to study the bond-forming reaction commencing with an enzyme from which the bond is largely absent. Nevertheless, it has been unambiguously demonstrated that the ester covalent links are formed via an autocatalytic mechanism (12). It has also been shown in MPO that only a carboxylate can trigger covalent tethering of the heme to the protein (13), and in LPO that the length of the carboxylate side chain is critical for the same bond to be formed (2).

It is not known, however, whether there are requirements other than the presence of an appropriate carboxyl side chain for ester bond formation. Despite their common function and the presence of some structural homologies, mammalian peroxidases share very little sequence identity with peroxidases from other organisms (14, 15). Is the uniqueness of covalent heme attachment in the mammalian peroxidases simply a result of the presence of appropriately positioned active site carboxyl side chains, for which there is no counterpart in other peroxidases, or is the participation of other residues required? To address this question, we have introduced a carboxylate into the active site of HRP, one of the best studied peroxidases and...
Horseradish Peroxidase Mutants Covalently Bind Heme

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes were purchased from New England Biolabs. Hemin, H$_2$O$_2$, ABTS, guaiacol, Pronase, bis-tris propane, trifluoroacetic acid, and tissue culture grade water were purchased from Sigma. All other chemicals, including buffer components and HPLC solvents, were purchased from Fisher Scientific. HRP was from Roche. The monohydroxylated hemin derivatives used as HPLC standards were isolated from recombinant HRP (19) and LPO (2) according to the published procedures. H$_2$PO$_4$ (95% 18O) was obtained from Cambridge Isotope Laboratories (Andover, MA). High-Five cells were grown in supplemented Express Five SFM medium, both purchased from Invitrogen. The SF9 cells (Invitrogen) were grown in Excell 420™ medium purchased from JRH Biosciences (Lenexa, KS). Penicillin/streptomycin and Fungizone were purchased from the UCSF Cell Culture Facility. UV-visible absorption measurements were undertaken on a Hewlett Packard 8422A diode array detector. Analytical and preparative HPLC separations were performed on a Hewlett Packard 1090 or 8452A diode array detector. The silica column was eluted with a 3-min isocratic wash of 15% solvent B, followed by a 0.9%/min linear gradient to 43% of B. For the analysis of undigested samples, the Vydac column was eluted with a 3-min isocratic wash of 15% solvent B, followed by a 0.9%/min linear gradient to 43% of B (as above). For the analysis of the 8-monohydroxydeheme derivative, the Kromasil column was eluted with a 3-min isocratic wash of 15% solvent B, followed by a 0.9%/min linear gradient to 43% of B. For the analysis of the 5-monohydroxydeheme derivative, the Vydac column was eluted with a 3-min isocratic wash of 15% solvent B, followed by a 0.9%/min linear gradient to 43% of B. Characterization of the F41E Mutant Peroxidase—The F41E mutant was treated with 6 × 2 equivalents of H$_2$O$_2$ in 100 mM bis-tris propane buffer, pH = 8.2, over a period of 35 min. Disodium EDTA, pH = 8, was added to a final concentration of 2 mM, and the resulting mixture was incubated for 15 min at room temperature before being transferred to a siliconized glass vessel (2). A freshly made 2.5 mg/ml solution of Pronase was then added, and the digestion was allowed to proceed for 36 h at 37 °C. The digest was then injected into an HPLC using the same eluant (water/acetonitrile with trifluoroacetic acid) as above with the following gradient: 0–3 min at 15% acetonitrile, and then a linear gradient of 15%/min. The porphyrin peaks were repeatedly collected in 50 mM KH$_2$PO$_4$/K$_2$HPO$_4$, pH = 7, and the RZ calculated as RZ = 2.2 for the oxidized ABTS product. Characterization of the F41E Mutant Peroxidase—The F41E mutant was treated with 6 × 2 equivalents of H$_2$O$_2$ in 100 mM bis-tris propane buffer, pH = 8.2, over a period of 35 min. Disodium EDTA, pH = 8, was added to a final concentration of 2 mM, and the resulting mixture was incubated for 15 min at room temperature before being transferred to a siliconized glass vessel (2). A freshly made 2.5 mg/ml solution of Pronase was then added, and the digestion was allowed to proceed for 36 h at 37 °C. The digest was then injected into an HPLC using the same eluant (water/acetonitrile with trifluoroacetic acid) as above with the following gradient: 0–3 min at 15% acetonitrile, and then a linear gradient of 15%/min. The porphyrin peaks were repeatedly collected in 50 mM KH$_2$PO$_4$/K$_2$HPO$_4$, pH = 7, and the RZ calculated as RZ = 2.2 for the oxidized ABTS product.
The solvents, gradient, and MS settings used were the same as de-
yalyzed by LC/MS. This procedure is required to allow a thorough dena-
precipitate. The supernatant was harvested after centrifugation, di-
mixture was allowed to stand for 5 min. An identical volume of 4:6 (v/v)
of MPO and the carbon of HRP. Two volumes of 100 mM disodium EDTA, pH
trated again 10-fold and rediluted 2-fold in water/methanol (9/1). Two

FIG. 1. View of the common secondary structures and spatially overlapping residues of MPO and HRP. a, view from the $\omega$-meso
carbon of MPO and the $\gamma$-meso carbon of HRP; b, view from the distal cavities; c, spatially overlapping residues, as viewed from the $\omega$-meso carbon
of MPO and the $\gamma$-meso carbon of HRP. Dark gray, MPO; light gray, HRP.

RESULTS

Structure Superposition and Choice of Mutations—MPO is the reference enzyme for the mammalian peroxidases because its crystal structure has been determined (1, 22). No other mammalian peroxidase structure is available. The MPO structure makes it possible to define the precise location and ar-
rangement of each of the three bonds that link the protein to the heme. To determine which residues in HRP might be suit-
able candidates for the introduction of active site carboxylate
side chains, the x-ray structure of HRP (18) was superimposed on that of MPO. The only constraint applied was conservation of the
average heme plane. As noted elsewhere for MPO/prostaglandin H synthase and yeast cytochrome c peroxidase/lignin
peroxidase (14), the superposition revealed striking similarities
in the secondary structures immediately surrounding the
iron porphyrin (Fig. 1, a and b). Helix H2 (residues 84–97) of
MPO and helix B (residues 31–44) of HRP, which bear the
corresponding distal histidines, coincided almost perfectly.
Likewise, MPO helix H8 (residues 326–338) superimposed
very well on HRP helix F and the residues immediately follow-
ing it (161–171); both segments bear the proximal histidines.
On the same side, the first segment of helix H12 of MPO (417–
423) was found in a similar position to helix H of HRP (244–250),
even if the length and orientation of the two helices differed as the
distance from the heme increased. It is to be noted that these
two segments include Asn421 of MPO and Asp247 of HRP, respec-
tively, which confer a partial imidazolyl character to their re-
spective proximal histidine.

At the approximate position of the MPO 234–243 segment bearing two of the three heme covalent bonds, we found in HRP a similar poorly defined loop (residues 67–78), a region that is among the most variable in class III
peroxidases (23). Finally, and to a lesser degree, two additional
helical segments further away from the heme were found in
comparable positions: part of helix H6 of MPO (287–300) and
helix D of HRP (97–112), and part of helix H17 of MPO (493–504)
and helix J of HRP (273–283). The HRP segments identified by
this superposition of structures are almost the same as the ones
invoked as being the remnants of an ancient gene duplication
(24). As previously noted (14), the heme in HRP was “flipped” by a 180°
rotation about the $\beta$-meso/$\delta$-meso axis relative to that in
MPO. The result of this was an exchange of pyrrole rings A
and B of MPO with pyrrole rings D and C of HRP.
The proximal His\textsuperscript{95} and His\textsuperscript{42} of MPO and HRP, respectively, as well as the distal His\textsuperscript{336} and His\textsuperscript{170}, and Asn\textsuperscript{421} and Asp\textsuperscript{247} superimpose almost perfectly (Fig. 1c). Surprisingly, Arg\textsuperscript{239} of MPO is found at a position roughly equivalent to that of Asn\textsuperscript{70} of HRP, a residue that is known to interact directly with His\textsuperscript{42} to increase the basicity of the latter. Arg\textsuperscript{239} of HRP was found at the same spatial location as Glu\textsuperscript{64} of MPO, one of the key residues in the active site hydrogen bond network of MPO (1). The chain of hydrogen bonds leading from the distal histidines is quite different in the two; in MPO, His\textsuperscript{95} interacts via a water molecule with Asp\textsuperscript{237} and His\textsuperscript{250}, whereas in HRP, His\textsuperscript{42} forms a hydrogen bond with Asn\textsuperscript{70}, which in turn interacts with Glu\textsuperscript{64} (25). Finally, the location of the distal calcium binding sites was strikingly similar in the two enzymes (data not shown).

We surveyed the residues in HRP closest to the methyl heme substituents in the superimposed MPO structure, reasoning that a covalent ester link might be formed between any of them if it were correctly located relative to a protein carboxylate group. We limited our search to the distal cavity, as the covalent heme-protein links in the mammalian peroxidases appear to be located on this side of the heme. The closest residue lying under the 1-methyl is Gln\textsuperscript{91} of MPO, one of the key residues in the active site hydrogen bond network of MPO (1). The chain of hydrogen bonds leading from the distal histidines is quite different in the two; in MPO, His\textsuperscript{95} interacts via a water molecule with Asp\textsuperscript{237} and His\textsuperscript{250}, whereas in HRP, His\textsuperscript{42} forms a hydrogen bond with Asn\textsuperscript{70}, which in turn interacts with Glu\textsuperscript{64} (25). Finally, the location of the distal calcium binding sites was strikingly similar in the two enzymes (data not shown).

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H2O2 results in covalent attachment of the heme to the protein.

The commercial enzyme is included for comparison. CT1 and CT2, charge transfer bands 1 and 2; SA, specific activity in micromoles of ABTS/min/μmol of enzyme; NA, not applicable; ND, not determined. Yields are in mg/liter of culture.

| Yield | Soret | ε | CT1 | CT2 | RZ | SA |
|-------|-------|---|-----|-----|----|----|
| mg/liter | nm | nm | nm | nm |
| Commercial | NA | 402 | 102a | 498 | 643 | 3.2 | 15.2 |
| Wild-type | 5 | 402 | 105 | 498 | 638 | 2.9 | 18.7 |
| L37E | 0.02 | 404 | 50b | 498 | ND | 0.5 | 7.5 |
| F41E | 2 | 406 | 170 | 498 | 630 | 4.3 | 6.1 |
| S73E | 5 | 404 | 100 | 498 | 637 | 2.8 | 11.5 |

a From Ref. 15.
b The protein was obtained in too low a yield to accurately determine the absorption value.

c The protein cross-linking under oxidative conditions, the enzymes were treated with 6 × 2 equivalents of H2O2. Analysis of the resulting proteins by SDS-PAGE (Fig. 5) shows that reaction with H2O2 did not alter their electrophoretic mobility and did not cause significant degradation of the protein.

The catalytic properties of the commercial and wild-type enzymes were not significantly modified by H2O2 treatment, but the specific activity of the F41E mutant increased 10-fold, whereas the S73E mutant lost approximately 50% of its activity (Table II). The UV-Vis spectra revealed no significant changes in the commercial or wild-type enzymes, but the F41E and S73E mutants lost 40 and 20%, respectively, of their total heme content (data not shown). Furthermore, the Soret band of the F41E mutant shifted from 406 to 409 nm as a result of the exposure to H2O2, whereas that of the S73E mutant shifted from 404 to 406 nm (data not shown).

Finally, the H2O2-treated enzymes were analyzed by HPLC to determine whether covalent bond formation had occurred. Control experiments showed that neither the commercial nor the recombinant enzyme underwent any significant cross-linking between the heme and protein (Fig. 6, a and b). For the F41E mutant, the heme absorbance was found to co-elute with the protein peak of the L37E, F41E, and S73E mutants. The origin of this phenomenon remains unclear, and we decided to treat both samples as if they were homogeneous. In the case of the wild type, because there was no apparent change in the enzyme properties upon treatment, this was probably justified. In the case of the F41E mutant, it appears from the chromatogram in Fig. 7c that the second peak is associated with 400 nm absorption. The first peak therefore represents heme-free, inactive enzyme, as virtually no free heme is detected. Allowance...
was made for this by calculating the enzyme concentration from the absorption at 400 nm.

Analysis of the Porphyrins in the F41E Mutant—To examine the type and number of bonds between the enzyme and the porphyrin, the F41E mutant was subjected to complete proteolysis. Preliminary experiments showed that it was necessary to add EDTA to partially denature the protein to achieve efficient digestion of the HRP mutant by Pronase. The digestion medium was injected into the HPLC (Fig. 7a), and the elution time of the new heme-like peak compared with those of authentic 1-hydroxy, 5-hydroxy, and 8-hydroxymethyl heme (2, 19). None of these standards co-eluted with the porphyrin derivative isolated here (data not shown). Nevertheless, LC/MS of the new product (Fig. 7b), gave a mass of 632.2 amu, as expected for a monohydroxylated heme. Its absorption spectrum, HPLC properties, mass spectrum, and nonidentity with the three available hydroxymethylheme standards identifies it as the fourth possible hydroxymethyl heme isomer, i.e., 3-hydroxyethyl heme. Thus, the enzyme and the porphyrin in the F41E mutant are linked via an ester bond to the 3-methyl, as predicted by our original modeling studies.

Analysis of the Porphyrins in the S73E Mutant—Even though a heme-protein covalent bond was not formed with this mutant, a more polar species absorbing strongly at 400 nm was formed in its reaction with H2O2 (Fig. 6d). The compound eluting at 6.7 min in Fig. 6d was isolated from a large incubation of the S73E mutant with H2O2 (Fig. 8a), and, upon co-injection with 1-hydroxy-, 3-hydroxy-, and 5-hydroxyheme was shown to co-elute with the 8-hydroxy derivative (data not shown). LC/MS gave a mass of 632.1 amu for the new product (Fig. 8b), consistent with its identification as 8-hydroxymethylheme. Interestingly, although the predicted formation of a covalent bond to the 8-methyl group was not observed, the carboxylate in the mutant nevertheless triggered a partial hydroxylation of that position.

Formation of the 8-hydroxymethylheme derivative but not of a heme-protein covalent bond could arise from competition of a water molecule with the protein carboxylate for the postulated 8-methylene carbocation intermediate in the bond-forming reaction. To test this hypothesis, the experiment was repeated with an enzyme that had been extensively pre-equilibrated in buffer made with 18O-labeled water. The 8-hydroxymethylheme peak at 6.7 min was obtained as usual on treatment of the enzyme in the same 18O-labeled buffer with H2O2. The compound in this instance gave a mass of 634 amu, establishing that the new oxygen atom derived from the bulk medium (Fig. 9a). As an internal control, the unmodified heme present in the same sample was analyzed by ESI mass spectrometry and was found to give the expected mass of 616 amu, confirming that the increased mass of the new product was not simply because of exchange of the carboxylate groups of the heme with the medium (Fig. 9b).

Influence of the Number of H2O2 Equivalents—It has been shown in recombinant LPO that the extent of bond formation is directly related to the amount of H2O2 that is added (2). To explore the relationship of heme modification to H2O2 equivalents in the HRP mutants, the area of the 400 nm peak co-eluting with the protein was measured by HPLC for the F41E

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TABLE II

Comparison of specific activities of HRP and its mutants before and after H2O2 treatment

|          | Commercial | Wild-type | F41E | S73E |
|----------|------------|-----------|------|------|
| Before H2O2 | 1390       | 1490      | 51   | 365  |
| After H2O2  | 1560       | 1380      | 477  | 182  |
hydroxylated heme obtained with the S73E mutant does not rise above 9–10%. The majority of the heme in this latter mutant remains unmodified.

As indicated by the original spectroscopic studies (Table II), there is some net heme loss with both mutants and this loss increases as the H$_2$O$_2$ concentration increases. We attribute this phenomenon to bleaching, i.e., destruction, of the heme because of increased sensitivity of the mutant proteins to H$_2$O$_2$.

Influence on the Elementary Rate Constants—The heme moiety of peroxidases oscillates between three different oxidation states as it traverses the catalytic sequence. The order of the rates of formation of the different states in HRP is usually compound I to compound II to resting state (15). Even though compound I and compound II of HRP have similar redox potentials, compound II is reduced more slowly than compound I through the arginine at a site that corresponds to a key auxiliary residue hydrogen-bonded to the distal histidine, $k_2$ values for guaiacol oxidation are within the same order of magnitude as the published value for the wild-type enzyme (25, 32), although the value for the commercial enzyme measured here was 10–30% lower than that in the literature. The values recorded for the wild-type enzyme before and after treatment are consistent with the ones obtained for the commercial enzyme, and show no significant influence of the treatment with peroxide. In contrast, the F41E mutant gave significantly higher values before (50% higher than the wild type) and after (160% higher) treatment with peroxide. Unexpectedly, in the case of the F41E mutant after treatment with H$_2$O$_2$, no red shift was observed upon mixing compound I with varying concentrations of guaiacol. Instead, we simply observed a gradual increase of the absorbance of the peak, giving the appearance of direct reversion to the resting state. The use of a slower reducing substrate, p-aminobenzoic acid, did not alter this behavior. We therefore undertook the calculations assuming that the compound II of this enzyme has a spectrum indistinguishable from that of the resting state under the conditions employed. The S73E mutant for its part gave values approximately 3 times lower than the wild type, and this was not significantly changed by the oxidative treatment. The instability of compound II precludes the measurements of $k_3$ by stopped flow kinetics under the conditions used, so we tried to obtain $k_3$ values through steady state kinetic measurements as described elsewhere (25, 32, 33). Unfortunately, in our hands the model did not permit the calculation of reliable values by this method. Further work will therefore be necessary to obtain these values by the pH jump technique (34, 35).

**DISCUSSION**

Superposition of the structure of MPO with that of HRP revealed significant similarities in their three-dimensional structures in the immediate vicinity of the heme despite the fact that they belong to different superfamilies and share little sequence identity. Five helices or helical segments and one loop were found at spatially equivalent positions. More interestingly, both catalytic histidines and the proximal carboxyl side chain responsible for increasing the proximal histidine basicity were found at almost identical spatial positions. The MPO catalytic arginine was located at a position where HRP has a key auxiliary residue hydrogen-bonded to the distal histidine, and the HRP arginine at a site that corresponds to a key
component of the MPO active site hydrogen-bonding network. As far as the heme is concerned, the general plane of the tetrapyrrolic assembly is conserved, but not the positions of the heme substituents. This conservation of active site structure in MPO and HRP validates our use of superposition in identifying potential HRP residues to mutate in efforts to introduce a heme-protein covalent bond.

Four positions were thus identified, which, if mutated to a carboxyl-bearing amino acid, might result in covalent bonding of the heme to the protein: Leu37, Phe41, Gly69, and Ser73. From these, only Phe41 and Ser73 yielded mutants that could be isolated and studied, as the mutations at the other two sites did not yield detectable proteins. The failed mutants show, as might be expected, that the introduction of a negatively charged carboxylic acid residue into the active site can cause serious structural perturbations. The introduction of a carboxylate into the active site of HRP has been achieved earlier for other purposes, notably the substitution of His42 by a glutamate in efforts to prepare a chloroperoxidase mimic (29, 32, 36) or to disrupt the active site hydrogen-bonding network associated with a bound benzenedimac acid molecule (37). Asn70, which interacts with His42, has also been successfully replaced by a glutamate (17, 38, 39). The H42E mutation decreased the catalytic activity 300-fold (32) and the N70D mutation 2–6-fold (17). Finally, a glutamate has been introduced at position 143, in the postulated aromatic reductant binding site, leading to an enzyme with reduced activity (40).

Mutagenesis of Ser73 has not been reported, but Phe41 has been replaced by a variety of residues: glycine (41), alanine (16, 31, 41, 42), valine (30, 31, 41), leucine (41, 43), threonine (43), tyrosine (41), histidine (40, 42), and tryptophan (31, 41). Indeed, Phe41 has emerged as a key residue in HRP. In all the sequence-related plant peroxidases (24), an aromatic side chain is found at the corresponding position: a tryptophan in cytochrome c peroxidase and ascorbate peroxidase and a phenylalanine in the others. Phe41 restricts access of reducing substrates to the oxoferryl moiety and thus limits the peroxygenase activity of the enzyme (19, 27). We examined the ability of our mutants to epoxidize styrene as a test for peroxygenase activity (43), but in none of the variants, before or after H2O2 treatment, could we detect any styrene oxide formation (data not shown). Replacement of Phe41 by a glutamate therefore does not increase access to the ferryl oxygen. This aromatic position governs the resting state spin state in both cytochrome c peroxidase and A. ramosus peroxidase and the stability of compound I in cytochrome c peroxidase. A red shift of the HRP Soret band and an increase in its molar absorption coefficient have been observed when Phe41 is mutated to a Val or Ala (30, 31, 37). This shift has been attributed to an increase in 6-coordinate high spin character at the expense of the 5-coordinate high spin character of the wild-type enzyme. Our observations for the F41E mutant are consistent with the literature; introducing a glutamate at position 41 sharpens the Soret band, shifts it by 3 nm, and increases its molar absorption coefficient. Introducing a glutamate at position 73, further away from both the heme and the iron, only causes minor changes in the UV-visible spectrum.

Not surprisingly, the mutations alter the catalytic properties of the enzyme. Under steady state conditions, the mutant enzymes as isolated from the insect cell cultures exhibited a moderately reduced ABTS oxidizing activity (5 and 2 times lower for F41E and S73E, respectively), but the influence on the guaiacol oxidation activity was stronger (Table II, before H2O2). This difference in sensitivity between ABTS and guaiacol has been attributed to the existence of additional limiting association/dissociation steps in the case of ABTS (30). However, we found a much stronger decrease of the guaiacol oxidation rates, by a factor of 100 rather than 8, which was paralleled by a similar reduction of the rate of compound I formation (see below). In contrast, the mutation at position 73 only modestly reduced the specific activity of the enzyme despite the fact that Ser73 has been postulated to interact with Arg58 and one of the heme propionates (29).

Analysis of the individual catalytic steps is informative. In the case of the F41E mutant, a decrease by a factor of 100 of the second order rate constant for the formation of compound I is observed, whereas in the case of the S73E mutant the decrease is only 2–3-fold. After preincubation with H2O2, the velocity constant for oxidation of F41E to compound I increases greatly, reaching a value in the same range as that for the wild type. In contrast, the value for the S73E mutant is barely changed. The rates for the single electron reduction of compound I by guaiacol are less affected by the mutations, because the measured variations among all the recombinant enzymes do not exceed a factor of 3. These results tend to show that the major change brought about by the substitutions is a decrease of the rate of reaction with H2O2, whereas the subsequent reduction of the oxidized states is relatively unaffected. In the case of the F41E mutant, one could envision either an ionic or steric interaction between the distal histidine at position 42 and the glutamate at position 41. In the case of the H2O2 pretreated F41E mutant, we were unable to detect the characteristic red shift upon conversion of compound I to compound II. The two obvious possibilities are that either no compound II forms, or it forms but has spectral properties that cannot be differentiated from those of the resting state. The first possibility seems unlikely, however, as it implies simultaneous reduction of the two oxidizing equivalents of compound I. These reducing equivalents would have to come from two different sources, either two substrate molecules or one substrate molecule and the protein itself. Because in the absence of added reductant, compound I of the H2O2-treated F41E mutant is stable (data not shown), the second hypothesis requires the improbable assumption that reaction with guaiacol (or p-aminobenzoic acid) triggers simultaneous oxidation of the protein. We have therefore proceeded on the assumption that compound II of the H2O2-treated F41E mutant has spectral characteristics indistinguishable from those of the resting state. In the case of the S73E mutant, the glutamate side chain is further away from the iron center and its perturbations of the catalytic cycle are understandably less pronounced.

The most important result of this work, however, is the finding that both mutants differ from the wild-type and native enzymes in their reactions with H2O2. In the absence of any other factor, the F41E mutant underwent nearly quantitative covalent linking of its heme 3-methyl group to the protein.

| TABLE III | k1 and k2 are expressed in 106 M–1 s–1. |
|-----------|---------------------------------|
| Commercial | Wild-type | F41E | S73E |
| k1         | Before   | 14.4 | 16.1 | 17.7 | 16.9 | 0.16 | 9.88 |
| k2         | After    | 1.02 | 1.13 | 2.51 | 2.36 | 3.76 | 6.18 |

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presumably via Glu41, and the S73E mutant partially hydroxylated the 8-methyl substituent of its prosthetic heme group. Thus, as in the mammalian peroxidases, heme covalent binding occurs via an autocatalytic mechanism (2, 11, 12). Both of these modifications most likely arise via a common mechanism (12). After formation of compound I, a carbocation is formed either by transient oxidation of the carboxylate and subsequent hydrogen abstraction from the methyl, or via direct deprotonation of the methyl acidified by the porphyrin radical cation (11). The resulting cation is then quenched by a nearby nucleophile, either the carboxylate anion or a nearby molecule of water (Fig. 11). In these mechanisms, it is the very presence of the carboxylate that allows the transfer of oxidizing equivalents from the iron and the π-system to the methyl substituent. In the F41E mutant, for which modeling predicted a distance of 2.5 Å between the glutamate oxygen and the 3-methyl carbon, quenching gives the ester. In the S73E mutant, where the distance to the 8-methyl carbon was predicted to be ~5 Å, the carboxylate is unable to compete with an active site water molecule in trapping of the cation, so that hydroxylation of the heme rather than covalent binding to the protein is observed. In accord with this postulated mechanism for the formation of 8-hydroxymethylheme, the hydroxyl oxygen is shown by incubation in 18O-labeled water to derive from the solvent. The same type of competition between a carboxylate and a water molecule was postulated in the cytochrome P450 system (3, 5) and was later confirmed for CYP4B1 (6). It is to be noted that the heterogeneity of heme species in the S73E mutant after H2O2 treatment does not appear to affect its catalytic properties. This is consistent with the previous finding that HRP with an 8-hydroxymethylheme group has the same catalytic properties of the enzyme with a conventional heme group (44). Fi-

**Fig. 11.** Radical mechanism, starting with the compound I obtained by reaction with H2O2, proposed to explain autocatalytic covalent binding of the glutamic acid to the 3-methyl group in the F41E HRP mutant. The structure of the 8-hydroxyheme formed with the S73E mutant because of effective competition of water for trapping of the carbocation analogous to that shown for the F41E mutant is shown on the bottom far right.
nally, we undertook the same type of $H_2O_2$ treatment in the presence of guaiacol, reasoning that if an oxidized form of the enzyme is indeed needed for the iron porphyrin to be modified, then a competition between porphyrin and guaiacol oxidation should take place. Indeed, we found that 20 equivalents of guaiacol were enough to completely inhibit covalent linking of the heme to the protein during the $H_2O_2$ pretreatment (data not shown). This finding emphasizes the close similarities between the bond-forming process (porphyrin oxidation) and the normal catalytic cycle (exogenous substrate oxidation).

Perhaps the most important conclusion of this study is that all that is needed for the formation of a protein-heme covalent link is the proper juxtaposition of a carboxylate side chain and a heme methyl group within the hemoprotein active site. Previous data suggested this conclusion, but in those instances the carboxyl residue was introduced into an active site that was very closely related in sequence to that of the reference protein, for example, in the CYP4F5 G330E mutant (5). In the case of MPO and HRP, there is no sequence identity in the active site region even though both proteins employ similar iron ligands for the heme to the protein during the $H_2O_2$ pretreatment (data not shown).

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Horseradish Peroxidase Mutants That Autocatalytically Modify Their Prosthetic Heme Group: INSIGHTS INTO MAMMALIAN PEROXIDASE HEME-PROTEIN COVALENT BONDS

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