Horseradish Peroxidase Phe^{172} → Tyr Mutant

SEQUENTIAL FORMATION OF COMPOUND I WITH A PORPHYRIN RADICAL CATION AND A PROTEIN RADICAL*

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A gene coding for the F172Y mutant of horseradish peroxidase isozyme C (HRP) has been constructed and expressed in both *Spodoptera frugiperda* (SF-9) and *Trichoplusia ni* egg cell homogenate (HighFive®) cells. Homology modeling with respect to three peroxidases for which crystal structures are available places Phe^{172} on the proximal side of the heme in the vicinity of porphyrin pyrrole ring C. The pH optimum and spectroscopic properties of the F172Y mutant are essentially identical to those of wild type HRP. V_{max} values show that the mutant protein retains most of the guaiacol oxidizing activity. Stopped flow studies indicate that Compound I is formed with H_2O_2 at the same rate ($k_1 = 1.6 \times 10^7$ M^{-1} s^{-1}) at both pH 6.0 and 8.0 as it is with the wild type enzyme. This Compound I species decays rapidly at a rate $k_2 = 1.01$ s^{-1}, pH 7.0, to a second two-electron oxidized species that retains the ferryl (Fe^{IV} = O) absorption. EPR studies establish that a ferryl porphyrin radical cation is present in the initial Compound I, but electron transfer from the protein results in formation of a second Compound I species with an unpaired electron on the protein (presumably on Tyr^{172}). The presence or absence of oxidizable amino acids adjacent to the heme is thus a key determinant of whether the second oxidation equivalent in Compound I is found as a porphyrin or protein radical cation.

Hemoprotein peroxidases catalyze the H_2O_2-dependent one-electron oxidation of proteins and/or small organic or inorganic molecules (1, 2). Horseradish peroxidase isozyme C (HRP)[1] catalyzes the oxidation of small substrates to free radical products, most notably the conversion of phenols to phenoxy radicals (3). In contrast, cytochrome *c* peroxidase (CcP) only slowly oxidizes small molecules but very efficiently oxidizes cytochrome *c*, a 12-kDa protein that is its natural substrate (4, 5). The catalytic mechanisms of these two prototypical peroxidases have been extensively studied, and the mechanistic understanding thus obtained provides the foundation for current views on the structure and function of all hemoprotein peroxidases, including myeloperoxidase (6), eosinophil peroxidase (7), and thyroid peroxidase (8).

The first step of the normal catalytic sequence is reaction of the heme prosthetic group of the peroxidase with H_2O_2 to give a two-electron oxidized species known as Compound I (2, 3). Compound I is reduced to Compound II by a substrate-derived electron and to the resting ferric state by a second electron. All known hemoprotein peroxidases have a common Compound II structure in which the single oxidation equivalent is stored as a ferryl (Fe^{IV} = O) species (1, 2). The same ferryl moiety is present in Compound I, but there are two possible sites for the location of the second oxidation equivalent. Diverse spectroscopic and physical measurements, including magnetic circular dichroism, electron nuclear double resonance, magnetic susceptibility, and resonance Raman spectroscopy, clearly establish that the second oxidation equivalent in Compound I of HRP is stored as a porphyrin radical cation (2). The HRP Compound I structure therefore can be represented as [Por$^+$·Fe^{IV} = O]. In contrast, the second oxidation equivalent in the case of CcP is stored as an unpaired electron on a tryptophan residue (Trp^{191}) located on the proximal side of the heme (9). The CcP Compound I structure therefore can be represented as [Prot$^+$·Fe^{IV} = O]. Some of the structural features that promote the formation of a protein radical in CcP have been determined (10–12), but the factors that stabilize a porphyrin radical cation in HRP and other peroxidases with respect to oxidation of nearby residues remain obscure.

A high resolution crystal structure is not available for HRP but such structures are available for CcP (13), LiP (14, 15), and the peroxidase from *Arthromyces rhamosus* (or *Coprinus cinereus*) (16, 17). However, alignment of the highly conserved sequences of HRP and the three crystalline peroxidases suggests that the structure of HRP is very similar to that of the other peroxidases (13–19). These alignments have tentatively identified the proximal (His^{170}) and distal (His^{42}) histidines and the arginine (Arg^{38}) of HRP suggested by the structures of the crystalline peroxidases (13–17) to be involved in the formation and stabilization of Compounds I and II. Sequence alignments have also been used, in conjunction with NMR data, to tentatively identify residues in the vicinity of the heme group. An aromatic residue adjacent to the 1- and 8-methyl groups of the heme was initially identified by such arguments as Tyr^{185} (20, 21), but more recent NMR studies suggest that the residue is a phenylalanine rather than a tyrosine (22, 23).[2]

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[1] The abbreviations used are: HRP, horseradish peroxidase isozyme C; CcP, cytochrome *c* peroxidase; LiP, lignin peroxidase; heme, iron protoporphyrin IX regardless of oxidation or ligation state; ee, enantiomeric excess.

[2] A gene coding for the Y185F mutant of HRP has been constructed and expressed in the baculovirus/*S. frugiperda* system described here and has been found to have spectroscopic and catalytic properties indistinguishable from those of the wild type enzyme (V. Miller and P. R. Ortiz de Montellano, unpublished results).

Protein Radical in Horseradish Peroxidase F172Y

We have expressed a synthetic HRP gene in a baculovirus/insect cell system and are carrying out site-specific mutagenesis studies of the structure and function of this enzyme (24–26). We demonstrate here that reaction of H_2O_2 with the F172Y mutant of HRP, in which a tyrosine has been introduced into the structure near the heme group, initially yields a normal Compound I intermediate. The porphyrin radical cation of this Compound I, however, oxidizes the protein to give a CcP-like Compound I in which the second oxidation equivalent is present as a protein radical. The results demonstrate that one of the parameters that determine whether the second oxidation equivalent is on the porphyrin or the protein is the presence or absence of oxidizable residues near the heme.

EXPERIMENTAL PROCEDURES

Materials—Guaiacol was purchased from Sigma. HRP (grade 1) was from Boehringer Mannheim. Restriction enzymes were purchased from New England Biolabs (Beverley, MA) or Boehringer Mannheim. S Fast Flow and Q Fast Flow resins were from Pharmacia. Oligonucleotides were synthesized at the University of California, San Francisco, Biomolecular Resource Center using an Applied Biosystems 380B DNA synthesizer. Heat-inactivated fetal serum, penicillin, streptomycin, and *Escherichia coli* strain DH5α were obtained from the University of California, San Francisco, Cell Culture Facility. DNA miniprep columns were from Promega (Madison, WI). All insect cell culture media contained 100 μg/ml streptomycin sulfate and 100 units/ml penicillin "G." Guaiacol peroxidation and thioanisole sulfoxidation activities were assayed as reported elsewhere (26).

Construction of the F172Y HRP Gene—The F172Y mutant gene was constructed by cassette mutagenesis between the *Bsp*EI and *Bst*EII restriction sites of a synthetic HRP gene (British Biotechnologies, Ltd.). The cassette sequence was identical to the gene sequence except it contained the tyrosine codon TAC at amino acid position 172:

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5'-CC GGA GGA CAC ACA TAC GGA AAG AAC CAG TGT AGG TTC ATC ATG GAT
       T CCT GTG TGT ATG CCT TTC TTG GTC ACA TCC AAG TAG TAC CTA

AGG CTC TAC AAT TTC AGC AAC ACT GG-3'
TCC GAG ATG TTA AAG TCG TTG TGA CCC AAT G
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An equal molar mixture of oligonucleotides (55 pmol) in ligation buffer (66 mM Tris-HCl, pH 7.6, 6.6 mM MgCl$_2$, 10 mM dithiothreitol, and 0.4 mM ATP) was annealed by heating at 100 °C for 3 min and cooling slowly to room temperature over a period of 1 h. The plasmid pUC-HRP containing the synthetic gene cloned into pUC19 was digested with *Bsp*EI and *Bst*EII, gel purified, and ligated with the annealed cassette. The ligation mixture was used to transform *E. coli* strain DH5α to ampicillin resistance. Positive clones were screened by restriction digests and verified by DNA sequencing. A 762-base pair *SacI/NcoI* fragment of the HRP gene containing the mutation was then exchanged into the baculovirus vector pVLHRP2 (24) which contains the same HRP gene as well as the HRP 5'-leader sequence. Positive clones were identified by restriction digestion. The purified plasmid DNA from one clone (pVLHRPF172Y) was used for HRP gene incorporation into baculovirus.

Production of Recombinant Virus—Recombinant virus was generated in Sf9 cells growing in Hink's TNM-FH insect tissue culture media (JRH Biosciences, Lenexa, KA) containing 10% heat-inactivated fetal calf serum using the BaculoGold® transfection kit (Pharmingen, San Diego). Plaque-purified virus was used to infect cell monolayers in 3 ml of media. After 3 days the medium was removed and used as first passage virus stock (pass 1 stock). Genomic viral DNA from 0.75 ml of this media was screened for the presence of HRP gene insertion using polymerase chain reaction procedures and primers from Invitrogen. HRP gene insertion was verified by sequencing the polymerase chain reaction-amplified fragment. A 400-μl aliquot of pass 1 stock was then used to infect 1×10^8 cells in a 100-ml culture. After 4 days of growth, the medium was harvested and used for large scale expression after the viral titer had been determined.

Purification of F172Y HRP—Recombinant enzyme was expressed using *Trichoplusia ni* egg homogenate (HighFive®) cells (Invitrogen) growing in shaker flasks at 27 °C with Sf-900 II SFM serum-free insect cell media (Life Technologies Inc.). Pass 2 stock was added to 300 ml of *T. ni* cells (~2×10^6 cells/ml) in a Fernbach culture flask to give a multiplicity of infection of 5. After 1 h at 27 °C the volume was increased to 800 ml with serum-free media. Two h later, 10 ml of a 500 μM hemin solution was added to the expression flask. The hemin solution

was made by dissolving 3.3 mg of hemin in 10 ml of 10 mM NaOH. Immediately after the hemin was dissolved, the solution was carefully neutralized with a few drops of 0.5 N HCl. The hemin solution was filter sterilized before addition to the expression flask. Three days post-infection another 10 ml of hemin solution was added to the expression flask.

The media was harvested 4–6 days post-infection. Following removal of the cells by centrifugation, the media was concentrated to ~50 ml using a spiral channel ultrafiltration apparatus (YM-10 membrane, Amicon, Beverley, MA). The crude concentrated media was dialyzed against 10 mM NaOAc, pH 5.0, and applied to a column of S Fast Flow (1.5 × 40 cm) equilibrated with 10 mM NaOAc, pH 5.0, buffer. The column was washed with the same buffer (50 ml) and then eluted with 20 mM NaCl in NaOAc buffer. The fractions containing peroxidase activity were pooled and dialyzed against 10 mM Tris, 5 mM CaCl$_2$, pH 7.5, buffer. The protein was then washed through a small column (1.5 × 2 cm) of Q Fast Flow. HRP does not bind to anion-exchange media under these conditions, but the remaining impurities bound tightly. The pure enzyme was stored at −70 °C and lyophilized when higher concentrations of enzyme were desired.

Stopped Flow Kinetics—Stopped-flow experiments were performed on an Applied Photophysics Sequential DX-17 MV stopped-flow instrument. The sample handling unit was fitted with two drive syringes mounted inside a thermostated-bath compartment to allow for variable temperature experimentation. A 1.0-cm path-length cell was employed. First- and second-order curve fitting and rate constants were calculated from the average of at least five traces, using a Marquardt algorithm based on a routine "curfit" (27, 28). The absorption spectra at indicated time points were calculated with software provided by Applied Photophysics. This consisted of slicing the appropriate time points across a series of kinetic traces at different wavelengths and then splining the points of a specific time group (29). The concentrations of HRP and the F172Y HRP mutant in these studies were between 1×10^{-6} and 5×10^{-7} M. The concentrations were calculated assuming the molar absorptivity for the F172Y mutant is the same as that of native HRP ($\epsilon = 1.02 \times 10^5$ M^{-1} s^{-1}). The concentration of H_2O_2 was either 10-fold that of either HRP or F172Y HRP for pseudo-first-order conditions (*i.e.* k_1 for HRP → Compound I), or the concentration was matched 1:1 to H_2O_2-enzyme for second-order experiments for k_1 and the subsequent measurement of k_2 (*i.e.* for the decay of Compound I). All experiments involving the formation of Compound I were followed at 402 nm, the largest absorbance change for this transition. All experiments involving the conversion of Compound I to final product were followed at 420 nm (greatest ΔA). All solutions contained 100 mM KCl buffered at a concentration of 25 mM with either acetate, <pH 6, phosphate, pH 6–8, or Tris-OH, >pH 8. To solutions at pH 5.47, 7.1, and 8.56, Ca^{2+} (5.0 mM) was added to ensure that the calcium sites were filled.

EPR Spectra—A 1.3-fold molar excess of H_2O_2 was added to solutions of either HRP (313 μM) or F172Y HRP (329 μM) in 25 mM Tris buffer, pH 7.0, containing 100 mM KCl and, for some experiments, 5 mM Ca^{2+}. Within less than 30 s, the samples were transferred to EPR tubes and were quickly frozen at 77 K. After the EPR spectra were recorded, the samples were quickly thawed to 298 K, allowed to stand for 5 min, and refrozen to 77 K before again recording their EPR spectra. Finally, the samples were thawed to 298 K for 30 min before refreezing and recording the final set of EPR spectra. EPR spectra were recorded in 3 mm O.D. quartz tubes on a Bruker ESP300 spectrometer at X-band using 100 KHz field modulation. Low temperature control was achieved by an Air Products LTR-3 Helitran liquid helium transfer line and cryostat. Temperatures below 4 K were achieved by maintaining a measured vacuum above the helium flow. Background spectra on blank samples collected under identical conditions were subtracted from each spectrum. EPR simulations were performed using the program qpowder (30) on a 486/66 DX2 personal computer.

RESULTS

Alignment of the HRP amino acid sequence with the sequences of three peroxidases for which crystal structures are available indicates that Phe^{172} corresponds to Leu^{176} in CcP, Val^{178} in LiP, and Leu^{185} in the *A. rhamosus* peroxidase (31). These residues are located in all three crystal structures on the proximal side of the heme close to pyrrole ring C (Fig. 1) (13–17). The sequence and structural relationships between HRP and the three crystalline peroxidases suggest that Phe^{172} occupies a similar site in the HRP structure. We have therefore chosen to replace Phe^{172} with a tyrosine residue to determine the consequences of placing a structurally similar but more

oxidizable residue close to the heme group. It should be noted that the residue equivalent to Phe[172] in the peroxidase crystal structures does not interact directly with the proximal histidine iron ligand or with its hydrogen bonding network, so that a mutation of Phe[172] should not directly influence the axial ligand-heme interaction (13–17).

Expression and Kinetic Characterization of the F172Y HRP Mutant—A gene coding for the F172Y HRP mutant was constructed by cassette mutagenesis between the *Bsp*EI and *Bst*EII restriction sites of a commercially available HRP gene. The cassette sequence was identical to the gene sequence except for substitution of the tyrosine codon TAC for the phenylalanine codon at position 172. A 762-base pair *Sac*I/*Nco*I fragment of the HRP gene encompassing the mutated region was then placed into the previously constructed baculovirus vector pVLHRP2 (24). This vector contains the HRP gene and a 5'-leader sequence that directs excretion of the protein into the medium. The mutated vector was incorporated into baculovirus and was then used to express the F172Y HRP protein. Viral stocks were obtained by expressing the mutant baculovirus in Sf9 cells, but large scale protein expression was done in shaker flasks with HighFive® cells. The use of serum-free media in the expression system simplified protein purification.

The protein was purified to homogeneity from baculovirus infected HighFive® cell culture media by ion exchange chromatography (Table I). The concentrated culture medium was applied to a long thin column of the cation-exchanger S Fast Flow at pH 5.0. Although binding of the protein to the column is not optimal at this pH, F172Y HRP denatures at lower pH values.
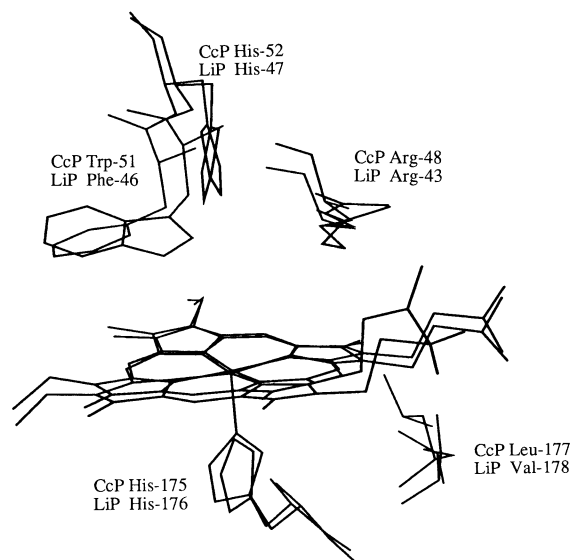

FIG. 1. **Superposition of key active site residues in the crystal structures of CcP and LiP.** The catalytic histidine (CcP, His[52]; LiP, His[47]) and arginine (CcP, Arg[48]; LiP, Arg[43]) and the adjacent aromatic residue (CcP, Trp[51]; LiP, Phe[46]) are on the distal side above the heme plane. The proximal histidine iron ligands (CcP, His[175]; LiP, His[176]) are below the heme plane, as are Leu[177] of CcP and Val[178] of LiP, the residues that correspond to Phe[172] of HRP (14). These latter residues are off the edge of, and below, heme pyrrole ring C.

Following elution with a small amount of NaCl (20 mM), the active fractions were combined, equilibrated into pH 7.0 Tris buffer, and passed through a plug of the anion-exchanger Q Fast Flow. HRP does not bind to anion exchangers at neutral pH, but the remaining impurities bind strongly and are easily removed. Ca^{2+} was added to the neutral Tris buffer to help stabilize the protein (32). The F172Y HRP mutant was thus obtained as a pure protein, as judged by SDS-polyacrylamide gel electrophoresis, in a yield of 15 mg/liter of culture (Fig. 2). The R_z value of the purified protein was 2.9, and its specific activity, determined by guaiacol oxidation, was 86 μmol min^{-1} mg^{-1}. The R_z value suggests that a small fraction of the protein does not contain a heme prosthetic group because the R_z value for pure, native HRP is between 3 and 3.4 (3). The kinetic parameters for the oxidation of guaiacol by recombinant wild type HRP and the F172Y mutant are similar: HRP, $K_m = 7.63$ mM, $V_{max} = 0.71$ nmol s^{-1} pmol^{-1}; F172Y, $K_m = 2.62$ mM, $V_{max} = 0.41$ nmol s^{-1} pmol^{-1}. The protein concentrations used for these experiments were based on the Soret absorbance of the catalytically active enzyme. The V_{max} for the oxidation of guaiacol by the F172Y mutant is thus approximately half that for the wild type enzyme.

pH Profile for Guaiacol Oxidation—Mutations within the active site of HRP may affect the pH optimum for guaiacol oxidation. The pH profile for guaiacol oxidation was therefore examined by determining the activity of the enzyme in buffers of different pH values adjusted to an ionic strength of 0.1 with NaCl (Fig. 3) (33, 34). No difference in pH profile was found between wild type HRP and the F172Y mutant. The pH optimum is approximately 6.4 for both wild type and F172Y HRP.

Thioanisole Sulfoxidation—Stereoselective thioanisole sulfoxidation is catalyzed by native HRP, but the rate and stereoselectivity of the reaction are increased in the F42V mutant (25, 26). The stereospecificity of thioanisole sulfoxidation by F172Y HRP was therefore examined to determine if the mutation alters the topology in the vicinity of the ferryl oxygen. However, the enantiomeric excess obtained with F172Y HRP (ee 76%) is similar to that observed with the wild type enzyme (ee 70%). These results suggest that the distal active site pocket is not significantly altered by the F172Y mutation.

Spectroscopic Properties of the F172Y HRP Mutant—The spectrum of ferric F172Y HRP is identical to those of the native enzyme and the recombinant wild type protein. The same spectroscopic identity characterizes the ferric cyano complexes of native (λ_{max} 362, 422, 538), recombinant wild type (λ_{max} 362, 422, 538), and F172Y HRP (λ_{max} 362, 422, 540).

Formation and Decay of Compound I—Reaction of the F172Y HRP mutant with one equivalent of H_2O_2 initially gives a Compound I spectrum that changes with time to a Compound II-like spectrum (Fig. 4). Similar treatment of native HRP gives a Compound I spectrum that does not decay significantly within 1 h (42).

The rate of Compound I formation for F172Y as determined by stopped flow methods was $k_1 = 1.6 \times 10^7$ M^{-1} s^{-1} at both pH 6 (100 mM phosphate buffer) and pH 8 (50 mM Tris buffer, 100 mM KCl). This value is the same as those reported in the literature for native HRP ($k_1 = 1.0$ to 2.0×10^7 M^{-1} s^{-1})

TABLE I
Purification of F172Y HRP

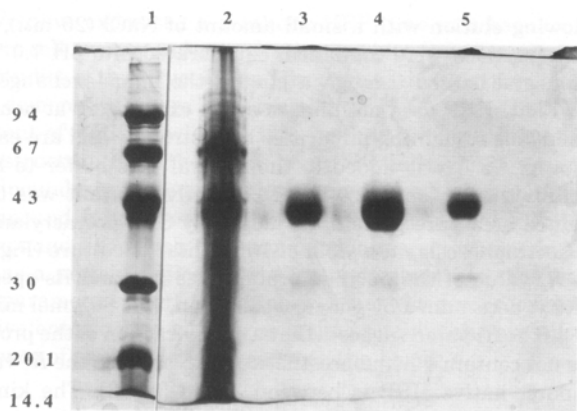| Purification step	Total protein	Specific activity	Total activity	Purification fold	R_z
	mg	*μmol min^{-1} mg^{-1}*	*μmol min^{-1}*		
Concentrated medium (800 ml → 50 ml)	592	3	1538	1	ND[a]
Dialysis and centrifugation	206	7	1442	3	0.3
S-Fast Flow (10 mM NaOAc, pH 5.0)	12	80	919	31	2.5
Q-Fast Flow (10 mM NaPi, pH 6.0)	12	86	990	33	2.9

[a] ND, not done.

FIG. 2. **SDS-polyacrylamide gel electrophoresis (12% gel) analysis of the F172Y HRP mutant purified as reported in Table I.** *Lane 1*, molecular mass standards in kDa; *lane 2*, medium after dialysis and centrifugation; *lane 3*, eluate from S-Fast Flow column; *lane 4*, eluate from Q-Fast Flow column; *lane 5*, native HRP standard.
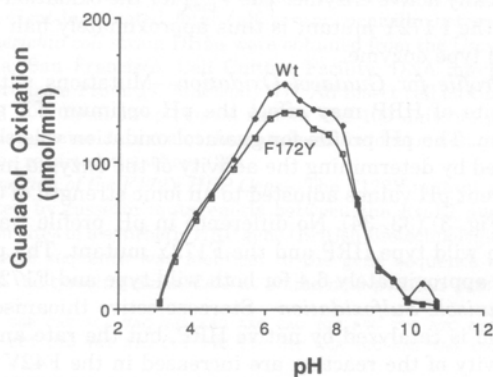

FIG. 3. **pH profile for the oxidation of guaiacol by wild type and F172Y HRP.** Slightly different protein concentrations were used in these assays to clearly differentiate the two sets of data.

(35–37). This was determined using the equation $rate_1 = k_{obs}[HRP]$ where $k_{obs} = k_1[H_2O_2]$ under pseudo-first-order conditions.

In contrast to native HRP which has a stable Compound I,[3] Compound I formed from F172Y decays and the decay exhibits a modest pH dependence (Fig. 5). At pH 7, the rate of decay in the absence of added substrate is 1.01 s^{-1} ([F172Y] = 5.0×10^{-7} M) and, as shown by a linear dependence of $rate_2$ on the concentration of F172Y (not shown), follows the equation $rate_2 = k_2$[F172Y Compound I].

The same value for the rate is obtained in the presence or absence of 5.0 mM Ca^{2+}. This suggests either that the calcium site on the protein is filled even in the absence of added calcium, or that the degree of saturation of this site does not influence the rate of decay of Compound I.

EPR Studies—The decay of the Compound I chromophore in the absence of an external electron donor suggests that the electron required to reduce the ferryl species is provided by the protein. EPR studies have therefore been carried out to determine whether the decay of compound I is linked to the formation of a protein radical. Indeed, a protein radical is detected when H_2O_2 is added to F172Y HRP, and the sample is then rapidly frozen to 10 K (Fig. 6A). Control experiments show that an analogous protein radical signal is not generated when native or wild type recombinant HRP is treated with H_2O_2 under the same conditions (Fig. 6B). The protein radical was

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[3] Compound I formed from native HRP is stable for hours in the absence of reducing agents.

quantitated by spin integration with respect to a known Cu^{2+} standard under conditions that showed no evidence of saturation for either sample. The signal observed 30 s after the addition of H_2O_2 corresponded to 11% of the F172Y mutant protein in the solution. If the sample was warmed to ambient temperature for 5 min and then refrozen, the signal intensity decreased to 6% of the total protein, and decreased slightly to 5% of the protein if the sample was held at ambient temperature for 30 min. To determine whether incomplete saturation of the Ca^{2+} sites in the recombinant protein contributed to radical formation and decay, the same experiment was run in the presence of 5 mM Ca^{2+}. Essentially identical results were obtained with the protein radical observed corresponding to 11, 8, and 6% of the total F172Y HRP protein in the sample.

The narrow signal observed at 6 K for F172Y is consistent with an isolated $S = 1/2$ radical on a tyrosine residue. The lineshape appears isotropic with a g value near 2 as expected for an isolated $\mathbf{S} = 1/2$ spin, and does not show the characteristic broadening that is observed for the porphyrin π-cation radical of HRP Compound I or the Trp[191] radical of CcP Compound I. These broad anisotropic signals arise from exchange coupling of the radical species with the $S = 1$ ferryl center (10). In addition, the narrow signal for F172Y becomes saturated above 50 microwatts at 6 K, indicating that it is not efficiently relaxed by the ferryl center as is observed for the radicals of wild type HRP and CcP. Finally, the narrow signal observed for F172Y exhibits a complex hyperfine structure (Fig. 7A). No evidence for such a signal is observed with samples of recombinant wild type HRP (Fig. 7C), demonstrating that this signal is not an artifact of heterologous protein expression. A simulation of this signal, using the g value and proton hyperfine tensors that have previously been established for tyrosine radicals in proteins (38–40), is shown in Fig. 7B. Such radicals typically exhibit a small g anisotropy and an anisotropic hyperfine coupling to the C_3 and C_5 ring protons that are not expected to vary significantly with sidechain conformation. The great variability in observed tyrosine radical spectra arises from differences in conformation of the aromatic ring about the C_β-C_γ dihedral angle, which dramatically alters the proton hyperfine couplings to the two methylene protons on the C_β carbon. Spectra were simulated for all possible values of this dihedral angle and at several values for ρ_{c1}, the unpaired spin density on the C_1 ring carbon π-orbital. The simulated spectrum in Fig. 7B used a value of $\phi_{c1} = 0.37$, which is that found previously for the Y_D radical of photosystem II (40), the g tensor determined for the *E. coli* ribonucleotide reductase tyrosine radical ($G_{x,y,z} = 2.0091, 2.0046, 2.0023$) (39), and values for the hyperfine coupling tensors to the C_3 and C_5 protons as determined for *E. coli* ribonucleotide reductase (38). The simulation implies a conformation for the tyrosine of HRP F172Y in which the two methylene protons make angles, $\theta_1 = 20°$ and $\theta_2 = 140°$ with the tyrosine ring normal.

At 2.3 K, the porphyrin radical cation of a conventional Compound I structure is observed with both native and F172Y HRP (Fig. 8). The signals are broad, weak, and very temperature dependent and are therefore difficult to quantitate accurately. Nevertheless, the porphyrin radical cation signals are of approximately equal intensity for both native and F172Y HRP, but the porphyrin radical cation of the F172Y mutant appears to decay more rapidly. Very little residual ferric HRP or F172Y HRP is detected by EPR, showing that 1.3 equivalents of H_2O_2 are sufficient to completely oxidize the protein. No more than a slight increase is observed in the ferric EPR signal of either protein during the course of these experiments. The iron in both proteins thus remains in the ferryl state during the 30-min course of the experiment despite the decrease in the por-
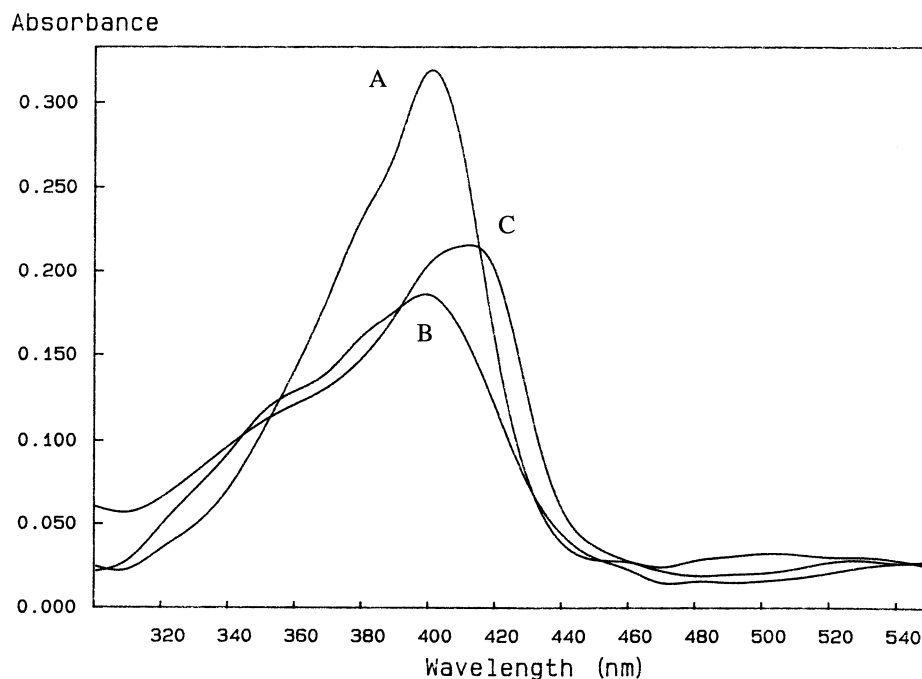
Absorbance

FIG. 4. **Spectra of wild type F172Y HRP at ambient temperature before addition of H_2O_2 (A), immediately after addition of one equivalent of H_2O_2 (B), and 5 min after the addition of one equivalent of H_2O_2 (C).**
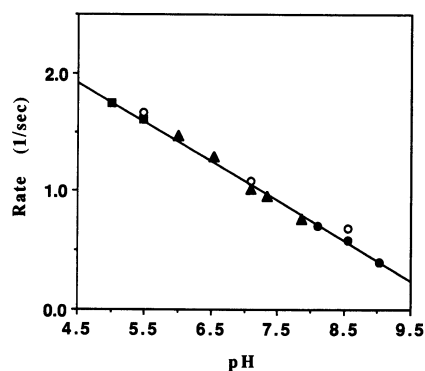

FIG. 5. **Rate of decay of the ferryl porphyrin radical cation (Compound I) of F172Y HRP as a function of pH as determined by stopped flow absorption spectroscopy.** The buffer used to obtain the individual values is indicated: acetate buffer (■), phosphate buffer (▲), and Tris buffer (●). Some values (○) were obtained in buffer used for the other point at the same pH but in the presence of 5 mM Ca^{2+}.

phyrin and protein radical signals observed during this time period. This is consistent with the fact that the ferryl species is observed in the absorption spectrum for more than 2 h.

Protein Cross-linking Studies—The formation of a tyrosine-free radical in the reaction of sperm whale myoglobin with H_2O_2 leads to the formation of protein dimers due to coupling of the tyrosine radicals from two protein molecules (41, 42). Dimer formation accounts, in part, for quenching of the protein radical generated in the reaction. To determine if the tyrosine radical formed from the F172Y mutant gives rise to similar protein-protein cross-linking, the protein was analyzed by SDS-polyacrylamide gel electrophoresis after reaction with H_2O_2. Intermolecular protein cross-linking was not detected in these experiments (not shown). Tyr[172] is thus located within the protein in a site that is sterically or electrostatically protected from interaction with a reactive site in a second protein molecule. This is reminiscent of the tyrosine radicals formed with horse rather than sperm whale myoglobin, which are also
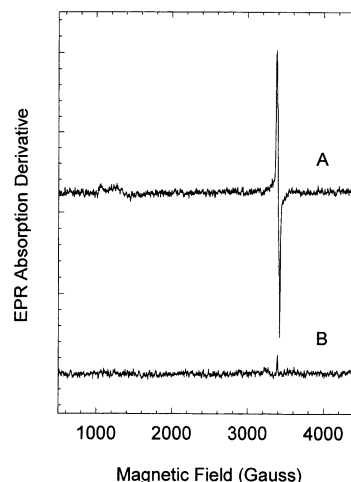

FIG. 6. **EPR spectra at 10 K of F172Y HRP (A) and wild type, recombinant HRP (B) which were frozen 5 min after addition of 1.3 equivalents of H_2O_2.** Addition, mixing, and 5 min of incubation were performed in 3-mm quartz EPR tubes at ambient temperature before freezing to 77 K for storage. Instrument conditions were 100 microwatts microwave power at 9.51 GHz, 1 Gauss field modulation at 100 KHz, 328 ms time constant, and 1×10^6 receiver gain.

prevented by their protein environment from dimerizing to form dityrosine links (41, 42).

DISCUSSION

A gene coding for the F172Y mutant of HRP has been expressed via a baculovirus vector in *Spodoptera frugiperda* and *T. ni* cells. The spectra of the mutant protein purified from the incubation medium in the ferric and ferric cyano states are essentially identical to those of the corresponding spectra of either native HRP or recombinant, wild type HRP. The catalytic activity of the F172Y mutant toward guaiacol peroxidation, as revealed by K_m and V_{max} measurements, is similar to that of native HRP and recombinant, wild type HRP. Furthermore, the pH profile for the oxidation of guaiacol by F172Y HRP is essentially identical to that for the native protein.
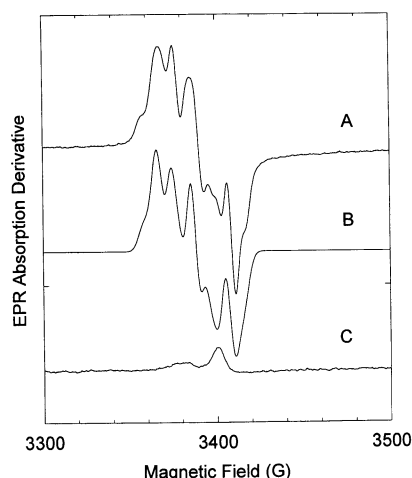
FIG. 7. **EPR spectra at 6 K of F172Y HRP (A) and recombinant wild type HRP (C) 30 s after the addition of 1.3 equivalents of H$_2$O$_2$, acquired under conditions that show hyperfine structure.** Instrument conditions: 10 microwatts microwave power at 9.51 GHz, 5.2 Gauss field modulation at 100 KHz, 164 ms time constant, and 1.25 × 10^5 receiver gain. Shown in B is an EPR simulation using an anisotropic g tensor ($g_x = 2.0091$, $g_y = 2.0046$, $g_z = 2.0023$), and anisotropic a tensors for the C$_3$ and C$_5$ ring protons ($a_x = -26.9$, $a_y = -7.8$, $a_z = -19.6$ MHz) and using the Euler angles, $\alpha = \pm 30$, $\beta = 0$, and $\chi = 0$ relating the principal axes of the hyperfine and g tensors (37–39). In addition, a variable dihedral angle about the C$_\beta$-Cγ bond was assumed to result in isotropic hyperfine constants for the two methylene protons of $a_1 = B_1\rho_{c1}\cos^2\theta_1$ and $a_2 = B_1\rho_{c1}\cos^2\theta_2$, where $\theta_1 + \theta_2 = 120°$ and $\rho_{c1} = 0.37$ and $B_1 = 162$ MHz (37). The simulation shown represents a conformation with $\theta_1 = 20°$ and $\theta_2 = 140°$.
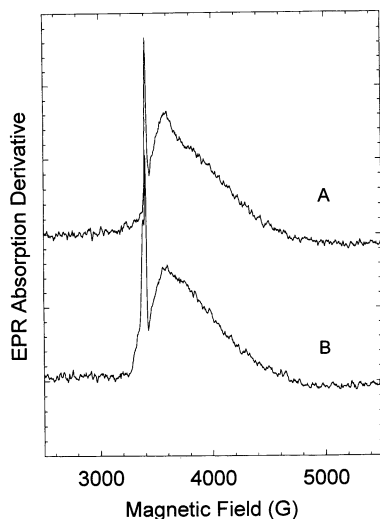

FIG. 8. **EPR spectra at 2.3 K of the porphyrin radical cations obtained from native HRP (A) and F172Y HRP (B) 30 s after reaction with 1.3 equivalents of H$_2$O$_2$.** Instrumental conditions were 20 milliwatts microwave power at 9.51 GHz, 2.6 Gauss field modulation at 100 KHz, 1310 ms time constant, and 3.2 × 10^5 receiver gain. Background spectra were collected under identical conditions for blank samples and subtracted before display.

Finally, the absolute stereochemistry of the sulfoxide produced from thioanisole, a sensitive monitor of the active site structure and topology (25, 26), is not significantly changed by the F172Y mutation. These results indicate that the F172Y HRP mutant folds properly and has an active site that differs very little in structure or topology from that of native HRP.

Striking differences are observed in the activated states of the F172Y mutant despite its high degree of structural identity with the active site of native HRP. Reaction of the F172Y mutant with one equivalent of H$_2$O$_2$ in the absence of an added substrate yields initially a Compound I spectrum that decays

with time to a Compound II-like spectrum (Fig. 4). Reaction of native or recombinant wild type HRP under the same conditions produces the normal Compound I spectrum. Determination of the kinetic constants for the reactions of these proteins with H$_2$O$_2$ by stopped flow methods shows that Compound I is formed at essentially the same pH-independent rate by the F172Y mutant ($k_1 = 1.6 \times 10^7$ M^{-1} s^{-1}) as by native HRP (1.1–2.0 × 10^7 M^{-1} s^{-1}) (35–37). However, Compound I of F172Y HRP decays much more rapidly than that of native HRP in the absence of added substrates. At pH 7, the decay rate for F172Y HRP is $k_2 = 1.01$ s^{-1} (Fig. 5) whereas little decay is observed for Compound I of native HRP within the time frame of these experiments (43). The basis for the decay of the F172Y HRP species with a Compound I chromophore is unmasked by freeze-quench EPR studies which show that the normal Compound I porphyrin radical cation is rapidly formed in the reaction of both native and F172Y HRP with H$_2$O$_2$. A protein radical, however, is simultaneously formed in the F172Y but not native HRP reaction (Fig. 6). Spin quantitation indicates that approximately 10% of the F172Y protein carries a protein radical 30 s after peroxide addition. The intensity of the protein radical signal decays with time and accounts for 5% rather than 10% of the protein after 30 min. Although it has not been possible from the EPR to accurately determine the rate of decay of the porphyrin radical cation due to the nature of the signal, qualitative comparison of the data suggests that the porphyrin radical cation is more rapidly lost from the F172Y mutant than from the native protein. This is supported by the observation that the F172Y mutant gives a Compound II spectrum 2.5 h after addition of one equivalent of H$_2$O$_2$ whereas native HRP gives the normal Compound I spectrum.

One possible explanation for these data is that there are two populations of F172Y that differ in extent of saturation of the calcium-binding sites on the protein (32, 44) and that insufficient calcium saturation of one of the two populations makes it less stable and gives rise to the observed instability of Compound I. This possibility is ruled out by the demonstration that the rates of Compound I formation and decay, and the amplitude of the protein EPR signal, are insensitive to the presence or absence of 5 mM Ca^{2+} in the incubation buffer.

The results clearly show that oxidation of F172Y HRP by H$_2$O$_2$ produces a ferryl/porphyrin radical cation Compound I structure analogous to that obtained with the native protein. However, electron transfer from a protein residue gradually reduces the porphyrin radical cation with concomitant formation of a protein radical. The residue involved in this intramolecular electron transfer reaction is presumably Tyr[172] because (a) the corresponding reaction is not observed for native HRP within the 30-min time frame of the EPR experiments, and (b) the radical signal can be adequately simulated with the parameters for a tyrosine residue (Fig. 7). Quantitative conversion of the porphyrin radical cation to the protein radical is not observed. Indeed, only approximately 10% of the protein is found as the protein radical after 30 s, and the extent of protein radical formation decays as the incubation time is lengthened. These results suggest that the protein radical is in equilibrium with the porphyrin radical cation, and/or is quenched by intra- or intermolecular reactions that occur at rates competitive with its formation, so that only a steady state level of protein radical is observed. The finding that reaction with H$_2$O$_2$ at ambient temperature produces a Compound I species that decays to a species with a Compound II-like spectrum is consistent with the hypothesis that the porphyrin radical cation characteristic of Compound I is gradually quenched by oxidation of Tyr[172] and that the Tyr[172] radical is quenched, in turn, by reactions that do not produce stable radicals.

The present results indicate that the presence or absence of a highly oxidizable residue vicinal to the heme group is a major factor in determining whether the second oxidation equivalent required for the peroxidase-catalyzed reduction of H_2O_2 comes from the porphyrin or the protein. These results concur with earlier work on CcP which showed that a porphyrin cation radical is formed instead of the usual protein radical when Trp^{191}, the residue that is normally oxidized, is replaced by a phenylalanine (45).

The balance between porphyrin and protein oxidation is physiologically relevant and can be observed in intact peroxidases. Thus, oxidation of lactoperoxidase yields a porphyrin radical cation species that is transformed with time into a CcP-like protein radical (46–48). Evidence for this transformation includes the demonstration by titration with ferrocyanide that two oxidizing equivalents are retained in the second species despite its Compound II-like spectrum and the observation that the initial species catalyzes the iodination of tyrosine and the dimerization of iodotyrosine, whereas the second Compound I species only catalyzed the dimerization reaction.

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