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Oxidation of Carboxylic Acids by Horseradish Peroxidase Results in Prosthetic Heme Modification and Inactivation

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Abstract: Hemoproteins are powerful oxidative catalysts. However, despite the diversity of functions known to be susceptible to oxidation by these catalysts, it is not known whether they can oxidize carboxylic acids to carboxylic radicals. We report here that incubation of horseradish peroxidase (HRP) at acidic pH with H_2O_2 in acetate buffer results in rapid modification of the heme group and loss of catalytic activity. Mass spectrometry and NMR indicate that an acetoxy group is covalently bound to the δ-*meso*-carbon in the modified heme. A heme with a hydroxyl group on the 8-methyl is also formed as a minor product. These reactions do not occur if protein-free heme and H_2O_2 are co-incubated in acetate buffer, if the HRP reaction is carried out at pH 7, in the absence of H_2O_2, or if citrate rather than acetate buffer is used. A similar heme modification is observed in incubations with *n*-caproic and phenylacetic acids. A mechanism involving oxidation of the carboxyl group to a carboxylic radical followed by addition to the δ-*meso*-position is proposed. This demonstration of the oxidation of a carboxylic acid solidifies the proposal that a carboxylic radical mediates the normal covalent attachment of the heme to the protein in the mammalian peroxidases and CYP4 family of P450 enzymes. The hemoprotein-mediated oxidation of carboxylic acids, ubiquitous natural constituents, may play other roles in biology.

Unlike the plant and fungal peroxidases, the mammalian peroxidases, including myeloperoxidase,[1] lactoperoxidase,[2,3] eosinophil peroxidase,[4] and probably thyroid peroxidase,[5] covalently bind their heme to the protein through ester links between two of the heme methyl groups and aspartate or glutamate residues of the protein.[6] Using lactoperoxidase expressed in a baculovirus system, we demonstrated earlier that the heme covalent bonds are formed autocatalytically upon exposure of the immature protein to H_2O_2.[3,7] Interestingly, a similar process has been demonstrated to occur with most members of the CYP4 family of cytochrome P450 enzymes.[8−12] For both of these classes of enzymes, covalent attachment of the heme is a normal step in formation of the mature, catalytically active proteins. We have proposed a mechanism for this autocatalytic process that involves oxidation of the carboxylic acid group of the relevant aspartate or glutamic acid residue to the carboxyl radical, followed by hydrogen abstraction from a vicinal heme methyl, conversion of the resulting methylene radical to the carbocation by transfer of an electron to the hypervalent iron, and collapse of the cation with the carboxylic acid group to give the ester.[3,6−10] However, an alternative mechanism can be formulated involving abstraction of a proton from a heme methyl by the protein carboxylic acid group. This might be possible if conversion of the ferric heme to the ferryl porphyrin radical cation by reaction with H_2O_2 lowers the pK_a of the heme methyl sufficiently to enable a carboxylate group in a protein environment to remove one of its protons.

The postulate that a carboxylic radical is involved in autocatalytic covalent binding of the heme suffers from the lack of independent evidence that hemoproteins can oxidize carboxylic acid groups. Early literature reported the decarboxylation of retinoic acid by HRP/H_2O_2 to give multiply oxidized products,[13,14] but this reaction was subsequently shown to result from a nonenzymatic heme-catalyzed reaction.[15] The decarboxylation of ibuprofen by HRP and H_2O_2 in reverse micelles has also been reported,[16] but in the absence of reverse micelles

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we have been unable to detect the decarboxylation of phenylacetic acid by HRP/H_2O_2 (C. Colas, unpublished). Administration of ketoprofen to rats has been reported to result in excretion in the bile of small amounts of decarboxylated metabolites, but the enzymatic system responsible for their formation was not defined.[17] Better evidence exists in the case of metalloporphyrin model systems, as both iron and manganese porphyrins have been reported to promote the periodate- or iodosobenzene-dependent decarboxylation of highly substituted carboxylic acids,[17−19] but the relationship of these observations to the normal oxidation reactions catalyzed by peroxidases and other hemoproteins remains unclear. Thus, clear evidence that a peroxidase or other catalytic heme protein can oxidize a carboxylic acid, particularly one without α-alkyl or α-aryl substituents, is lacking.

We report here that HRP catalyzes the oxidation of acetic acid and other linear alkyl carboxylic acids, presumably to the carboxylic radical, that adds to the prosthetic heme of the enzyme to give a *meso*-substituted acetoxy (or acyloxy) derivative. It also removes a hydrogen from the 8-methyl to give small amounts of the 8-hydroxymethylheme derivative. These findings not only demonstrate that a hemoprotein can oxidize the carboxylic acid function, but also critically expands the range of functions whose oxidation results in addition to the heme and inactivation of the enzyme to include endogenous substrates.

Experimental Section

Materials. Horseradish peroxidase (grade I) was obtained from Boehringer Mannheim GmbH and Roche. The 30% (w/w) hydrogen peroxide, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)diammonium salt (ABTS), catalase, sodium ascorbate, 2-butanone, trifluoroacetic acid, tin(II) chloride, pyridine-d_5, and peracetic acid (32 wt % in 40−45% acetic acid) were purchased from Sigma-Aldrich. Acetic acid, phosphoric acid, potassium hydroxide, and acetonitrile were obtained from Fisher. Acetate buffer ($CH_3CO_2^-/K^+$) was prepared from acetic acid titrated to pH 4.4 with 1 M KOH. Citrate buffer was prepared from citric acid titrated to pH 4.4 with 1 M KOH. Phosphate buffer (25 mM) was from dibasic potassium phosphate titrated to pH 7.0 with 0.1 M KOH. Water was double distilled prior to use. 8-Hydroxymethylheme was prepared as previously described.[29]

Spectrophotometric measurements were performed on a Hewlett-Packard 8450A diode array spectrophotometer. The concentration of HRP was determined by using $\epsilon_{402} = 102\,000\ M^{-1}\ cm^{-1}$, MW = 44 000.[20] The H_2O_2 concentration was standardized spectrophotometrically at 240 nm by using a molar extinction coefficient of $\epsilon = 43.6\ M^{-1}\ cm^{-1}$.[21] High-pressure liquid chromatography (HPLC) was performed on a Hewlett-Packard 1090 Series II instrument equipped with a photodiode array detector. Liquid chromatography-mass spectrometry (LC−MS) was performed on a Waters Micromass ZQ coupled to a Waters Alliance HPLC system (2695 separations module, Waters 2487 Dual λ Absorbance detector) employing an Xterra MS C_{18} column (2.1 × 50 mm, 3.5 μm). ^{1}H NMR spectra were obtained on a Varian 400-MHz instrument in pyridine-d_5. Chemical shifts are reported in parts per million relative to tetramethylsilane.

HPLC Analysis. Unless otherwise mentioned, HPLC was performed with a Vydac protein C_4 column (4.6 × 150 mm, 5 μm) fitted with a guard column. Solvent A was water containing 0.1% trifluoroacetic acid (TFA), and solvent B was acetonitrile containing 0.1% TFA. The gradient program consisted of linear segments with 33% B (0−3 min), from 33 to 43% B (3−23 min), 95% B (23.1−28 min), and 33% B (28.1−30 min) at a flow rate of 1 mL/min. The eluent was monitored at 278 and 400 nm.

Formation of the Heme Adduct: HPLC and Mass Spectrometry. A 31 μL aliquot of 98 mM H_2O_2 aqueous solution (150 equiv) was added to 0.9 mL of 23 μM HRP in 50 mM acetate buffer at pH 4.4 and mixed well. After 3 h at ∼25 °C, the solution was analyzed by HPLC. Fractions containing the peak at $t = 13.0$ min were collected, concentrated, and analyzed by mass spectrometry. The settings of the mass spectrometer were as follows: capillary voltage, 3.5 kV; cone voltage, 25 V; desolvation temperature, 350 °C; source temperature, 120 °C. Experiments were also performed by using (a) heme instead of HRP, (b) citrate instead of acetate buffer, and (c) ammonium acetate at pH 7 instead of acetate buffer at pH 4.4.

pH Dependence of Heme Adduct Formation. To 50 μL of 0.98 mM H_2O_2 in 1 M acetate solutions of pH = 3.0, 4.0, 4.4, 4.8, 5.5, 6.1, 6.5, or 7.0, respectively, was added 50 μL of HRP (2 mg/mL in water). The resulting mixtures were mixed well and were then incubated at ∼25 °C for 30 min before the reaction mixtures (70 μL) were analyzed by HPLC. The peaks of the heme adduct (retention time: 13 min) and unmodified heme (retention time: 14 min) were individually integrated, and the data acquired were analyzed with kaleidaGraph 3.5.

Dependence of Heme Adduct Formation on the H_2O_2 and Acetate Concentrations. To 100 μL aliquots of 45 μM HRP in 1 M acetate buffer at pH 4.4 was added 100 μL of 90 μM, 0.45 mM, or 2.3 mM (2, 10, or 50 equiv) H_2O_2 in 1 M acetate buffer at pH 4.4. The solutions were mixed well and were then incubated for time periods from 15 min to 5 h. The resulting solutions were analyzed by HPLC. To 50 μL aliquots of 45 μM HRP in 1 M citrate buffer at pH 4.4 was added 50 μL of 0.98 mM H_2O_2 (22 equiv) in 0.1, 0.2, 0.5, 1, or 2 M acetate buffer at pH 4.4, respectively, and the solutions were mixed well. After 35 min, the reaction mixtures were analyzed by HPLC. The peaks of the heme adduct (retention time: 13 min) and unmodified heme (retention time: 14 min) were integrated. The data were analyzed with kaleidaGraph 3.5.

Inactivation of HRP by Acetate. A 100 μL solution of HRP (2 mg/mL, 45 μM) in 0.5 M citrate buffer, pH 4.4, was mixed with 100 μL of H_2O_2 (2 mM) in 1 M acetate buffer, pH 4.4. A 2 μL aliquot of the reaction solution was taken after 10 and 20 min and was diluted to 50 μL with 50 mM citrate buffer (pH 4.4). Into a disposable cuvette were added 2 μL of the diluted solution (containing 40 μg/mL HRP), 2 μL of 49 mM H_2O_2, and 50 μL of 2.5 mM ABTS. The solution was then mixed with 950 μL of 50 mM citrate buffer, and the absorbance at 414 nm was immediately recorded as a function of time. The initial rate was calculated from the change in absorbance between 2 and 22 s.[22] Two control experiments were performed. First, 100 μL of HRP (45 μM) in 0.5 M citrate buffer, pH 4.4, was mixed with 100 μL of H_2O_2 (2 mM) in 0.5 M citrate buffer, pH 4.4, and the same procedure as above was then followed. Second, 100 μL of HRP (2 mg/mL, 45 μM) in 0.5 M citrate buffer, pH 4.4, was mixed with 100 μL of 1 M acetate buffer, pH 4.4, but without H_2O_2. The spectroscopic changes were then followed as already described.

Formation of Heme Adduct from HRP Compound I. In a 100 μL microcuvette, 50 μL of 22 μM HRP in water was mixed with 50

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μL of 22 μM H_2O_2 in 50 mM citrate buffer at pH 4.4. Compound I, as judged by absorption spectroscopy, was formed within 1 min. A 50 μL aliquot was taken and mixed with 50 μL of 1 M acetate at pH 4.4. After 15 min, the solution was analyzed by HPLC.

Incubation of Peracetic Acid with HRP. HRP (0.1 mM, 10 μL) was mixed with 1 equiv of peracetic acid in 0.5 M citrate buffer at pH 4.4 (final volume was 100 μL). Formation of Compound I was observed within 15 s. After incubation at ~25 °C for 90 min, the solution was analyzed by HPLC.

Removal of Heme from HRP. ApoHRP was prepared according to the method of Teale with some modifications.[23] HRP (15 mg) was dissolved in 1 mL of 25 mM phosphate buffer (pH 7.0), and into this was added 40 μL of 3 N HCl to give a solution with pH = 1.7. The HRP solution was extracted with 2-butanone (2 mL × 2) to remove the heme, and was then immediately neutralized with 1 N NaOH. The apoprotein in the aqueous phase was passed through a PD-10 column eluted with 25 mM phosphate buffer (pH = 7.0). Fractions containing apoHRP were combined and condensed with an ultracentrifugal filter device (10 000 MWCO, Millipore Amicon), affording ~0.7 mL of apoHRP with AU_{280} = 1.04 after 4-fold dilution, R_z = 0.04 (R_z = Reinheitzahl). The residual heme in the apoHRP was less than 2%.

Reconstitution of HRP with *meso*-Acetoxyheme. To a 200 μL solution of apoHRP (AU_{280} = 1.41) in 25 mM phosphate buffer (pH 7.0) was added 2 μL (estimated to be more than 1 equiv) of *meso*-acetoxyheme in methanol. The *meso*-acetoxyheme was isolated from inactivated HRP and prepared for reconstitution as described in the section on ^{1}H NMR characterization. After 2 h of incubation at ~25 °C, the solution was passed through a DEAE column (DE52) with 25 mM phosphate buffer (pH 7.0) as the eluent to remove the excess of the heme adduct. Three independent reconstitution experiments were performed. The resulting HRP solutions had R_z values of 0.47, 0.50, and 0.69, respectively. Heme-reconstituted HRP (R_z = 0.5) was also prepared as a control.[24] The activity of the reconstituted HRP was determined as follows: into a disposable cuvette were added 50 μL of 2.5 mM aqueous ABTS and 2 μL of HRP (20 μg/mL) in 25 mM phosphate buffer (pH 7.0) before mixing with 950 μL of 50 mM citrate buffer. The procedure already described was then followed.

Comparison with 8-Acetoxymethylheme. 8-Hydroxymethylheme (~0.5 mg) was dissolved in 100 μL of acetic anhydride-pyridine (1:1, v/v), stirred at ~25 °C for 0.5 h.[25] The resulting solution was analyzed by LC−MS with the following LC settings where solvent A was water containing 0.1% formic acid and solvent B was methanol containing 0.1% formic acid. The gradient profile used was: (1) 40% B for 2 min (0−2 min); (2) 40−70% B in 15 min (2−17 min); (3) 70% B for 5 min (17−22 min); (4) 95% B for ~3 min (22.1−25 min); (5) 40% B for ~1 min (25.1−26 min). The flow rate was 0.2 mL/min, and the detector was set at 400 nm with the following MS settings: capillary voltage, 3.5 kV; cone voltage, 25 V; desolvation temperature, 350 °C; source temperature, 120 °C. The reaction solution was also analyzed by HPLC and co-injected with the heme adduct. The remaining reaction solution was dried under a stream of air. The residue (8-acetoxymethylheme) was hydrolyzed by dissolving it in 80 μL of tetrahydrofuran (THF) and 80 μL of 2 N aqueous KOH and stirring overnight.[26] The solution was dried under a stream of air. The final residue was dissolved in 100 μL of acetonitrile−water (3:2) and was analyzed by HPLC. Authentic 8-hydroxymethylheme was injected as a control.

Reaction of Acetate with Mesoheme-Reconstituted HRP. The dimethyl ester of mesoporphyrin IX was hydrolyzed with 2 N aqueous KOH−THF (1:1 v/v) overnight, and then Fe^{3+} was inserted as described previously.[27] Briefly, the mesoporphyrin IX (~1 mg), dissolved in 0.5 mL of pyridine, was combined with 5 mL of glacial acetic acid, the mixture was purged with N_2, and then the mixture was heated to 80 °C. Saturated $FeSO_4$ solution (1 mL) was added, and the mixture was stirred for 10 min before it was exposed to air, cooled to room temperature, and poured into 20 mL of ethyl ether. It was then washed with brine (20 mL), 25% HCl (12 mL), and water (12 mL). After drying over Na_2SO_4, the solvent was removed and the residue was redissolved in MeOH (~0.2 mL). A 10 μL aliquot of mesoheme in MeOH was mixed with 40 μL of apoHRP (AU_{280} = 1.04 after 4-fold dilution) and 350 μL phosphate buffer (pH 7), and the mixture was incubated at ~25 °C for 2 h. The solution was passed through a DEAE (DE52) column. Fractions containing HRP were combined and concentrated, affording mesoheme reconstituted HRP with R_Z = 2.8 and AU_{394} = 1.8. This HRP solution (50 μL) was mixed with 50 μL of 1.6 mM H_2O_2 in 1 M acetate buffer, incubated at ~25 °C for 20 min, and analyzed by HPLC and LC−MS.

Isolation and ^{1}H NMR Characterization of the Heme Adduct. HRP (44 mg, 0.1 mM) in 10 mL of 1 M potassium acetate buffer (pH 4.4) was added to 10 mL of H_2O_2 (2 mM) in water. The solution was well mixed and was then incubated in a shaker at ~25 °C for 15 min before adding 5 μL of 5.6 mg/mL catalase to destroy the excess H_2O_2 and 0.2 mL of 50 mM sodium ascorbate to reduce the enzyme to the ferric state. The resulting solution was acidified with 42% H_3PO_4 to a pH of 1.6 before being extracted with 2-butanone (15 mL × 2). The 2-butanone solutions were combined, and $NaHCO_3$ was added to neutralize the residual H_3PO_4. The 2-butanone phase was dried over Na_2SO_4 before the 2-butanone was removed by evaporation. The residue was dissolved in 0.5 mL of acetonitrile−water (3:2), and the solution was purified by HPLC on a C_{18} semipreparative column (10 × 150 mm, 5 μm, Alltech Alltima). The HPLC settings were as follows, where solvent A was water containing 0.1% TFA and solvent B was acetonitrile containing 0.1% TFA: 0−2 min, 40% B; 2−18 min, 40−48% B; 18−19 min, 48−95% B; 19−22 min, 95% B; and 22.1−25 min, 40% B. The flow rate was 4 mL/min. The eluent was monitored at 278 and 400 nm. Fractions containing the heme adduct (retention time: 15 min) and other heme derivatives were pooled separately. Removal of the solvents by rotary evaporation and drying under vacuum overnight afforded 1.0 mg of the heme adduct. According to a reported method,[28] $SnCl_2$ (2.7 mg, 10 equiv) was added to the flask containing the product, and air in the flask was removed by placing the flask under vacuum and then under N_2. Pyridine-d_5 (0.5 mL) was added and stirred for a while. The $SnCl_2$ reduces the ferric iron to the ferrous state. The final solution was examined by ^{1}H NMR spectroscopy on a Varian 400-MHz instrument. The chemical shifts of the peaks were determined with reference to the chemical shift of pyridine at 8.70 ppm. Another heme derivative (retention time: 9.5 min) was examined by mass spectrometry. The heme adduct from deuterated acetate was prepared by following the same procedure as above employing trideuterated (CD_3CO_2H) acetate buffer.

LC−MS of HRP Treated with *n*-Caproic Acid. A 100 μL aliquot of HRP (4 mg/mL) in 1 M citrate buffer at pH 4.4 was added to 200 μL of 10:1 water−ethanol containing 0.98 mM H_2O_2 and 0.1 M *n*-caproic acid. After mixing well, the mixture was incubated at ~25 °C for 20 min and was then directly analyzed by LC−MS employing an Xterra MS C_{18} column (2.1 × 50 mm, 3.5 μm) at a flow rate of 0.2 mL/min. Solvent A was water containing 0.1% formic acid, and solvent B was methanol containing 0.1% formic acid. The following gradient was employed: 40−44% B, 0−4 min; 65−75% B, 4.1−9.1 min; 95% B, 9.2−13 min; 40% B, 13.1−17 min. The eluent was monitored at 400 nm. The mass spectrometer settings were as above.

LC−MS of HRP Treated with Phenylacetic Acid. A 100 μL aliquot of aqueous HRP (2 mg/mL) was added to 200 μL of 4:1 water−ethanol containing 0.98 mM H_2O_2 and 0.4 M phenylacetic acid at pH 5. After mixing well, the mixture, having pH ≈ 4.6, was incubated at ~25 °C for 30 min and then analyzed by LC−MS employing an Xterra MS C_{18} column (2.1 × 50 mm, 3.5 μm) at a flow rate of 0.2 mL/min. Solvent A was water containing 0.1% formic acid, and solvent B was methanol containing 0.1% formic acid. The following gradient was employed: 40% B, 0−2 min; 40−70% B, 2−10 min; 95% B, 10.1−13.1 min; 40% B, 13.2−15 min. The eluent was monitored at 400 nm. The mass spectrometer settings were as follows: mode, ES^+; capillary voltage, 3.5 kV; cone voltage, 25 V; desolvation temperature, 250 °C.

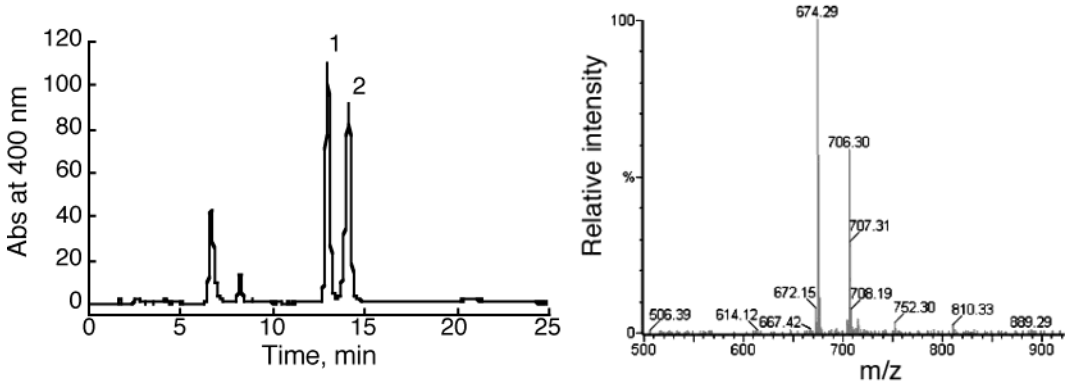

Figure 1. Formation of the heme adduct. (Left panel) HPLC trace of HRP (23 μM) after incubation with 150 equiv of H_2O_2 in 50 mM acetate buffer at pH 4.4 for 3 h. The reaction mixture was analyzed by HPLC with the detector set at 400 nm. Peak 1 is the product, and peak 2 is normal heme. (Right panel) The mass spectrum of the acetate-heme adduct.

Results

HPLC and Mass Spectrometric Identification of the Heme Adduct. Incubation of HRP with a large excess of H_2O_2 (150 equiv) in 50 mM acetate buffer (pH 4.4) at ~25 °C for 3 h produced a new compound which on HPLC eluted ~1 min earlier than heme (Figure 1). When the incubation was performed with heme instead of HRP, no such modified heme product was detected. When HRP was incubated with H_2O_2 (150 equiv) in citrate buffer at the same pH, or in ammonium acetate buffer at pH 7, the new product also was not detected, suggesting that the reaction can only occur between HRP and a linear carboxylic acid under acidic conditions. The mass spectrum of the new compound exhibits a molecular ion at *m*/*z* 674 (100% intensity). Comparison with the mass spectrum of heme, which has a molecular ion at 616, indicates that the new compound is an acetoxy-heme adduct [MW 616 − 1 (H) + 59 (CH_3CO_2)]. The UV−visible spectrum of the new compound in MeOH (Soret band at 398 nm and bands at 494 and 630 nm) is very similar to that of heme itself with a Soret band at 398 nm and bands at 504 and 628 nm.

The pH Dependence of Heme Adduct Formation. The heme adduct was first identified in an incubation of HRP in 50 mM acetate buffer at pH 4.4. Subsequent experiments with 0.5 M acetate buffer over a range of pH values demonstrated that pH 4.4 is fortuitously optimal for formation of the acetate-heme adduct (Figure 2). At pH 7.0, very little adduct is obtained, and at pH < 3.0, no products with 400 nm absorbance are detected.

Dependence of Heme Adduct Formation on the H_2O_2 and Acetate Concentrations. An excess of H_2O_2 is required for formation of the heme adduct. When HRP was incubated with 2 equiv of H_2O_2 and 1 M acetate for 15 min, the reaction only went partially to completion. Even if the incubation was extended to 5 h, the unreacted heme did not decrease further significantly, but the peak intensity of the heme adduct decreased, indicating that the heme adduct was degraded under the reaction conditions. When HRP was incubated with more than 10 equiv of H_2O_2 in 1 M acetate, essentially no residual heme was detected. Because a high concentration of H_2O_2 causes degradation of both heme and the heme adduct, it is preferrable to increase the acetate concentration to drive the reaction to completion. Increasing concentrations of acetate decrease the amount of unreacted heme isolated from the incubation but without significantly increasing the amount of the heme adduct (Figure 3). The constant level of adduct in Figure 3 is presumably the result of a steady state in which the higher the concentration of acetate, the faster the heme adduct forms but also the faster it is degraded, resulting in a progressively lower concentration of the parent heme. The yield of heme adduct

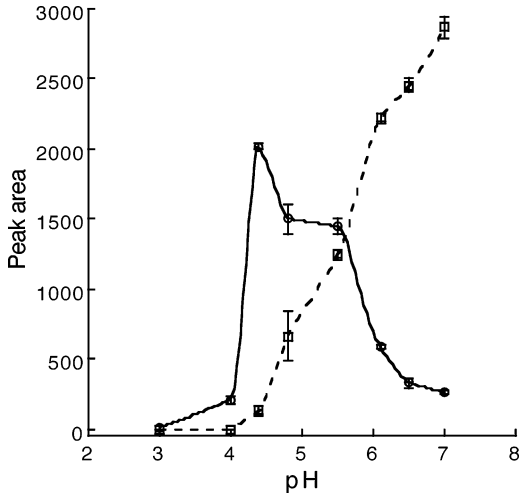

Figure 2. The pH dependence of heme-acetate adduct formation. The data represents the average of three experiments, with the error bars indicating the standard deviation. The solid line represents the amount of acetate-heme adduct, and the dashed line represents unreacted heme.

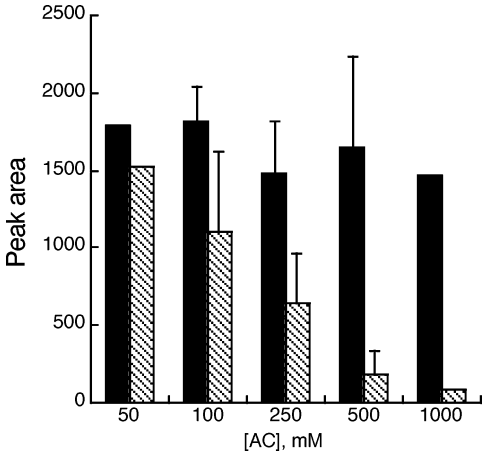

Figure 3. Effect of the acetate concentration on the heme modification reaction. The black columns represent the amount of the heme adduct, and the slashed columns represent the remaining unmodified heme. The *Y*-axis represents the integrated peak area from HPLC. The error bars represent the standard deviation.

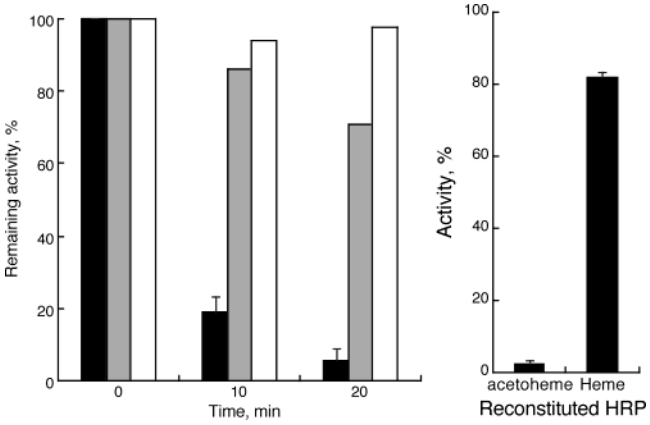

Figure 4. Inactivation of HRP by acetate. (Left panel) Catalytic activity was tested after preincubation of HRP with 0.5 M acetate (black bar) or 0.5 M citrate (gray bar) in the presence of H_2O_2, or in 0.5 M acetate in the absence of H_2O_2 (white bar). (Right panel) Relative activity of acetoxyheme-reconstituted HRP and heme-reconstituted HRP. The activity (%) is calculated relative to the initial rate of natural (not reconstituted) HRP. The black bar represents the average of three independent experiments, and the error bar corresponds to the standard deviation.

can be increased by using high concentrations of acetate and a reduced reaction time.

Formation of the Heme Adduct by HRP Compound I. Compound I, obtained by treatment of HRP with 1 equiv of H_2O_2 in 50 mM citrate buffer at pH 4.4, was stable for at least 15 min. Incubation of the Compound I thus obtained with 0.5 M acetate for 15 min yielded the acetoxyheme adduct and unmodified heme in a 2.1:1 ratio. This result not only indicates that Compound I plays a major role in heme adduct formation and that the heme acetoxylation reaction is highly efficient, but also provides direct evidence that the heme modification is not mediated by peroxyacetic acid formed in the incubation medium from acetic acid and H_2O_2. A role for peroxyacetic acid is furthermore precluded by the observation that incubation of HRP with peracetic acid in citrate buffer at pH 4.5 gives Compound I, but no acetoxy adduct, whereas addition of acetate to the Compound I formed with peroxyacetic acid gives the acetoxy adduct in good yield.

Inactivation of HRP by Acetate. Incubation of HRP with 0.5 M acetate at pH 4.4 in the presence of H_2O_2 resulted in rapid inactivation of HRP (Figure 4). The residual activity after 20 min was only 5.6 ± 3.4% of the original wild-type activity. In contrast, in 0.5 M citrate buffer at pH 4.4, the activity of HRP only decreased slowly due to the bleaching effect of H_2O_2. In the absence of H_2O_2, the activity of HRP remained essentially unchanged. Furthermore, we reconstituted HRP with the *meso*-acetoxyheme purified from a separate incubation. The resulting reconstituted HRP was virtually inactive (2.5 ± 0.8%), whereas heme-reconstituted HRP, as a control, exhibited 82.0 ± 1.2% of the original wild-type activity (Figure 4, the right panel).

Comparison with 8-Acetoxymethylheme. One possibility was that the acetate added to the 8-methyl position, as reaction of HRP with phenylhydrazine-derived phenyl radicals results in the formation of the 8-hydroxymethylheme.[29] Efforts to hydrolyze the acetoxy-heme adduct for a direct comparison were unsuccessful. We therefore synthesized the 8-acetoxymethylheme by reacting authentic 8-hydroxymethylheme with acetic anhydride/pyridine.[25] The mass spectrum of the synthetic product (Figure 5, the major peak at $t = 13.5$ min) exhibits a

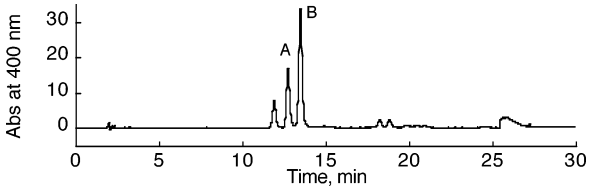

Figure 5. HPLC comparison of authentic 8-acetoxymethylheme and the acetate-heme adduct from the HRP incubations in acetate buffer. The experimental acetate-heme adduct (peak A) and authentic 8-acetoxymethylheme (peak B) have different retention times (12.7 and 13.5 min, respectively), establishing that the two compounds are different.

molecular ion at *m*/*z* 674, as expected for 8-acetoxymethylheme. However, co-injection experiments indicated that the HRP-derived adduct differed from authentic 8-acetoxymethylheme (Figure 5). Furthermore, as the synthetic 8-acetoxymethylheme could be hydrolyzed to 8-hydroxymethylheme under basic conditions (2 N KOH−THF, 1:1), the instability of the HRP-derived adduct under the same conditions indicates that the acetoxy group is not attached to any of the other three methyl groups of the heme, as they would all be expected to have similar chemical properties.

Mesoheme-Reconstituted HRP. To check if the acetate is attached to the 2- or 4-vinyl group, we reconstituted apoHRP with mesoheme, in which the vinyl groups are replaced by ethyl groups, and incubated the reconstituted enzyme under the conditions that normally lead to adduct formation. A modified porphyrin product with a retention time ~1 min less than that of mesoheme itself was identified by HPLC in these incubations. The mass spectrum of the new product exhibited a molecular ion at *m*/*z* 678, in accord with its identification as an acetoxy adduct of mesoheme (data not shown).

Characterization of the Heme Adduct by ^{1}H NMR Spectroscopy. The acetoxyheme adduct was converted to its chloroiron(III) form by partitioning between 2-butanone and a solution of 0.1 N DCl in NaCl-saturated D_2O. However, this causes partial decomposition of the sample due to acid instability of the heme adduct. To avoid this, the heme adduct from trideuterated acetate was dissolved in pyridine-d_5 without first converting it to the chloroiron(III) form. NMR data were obtained after the sample was reduced to the iron(II) form with $SnCl_2$. The NMR spectrum of the heme adduct (Figure 6 and Table 1) is similar to that of the previously reported *δ-meso*-azidoheme adduct.[28] The presence of the 4 methyl groups (3.629, 3.527, 3.514, 3.478) and of all of the vinyl protons (internal H, 8.443, dd, 8.275,dd, $J = 11.6$, 17.6 Hz; external H, trans, $J =$ 17.6 Hz, 6.284 and 6.163, cis, $J = 11.6$ Hz, 6.014 and 5.970), and the absence of a *meso*-proton, confirmed that the acetoxy group was bound to a *meso*-carbon. Comparison with the spectrum of heme indicates that the *δ-meso*-proton signal at 9.939 ppm is missing. *δ-meso*-Substitution is consistent with the finding that two methyl groups (assigned to the 1- and 8-methyls) are shifted while the other two methyls remain essentially unchanged. Finally, comparison of the ^{1}H NMR spectra of the acetate-heme adducts obtained with acetate and 2,2,2-trideuterioacetate identifies the peak at 3.587 ppm as that derived from the acetate methyl group.

Identification of 8-Hydroxymethylheme as a Minor Product. In addition to the heme adduct isolated from acetate-treated HRP by HPLC (retention time: 15 min), two minor products that absorbed at 400 nm were detected (Figure 7). The larger

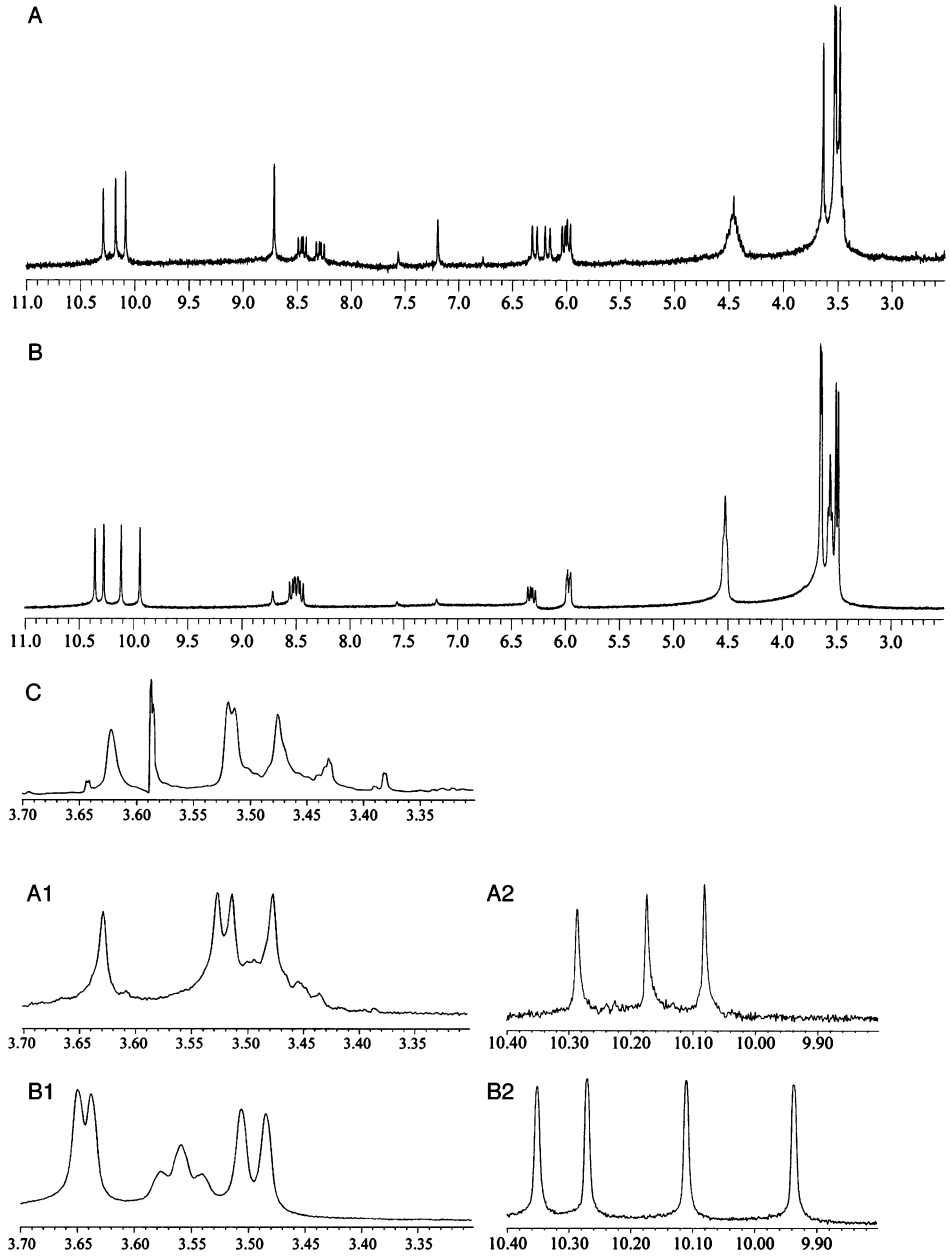

Figure 6. ^{1}H NMR (400 MHz) spectra of heme and the heme adducts obtained with acetate and deuterated acetate. (A) The full spectrum of deuterated acetate-heme adduct; (B) the full spectrum of heme. The pertinent regions of the NMR spectra for acetate-heme adduct (C), deuterated acetate-heme adduct (A1 and A2), and heme (B1 and B2) are presented.

of the two eluted at $t = 5$ min but was too unstable to characterize. The second minor product at $t = 9.5$ min (~5% relative to the *δ-meso*-acetoxy adduct) exhibits a molecular ion at *m*/*z* 632, as expected for a hydroxyheme, and has the same HPLC retention time and spectroscopic properties as authentic 8-hydroxymethylheme. The amount of 8-hydroxymethylheme decreased when the concentration of acetate was increased.

Reaction with Other Alkyl Carboxylic Acids. To test if HRP can react with other alkyl carboxylic acids, HRP was incubated at pH 4.4 with *n*-caproic acid in the presence of H_2O_2. LC and LC−MS analysis shows the presence of a new peak with a retention time of 12.6 min and a molecular weight of *m*/*z* 730 (Figure 8A), in accord with its identification as an adduct of heme (MW 616) with *n*-caproic acid (MW 116). Similar incubation of HRP with phenylacetic acid produced a phenylacetic acid (MW 136) heme adduct with a molecular ion at *m*/*z* 750 (Figure 8B). The similarity of the spectroscopic properties of these adducts to those of the acetoxy adduct suggests that they, too, are substituted at the *δ-meso*-position.

Discussion

The present study demonstrates that the prosthetic heme group of HRP undergoes a pH- and H_2O_2-dependent reaction with acetate. Addition of acetate to the heme is most efficient at pH 4.4 (Figure 2) but is greatly attenuated below pH 4.0 and above pH 7.0. The lack of heme modification at pH 7.0 is not due to failure to form the enzymatic oxidizing species, as *meso*-phenyl and *meso*-alkyl heme adducts are formed in the H_2O_2-dependent reaction of HRP at pH 7.0 with phenylhydrazine[29] and alkylhydrazines,[30] respectively. This is consistent with kinetic studies of HRP which show that Compound I is readily formed with H_2O_2 in the pH range from 4.0 to 8.0.[31] These observations

Table 1. Assignment of the NMR Signals of the Heme Adducts from Normal Acetate and Trideuterated Acetate

proton	heme	acetoxy-d_3-heme adduct	acetoxy-heme adduct
meso	10.354 (γ)	10.287 (s)	10.292 (s)
	10.273	10.175 (s)	10.171 (s)
	10.112	10.082 (s)	10.081 (s)
	9.939 (δ)		
inner vinyl	8.515 (dd, J = 18, 11.6)	8.448 (dd, J = 17.6, 11.6)	8.443 (dd, J = 17.6, 11.6 Hz)
	8.462 (dd, J = 18, 11.6)	8.282 (dd, J = 17.6, 11.6)	8.275 (dd, J = 17.6, 11.6 Hz)
ext vinyl	6.323 (d, J = 18)	6.288 (d, J = 17.6)	6.284 (d, J = 17.6 Hz)
(trans)	6.296 (d, J = 18)	6.168 (d, J = 17.6)	6.163 (d, J = 17.6 Hz)
ext vinyl	5.970 (d, J = 11.6)	6.018 (d, J = 11.2)	6.014 (d, J = 11.6 Hz)
(cis)	5.962 (d, J = 11.6)	5.972 (d, J = 11.6)	5.970 (d, J = 11.6 Hz)
methyls	3.643 (1 or 8)	3.629 (s)	3.621 (s)
	3.630 (3 or 5)	3.527 (s)	3.519 (s)
	3.497 (1 or 8)	3.514 (s)	3.514 (s)
	3.475 (3 or 5)	3.478 (s)	3.476 (s)
inner CH_2's	4.516 (br)	4.449 (m)	4.45 (m)
external CH_2's	3.559 (t)	3.39−3.49	3.35−3.55
acetyl CH_3			3.587 (s)

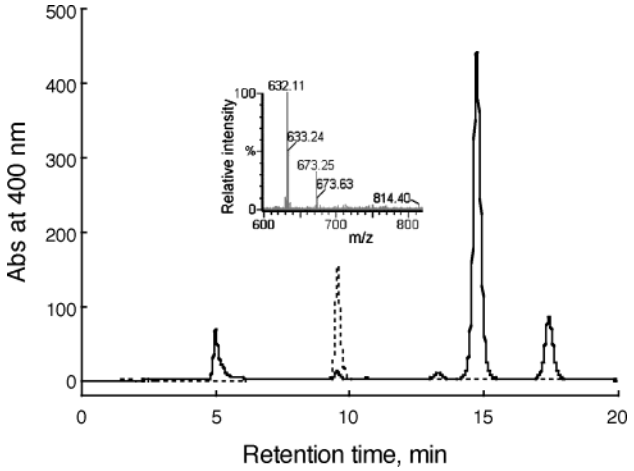

Figure 7. Identification of 8-hydroxymethylheme as a minor product. In addition to the major peak at t = 15 min, identified as the heme adduct, and the peak at t = 17.4 min for the unmodified heme, the minor peak at t = 9.5 min shows the same retention time as that for 8-hydroxymethylheme (dash line) and exhibits a molecular ion at 632. The peak at t = 5 min is unstable and decomposed upon concentration.

and the pK_a of 4.8 for acetic acid suggest that CH_3CO_2H rather than $CH_3CO_2^-$ is involved in the heme modification reaction. The decline in adduct formation at very acidic pH values is due to chemical instability of the adduct under those conditions, and at higher pH values it is due to lower concentrations of the protonated acetic acid. However, the kinetic data are complex and could include a change in protonation state of one or more protein residues. Furthermore, it appears that the adduct may be oxidatively unstable, as high concentrations of H_2O_2, long incubation times, and low concentrations of acetate lead to gradual disappearance of the prosthetic heme without a concomitant detectable increase in the amount of acetate heme adduct.

Adducts appear to be readily formed with linear but not nonlinear acids, as heme modification occurs not only with acetate but also with *n*-caproic and phenylacetic acids but not with citrate. In all three cases, the adducts involve replacement of a hydrogen of the heme by the corresponding carboxylate moiety. The adduct with acetic acid has been unambiguously identified as *δ-meso*-acetoxyheme by the fact that (a) the adduct is still formed when HRP is reconstituted with mesoheme, (b) it is not 8-acetoxymethylheme, (c) it is not hydrolyzed to detectable products, whereas 8-acetoxymethylheme (and presumably the other acetoxymethylheme isomers) is readily hydrolyzed to 8-hydroxymethylheme, and (d) the NMR indicates the heme is *meso*-substituted.

In addition to *δ-meso*-acetoxyheme, a small amount of another modified heme was isolated from the reaction of HRP with H_2O_2 in acetate buffer. The second product was identified by its mass spectrum, coelution by HPLC with an authentic standard, and its spectroscopic properties as 8-hydroxymethylheme. However, unlike *δ-meso*-acetoxyheme formation, the amount of 8-hydroxymethylheme did not increase as the pH decreased from 7 to 4.4. One reason for this is that the complex of HRP with 8-hydroxymethylheme remains catalytically active[32] and thus can undergo secondary transformations such as *meso*-acetoxylation. The simultaneous formation of a *δ-meso*-phenylheme adduct and 8-hydroxymethylheme was observed previously in the reactions of HRP with phenylhydrazine.[29] This dual product formation was attributed to the formation of a phenyl radical that either added to the *δ-meso*-carbon or abstracted a hydrogen from the vicinal 8-methyl group. This analogy is relevant to the proposal (see below) that the acetate reaction proceeds via oxidation of the acid to a carboxylate radical.

A rationale for specific *δ-meso*-substitution is provided by the crystal structure of the HRP-acetate complex formed when the protein is crystallized from a calcium acetate solution (PDB 1h5a).[33] The acetate group is located above the heme plane close to the *δ-meso*-carbon (O_1-C_δ = 3.7 Å) (Figure 9), as compared to its distance from the other *meso*-carbons (O_1-C_α = 6.6 Å, O_1-C_β = 7.7 Å, O_1-C_γ = 5.5 Å). The $H_\delta-C_\delta-O_1$ angle is 91.8°. An oxygen atom of the acetate is also close to an 8-methyl proton (H_{8a}): the distance between them ($H_{8a}-O_1$) is 3.6 Å, and the $C_8-H_{8a}-O_1$ angle is 118.6°. The other oxygen atom on the acetate is at a distance of 3.6 Å from the Fe^{3+}. The space proximity readily accounts for the C_δ and the 8-methyl regioselectivity of the reaction. Presumably, the caproic and phenylacetic acids bind and react with the same heme periphery,

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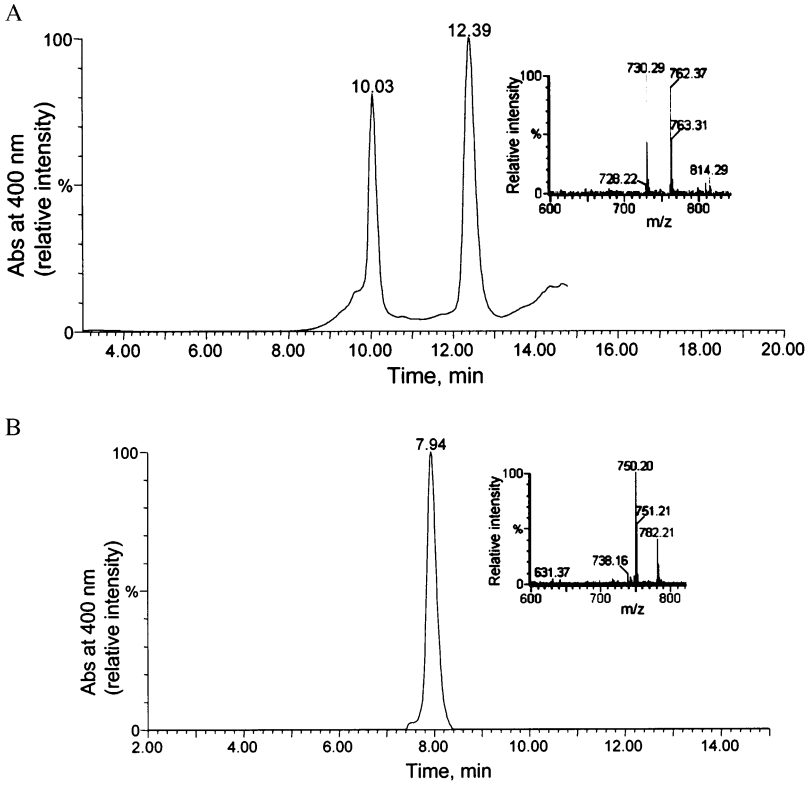

Figure 8. LC−MS analysis of the heme adduct of (A) *n*-caproic acid (30 μM HRP and 67 mM caproic acid, 30 min) and (B) phenylacetic acid (15 μM HRP and 267 mM phenylacetic acid, 20 min) after the indicated time of incubation with 0.65 mM H_2O_2 at ~25 °C. The incubations were then analyzed by LC−MS. The figures show the LC trace of the reaction mixture as detected at 400 nm. The *Y*-axis represents the relative absorbance intensity at 400 nm. The inset in (A) shows the mass spectrum of the heme adduct at $t = 12.39$ min, and the inset in (B) shows that of the heme adduct at $t = 7.94$ min.

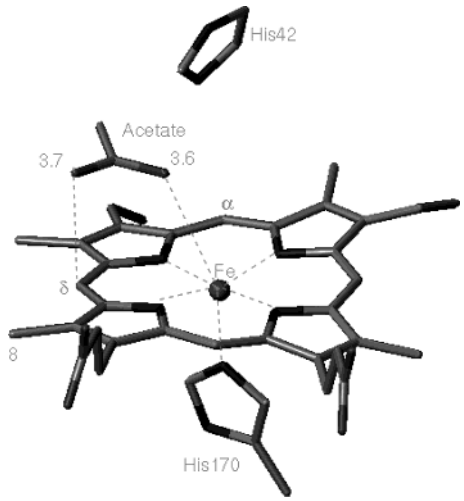

Figure 9. Partial active site structure of the HRP-acetate complex (PDB 1h5a). An acetate is located above the *δ-meso*-carbon (C_δ) of the heme. The figure was generated by Sybyl 6.9.1.

in accord with the crystal structure of the enzyme and our original proposal that in HRP substrates are generally restricted to reactions with the *δ-meso*-edge of the heme.[29,34]

δ-meso-Acetoxyheme adduct formation results in inactivation of HRP, as judged from a decrease in its ability to oxidize ABTS. This inactivation is consistent with the previous observation that the *δ-meso*-azido-, *δ-meso*-phenyl-, and *δ-meso*-alkylheme adducts of HRP are also inactive.[28−30] However, as incubation with excess H_2O_2 and acetate leads to degradation of the *δ-meso*-acetoxyheme, the modified heme group appears to still be able to react with H_2O_2 in a self-destructive manner.

The striking similarities among the reactions of HRP with acetate, azide,[28] phenylhydrazine,[29] and alkylhydrazines[30] suggest that they proceed via a common mechanism (Scheme 1). In the present situation, the first step is proposed to be oxidation of protonated acetic acid to the acetate radical with concomitant reduction of Compound I to Compound II. Addition of the resulting acetoxy radical to the *δ-meso*-carbon of the heme, followed by internal electron transfer of the unpaired electron to the iron, reducing it to the ferric state, produces an isoporphyrin cation that loses a proton to give *δ-meso*-acetoxyheme. Alternatively, the acetoxy radical can abstract a hydrogen atom from the 8-methyl group to give the corresponding carbon radical. Electron transfer to the iron then yields the methylene cation that is trapped by water to give the 8-hydroxymethyl heme derivative.

The oxidation of phenylacetic acid was examined because its carboxyl radical readily decarboxylates to give the benzyl radical.[35] Interestingly, the heme adduct formed with this substrate resulted from addition of the intact phenylacetoxy rather than benzylic moiety to the heme. This result suggests that the carboxylic acid radical is trapped by addition to the *δ-meso*-carbon at too rapid a rate for decarboxylation to be competitive. The rates of decarboxylation of the acetoxy and phenylacetoxy radicals have been reported to be $2-3 \times 10^9$ and 1.8×10^9 s^{-1}, respectively,[36,37] which would require the

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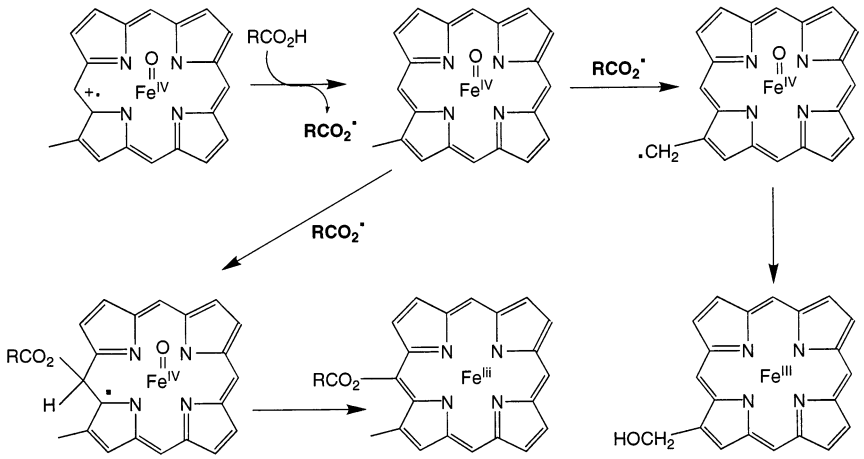
Scheme 1. Proposed Mechanism for Formation of the *δ-meso*-Acetoxy Heme and 8-Hydroxymethylheme in the Reaction of HRP with Acetate in the Presence of H_2O_2[a]



[a] The heme substituents are not shown except for the 8-methyl to simplify the figure.

heme of Compound II to trap the phenylacetoxy radical at a rate in the order of 10^{10} s^{-1}. The efficiency of this reaction is consistent with the finding that preformed Compound I reacts with acetate to give largely the acetoxyheme adduct. Decarboxylations have been previously reported in the HRP-catalyzed oxidation of amines such as indole-3-acetic acid[38] and *N*-alkyl-*N*-phenylglycines,[39,40] but these reactions involve enzymatic oxidation of a nitrogen to a nitrogen radical cation, followed by a decarboxylation that neutralizes the radical cation. These reactions are therefore completely different from those reported here. In the present reactions, the carboxylic acid is itself oxidized.

An alternative mechanism can be envisioned in which the acetoxy anion either adds to the *δ-meso*-position or deprotonates the 8-methyl of Compound I. However, this alternative is inconsistent with the fact that the reaction is favored by low rather than high pH, conditions that favor the protonated acetic acid over the acetate anion. Furthermore, incubation of HRP with H_2O_2 in the absence of acetate even at pH 8 does not result in formation of the 8-hydroxymethylheme (unpublished work).

A key finding of this study is that carboxylic acids are oxidized to carboxyl radicals by HRP Compound I. The demonstration that a hemoprotein ferryl species can oxidize simple carboxylic acids provides strong support for the mechanism we have proposed for the maturation process that results in covalent linking of the heme group to carboxylic acid residues in the mammalian peroxidases and in the CYP4 family of P450 enzymes.[6−12] The present results demonstrate the feasibility of the key step in the process, formation of a carboxylic acid radical. Furthermore, we have also observed the proposed first step in the heme covalent linking process − that is, abstraction of a hydrogen from a heme methyl substituent by the carboxylic radical. In the mechanism proposed for heme covalent binding, the methylene cation that results from transfer of the unpaired electron to the iron is trapped by the carboxyl group or, in the present instance, by a water molecule. Indeed, altering the length of the side chain by replacing the glutamic acid by an aspartate residue in the lactoperoxidase active site results in formation of a hydroxyheme rather than in covalent binding to the protein, presumably because water can now compete effectively with the carboxyl group for trapping of the methylene cation. The present results thus provide additional evidence that the initial step in all of these reactions is oxidation of the carboxylic acid to the carboxylic radical.

It is not known whether carboxylic radical formation by hemoproteins is of wider biological relevance, but it is of interest that at least two classes of catalytic hemoproteins, chloroperoxidase and the KatG catalase peroxidases, have active site carboxylic acid residues.[41] Glu318 in chloroperoxidase is located 5.1 Å from the heme iron and influences the product formation ratio, but is not absolutely required for catalysis.[42] It has been postulated to act as an acid−base catalyst. Asp152 in the catalase-peroxidase from *Synechocystis* is 7.8 Å from the heme iron atom.[43] Mutation of Asp152 to alternative residues virtually suppresses catalytic activity.[44] The aspartate is conserved in all of the catalase-peroxidases for which structures have been determined. The carboxylic acid residues in these proteins are presumably protected from oxidation by distance from the ferryl species or by the placement of water residues between the carboxylate group and the ferryl species, but it is possible that in some instances they are oxidized to transient radical species.

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