Catalytic Properties of Horseradish Peroxidase Reconstituted with the 8-(Hydroxymethyl)- and 8-Formylheme Derivatives†

Robert Z. Harris,‡ Paul A. Liddell,§ Kevin M. Smith,§ and Paul R. Ortiz de Montellano*,‡

Department of Pharmaceutical Chemistry, School of Pharmacy, University of California at San Francisco, San Francisco, California 94143-0446, and Department of Chemistry, University of California at Davis, Davis, California 95616

Received August 28, 1992; Revised Manuscript Received February 2, 1993

ABSTRACT: Recent studies suggest that 8-(thiomethyl)- and 8-formylheme modifications may be present in, respectively, lactoperoxidase and myeloperoxidase. To examine whether these heme modifications contribute to the unusual catalytic properties of the mammalian peroxidases, we have reconstituted apohorseradish peroxidase (HRP) with 8-(hydroxymethyl)heme (8HM-HRP) and 8-formylheme (8F-HRP) and have characterized the reconstituted enzymes. Native HRP and 8HM-HRP have identical spectra in the ferric, compound I, and compound II states. In contrast, the Soret band of 8F-HRP is at 417 rather than 402 nm and that of its compound II species is at 436 rather than 416 nm. Compound I was observed as a transient species with 8F-HRP. The rate of formation of compound I was the same for native and 8HM-HRP, but the pseudo-first-order constant for decay of compound I was 0.021 s^{-1} for 8HM-HRP and 0.010 s^{-1} for native HRP. The rates of oxidation of guaiacol, iodide, and thioanisole are the same for native HRP and 8HM-HRP but are significantly slower for 8F-HRP. The stereospecificity of thioanisole oxidation is the same for native and 8HM-HRP, but differs for 8F-HRP. For guaiacol, which was studied in detail, K_m = 2.3 mM and k_{cat} = 33 s^{-1} for 8F-HRP versus K_m = 1.8 mM and k_{cat} = 104 s^{-1} for native HRP. 8HM-HRP oxidizes ethylhydrazine and azide to the ethyl and azidyl radicals, respectively, and is simultaneously inactivated. 8F-HRP is also slowly inactivated by ethylhydrazine and azide. Inactivation of 8HM-HRP by azide is associated with the formation of a heme adduct with the electronic absorption and mass spectrometric properties expected of δ-*meso*-azido-8-(hydroxymethyl)heme. Neither of the reconstituted enzymes oxidizes styrene, chloride, or bromide at detectable rates. The catalytic properties of HRP and 8HM-HRP are thus similar whereas those of 8F-HRP are altered, but in neither instance does the modification convey the ability to oxidize bromide or chloride ions.

The catalytic properties of heme-dependent peroxidases[1] are governed by the nature of the prosthetic group, the ligands to the iron atom, the catalytic residues provided by the protein, and the general topological and physicochemical properties of the active site (Ortiz de Montellano, 1992; Dunford, 1991; Bosshard et al., 1991; Thomas et al., 1991; Magnussen, 1991; Hurst, 1991; Henderson, 1991). The crystal structure of cytochrome *c* peroxidase (Finzel et al., 1984), the only peroxidase for which a high-resolution crystal structure is available, indicates that the iron is coordinated to a histidine residue and suggests that cleavage of the peroxide dioxygen bond to yield the catalytically active ferryl (Fe^{IV}=O) species is promoted by distal histidine and arginine residues (Poulos & Kraut, 1980). The distal histidine is thought to function as an acid–base catalyst and the arginine as a polarizing residue that facilitates dissociation of the terminal oxygen as a molecule of water. Spectroscopic and kinetic evidence suggests that the proximal and distal histidines and distal arginine are also part of the catalytic mechanisms of other classical peroxidases (Ortiz de Montellano, 1992; Dunford, 1991; Bosshard et al., 1991; Thomas et al., 1991; Magnussen, 1991; Hurst, 1991; Henderson, 1991). These three amino acid residues and the prosthetic group thus represent the catalytic core of classical peroxidases, although hydrogen-bonding and other interactions appear to modulate their detailed function (Thanabal et al., 1988). We have recently provided evidence that peroxidase catalysis is also controlled, at least in some instances, by structural and topological features that promote reaction of substrates with the δ-meso edge of the heme group rather than the ferryl oxygen (Ortiz de Montellano, 1987, 1992). Despite the apparent conservation of the catalytic mechanism, there are important differences in the catalytic potentials of the individual peroxidases.

The primary physiological function of lactoperoxidase and myeloperoxidase appears to be oxidation of small endogenous ions to reactive, diffusible species that kill pathogenic organisms (Thomas 1985; Klebanoff, 1991). The antibacterial activity of lactoperoxidase is due primarily to oxidation of thiocyanate to hypothiocyanous acid (HOSCN) (Thomas, 1985) and that of myeloperoxidase to oxidation of chloride to hypochlorous acid (HOCl) (Hurst, 1991; Klebanoff, 1991). Hypochlorous acid is not involved in the action of lactoperoxidase because it can oxidize iodide and bromide but not chloride (Thomas, 1985). HRP, like other plant peroxidases, oxidizes iodide but not bromide or chloride (Thomas, 1985).

The mechanistic or structural basis for the exceptional ability of lactoperoxidase and myeloperoxidase to oxidize halogens or pseudohalogens other than iodide is not known. It is

† This work was supported by Grants GM32488 (P.O.M.) and HL22252 (K.M.S.) from the National Institutes of Health. The UCSF Liver Center (R. Ockner, Director) spectrophotometry core facility is supported by Grant 5 P30 DK26743. The Biomedical, Bioorganic Mass Spectrometry Facility of the University of California at San Francisco (A. Burlingame, Director) is supported by National Institutes of Health Grants RR 01614 and 5 P30 DK26743.

* Author to whom correspondence should be addressed.

‡ University of California at San Francisco.

§ University of California at Davis.

[1] Abbreviations: heme, iron protoporphyrin IX regardless of the oxidation and ligation state of the iron; HRP, horseradish peroxidase; 8HM-HRP, HRP reconstituted with the 8-hydroxymethyl derivative of heme; 8F-HRP, HRP reconstituted with the 8-formyl derivative of heme; HPLC, high-pressure liquid chromatography; POBN, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone.

0006-2960/93/0432-3658$04.00/0

probable, however, that the differences between the prosthetic groups of lactoperoxidase, myeloperoxidase, and horseradish peroxidase are relevant to the catalytic differences. The prosthetic group of HRP is heme itself, but the heme is modified and covalently bound in the two mammalian enzymes. The prosthetic group extracted from lactoperoxidase under relatively mild conditions is a derivative of heme with a thiol function attached to either the 1- or 8-methyl group (Nichol et al., 1987). This has led to the proposal that the heme is covalently bound to the protein via a thioether link between the appropriate methyl group and a cysteine residue (Nichol et al., 1985). The structure of the prosthetic group of myeloperoxidase continues to be difficult to establish. Early resonance Raman studies suggested that the prosthetic group was a chlorin derived by reduction of one of the double bonds of heme (Sibbett & Hurst, 1984; Babcock et al., 1985), but recent work provides strong evidence that it is a covalently bound derivative of heme in which one of the peripheral heme substituents is a formyl group (Wever et al., 1991; Sono et al., 1991). Proton NMR studies indicate that the number, resonance patterns, and metal ion-induced relaxation properties of the well-resolved prosthetic group signals are very similar for myeloperoxidase and lactoperoxidase, suggesting that the prosthetic groups of both proteins are similar (Dugad et al., 1990). Extraction of the prosthetic group from the protein after modification by reaction with photochemically generated ketyl radicals yields a modified heme group that retains two vinyl and three (rather than four) methyl substituents (Hori & Ikeda-Saito, 1990). These results suggest that the prosthetic group of myeloperoxidase bears some resemblance to heme *a*, the prosthetic group of cytochrome *c* oxidase, in which the 8-methyl is replaced by a formyl group and an isoprenyl moiety is attached to the 2-vinyl substituent (Battersby et al., 1985). However, the crystal structure of canine myeloperoxidase at a resolution of 3 Å shows that the major unidentified electron density in the vicinity of the prosthetic group is near pyrrole ring B rather than pyrrole ring D (Zeng & Fenna, 1992). The heme is therefore probably attached to the protein via a link to the 3-methyl or 4-vinyl of pyrrole ring B. Little unaccounted electron density is detected in the difference map in the vicinity of pyrrole ring D, but the relatively low resolution of the data does not unambiguously establish the position of the formyl group (Zeng & Fenna, 1992).

Prosthetic group modifications may contribute to the unique oxidative activities of lactoperoxidase and myeloperoxidase. In order to examine the consequences of modification of the 8-methyl group on peroxidase function, we have synthesized the 8-hydoxymethyl and 8-formyl derivatives of heme and used them to reconstitute apo-HRP. The results of spectroscopic and catalytic studies indicate that modification of the 8-methyl group, particularly its conversion to a formyl group, alters the catalytic properties of HRP but does not, of itself, convey the ability to oxidize chloride ions or to catalyze peroxygenase reactions.

EXPERIMENTAL PROCEDURES

Materials. Thioanisole, H_2O_2, POBN, phenylhydrazine hydrochloride, styrene, tetrapropylammonium perruthenate, and 4-methylmorpholine *N*-oxide were purchased from Aldrich (Milwaukee, WI). HRP (type VI), monochlorodimedone, and guaiacol were purchased from Sigma (St. Louis, MO). Ethylhydrazine oxalate was from Fluka (Ronkokoma, NY). Stock solutions of ethylhydrazine were made in 0.01 N HCl to minimize autoxidation. Buffers were made with deionized, glass-distilled water that had been stirred overnight with 5 g/L Chelex 100 beads (Bio-Rad). Incubations were carried out in 50 mM sodium phosphate buffer (pH 7.0).

Analytical Methods. HPLC was performed on a Hewlett-Packard Model 1040A system equipped with a diode array detector and a Varian 9010 solvent pump. Guaiacol assays were performed on a Hewlett-Packard Model 8450A diode array spectrophotometer. Absorption spectra were recorded on an Aminco DW-2000 spectrophotometer. Fast kinetics were measured on an Applied Photosystem stopped-flow spectrometer Model SF.17MV equipped with a xenon arc lamp and interfaced to an Acorn Archimedes 400 series computer. EPR spectra were recorded on a Varian E-104 instrument interfaced with an IBM XT-computer. Guaiacol, iodide, and thioanisole oxidation activities were measured as previously reported (Ator et al., 1987; Harris et al., 1993). The guaiacol activity was measured in incubations containing 30 nM enzyme, 600 μM H_2O_2, and 0.1–10 mM guaiacol. Monochlorodimedone chlorination and bromination were assayed by the procedure of Hager et al. (1967). The enzyme (0.4 nmol) was added to incubation mixtures containing monochlorodimedone (100 μM), H_2O_2 (200 μM), and NaCl or NaBr (100 mM) in 50 mM sodium phosphate buffer (pH 7.0). The decrease in the monochlorodimedone absorbance at 290 nm (ϵ = 18 000 M^{-1} cm^{-1}) was used as a measure of halide oxidation.

8-(Hydroxymethyl)heme. 8-(Hydroxymethyl)heme was prepared synthetically (Snow & Smith, 1989) or was isolated from incubations of phenylhydrazine with HRP (Ator & Ortiz de Montellano, 1987). Two 18-μL aliquots of 4 M H_2O_2 (1.0 mM final concentration) were added 10 min apart to 140 mL of sodium phosphate buffer (pH 7.0) containing 168 mg (30 μM) of HRP and 15 mg (750 μM) of phenylhydrazine. After an additional 15 min at 25 °C, 20 μL of a 10 mg/mL solution of catalase was added to quench excess peroxide, followed 5 min later by 600 μL of 50 mM sodium ascorbate to reduce oxidized forms of HRP. The solution was then acidified with acetic acid, and the heme derivatives were extracted into diethyl ether and purified by HPLC as previously reported (Ator & Ortiz de Montellano, 1987).

8-Formylheme. The oxidation of 8-(hydroxymethyl)heme to 8-formylheme was accomplished with tetrapropylammonium perruthenate (Griffith et al., 1987). A solution of 8-(hydroxymethyl)heme (1 mg, 1.5 μmol) in a few drops of pyridine was combined with 1 mL of CH_2Cl_2, a small amount of tetrapropylammonium perruthenate was added, and the resulting solution was stirred in the dark at room temperature under argon. The shift of the Soret absorbance from 397 to 408 was used to monitor the extent of the reaction. Additional tetrapropylammonium perruthenate, or the perruthenate regenerator 4-methylmorpholine *N*-oxide, was periodically added until the reaction appeared to be complete (approximately 2.5 h). The mixture was then diluted with 20 mL of CH_2Cl_2, and the solution was washed sequentially with 20 mL each of sodium sulfite, saturated NaCl solution, saturated cupric sulfate solution, and saturated NaCl solution. The CH_2Cl_2 was removed on a rotary evaporator, and the residue was taken up in 55:45:10 acetonitrile/water/acetic acid and was subjected to HPLC on a Whatman Partisil ODS-3 column eluted with the same solvent. The column effluent was monitored at 408 nm. The 8-formylheme had a Soret maximum at 408 nm and, as expected, an electrospray mass spectrometric molecular ion at m/z 632.

Kinetic Analysis of the Reaction of 8HM-HRP and 8F-HRP with H_2O_2. HRP was reconstituted with 8-(hydroxymethyl)heme or 8-formylheme as reported earlier for recon-

stitution of the enzyme with modified hemes (Ator et al., 1989). The rate of formation and decay of the compound I species was measured by stopped-flow spectrophotometry. Equal volumes of 20 μM 8HM-HRP and 200 μM H_2O_2 were delivered into the mixing chamber of the stopped-flow apparatus, giving final enzyme and H_2O_2 concentrations of 10 and 100 μM, respectively. The concentration of H_2O_2 was an order of magnitude higher than that of HRP to ensure that compound I was formed under pseudo-first-order conditions. The reaction was monitored at 412 nm, the isosbestic point between the absorption spectra of 8HM-HRP and the compound II species produced from it. Changes in the absorption at 412 nm therefore reflect only the formation and decay of compound I. The kinetics of compound I formation with 8F-HRP was similarly investigated with the monochrometer set at 427 nm, the isosbestic point between native 8F-HRP and its compound II complex.

Spin-Trapping of Ethyl and Azidyl Radicals. A 50-μL aliquot of an incubation of 8HM-HRP (12 μM), 175 μM H_2O_2, 45 mM POBN, 5 μM DETAPAC, and either 10 mM sodium azide or 20 mM ethylhydrazine in 50 mM sodium phosphate (pH 7.0) buffer was transferred by syringe to a capillary tube. The capillary tube was then dropped into a quartz tube aligned in the EPR cavity. The azide incubations were scanned immediately after the reaction was initiated, whereas the ethylhydrazine reactions were scanned at various times during the course of the reaction. Control reactions were carried out in the absence of the enzyme or H_2O_2. The EPR parameters were as follows: field, 3400 G; scan range, 10 × 10 G; microwave frequency, 9.52 GHz; power, 20 mW; 2nd harmonic, 100 kHz; modulation amplitude, 1 × 1; scan time, 2 min; time constant, 0.25.

Enzyme Inactivation. To a solution of HRP, 8HM-HRP, or 8F-HRP (1 μM) in 1 mL of pH 7.0 sodium phosphate buffer at 25 °C were added 30 μL of a 50 mM solution of ethylhydrazine oxalate in 0.01 M HCl and 2 μL of a 70 mM solution of H_2O_2. The final ethylhydrazine and H_2O_2 concentrations were 1.5 mM and 140 μM, respectively. Aliquots (5 μL) were removed periodically and were assayed for guaiacol oxidizing activity. Inactivation of the enzyme by sodium azide was determined with incubation mixtures containing modified HRP (2.5 μM), H_2O_2 (120 μM), and sodium azide (2.5–120 μM). The residual guaiacol oxidizing activity at each concentration of sodium azide was determined after preincubation for 2 h at 25 °C.

Isolation of Modified Heme from Incubations of 8HM-HRP with Azide. A solution of 8HM-HRP (20 μM), H_2O_2 (1.5 mM), and sodium azide (2 mM) in 4 mL of 50 mM sodium phosphate buffer, pH 7.0, was incubated for 10 min at room temperature. Catalase was then added, and 5 min later the mixture was acidified with acetic acid and extracted with diethyl ether. Solvent removal under vacuum yielded a residue that was subjected to HPLC on an Alltech Partisil ODS-3 5-μm column eluted with the following gradient: 0 to 20 min, 95% solvent A, 5% solvent B; 20 to 30 min, a linear gradient of solvent B from 5% to 100%; and 30 to 35 min, 100% solvent B. Solvent A is 60:40:10 methanol/water/acetic acid, and solvent B is 100:1 methanol/acetic acid. The mass spectrum of the modified porphyrin thus isolated from the incubation mixture was obtained by microbore (1.0 mm i.d. × 100 mm) HPLC-ESIMS on a VG Bio-Q triple quadrupole instrument.

RESULTS

8HM-HRP and 8F-HRP. Reconstitution of apo-HRP with the 8-hydroxymethyl or 8-formyl derivatives of heme (Figure 1) was achieved by the procedure used to reconstitute apo-HRP with hemin (Ator et al., 1989). The 8-(hydroxymethyl)-heme derivative was obtained by total chemical synthesis (Snow & Smith, 1989) and, to a minor extent, by extraction and purification of the prosthetic group from incubations of HRP with phenylhydrazine (Ator & Ortiz de Montellano, 1987). The spectra of 8HM-HRP, the resulting reconstituted enzyme, and the compound I and II states obtained by reaction with low to moderate amounts (up to 100-fold excess) of H_2O_2 are indistinguishable from the corresponding states of native HRP. In contrast, the Soret band of HRP reconstituted with 8-formylheme (8F-HRP) is shifted from 402 to 417 nm by the formyl substituent on the prosthetic group (Figure 2). The 8-formyl derivative of heme required for the reconstitution was generated by oxidation of the 8-hydroxymethyl derivative with tetrapropylammonium perruthenate (Griffith et al., 1987). This was the only oxidizing agent tried that gave acceptable yields of the 8-formylheme derivative, the structure of which is confirmed by a shift in the absorption spectrum from 398 to 408 nm and a shift of the electrospray molecular ion from m/z 616 to 632. At room temperature, reaction of 8F-HRP with H_2O_2 does not detectably give compound I, but does give compound II with a Soret band at 432 rather than 416 nm (Figure 2).

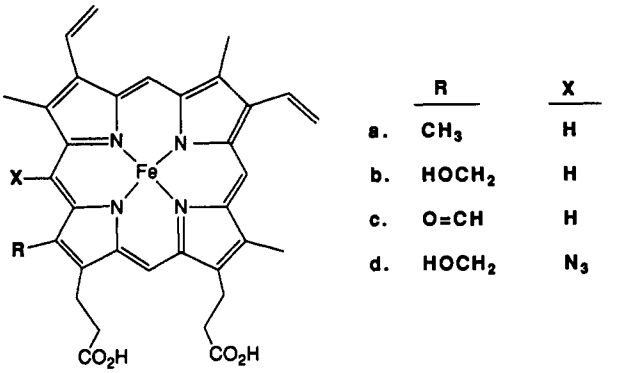

FIGURE 1: Structures of (a) heme, (b) 8-(hydroxymethyl)heme, (c) 8-formylheme, and (d) the δ-*meso*-azido derivative of 8-(hydroxymethyl)heme.

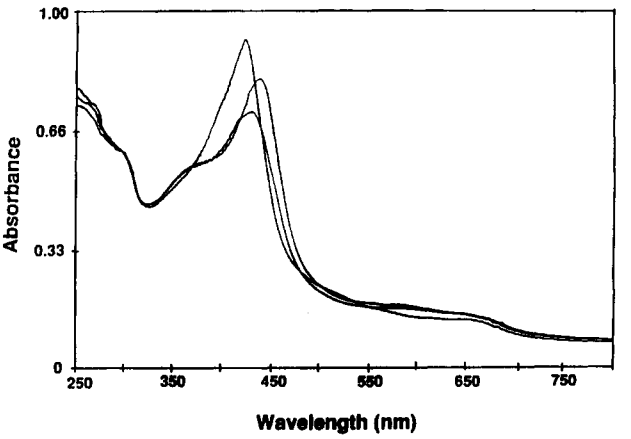

FIGURE 2: Absorption spectrum of 8F-HRP (highest maximum) and the compound I (weakest maximum) and compound II (intermediate maximum) generated by reaction with H_2O_2 at 5 °C. The maximum of compound I is at a slighly longer wavelength than expected, presumably because of a small extent of decay to compound II.

Rates of Formation and Decay of Compound I of 8HM-HRP and 8F-HRP. The rates of formation and decay of compound I from HRP and 8HM-HRP were measured by stopped-flow spectrophotometry. The monochrometer was set at 412 nm, the isosbestic point between the ferric state and

compound II of both native and 8HM-HRP, so that only the formation and decay of compound I were monitored. The rate of formation of compound I is given by the equation, $V = k_1[\text{enzyme}][H_2O_2] = k_1'[\text{enzyme}]$. Under the reaction conditions, k_1' was found to be approximately 860 s^{-1} for both native and 8HM-HRP. This gives a second-order rate constant of 8×10^6 M^{-1} s^{-1}, which agrees well with the literature value of 2.0×10^7 M^{-1} s^{-1} (Dunford & Nadezhdin, 1982). The rate of decay of compound II is given by $V = k_2[\text{enzyme}][\text{reducing substrate}]$, where the reducing substrate is an impurity in the buffer or the protein itself because no external substrate was added. Thus, $V = k_2'[\text{enzyme}]$, where k_2' is a pseudo-first-order rate constant. For native HRP and 8HM-HRP, k_2' was found to be 0.010 and 0.021 s^{-1}, respectively. Compound I of 8HM-HRP thus forms at the same rate as that of native HRP but decays approximately twice as fast to compound II.

Reaction of 8F-HRP with a 20-fold excess of H_2O_2, conditions which allow the observation of compound I of native HRP for at least 1 min, did not give a detectable compound I spectrum. The spectrum of compound II was directly observed (Figure 2). The spectrum of a mixture of compounds I and II was observed immediately after the modified enzyme was mixed with a 2-fold excess of H_2O_2, but only the spectrum of compound II was observed after 15 s. Under these conditions, the conversion of native HRP compound I to compound II requires more than 7 min. If the solution of 8F-HRP is cooled to 5 °C, compound I can be transiently detected (Figure 2). However, compound I of 8F-HRP decays to compound II within a few seconds, even at 5 °C. Due to the amount of sample required for stopped-flow experiments, the kinetic parameters for formation and decay of the 8F-HRP compound I have not been obtained. A freeze–quench EPR study detected no more than a small, transient signal assignable to a protein radical in incubations of 8F-HRP with H_2O_2.[2]

Catalytic Activities of Modified HRP. 8HM-HRP catalyzes the oxidation of guaiacol, iodide, and thioanisole at rates essentially identical to those of the native enzyme, but does not detectably oxidize chloride or bromide ions. Futhermore, the stereospecificity of the sulfoxidation reaction (R:S = 5:1) is the same for 8HM-HRP as for native HRP. In contrast, 8F-HRP oxidizes guaiacol, iodide, and thioanisole at significantly slower rates. The k_{cat} and K_m values for the oxidation of guaiacol are 33 s^{-1} and 2.3 mM for the modified enzyme versus 104 s^{-1} and 1.8 mM for native HRP, respectively. The decreased rate of guaiacol turnover is therefore primarily due to a decrease in the catalytic constant rather than a decrease in binding affinity. 8F-HRP catalyzes thioanisole oxidation at less than half the rate of the native enzyme, and iodide at only 13% of the rate of the native enzyme, and produces a different ratio (R:S = 2.9:1) of the thioanisole sulfoxide enantiomers. Like native HRP, neither 8F-HRP nor 8HM-HRP catalyzes the epoxidation of styrene or the detectable oxidation of chloride or bromide as measured by halogenation of monochlorodimedone (not shown) (Hager et al., 1967).

Inactivation of Modified HRP by Alkylhydrazines and Azide. Incubation of 8HM-HRP with ethylhydrazine results in the formation of POBN-trappable ethyl radicals (Figure 3) in a reaction that also results in inactivation of the enzyme. The enzyme is inactivated half as fast as native HRP, however (Figure 4), suggesting that the 8-hydroxymethyl group conveys some resistance to inactivation. 8F-HRP is inactivated even more slowly by ethylhydrazine than 8HM-HRP (Figure 4). The slower rate of inactivation of 8F-HRP may simply reflect the facts that ethylhydrazine oxidation is required for inactivation and that the formyl enzyme oxidizes substrates more slowly than native or 8HM-HRP.

8HM-HRP also oxidizes azide to the azidyl radical (Figure 3) and, like native HRP (Ortiz de Montellano et al., 1988), is inactivated in the process. Inactivation occurs too rapidly for the rates to be measured, but the partition ratio between metabolite formation and enzyme inactivation can be determined by measuring the extent of inactivation with various limiting concentrations of azide (Figure 5). A biphasic plot is obtained for the inactivation of 8F-HRP by azide, in accord with our previous observation of a biphasic plot for the inactivation of HRP itself (Ortiz de Montellano et al., 1988). The resistance to inactivation at higher azide-to-enzyme ratios may be due to coordination of the azide to the heme with

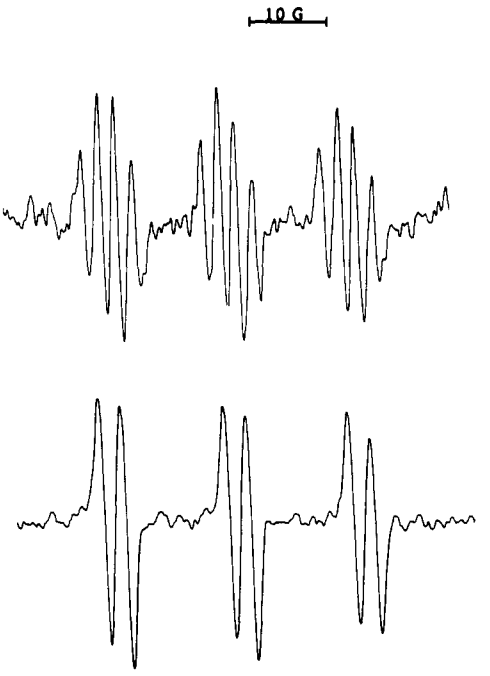

FIGURE 3: EPR spectrum of the radical trapped by POBN in the reaction of 8HM-HRP with (upper) sodium azide and (lower) ethylhydrazine. The incubation details and provided in the Experimental Procedures.

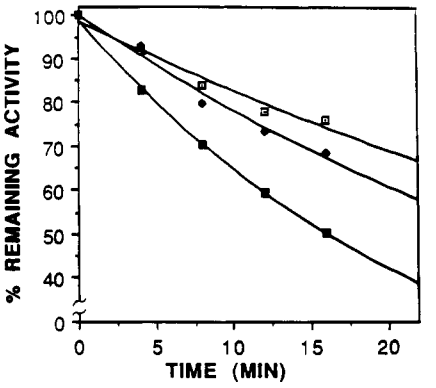

FIGURE 4: Inactivation of HRP (■), 8HM-HRP (◆), and 8F-HRP (⊡) by ethylhydrazine. The incubation details are provided in the Experimental Procedures.

[2] Electron transfer to the heme from the protein associated with the formation of compound II would be expected to result in the formation of a protein radical. Addition of H_2O_2 (400 μM final concentration) to 8F-HRP (100 μM) in a quartz EPR tube followed by immediate freezing in liquid nitrogen produced a very weak but reproducible EPR signal at approximately g = 2.0 (not shown). The nature of this weak signal is unknown.

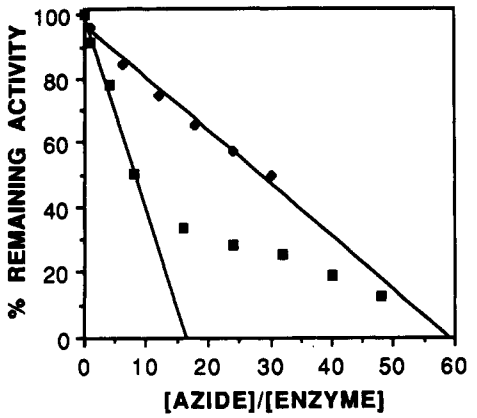

FIGURE 5: Partition ratios for the inactivation of HRP (◆) and 8HM-HRP (■) by sodium azide. The incubation details are provided in the Experimental Procedures.

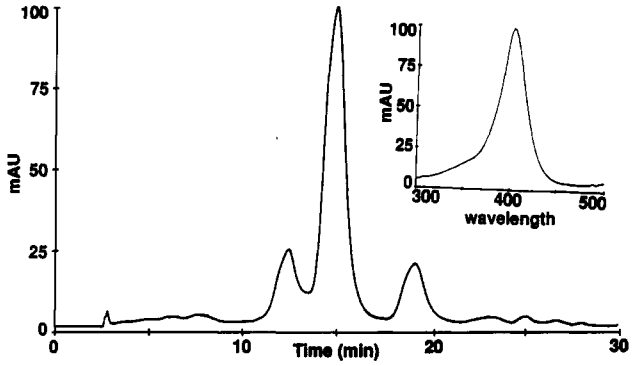

FIGURE 6: HPLC analysis of the prosthetic group recovered from the reaction of 8HM-HRP with sodium azide under turnover conditions. The absorption spectrum of the principal peak is shown in the inset (wavelength in nanometers). HPLC conditions are described in the Experimental Procedures.

consequent inhibition of compound I formation. The difference between the partition ratios deduced from Figure 5, 16 for the modified enzyme and 58 for native HRP, suggests that the 8-hydroxymethyl group increases the efficiency of the inactivation process. 8F-HRP is also inactivated during catalytic turnover of azide, but at a much slower rate than native HRP or 8HM-HRP (not shown).

The mechanism of the azide inactivation reaction appears to be the same in the case of 8HM-HRP and native HRP. Incubation of the reconstituted enzyme with azide and H_2O_2, followed by HPLC analysis of the prosthetic group, shows that the 8-(hydroxymethyl)heme has been modified (Figure 6). The modified heme has a Soret maximum at 406 nm (Figure 6) and a mass spectrometric molecular ion at m/z 673. The molecular ion at m/z 673 corresponds to addition of azide and loss of a hydrogen. These results, in view of our earlier demonstration that azide adds to the δ-meso carbon of the heme group in native HRP, leave little doubt that the adduct obtained with 8-(hydroxymethyl)heme is the corresponding δ-*meso*-azidyl adduct (Figure 1).

DISCUSSION

Replacement of the prosthetic heme group of HRP with 8-(hydroxymethyl)heme yields an enzyme that differs only in minor ways from the native enzyme. Thus, the spectra of the reconstituted and native enzymes in the ferric, compound I, and compound II states are indistinguishable. The rate constant for formation of compound I is the same for both enzymes, although compound I decays to compound II twice as fast in the modified as in the native enzyme. This is not surprising because the decay occurs in the absence of an added electron donor. A protein residue therefore probably provides the required electron, a process that, if anything, is likely to be facilitated by structural perturbation of the active site. The finding that 8HM-HRP oxidizes guaiacol, iodide, and thioanisole at rates, and in the latter case with a stereochemistry, very similar to those catalyzed by the native enzyme indicates that the 8-hydroxymethyl modification does not significantly alter the interaction of the enzyme with the three substrates, even though evidence suggests that iodide and guaiacol bind in distinct sites near the 8-methylheme group (Harris et al., 1993; Sakurada et al., 1986, 1987). Finally, 8HM-HRP, like native HRP, oxidizes ethylhydrazine and azide to the corresponding radicals and is inactivated by them. The latter inactivation involves addition of the azidyl radical to the heme, presumably at the δ-meso carbon, exactly as it does in the inactivation of native HRP (Ortiz de Montellano et al., 1988). It is therefore clear that attachment of a heteroatom to the 8-methyl group alters the intrinsic properties of the prosthetic heme group very little. The unusual catalytic properties of lactoperoxidase, among them the ability to oxidize bromide ions, therefore appear not to derive from the probable presence of a heteroatom link between the heme 8-methyl group and the protein. Although oxygen and sulfur differ, the effects of the two heteroatoms on the properties of the heme group are likely to be primarily inductive, since the heteroatom is not conjugated to the porphyrin π-system, and therefore not very different.

In contrast to the 8-hydroxymethyl group, an 8-formyl moiety causes major differences in the properties of the reconstituted and native enzymes. As expected, the Soret bands in the spectra of 8F-HRP and its compound I and compound II species are considerably red-shifted due to conjugation of the formyl group (Figure 2). Previous spectroscopic studies have shown that reconstitution of HRP with diacetyldeuterioheme, a prosthetic group having two carbonyl substituents, causes a red shift of approximately 10 nm in the Soret band of the resting enzyme (Makino & Yamazaki, 1972). Compound I of 8F-HRP is much less stable than that of native HRP and is detected only as a transient species.[2] This is consistent with previous studies showing that carbonyl substituents at the 2- and 4-positions of the heme group in HRP strongly destabilize compound I (Makino & Yamazaki, 1972; DiNello & Dolphin, 1981). The oxidations of guaiacol, iodide, and thioanisole are slower for the 8-formyl enzyme than for native HRP, as is inactivation of the enzyme during catalytic turnover of ethylhydrazine and azide.

The finding that the 8-formyl enzyme, like HRP and 8HM-HRP, does not catalyze the oxidation of chloride or bromide ions clearly shows that the increase in the oxidation potential caused by placing an electron-withdrawing substituent on the heme periphery in the position normally occupied by the 8-methyl group does not, of itself, account for the unusual ability of enzymes like myeloperoxidase to oxidize chloride ions to hypochlorous acid. This is true whether the putative formyl substituent on the prosthetic group of myeloperoxidase is located at position 8, as suggested by NMR and inferential data (Dugad et al., 1990), or at position 3, as suggested by the 3-Å crystallographic results (Zeng & Fenna, 1992), because the electronic effects should be comparable at both positions. This conclusion is consistent, furthermore, with the finding that a chemical modification of the prosthetic group that suppresses the characteristic spectroscopic prop-

erties of myeloperoxidase does not interfere with chloride oxidation (Hori & Ikeda-Saito, 1990).

ACKNOWLEDGMENT

We thank James DeVoss for helpful advice on the synthesis of 8-formylheme and Julie Fruetel and David Maltby for obtaining the mass spectra.

REFERENCES

Ator, M. A., & Ortiz de Montellano, P. R. (1987) *J. Biol. Chem. 262*, 1542–1551.

Ator, M. A., David, S. K., & Ortiz de Montellano, P. R. (1989) *J. Biol. Chem. 264*, 9250–9257.

Babcock, G. T., Ingle, R. T., Oertling, W. A., Davis, J. C., Averill, B. A., Hulse, C. L., Stufkens, D. J., Bolscher, B. G. J. M., & Wever, R. (1985) *Biochim. Biophys. Acta 828*, 58–66.

Battersby, A. R., McDonald, E., Thompson, M., Chaudhry, I. A., Clezy, P. S., Fookes, C. J. R., & Hai, T. T. (1985) *J. Chem. Soc., Perkin Trans. 1* 135–143.

Bosshard, H. R., Anni, H., & Yonetani, T. (1991) in *Peroxidases in Chemistry and Biology* (Everse, J., Everse, K. E., & Grisham, M. B., Eds.) Vol. 2, pp 51–84, CRC Press, Boca Raton, FL.

DiNello, R. K., & Dolphin, D. H (1981) *J. Biol. Chem. 256*, 6903–6912.

Dugad, L. B., La Mar, G. N., Lee, H. C., Ikeda-Saito, M., Booth, K. S., & Caughey, W. S. (1990) *J. Biol. Chem. 265*, 7173–7179.

Dunford, H. B. (1990) in *Peroxidases in Chemistry and Biology* (Everse, J., Everse, K. E., & Grisham, M. B., Eds.) Vol. 2, pp 1–24, CRC Press, Boca Raton FL.

Dunford, H. B., & Nadezhdin, A. D. (1982) in *Oxidases and Related Redox Systems* (King, T. E., Mason, H. S., & Morrison, M., Eds.) pp 653–670, Pergamon Press, Elmsford, NY.

Finzel, B. C., Poulos, T. L., & Kraus, J. (1984) *J. Biol. Chem. 259*, 13027–13036.

Griffith, W. P., Ley, S. V., Whitcombe, G. P., & White, A. D. (1987) *J. Chem. Soc., Chem. Commun.* 1625–1627.

Hager, L. P., Morris, D. R., Brown, F. S., & Eberwein, H. (1967) *J. Biol. Chem. 242*, 1769–1777.

Harris, R. Z., Newmyer, S., & Ortiz de Montellano, P. R. (1993) *J. Biol. Chem. 268*, 1637–1645.

Henderson, W. R. (1991) in *Peroxidases in Chemistry and Biology* (Everse, J., Everse, K. E., & Grisham, M. B., Eds.) Vol. 1, pp 105–121, CRC Press, Boca Raton FL.

Hori, H., & Ikeda-Saito, M. (1990) *Biochemistry 29*, 7106–7112.

Hurst, J. K. (1991) in *Peroxidases in Chemistry and Biology* (Everse, J., Everse, K. E., & Grisham, M. B., Eds.) Vol. 1, pp 37–62, CRC Press, Boca Raton, FL.

Klebanoff, S. J. (1991) in *Peroxidases in Chemistry and Biology* (Everse, J., Everse, K. E., & Grisham, M. B., Eds.) Vol. 1, pp 1–35, CRC Press, Boca Raton, FL.

Magnussen, R. P. (1991) in *Peroxidases in Chemistry and Biology* (Everse, J., Everse, K. E., & Grisham, M. B., Eds.) Vol. 1, pp 199–219, CRC Press, Boca Raton, FL.

Makino, R., & Yamazaki, I. (1972) *J. Biochem. 72*, 655–664.

Nichol, A. W., Angel, L. A., Moon, T., & Clezy, P. S. (1987) *Biochem. J 247*, 147–150.

Ortiz de Montellano, P. R. (1987) *Acc. Chem. Res. 20*, 289–294.

Ortiz de Montellano, P. R. (1992) *Annu. Rev. Pharmacol. Toxicol. 32*, 89–107.

Ortiz, de Montellano, P. R., David, S. K., Ator, M. A., & Tew, D. (1988) *Biochemistry 27*, 5470–5476.

Poulos, T. L., & Kraut, J. (1980) *J. Biol. Chem. 255*, 8199–8205.

Sakurada, J., Takahashi, S., & Hosoya, T. (1986) *J. Biol. Chem. 261*, 9657–9662.

Sakurada, J., Takahashi, S., & Hosoya, T. (1987) *J. Biol. Chem. 262*, 4007–4010.

Sibbett, S. S., & Hurst, J. K. (1984) *Biochemistry 23*, 3007–3013.

Snow, K. M., & Smith, K. M. (1989) *J. Org. Chem. 54*, 3270–3281.

Sono, M., Bracete, A. M., Huff, A. M., Ikeda-Saito, M., & Dawson, J. H. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 11148–11152.

Thanabal, V., de Ropp, J. S., & La Mar, G. N. (1988) *J. Am. Chem. Soc. 110*, 3027–3035.

Thomas, E. L. (1985) in *The Lactoperoxidase System. Chemistry and Biological Significance* (Pruitt, K. M., & Tenovuo, J. O., Eds.) pp 31–53, Marcel Dekker, New York.

Thomas, E. L., Bozeman, P. M., & Learn, D. B. (1991) in *Peroxidases in Chemistry and Biology* (Everse, J., Everse, K. E., & Grisham, M. B., Eds.) Vol. 1, pp 123–142, CRC Press, Boca Raton, FL.

Wever, R., Oertling, W. A., Hoogland, H., Bloscher, B. G. J. M., Kim, Y., & Babcock, G. T. (1991) *J. Biol. Chem. 266*, 24308–24313.

Zeng, J., & Fenna, R. E. (1992) *J. Mol. Biol. 226*, 185–207.