Substrate Oxidation by the Heme Edge of Fungal Peroxidases. Reaction of *Coprinus macrorhizus* Peroxidase with Hydrazines and Sodium Azide†

Gia D. DePillis and Paul R. Ortiz de Montellano*

Department of Pharmaceutical Chemistry, School of Pharmacy, and Liver Center, University of California, San Francisco, California 94143-0446

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ABSTRACT: The peroxidase from *Coprinus macrorhizus* is inactivated by phenylhydrazine or sodium azide in the presence of H_2O_2. Inactivation by phenylhydrazine results in formation of the δ-*meso*-phenyl and 8-hydroxymethyl derivatives of the prosthetic heme group and covalent binding of the phenyl moiety to the protein but not in the detectable formation of Fe-phenyl- or *N*-phenylheme adducts. Alkylhydrazines are catalytically oxidized but do not inactivate the enzyme. Catalytic oxidation of sodium azide produces the azidyl radical and results in its addition to the δ-meso position of the prosthetic heme group. Comparison of the heme adducts obtained with *C. macrorhizus* peroxidase with those generated by horseradish peroxidase shows that the regiochemistry of the addition reactions is the same in both cases. The results suggest that substrates interact primarily or exclusively with the heme edge rather than the ferryl oxygen of *C. macrorhizus* peroxidase and indicate that the interaction occurs with the same sector of the heme edge as in horseradish peroxidase. The active-site topologies of this pair of plant and fungal peroxidases thus appear to be similar, although the observation that alkylhydrazines add to the heme edge of horseradish but not *C. macrorhizus* peroxidase clearly shows that there are significant differences in the two active sites.

Hemoprotein monooxygenases and peroxidases are distinguished by the fact that the monooxygenases transfer an oxygen atom from molecular oxygen to their substrates, whereas the peroxidases simply abstract an electron. Similar activated oxygen species appear to be produced by both classes of enzymes, however (Marnett, 1986; Ortiz de Montellano, 1986). The two peroxidases for which the most information is available are horseradish peroxidase and cytochrome *c* peroxidase. Reaction of H_2O_2 with horseradish peroxidase produces a ferryl species and a porphyrin (P) radical cation that can be represented as [protein][$P^{\bullet+}$][Fe(IV)=O] (Penner-Hahn et al., 1986; Hashimoto et al., 1986; Roberts et al., 1981; Schulz et al., 1984; Dolphin et al., 1971). The same ferryl species is produced in the reaction of H_2O_2 with cytochrome *c* peroxidase, but it is associated with a protein rather than porphyrin radical and thus can be represented as [protein$^{\bullet+}$][P][Fe(IV)=O] (Edwards et al., 1987; Goodin et al., 1987; Yonetani, 1976). These compound I species are reduced stepwise by single-electron transfers, first to compound II [protein][P][Fe(IV)=O], in which the porphyrin or protein radical cation has been quenched, and finally to the resting state of the enzyme [protein][P][Fe(III)]. The catalytic species of cytochrome P450 is less well characterized but is thought to be a hypervalent iron–oxygen complex related to one or the other of the two peroxidase compound I species (Marnett, 1986).

The differences in the reactions catalyzed by cytochrome P450 and the classical peroxidases are often ascribed to the differences in the ligand trans to the activated ferryl oxygen in the two proteins: an imidazole in the peroxidases and a thiolate in the monooxygenases (Marnett, 1986; Ortiz de Montellano, 1986). Recent work suggests, however, that the protein structure plays a dominant role in differentiating the functions of monooxygenases and peroxidases. We have shown that horseradish peroxidase is inactivated when it oxidizes arylhydrazines (Ator & Ortiz de Montellano, 1987), alkylhydrazines (Ator et al., 1987), and sodium azide (Ortiz de Montellano et al., 1987) by addition of the aryl, alkyl, or azide moiety, respectively, to the δ-meso carbon of the prosthetic heme[1] group. In contrast, the reactions of aryl- and alkylhydrazines with cytochrome P450 result in covalent binding of the aryl (Jonen et al., 1982; Delaforge et al., 1986) or alkyl moiety (Ortiz de Montellano et al., 1983) to the iron and/or nitrogens of the prosthetic heme group. Hemoglobin, myoglobin (Ringe et al., 1984), and catalase (Ortiz de Montellano & Kerr, 1983), proteins in which the heme group is also known from crystallographic data to be accessible to substrates, also oxidize aryl- and alkylhydrazines to radicals that bind to the heme iron or nitrogen atoms. These results have led us to propose that the active-site structure of horseradish peroxidase only allows substrates to interact with the edge of the prosthetic heme group in the vicinity of the δ-meso carbon rather than, as in the monooxygenases, with the ferryl oxygen (Ator & Ortiz de Montellano, 1987; Ator et al., 1987; Ortiz de Montellano et al., 1988; Ortiz de Montellano, 1987). The oxygen transfer that defines the monooxygenases is thus suppressed in horseradish peroxidase by physical separation of the substrate-derived radical from the radical-like ferryl oxygen by the active-site architecture. Further evidence that substrates bind near the δ-meso carbon is provided by the observation of a nuclear Overhauser effect between a substrate and the 8-methyl of the heme of ferric horseradish peroxidase (Sakurada et al., 1986). Ambiguous but possibly contradictory

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[1] Abbreviations: heme, iron protoporphyrin IX regardless of the iron oxidation and ligation states; CMP, *Coprinus macrorhizus* peroxidase; PBN, α-phenyl-*N*-*tert*-butylnitrone; EPR, electron paramagnetic resonance; HPLC, high-pressure liquid chromatography.

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evidence is provided by the crystal structure of cytochrome *c* peroxidase because it suggests that cytochrome *c*, the normal substrate, interacts with the γ-meso carbon edge even though the δ-meso carbon appears to be the only one that is not fully buried in the protein (Finzel et al., 1984).

To determine the generality of the mechanism proposed for the catalytic action of horseradish peroxidase, a plant enzyme, we have investigated the interaction of phenylhydrazine, three alkylhydrazines, and sodium azide with the commercially available fungal peroxidase from *Coprinus macrorhizus*. The results suggest that electron transfer to the δ-heme edge, proposed initially for horseradish peroxidase, may be a common feature of classical peroxidases.

Materials and Methods

Materials. *C. macrorhizus* peroxidase was purchased from Chemical Dynamics Corp.; 30% H_2O_2, guaiacol, and PBN were from Sigma Chemical Co. Pyridine-d_5 (100 atom % ^2H) and phenylhydrazine hydrochloride were from Aldrich Chemical Co., and (2-phenylethyl)hydrazine and methylhydrazine sulfate were from ICN Pharmaceuticals. Ethylhydrazine oxalate was from Fluka. Methylhydrazine sulfate and phenylhydrazine hydrochloride were recrystallized from ethanol. Uniformly labeled [^{14}C]phenylhydrazine hydrochloride was purchased from ICN Radiochemicals. All enzyme incubations and assays were carried out in 50 mM sodium phosphate buffer (pH 7.0) pretreated with Chelex 100 (Bio-Rad) to remove transition-metal ions.

Analytical Methods. Electronic absorption spectra were recorded on a Hewlett-Packard 8450A diode array spectrophotometer. The extinction coefficient of the *C. macrorhizus* peroxidase was estimated by measuring the protein concentration. The value $\lambda = 94300\ M^{-1}\ cm^{-1}$ was obtained and was routinely used to determine the concentration of the peroxidase. Protein concentrations were determined by the Bio-Rad method. High-pressure liquid chromatography was performed on a system composed of two Beckman Model 110A pumps, a Model 420 controller, and a Hewlett-Packard 1040A diode array detector. A General Electric 500-MHz spectrometer was used to obtain high-field ^1H NMR spectra. EPR studies were carried out on a Varian E-104 instrument custom interfaced with an IBM XT computer.

Measurement of Inactivation Partition Ratios. *C. macrorhizus* peroxidase (2 μM) was incubated for 10 min at 0 °C with H_2O_2 (0.1 mM) and 4–80 μM sodium azide in a total volume of 100 μL. Aliquots of 5 μL were then removed and assayed for residual peroxidase activity by the previously described guaiacol assay (Ator & Ortiz de Montellano, 1987). Inactivation of CMP by phenylhydrazine was similarly analyzed except that the incubations were run for 5 min at 25 °C in the presence of 20 μM–0.35 mM phenylhydrazine. Since CMP was found to be sensitive to H_2O_2 concentrations greater than 50 times the concentration of the enzyme, H_2O_2 was added slowly in 0.1 mM increments to reach a nominal final concentration greater than that of the inactivator. To determine the H_2O_2 dependence of the inactivation, CMP (2 μM) was incubated for 10 min at 0 °C with 80 μM sodium azide and concentrations of H_2O_2 ranging from 0 to 70 μM. An analogous experiment was carried out at 25 °C with 400 μM phenylhydrazine and 0–350 μM H_2O_2.

Analysis of Heme Modification. Three aliquots of H_2O_2 were added at 1-min intervals to a mixture of CMP (0.1 mM) and sodium azide or phenylhydrazine (4 mM) in 3 mL of sodium phosphate buffer. The final nominal concentration of peroxide was 5 mM. After incubation for 10 min at 25 °C with phenylhydrazine or at 0 °C with sodium azide, the mixture was acidified with 1 mL of glacial acetic acid. The heme was extracted twice with diethyl ether, and the combined extracts were evaporated to dryness under a stream of nitrogen. The heme products were then analyzed by HPLC on a 4.6 × 250 mm Whatman Partisil 5 ODS-3 column. A 15-min linear gradient was employed from 0.1% trifluoroacetic acid (TFA) in 60:40 methanol:water (solvent A) to 0.1% TFA in 100% methanol (solvent B). The flow rate was 1 mL/min, and the effluent was monitored at 400 nm. Parallel incubations of horseradish peroxidase with phenylhydrazine and sodium azide were conducted and analyzed in the same manner, except that the phenylhydrazine and H_2O_2 concentrations were 2 and 3 mM, respectively. To determine whether adducts were formed with ethylhydrazine, *C. macrorhizus* peroxidase (0.1 mM) was allowed to react with H_2O_2 (6 mM) and ethylhydrazine (3.75 mM) in a volume of 800 μL. The prosthetic group was extracted and its composition analyzed as described above.

The analysis of *N*-phenylprotoporphyrin IX formation was carried out by acidifying the incubation of CMP with phenylhydrazine and H_2O_2 with 5 volumes of 5% (v/v) H_2SO_4. The acidified mixture was extracted with ethyl acetate either immediately or after standing 8 h at 4 °C. HPLC was performed by using the solvents described above as follows: 0–10 min, 25% solvent B in solvent A; 10–20 min, linear gradient from 25% to 100% solvent B; 20–30 min, 100% solvent B.

Isolation and Identification of Heme Adducts. To 190 mg of CMP (4.8 μmol) in 80 mL of sodium phosphate buffer was added 1 mL of 50 mM sodium azide. The reaction was initiated with 125 μL of 1 M H_2O_2. The final concentrations of CMP, azide, and H_2O_2 were 59 μM, 0.6 mM, and 1.5 mM, respectively. Following incubation for 10 min at 0 °C, the mixture was acidified with 12 mL of glacial acetic acid and extracted thrice with diethyl ether. The combined extracts were evaporated to dryness in vacuo. The heme products were chromatographed on a 9 × 250 mm Whatman Partisil 5 ODS-3 semipreparative column eluted with 0.1% TFA in 70:30 methanol:water at a flow rate of 4 mL/min. The peak with a retention time of 12.5 min was collected, the HPLC solvent removed by evaporation, the residue dissolved in diethyl ether, and the ether solution shaken with 0.1 N ^2HCl and NaCl-saturated deuterium oxide to form the respective chloroiron(III) complex. The ether solution was then washed with deuterium oxide, and the ether was removed under a stream of nitrogen. The modified heme was dissolved in pyridine-d_5 and was transferred to an NMR tube. A grain of stannous chloride was added to the tube to form the ferrous bis(pyridine) complex, and the NMR spectrum was recorded.

Reconstitution of CMP with meso-Azidoheme. CMP was reconstituted with *meso*-azidoheme isolated from azide-inactivated horseradish peroxidase as described for the analogous reconstitution of horseradish peroxidase (Ortiz de Montellano et al., 1988).

Identification of the Metabolites of Phenylhydrazine Formed by CMP. CMP (80 μM) was incubated with phenylhydrazine (5 mM) and H_2O_2 in a total volume of 1 mL. H_2O_2 was added in five equal portions at 1-min intervals to give a nominal final concentration of 5 mM. After a total of 15 min at 25 °C, 10 μL of a solution of toluene and biphenyl (0.2 M each) in diethyl ether was added. The incubation mixture was then extracted with 1 mL of diethyl ether, and a 1-μL sample of the organic layer was removed for analysis by gas–liquid chromatography as previously described (Ator & Ortiz de Montellano, 1987). Metabolite yields were calculated from standard curves prepared for the authentic compounds.

Covalent Binding of [^{14}C]Phenylhydrazine to the Protein. Radiolabeling experiments were performed under normal conditions or with argon-saturated buffers under an argon atmosphere to minimize phenylhydrazine autoxidation. CMP (5 μM) was incubated with 50–500 μM [^{14}C]phenylhydrazine (specific activity 1.25 × 10^6 cpm/μmol) and 0.5 mM H_2O_2 in a 0.5-mL volume. Peroxide was omitted from control experiments. After 5 min of reaction, 1 μL of a catalase solution (5.6 mg/mL) was added to quench excess peroxide. The enzyme was assayed for remaining activity 4 min after the addition of catalase. The reaction mixtures were immediately chromatographed on columns of Sephadex G-25 (1.5 × 7 cm). Fractions of 15 drops were collected, and their absorbance spectra were determined. The protein content of the fractions exhibiting a Soret absorbance at 404 nm was determined by using CMP as the standard, and 500-μL aliquots of the fractions were analyzed for protein-bound radioactivity by liquid scintillation counting.

Identification of the Metabolites of (2-Phenylethyl)-hydrazine Formed by CMP. To 1 mL of CMP (50 μM) and (2-phenylethyl)hydrazine (5 mM) was added H_2O_2 (final concentration, 5 mM) in five aliquots as described above. The mixture was incubated a total of 15 min at 25 °C before 10 μL of a 0.2 M solution of 3-phenyl-1-propanol in diethyl ether was added. The mixture was then extracted with 1 mL of diethyl ether, and 50 μL of BSTFA was added to the organic layer prior to gas–liquid chromatographic analysis on a DB-5 capillary column. The injector and detector temperatures were 100 and 150 °C, respectively, and the column was programmed to rise from 35 to 50 °C at 50 °C/min and then from 50 to 150 °C at 6 °C/min. The oven was maintained at 150 °C for an additional 10 min.

Spin Trapping of the Azidyl Radical. EPR spin-trapping experiments were performed essentially as described by Kalyanaraman et al. (1985) Sodium azide or ethylhydrazine, H_2O_2, and DETAPAC were added to a solution of PBN in 50 mM sodium phosphate (pH 7.0) buffer. CMP was then added to initiate the reaction. An aliquot of the final mixture was immediately transferred to a capillary tube that was dropped into a quartz EPR tube aligned in the sample cavity. All measurements were begun within 15 s of initiation. Spectrometer settings were the same as those reported by Kalyanaraman et al., with the following differences: scan range, 100 G; modulation amplitude, 1.0; time constant, 0.25; gain, 5 × 10^4; scan time, 2 min.

Results

C. macrorhizus Peroxidase. Commercially available CMP is shown by SDS–PAGE analysis to consist of a single protein with a molecular weight of approximately 40000. The enzyme, for which essentially no published information is available, oxidizes common peroxidase substrates and can be assayed, as here, by the oxidation of guaiacol. The enzyme reacts with H_2O_2 to give a compound I spectrum analogous to that of horseradish peroxidase which, in the presence of an electron donor, is replaced by a steady-state compound II spectrum also very similar to that of horseradish peroxidase (Figure 1). These spectroscopic data indicate that CMP reacts with H_2O_2 to give a compound I ferryl species which is associated, as in horseradish peroxidase, with a porphyrin radical cation rather than with the protein radical characteristic of cytochrome *c* peroxidase.

Reaction of CMP with Phenylhydrazine and Alkylhydrazines. Phenylhydrazine readily inactivates CMP (not shown). Maximal, albeit incomplete, inactivation of the enzyme in the presence of excess H_2O_2 is achieved with approximately a 125:1 molar ratio of phenylhydrazine to CMP. An analogous experiment with excess phenylhydrazine and increasing concentrations of H_2O_2 shows that approximately the same ratio of peroxide to enzyme is required for maximal inactivation. Phenylhydrazine and H_2O_2 thus appear to be consumed in a 1:1 ratio in the inactivation process. Incomplete inactivation is not due to exhaustion of either H_2O_2 or phenylhydrazine because no further inactivation is observed when supplements of these agents are added to the incubation mixture. It is possible to further inactivate the enzyme but only if it is passed through a Sephadex G-25 column before it is reincubated with phenylhydrazine and H_2O_2. If the same conditions are used for the two incubations, approximately the same percent loss of activity is observed in both incubations. The identity of the inhibitory material removed by Sephadex G-25 chromatography is not known. Benzene and *trans*-azobenzene, identified by comparison of their chromatographic retention times and absorption spectra with those of authentic samples, are formed as the major detectable phenylhydrazine metabolites. Approximately 300 nmol of benzene and 500 nmol of *trans*-azobenzene are formed in a 1-mL incubation containing 78 nmol of enzyme and 5000 nmol of phenylhydrazine. No other products were detected. An equivalent amount of benzene, but no *trans*-azobenzene, is formed in the absence of added H_2O_2 (peroxide is slowly generated by autoxidation of phenylhydrazine). Earlier studies showed that horseradish peroxidase is also only partially inactivated by phenylhydrazine and established that the peroxidase is not protected from inactivation by either benzene or *trans*-azobenzene (Ator & Ortiz de Montellano, 1987). Analogous studies of the inactivation of CMP in the presence of equal concentrations of phenylhydrazine and either benzene or *trans*-azobenzene confirm that it also is not protected from inactivation by these compounds.

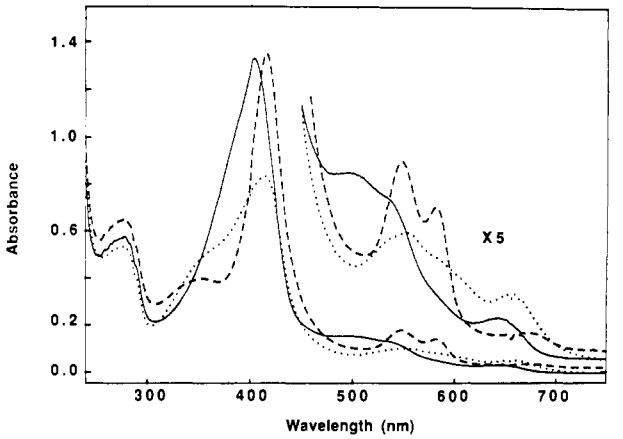

FIGURE 1: Spectra of CMP: (—) resting ferric enzyme; (···) compound I; (---) compound II. Compound II was obtained by reaction of CMP with H_2O_2 in the presence of ethylhydrazine. The regions of the spectra between 450 and 750 nm are shown with a 5-fold increase in the sensitivity of the spectrophotometer.

High-pressure liquid chromatographic analysis of the prosthetic group extracted from phenylhydrazine-inactivated CMP reveals the presence of two modified heme products (not shown). The retention times and absorption spectra of the modified hemes are identical with those of the *δ-meso*-phenyl- and 8-(hydroxymethyl)heme isolated from phenylhydrazine-treated horseradish peroxidase, although the two products are obtained in substantially lower yields than they are with horseradish peroxidase (Figure 2) (Ator & Ortiz de Montellano, 1987). No trace has been found of Fe-phenyl- or *N*-phenylheme adducts despite a specific search that would

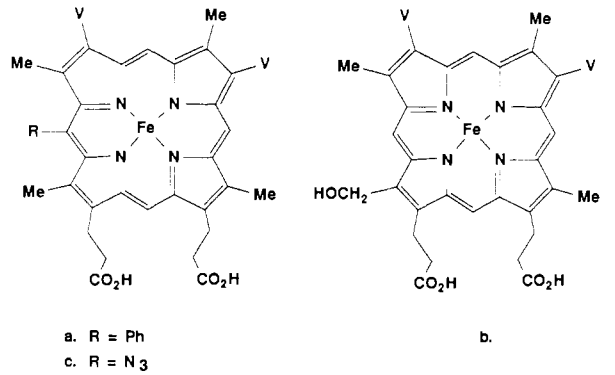

FIGURE 2: Structures of the modified heme products obtained in the reactions of CMP with phenylhydrazine and sodium azide: (a) δ-*meso*-phenylheme; (b) the 8-hydroxymethyl derivative of heme; (c) δ-*meso*-azidoheme.

have detected them if they represented as little as 1% of the inactivated enzyme. Loss of catalytic activity is not due exclusively to heme modification, however, because approximately 90% of the heme is recovered intact from enzyme that is 40% inactivated. This is consistent with the finding that 2–4 equiv of radiolabeled phenylhydrazine bind covalently to the protein in a H_2O_2-dependent manner when the enzyme is incubated with 10–100 equiv of [^{14}C]phenylhydrazine. Covalent binding of phenylhydrazine and inactivation of the enzyme occur under argon equally well as they do under a normal oxygen atmosphere.

In contrast to the findings with horseradish peroxidase (Ator et al., 1987), CMP is not detectably inactivated by 150-fold molar ratios of methyl-, ethyl-, or (2-phenylethyl)hydrazine even though these alkylhydrazines are oxidized by the enzyme. Catalytic oxidation of the alkylhydrazines, suggested by the observation of a compound II rather than compound I spectrum in incubations with the alkylhydrazines, is confirmed by the demonstration that the enzymatic oxidation of (2-phenylethyl)hydrazine yields 2-phenylethanol. The formation of 2-phenylethanol, demonstrated by gas–liquid chromatography after derivatization with BSTFA, is only observed in the presence of the enzyme and H_2O_2. No ethylbenzene, styrene, phenylacetaldehyde, or benzaldehyde was detected by gas–liquid chromatography even though control experiments show they would have been detected if they were present at $^1/_{10}$ the concentration of 2-phenylethanol. Further evidence that the alkylhydrazines are oxidized is provided by the fact that incubation of ethylhydrazine with the enzyme, H_2O_2, and PBN results in the accumulation of an EPR-detectable PBN-trapped carbon radical (not shown). The EPR parameters of the PBN radical adduct (α_N = 16, α_H = 3.5) are consistent with those observed previously for the PBN-trapped ethyl radical (Augusto et al., 1981). The carbon radical is not observed if CMP is omitted from the incubation but is observed, after a lag period, if H_2O_2 is omitted. However, addition of catalase to remove H_2O_2 produced by autoxidation of the alkylhydrazine inhibits the H_2O_2-independent reaction.

Inactivation of CMP by Sodium Azide. CMP, like horseradish peroxidase (Ortiz de Montellano et al., 1988), is inactivated when incubated with sodium azide and H_2O_2. Approximately 35 equiv of sodium azide (Figure 3) and 2 equiv of H_2O_2 (Figure 4) are required to maximally inactivate the enzyme. The dependence of the inactivation on the ratio of sodium azide to CMP is nearly identical with that for the comparable inactivation of horseradish peroxidase (Ortiz de Montellano et al., 1988). It has not been possible to carry out a detailed kinetic analysis of the inactivation reaction because

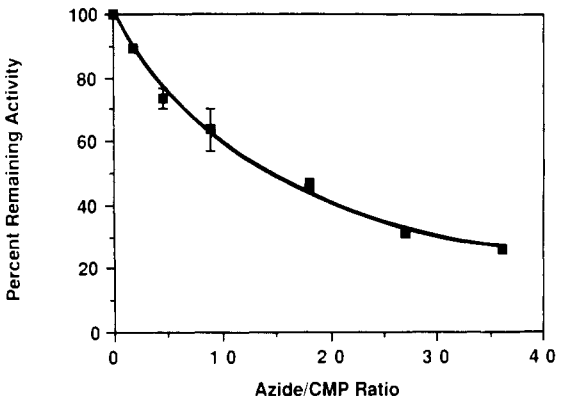

FIGURE 3: Inactivation of CMP as a function of the sodium azide:CMP ratio. The percent remaining guaiacol oxidase activity is plotted versus the sodium azide:CMP ratio. The incubation details are given under Materials and Methods. Standard deviations are given by the error bars.

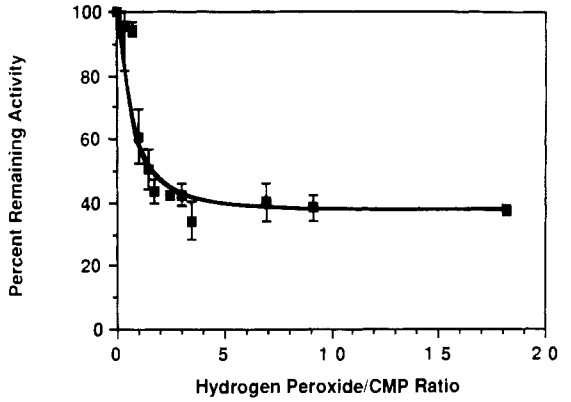

FIGURE 4: Inactivation of CMP as a function of the H_2O_2:CMP ratio. The percent remaining guaiacol oxidase activity is plotted versus the H_2O_2:CMP ratio. The incubation details are given under Materials and Methods. Standard deviations are given by the error bars.

the enzyme is degraded too rapidly by the concentrations of peroxide that are required to make the reaction a pseudo-first-order process.

Incubation of CMP with sodium azide and H_2O_2 yields a compound II like spectrum within 30 s that decreases in intensity as the enzyme activity is lost. No other intermediates are observed in the reaction by static spectroscopic methods. The linear plot obtained when loss of catalytic activity is correlated with loss of the compound II Soret band shows that the two events are closely linked (not shown). Loss of the Soret band is due to bleaching of the chromophore.

High-pressure liquid chromatography of the prosthetic group extracted by organic solvents from azide-inactivated CMP reveals the formation of a single nonpolar modified heme (Figure 5). This modified heme has been identified as δ-*meso*-azidoheme (Figure 2) by direct comparison of its HPLC retention time, electronic absorption spectrum, and NMR spectrum with those of an authentic sample isolated from azide-inactivated horseradish peroxidase (Ortiz de Montellano et al., 1988). Comparison of the meso-proton regions of the two spectra shows that the same three meso protons are present in both. This not only indicates that the azide is bound at a meso position but also confirms that the δ-meso position is involved (Figure 6). A polar heme derivative is also detected in the HPLC chromatograms (Figure 5). The identity of this product is not yet known, but studies (see below) of the reaction of CMP reconstituted with *meso*-azidoheme show that it is produced by degradation of *meso*-azidoheme in the secondary reaction of the modified enzyme with H_2O_2.

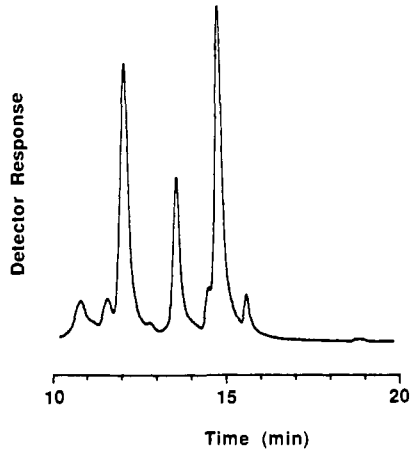

FIGURE 5: Analytical high-pressure liquid chromatogram of the prosthetic group mixture extracted from azide-inactivated CMP: unidentified polar product, 12 min; heme, 13.7 min; *meso*-azidoheme, 15 min. These analytical column retention times differ from those cited in the text for the preparative column.

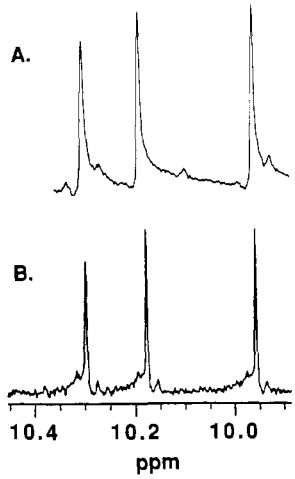

FIGURE 6: Comparison of the meso-proton region of the NMR spectra of δ-*meso*-azidoheme obtained from sodium azide inactivated CMP (A) and horseradish peroxidase (B).

meso-Azidoheme-Reconstituted CMP. CMP reconstituted with δ-*meso*-azidoheme was prepared from the apoenzyme as described previously for horseradish peroxidase (Ortiz de Montellano et al., 1988). The reconstituted enzyme has a Soret maximum at 408 nm that shifts to a compound II like maximum at 414 nm on addition of H_2O_2 in the presence or absence of sodium azide (not shown). The 414-nm absorbance maximum decays with time due to bleaching of the prosthetic group. The Soret maximum of the hypervalent reconstituted enzyme is therefore very similar to that of the intact enzyme. HPLC analysis of the prosthetic group extracted from the reconstituted enzyme after reaction with H_2O_2 indicates that the *meso*-azidoheme group is converted, at least in part, into the unidentified polar metabolite seen in Figure 5. These results differ from those obtained with horseradish peroxidase in that the compound II like spectrum obtained with the corresponding *meso*-azidoheme-reconstituted enzyme has a Soret maximum at 424 nm readily distinguished from that of the normal compound II (Ortiz de Montellano et al., 1988).

Spin Trapping of the Azidyl Radical. Incubation of CMP with sodium azide, H_2O_2, and the spin trap PBN results in observation by EPR of the PBN-trapped azidyl radical (Figure 7). Exactly the same EPR signal is obtained as is obtained in parallel incubations with horseradish peroxidase. Oxidation of azide to the azidyl radical by horseradish peroxidase was

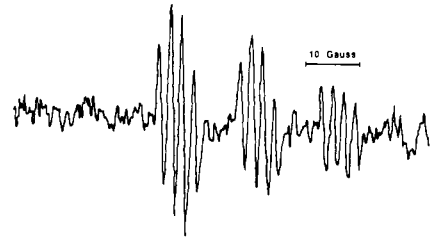

FIGURE 7: EPR spectrum of the PBN-trapped azidyl radical in incubations of CMP with sodium azide, PBN, and H_2O_2. No signal is seen in the absence of any of the incubation components.

first reported by Kalyanaraman et al. (1985). Both enzymes thus oxidize azide to the azidyl radical and are inactivated by it.

Discussion

CMP is oxidized by H_2O_2 to a horseradish peroxidase like compound I in which the ferryl oxygen species is matched with a porphyrin radical cation (Figure 1). Compound I of CMP is less stable than that of horseradish peroxidase because its formation is associated with slow inactivation of the enzyme and degradation of the prosthetic heme group. The enzyme is inactivated much more rapidly, however, if phenylhydrazine or sodium azide is present, although, as with horseradish peroxidase (Ator & Ortiz de Montellano, 1987; Ortiz de Montellano et al., 1988), only part of the enzyme activity is destroyed by even high concentrations of H_2O_2 and either phenylhydrazine or sodium azide (not shown). Further inactivation of the enzyme is possible, as in the case of horseradish peroxidase, if it is passed through a Sephadex G-25 column before it is reincubated with H_2O_2 and phenylhydrazine. Benzene and *trans*-azobenzene (PhN=NPh) are the principal metabolites of phenylhydrazine produced by CMP, although only the formation of the latter metabolite is strictly enzyme dependent. *trans*-Azobenzene presumably arises from coupling of the phenyldiazenyl (PhN=N$^\bullet$) and phenyl radicals produced in the oxidation sequence

$$Ph—NHNH_2 \rightarrow Ph—N{=}NH \rightarrow Ph—N{=}N^\bullet \rightarrow Ph^\bullet$$

The formation of *trans*-azobenzene requires a high flux of both phenyl and phenyldiazenyl radicals and is therefore only observed under catalytic turnover conditions. The low concentrations of these radicals produced by autoxidation are insufficient for *trans*-azobenzene formation and therefore only result in hydrogen abstraction by the phenyl radical to give benzene. CMP, as found earlier for horseradish peroxidase (Ator & Ortiz de Montellano, 1987), is not protected by either benzene or *trans*-azobenzene from complete inactivation by phenylhydrazine. It is unclear at this time why neither enzyme is completely inactivated by phenylhydrazine unless it is first passed through a Sephadex G-25 column.

Inactivation of CMP by phenylhydrazine is associated with conversion of its prosthetic heme group to the δ-*meso*-phenyl and 8-hydroxymethyl derivatives that are also formed in the reaction of phenylhydrazine with horseradish peroxidase (Figure 2) (Ator & Ortiz de Montellano, 1987). However, the loss of catalytic activity is primarily caused by modification of the protein because the activity loss is substantially greater than the extent of prosthetic heme modification. The analogous discrepancy between loss of catalytic activity and heme modification was shown in the case of horseradish peroxidase to result from binding of 2 equiv of radiolabeled phenylhydrazine to the protein (Ator & Ortiz de Montellano, 1987). The CMP reaction also results in covalent binding of 2–4 equiv of phenylhydrazine/mol of protein, although the extent of

protein radiolabeling correlates less well with the loss of catalytic activity. This may reflect a greater degree of nonspecific radiolabeling. In contrast to the reaction with phenylhydrazine, the inactivation of CMP by sodium azide is due to the formation of *δ-meso*-azidoheme (Figures 5 and 6) because a good correlation exists between the extent of heme modification and the loss of activity.

Three findings suggest that the gross active-site structure of CMP is similar to that of horseradish peroxidase. The absence of iron or nitrogen alkylation in the reaction with phenylhydrazine indicates, as argued earlier for horseradish peroxidase (Ator & Ortiz de Montellano, 1987), that these atoms of the prosthetic group are masked by protein residues because nitrogen and iron adducts are formed in the reactions of phenylhydrazine with hemoproteins that have open active sites (Ortiz de Montellano, 1987). The prosthetic heme group is converted instead, as with horseradish peroxidase (Ator & Ortiz de Montellano, 1987), into the *δ-meso*-phenyl and 8-hydroxymethyl derivatives. Furthermore, oxidation of azide by CMP results in formation of the azidyl radical and exclusive addition of an azidyl moiety to the δ-meso carbon of the heme group. The mechanisms of the 8-methyl hydroxylation and meso addition reactions are presumably identical with those postulated for the analogous reactions of horseradish peroxidase (Ator & Ortiz de Montellano, 1987; Ortiz de Montellano et al., 1988). The high specificities of these reactions for one of the four meso carbons and one of the four methyl groups argue that the active site of CMP is masked by the protein except for the heme edge that includes the δ-meso carbon and 8-methyl group (Ator & Ortiz de Montellano, 1987; Ator et al., 1987). A similar active-site structure was postulated earlier for horseradish peroxidase on the basis of analogous results (Ator & Ortiz de Montellano, 1987; Ator et al., 1987; Ortiz de Montellano et al., 1988; Ortiz de Montellano, 1987). The active sites of both enzymes thus appear to only allow substrates to interact with the δ-meso/8-methyl edge of the prosthetic heme group. Differences clearly exist between the topologies of the two active sites, however, because alkylhydrazine-derived carbon radicals readily inactivate horseradish peroxidase but not CMP, even though alkyl radicals are catalytically produced by both enzymes. This suggests that the heme edge of CMP is protected by the protein to a greater extent than that of horseradish peroxidase, although the nature of this protective mechanism is unknown. It is possible, for example, that steric factors allow $\pi-\pi$ overlap of the phenyl and porphyrin rings prior to tilting of the phenyl ring to initiate bond formation but do not allow alkyl radicals to approach along the trajectory perpendicular to the heme plane that is required for addition to the heme group.

The key feature of the proposed active-site structures of the horseradish and *C. macrorhizus* peroxidases is that they channel substrates to the heme edge rather than the ferryl oxygen, so that substrates are primarily or exclusively oxidized by electron transfer to the former rather than the latter. The physical separation of the catalytically generated substrate radical from the ferryl oxygen implied by this active-site geometry readily explains the virtual absence of oxygen-transfer reactions and universal production of diffusible free radicals (Ator & Ortiz de Montellano, 1987; Ator et al., 1987; Ortiz de Montellano, 1987). The finding that two phylogenetically distinct peroxidases have comparable active-site architectures strengthens the argument that they represent a general strategy for suppressing the monooxygenative function in favor of the peroxidative function of hemoproteins. The differences in the sensitivities of the two enzymes to inactivation suggests, however, that significant active-site differences are superimposed on a common active-site motif.

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