Magnetic Resonance Spectral Characterization of the Heme Active Site of *Coprinus cinereus* Peroxidase[†]

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ABSTRACT: Examination of the peroxidase isolated from the inkcap Basidiomycete *Coprinus cinereus* shows that the 42 000-dalton enzyme contains a protoheme IX prosthetic group. Reactivity assays and the electronic absorption spectra of native *Coprinus* peroxidase and several of its ligand complexes indicate that this enzyme has characteristics similar to those reported for horseradish peroxidase. In this paper, we characterize the H_2O_2-oxidized forms of *Coprinus* peroxidase compounds I, II, and III by electronic absorption and magnetic resonance spectroscopies. Electron paramagnetic resonance (EPR) and nuclear magnetic resonance (NMR) studies of this *Coprinus* peroxidase indicate the presence of high-spin Fe(III) in the native protein and a number of differences between the heme site of *Coprinus* peroxidase and horseradish peroxidase. Carbon-13 (of the ferrous CO adduct) and nitrogen-15 (of the cyanide complex) NMR studies together with proton NMR studies of the native and cyanide-complexed *Coprinus* peroxidase are consistent with coordination of a proximal histidine ligand. The EPR spectrum of the ferrous NO complex is also reported. Protein reconstitution with deuterated hemin has facilitated the assignment of the heme methyl resonances in the proton NMR spectrum.

In the pursuit of a comprehensive understanding of how the electronic and molecular structures of enzymes of the same class modulate reactivity, we have undertaken the characterization of a heme peroxidase isolated from the inkcap Basidiomycete *Coprinus cinereus*. Isolation of this peroxidase and some of its molecular characteristics such as its reactivity with hydrogen peroxide and guaiacol, amino acid and sugar composition, and secondary structure were reported (Morita et al., 1988) during the final phase of our investigation (Lukat et al., 1988). Magnetic resonance studies of the *Coprinus* peroxidase are described here for the first time along with selected ligand binding studies. Comparison of these results with those for other heme peroxidases will eventually contribute to general correlations between active-site environment and enzyme reactivity and specificity. It has been suggested that this inkcap Basidiomycete will be a new, practical source of peroxidase for clinical analyses (Shinmen et al., 1986; Uchida & Matsui, 1986), in addition to the peroxidase from horseradish root. Therefore, emphasis will be placed on the comparison of the characteristics of *Coprinus* peroxidase with those known for horseradish peroxidase.

EXPERIMENTAL PROCEDURES

The *Coprinus* peroxidase was purchased from Chemical Dynamics Corp. Protein samples as received were contaminated to varying degrees with a low-spin form of the protein identical in NMR spectral properties with the ferric cyanide complex. The samples were purified by reduction with sodium dithionite, followed by extensive dialysis and air oxidation back to the iron(III) form. The molecular weight and purity of the commercially available enzyme were examined with SDS-polyacrylamide gel electrophoresis in 10% gels in the presence of 10% β-mercaptoethanol. Our results (single polypeptide, 42 000 ± 1000 Da) were completely consistent with those previously reported (Morita et al., 1988). Spectroscopic examination of the pyridine hemochrome of *Coprinus* peroxidase also indicated that the prosthetic group is an iron protoporphyrin IX unit and yielded an extinction coefficient for the Soret at 403 nm (100 mM cm^{-1}) in agreement with the literature value (Morita et al., 1988). Isoenzyme content was determined by isoenzyme separation by affinity chromatography (Morita et al., 1988). The elution profile was identical with the published report with the exception of the distribution of isoenzyme content. The isoenzyme with 3.4% true sugar was not present in the purchased enzyme preparation. All the above physical characterization of the purchased enzyme and comparison of visible spectra of the native enzyme, its cyanide complex, compound I, and compound II with those reported by Morita and co-workers were done to ensure that the purchased enzyme was the same enzyme isolated and purified by Morita and co-workers (Morita et al., 1988).

The K_d values for the cyanide and thiocyanate complexes were determined by titration of the enzyme with the desired ligand monitored by visible spectroscopy (Sono et al., 1986).

Guaiacol (Maehly & Chance, 1954) and iodide ion (Hosoya & Morrison, 1967) oxidations as well as the monochlorodimedone chlorination (Hager, 1970) procedures were used to assay the *Coprinus* enzyme for peroxidase activity. These assays were also performed with horseradish peroxidase (Sigma type IV) for comparison purposes.

Compounds I, II, and III were prepared by addition of various amounts of hydrogen peroxide and characterization via electronic absorption, electron paramagnetic resonance (EPR),[1] and NMR spectroscopies.

Anion complexes of *Coprinus* peroxidase for EPR spectroscopy were generated by addition of excess of the desired reagent (as a solid) to protein samples or via titration with stock solutions prepared in the appropriate buffers. The ferrous NO complex of *Coprinus* peroxidase was prepared directly in an EPR tube. Once the aqueous buffered protein solution was thoroughly equilibrated with nitrogen gas, the

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[1] Abbreviations: NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; HRP, horseradish peroxidase; CPO, chloroperoxidase; LPO, lactoperoxidase.

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sample was reduced by addition of anaerobic stock solutions of sodium dithionite or ascorbic acid. Conversion to the NO complex was effected by anaerobic introduction of 1 atm of nitric oxide above the reduced protein solution at 0 °C. The EPR tube was quickly inverted 2 times and then flushed with nitrogen while the ferrous *Coprinus* peroxidase/NO complex was being frozen in liquid nitrogen. The NO complex was also generated by reduction of the *Coprinus* peroxidase/nitrite complex via the method of Yonetani and co-workers (Yonetani et al., 1972).

Concentrations for EPR samples ranged from 0.2 to 1.0 mM. The X-band EPR spectra were obtained on a Varian Model E-104A EPR spectrometer equipped with a 6-kG magnet and an Air Products helium cryostat system. The EPR signals were referenced to solid pitch for which $g = 2.002$. Spectra of anion complexes were run at 5.6 K; data for ferrous *Coprinus* peroxidase/NO complexes were collected at 70 K.

Protein reconstitution experiments were performed with 2-butanone according to the literature procedure (Yonetani & Asakura, 1969). Hemin and deuterohemin deuterated to various degrees at the methyl positions (Godziela et al., 1986) were incorporated into the protein. The proton NMR spectra of the reconstituted *Coprinus* peroxidase samples were examined in H_2O and D_2O. The reconstituted samples were allowed to equilibrate at 4 °C until no evidence of heme disorder was observed in the NMR spectrum (5 days).

Protein samples were concentrated (0.50–2.00 mM *Coprinus* peroxidase) by ultrafiltration for use in NMR experiments. Samples for proton NMR experiments were exchanged with D_2O (unless noted otherwise) containing 0.1 M phosphate buffer several times before the spectra were acquired. Dilute D_2SO_4 and NaOD were employed for pD adjustments. Uncorrected solution pD values (hereafter referred to as "pH" values) were measured in the NMR tube using a micro-NMR tube electrode. Solutions for ^{15}N NMR spectroscopy of *Coprinus* peroxidase/cyanide contained a 10-fold excess of 90 atom % $NaC^{15}N$ (Merck). For ^{13}C NMR spectroscopy, the reduced protein/CO complex was prepared with ^{13}CO (99% C-13, Iostech Inc.) as described for peroxidase/CO complexes by Behere and co-workers (Behere et al., 1985a).

Proton and ^{15}N NMR spectra were obtained at 300 and 30 MHz, respectively, on a Bruker MSL-300 spectrometer. For proton spectra, the residual water signal and the adjacent protein signals were suppressed by prepulse irradiation. External 4,4-dimethyl-4-silapentanesulfonate was used as a reference for proton chemical shift values; the residual HDO signal at 4.75 ppm provided a convenient secondary reference. Nitrogen-15 chemical shift values were referenced to external aqueous ammonium nitrate. Carbon-13 and deuterium NMR data were recorded on a Bruker WM-360 spectrometer at 90 and 55 MHz, respectively.

RESULTS

Activity Measurements. The reactivity of *Coprinus* peroxidase resembles in part that of the well-characterized HRP enzyme. Hence, *Coprinus* peroxidase with an R_z value of 2.3 yields 166 μmol of tetraguaiacol min^{-1} (mg of *Coprinus* peroxidase)$^{-1}$ with guaiacol as the substrate [compared to 217 μmol min^{-1} (mg of HRP)$^{-1}$]. *Coprinus* peroxidase has a specific activity of 20.9 μmol of triiodide min^{-1} (mg of *Coprinus* peroxidase)$^{-1}$ for the oxidation of iodide ion [compared to 15 μmol of triiodide min^{-1} (mg of HRP)$^{-1}$]. The *Coprinus* enzyme has a considerably lower specific activity [2.0 μmol min^{-1} (mg of *Coprinus* peroxidase)$^{-1}$] with monochlorodimedone as the substrate, but no chloroination is detectable with HRP.

Table I: Electronic Absorption Data of Several Peroxidases and Peroxidase Complexes

hemoprotein/X complex	absorption bands (nm)	reference
native enzyme		
Corprinus peroxidase[a]	403, 500, 643	this work
horseradish peroxidase[b]	403, 495, 640	Yonetani et al. (1972)
chloroperoxidase[c]	403, 515, 542, 650	Hager (1970)
lactoperoxidase[d]	412, 501, 541, 589, 631	Carlstrom (1969)
thyroid peroxidase[e]	413, 500, 543, 586, 636	Ohtaki et al. (1982)
Fe(III)/cyanide		
Coprinus peroxidase[a]	420, 543	this work
horseradish peroxidase[f]	423, 538	Keilin & Hartree (1951)
chloroperoxidase[g]	439, 557, 594 (sh)	Sono et al. (1986)
lactoperoxidase[d]	430, 555, 595	Carlstrom (1969)
Fe(III)/azide[h]		
Coprinus peroxidase	415, 538, 642	this work
horseradish peroxidase	418, 535, 570, 637	Tamura (1971)
Fe(III)/thiocyanate[i]		
Coprinus peroxidase	418, 500, 640	this work
Fe(II)		
Coprinus peroxidase[a]	438, 556	this work
horseradish peroxidase[f]	436, 555	Yonetani et al. (1972)
Fe(II)/CO		
Corprinus peroxidase[a]	424, 540, 568	this work
horseradish peroxidase[f]	423, 542, 572.5	Keilin & Hartree (1951)
chloroperoxidase[g]	445, 550, 576	Sono et al. (1986)
lactoperoxidase[d]	424, 543, 577.5	Carlstrom (1969)
Fe(II)/NO		
Coprinus peroxidase[a]	420, 544, 573	this work
horseradish peroxidase[b]	421, 543, 570	Yonetani et al. (1972)
chloroperoxidase[g]	444, 560, 587	Sono et al. (1986)
lactoperoxidase[j]	433, 550, 587	Lukat et al. (1987)
Fe(III)/NO_2^-		
Coprinus peroxidase[a]	417, 535, 570	this work
horseradish peroxidase[a]	417, 527, 560	this work
chloroperoxidase[g]	426, 544, 577	Sono et al. (1986)
lactoperoxidase[j]	425, 545, 583	Lukat et al. (1987)

[a] The *Coprinus* peroxidase samples were prepared in 0.1 M phosphate buffer, pH 7.0 at 25 °C, unless otherwise indicated. [b] Samples were prepared in 0.1 M phosphate buffer, pH 7.0. [c] The sample was prepared in 0.1 M phosphate buffer, pH 5.8. [d] Samples for reported spectra were prepared in 0.2 M phosphate buffer, pH 6.0. [e] The sample was prepared in 0.1 M phosphate, pH 7.4. [f] Samples for reported spectra were prepared in 0.25 M phosphate buffer, pH 5.6. [g] Samples for reported spectra were prepared in 0.1 M phosphate buffer, pH 6.0. [i] The thiocyanate complex was prepared in 0.1 M phosphate buffer, pH 4.8. [j] Samples for reported spectra were prepared in 0.1 M phosphate buffer, pH 6.8.

Electronic Absorption Spectra. The electronic absorption spectra of native *Coprinus* peroxidase, the reduced enzyme, and several *Coprinus* peroxidase/ligand complexes are very similar to those of HRP. The similarities between the *Coprinus* peroxidase data and the spectral properties of CPO or the animal peroxidases are less apparent (see Table I).

The titration of *Coprinus* peroxidase with cyanide ion and the determination of the dissociation constant (at 25 °C, 0.1 M phosphate buffer) at pH 4.6 ($K_d = 0.011$ mM) and pH 7.0 ($K_d = 0.014$ mM) indicate that the cyanide complex is not greatly affected over this pH range as determined by electronic absorption spectroscopy. Examination of the thiocyanate complex, however, reveals a marked pH dependence (K_d at pH 4.6 = 0.240 mM and K_d at pH 7.0 = 23.6 mM). The lack of a strong pH dependence of the cyanide complex K_d and the

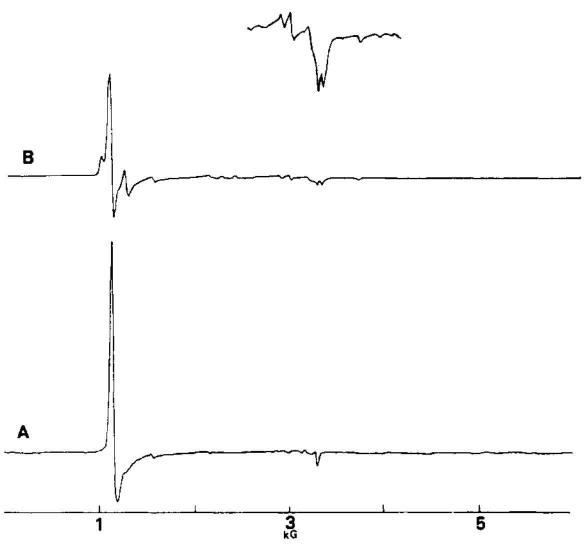

FIGURE 1: (A) EPR spectrum of 0.2 mM ferric *Coprinus* peroxidase in 0.1 M Tris/borate, pH 7.0, at 8.4 K. (B) EPR spectrum of sample described in (A) in the presence of excess sodium fluoride at 12.6 K. EPR conditions for both spectra were as follows: frequency, 9.191 GHz; power, 5 mW; modulation amplitude, 10 G at 100 kHz; sweep width, 6 kG.

presence of a significant pH dependence in the K_d value of the *Coprinus* peroxidase/thiocyanate complex is similar to the behavior reported for other peroxidases (Sono et al., 1986).

The electronic absorption spectra obtained for compounds I and II are in agreement with those reported by Morita and co-workers (Morita et al., 1988). Addition of excess hydrogen peroxide results in the formation of compound III (416, 548, and 584 nm). *Coprinus* peroxidase compound III has an electronic absorption spectrum very similar to those reported for compound III of HRP (Dunford & Stillman, 1976) and of lignin peroxidase (Reganathan & Gold, 1986).

Electron Paramagnetic Resonance Spectra. The EPR spectrum of native *Coprinus* peroxidase was examined in 0.1 M Tris/borate, pH 7.0, 0.1 M phosphate buffer, pH 5.8, and 0.05 M acetate buffer, pH 4.0. Under these conditions, the protein contains a high-spin Fe(III) ion with g values of 5.59 and 1.98. The fluoride complex of the enzyme also yields a high-spin Fe(III) EPR spectrum; its $g = 1.98$ feature is split into a doublet with a splitting of 50 G (see Figure 1). Cyanide, azide, and thiocyanate ion complexes of *Corpinus* peroxidase exhibit low-spin Fe(III) EPR spectra at 5.6 K (see Figure 2).

At 5 K, the EPR spectrum of *Coprinus* peroxidase compound I is not detected either in a derivative spectrum or in a rapid-passage spectrum. Under the same conditions, we were able to observed the rapid-passage spectrum of HRP compound I. This along with the relatively sharp proton NMR lines (discussed below) of *Coprinus* peroxidase compound I suggests that the isotropic coupling of the porphyrin radical and the metal-centered spins is larger in the *Coprinus* peroxidase case than in HRP compound I. Thus, lower temperatures will be required to observe the *Coprinus* peroxidase compound I EPR signal.

In order to further probe the nature of the proximal ligand of *Coprinus* peroxidase, the ferrous NO adduct of the enzyme has been examined by EPR spectroscopy. The EPR spectrum shows a hyperfine splitting pattern of three lines split by 17.5 G (see Figure 3). The EPR spectrum of the *Coprinus* peroxidase/NO adduct is not perturbed significantly by Tris buffer at pH 7.0 relative to acetate buffer at pH 5.2. Preparation of the NO complex via reduction of *Coprinus* peroxidase followed by addition of $NO_{(g)}$ or formation of the nitrite complex of *Coprinus* peroxidase followed by reduction yield identical EPR spectra.

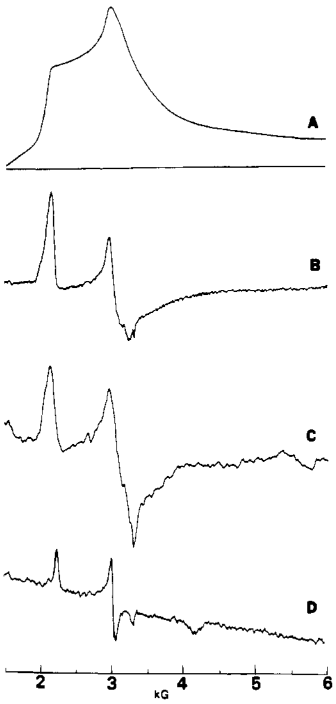

FIGURE 2: (A) Integration of the EPR spectrum of *Coprinus* peroxidase/cyanide. (B) EPR spectrum of 0.9 mM *Coprinus* peroxidase/cyanide in 0.1 M phosphate buffer, pH 6.4, at 5.4 K. EPR conditions: frequency, 9.188 GHz; power, 0.5 mW; gain, 4×10^3. (C) EPR spectrum of 0.4 mM *Coprinus* peroxidase/thiocyanate in 0.1 M phosphate buffer, pH 5.8, at 4.7 K. EPR conditions: frequency, 9.190 GHz; power, 2 mW; gain, 8×10^3. (D) EPR spectrum of 0.3 mM *Coprinus* peroxidase/azide in 0.05 M phosphate buffer, pH 6.8 at 12.5 K. EPR conditions: frequency, 9.190 GHz; power, 5 mW; gain, 1×10^4. All spectra were obtained with a modulation amplitude of 10 G at 100 kHz and a sweep width of 6 kG.

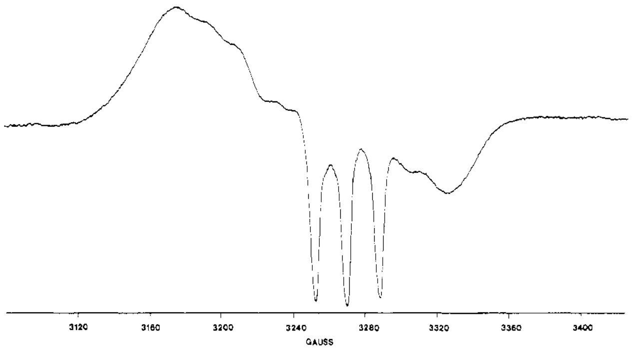

FIGURE 3: EPR spectrum observed for 0.5 mM ferrous *Coprinus* peroxidase/NO in 0.1 M phosphate buffer, pH 5.3. EPR conditions were as follow: temperature, 70.0 K; frequency, 9.189 GHz; power, 10 mW; modulation amplitude, 2 G at 100 kHz; sweep width, 400 G.

Heteronuclear Magnetic Resonance Spectra of Ligand Probes. The nitrogen-15 NMR spectrum of the *Coprinus* peroxidase/cyanide complex shows a signal corresponding to the heme-bound $C^{15}N^-$ at 578 ppm relative to nitrate ion as the reference. This resonance position is consistent with the classification of the protein as a peroxidase from comparison of the nitrogen-15 resonances reported for peroxidase enzymes relative to those observed for the hemoglobins. The 578 ppm resonance for *Coprinus* peroxidase is identical with that observed for HRP/cyanide (Behere et al., 1985b).

Carbon-13 NMR spectroscopy of the ^{13}CO ferrous hemoprotein adducts and model compounds has been shown to be

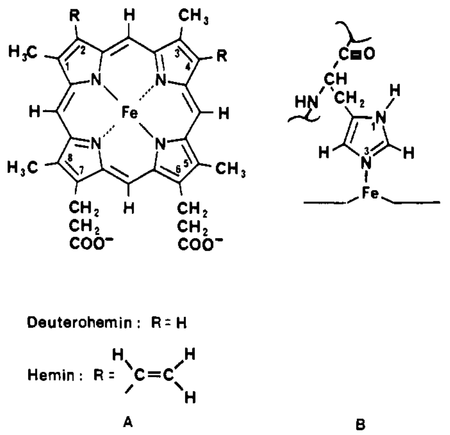

FIGURE 4: Structure and numbering system for (A) hemin and deuterohemin and (B) histidine coordinated to the iron.

sensitive to the identity of the proximal amino acid ligand. The signal for the *Coprinus* peroxidase/CO adduct is located at 207.2 ppm from the external tetramethylsilane reference. This resonance position is consistent with the coordination of a proximal histidine ligand in *Coprinus* peroxidase (Behere et al., 1985a). The visible spectrum of the ferrous CO adduct is also supportive of histidine as the proximal ligand (see Table I).

Proton and Deuterium NMR Spectroscopy. The proton NMR spectra of native *Coprinus* peroxidase resemble those of other high-spin ferric hemoproteins with numerous broad signals in the far-downfield spectral region. Proton NMR spectra of the three isoenzymes are identical and are superimposable on the spectrum of the mixture. Comparison of the proton NMR spectrum of native *Coprinus* peroxidase with the ^{2}H NMR spectrum of *Coprinus* peroxidase reconstituted with 1-, 3-, 5-, or 8-methyl-deuterated deuterohemin or with 1,3-dimethyl-deuterated protohemin confirms assignment of the peaks at 65.8, 73.6, and 87.0 ppm as heme methyl resonances (see Figures 4 and 5). Integration of these three methyl resonances in the proton NMR spectrum (Figure 5A) suggests that an exchangeable proton is present under the furthest downfield peak. In H_2O, the ratio of peak area is 2.00:0.96:1.34 (65.8 ppm:73.6 ppm:87.0 ppm). Therefore, the resonance at 65.8 ppm accounts for the protons of two methyl groups (which are resolved at temperatures higher than 33 °C); the 73.6 ppm peak represents one methyl group. The far-downfield resonance arises from four protons. When the integration is carried out on a proton NMR spectrum of the native enzyme which has been equilibrated in D_2O for 1 week, the ratio of peak areas is 2.00:0.90:1.09. This 19% decrease in relative area of the resonance at 87.0 ppm indicates that the fourth proton is slowly exchanged with the solvent. Further evidence for a single fourth proton comes from the proton NMR spectrum of *Coprinus* peroxidase reconstituted with deuterohemin with all four methyl groups highly deuterated. A broad peak of significant intensity is still observed at 87.0 ppm.

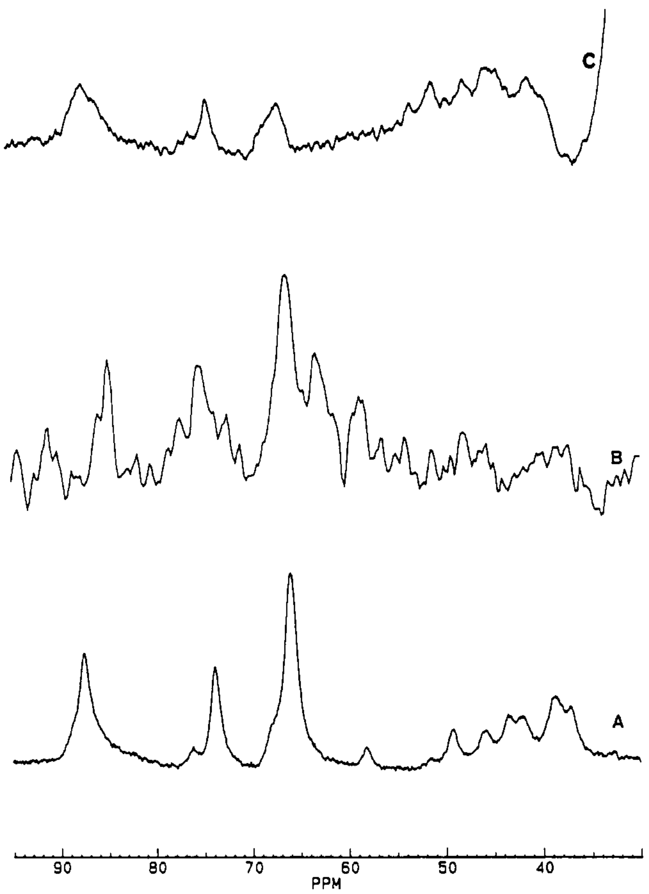

FIGURE 5: (A) 300-MHz proton NMR spectrum of the region downfield from H_2O of 0.5 mM native *Coprinus* peroxidase in 0.1 M phosphate buffer, pH 7.0 (H_2O), at 21 °C. (B) The 55-MHz deuterium NMR spectrum of *Coprinus* peroxidase reconstituted with 1- (86%), 3- (87%), 5- (69%), and (73%) 8-methyl-deuterated deuterohemin. Enzyme concentration was 0.4 mM in 0.1 M phosphate buffer, pH 7.0 (H_2O), at 21 °C. (C) The 300-MHz proton NMR spectrum of *Coprinus* peroxidase reconstituted with 1- (67%), 3- (78%), 5- (40%), and (29%) 8-methyl-deuterated protohemin. Enzyme concentration was 0.3 mM in 0.1 phosphate buffer, pH 7.5 (H_2O), at 21 °C.

Table II: Ratios of Methyl Peak Areas in the Proton NMR Spectrum Expected for Possible Methyl Assignments for *Coprinus* Peroxidase Reconstituted with Protohemin with Varying Percent Deuteration of the Four Methyl Groups

assignment of methyl resonances			ratio of theoretical peak areas[a]		
65.8 ppm	73.6 ppm	87.0 ppm	65.8 ppm	73.6 ppm	87.0 ppm
1 and 3	8	5	1.00	1.29	3.00
1 and 3	5	8	1.00	1.09	3.55
1 and 5	3	8	1.00	0.24	4.40
1 and 5	8	3	1.00	0.76	1.36
1 and 8	3	5	1.00	0.21	3.83
1 and 8	5	3	1.00	0.58	1.39
3 and 5	1	8	1.00	0.40	3.62
3 and 5	8	1	1.00	0.88	1.68
3 and 8	1	5	1.00	0.36	3.07
3 and 8	5	1	1.00	0.65	1.69
8 and 5	3	1	1.00	0.17	2.83
8 and 5	1	3	1.00	0.25	1.89
experimentally observed ratio			1.00	0.78	1.34

[a] Extent of methyl group deuteration: 1-CH_3, 67%; 3-CH_3, 78%; 5-CH_3, 40%; 8-CH_3, 29%. The presence of the N1 proton at 87.0 ppm is taken into consideration when calculating the relative peak areas.

Integration of the proton resonances observed for *Coprinus* peroxidase reconstituted with protohemin of varying fractional deuteration at the methyl positions also affords tentative assignments of the heme methyl proton resonances (see Figure 5C). Table II lists the calculated ratios of peak areas (65.8 ppm:73.6 ppm:87.0 ppm) for all the possible permutations of heme methyl group assignments. The area of the 65.8 ppm resonance is normalized to 1. The ratio calculated for the combination 1-CH_3 and 5-CH_3 at 65.8 ppm, 8-CH_3 at 73.6 ppm, and 3-CH_3 at 87.0 ppm, 1.00:0.76:1.36, is the best match to the experimental ratio of 1.00:0.78:1.34. Assignment of the 1-CH_3 and 3-CH_3 resonances at 65.8 and 87.0 ppm, respec-

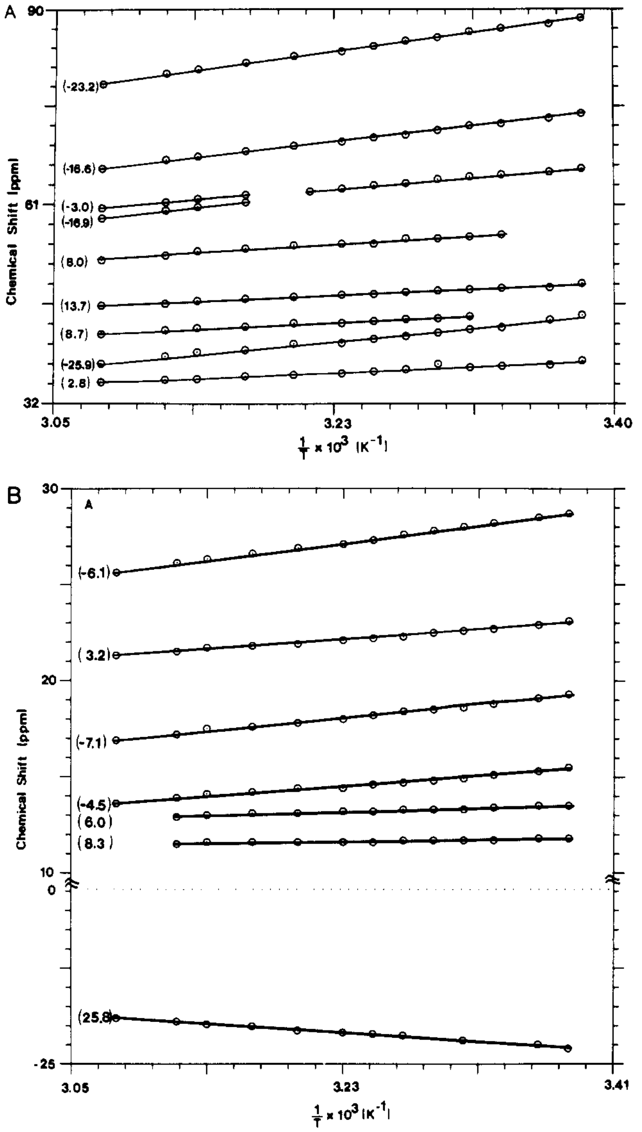

FIGURE 6: Curie plots (observed chemical shift versus reciprocal temperature) for the hyperfine-shifted resonances of (A) native *Coprinus* peroxidase and (B) *Coprinus* peroxidase/cyanide. The apparent intercepts at $T^{-1} = 0$ are given in the parentheses on the left. The uncertainties in the intercept are ±2.5 ppm for the native enzyme and ±1.5 ppm for the cyanide complex.

tively, is consistent with the deuterium NMR spectrum observed for the 1,3-dimethyl-deuterated mushroom peroxidase.

The chemical shifts observed in the proton NMR spectrum of native *Coprinus* peroxidase indicate that the protein is predominantly high spin at room temperature. However, over the temperature range of 278–325 K, the far-downfield heme methyl resonances do not adhere strictly to Curie's law (see Figure 6A). In contrast to behavior noted for myoglobin (La Mar et al., 1980a) and HRP (La Mar et al., 1980b), at least three of the methyl resonances of native *Coprinus* peroxidase have a negative $T^{-1} = 0$ intercept.

The proton NMR spectra of native ferric *Coprinus* peroxidase show only subtle changes when examined over the "pH" range 4.5–9.0. The features between 45 and 20 ppm are best resolved at pH values around 7.0. The line width of the 87.0 ppm signal decreases as the pH is increased from 4.5 to 7.6. This is consistent with the assignment of this resonance as overlapping signals of an exchangeable proton on an axial histidine and a heme methyl group. At very high pH, broadening of the all of the signals is observed, but not significant changes in chemical shift values are seen.

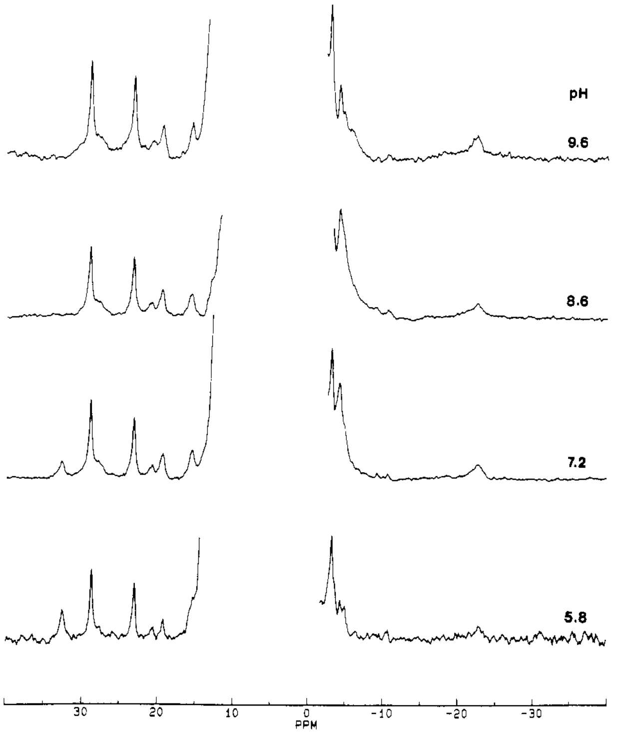

FIGURE 7: pH dependence of *Coprinus* peroxidase/cyanide in 0.1 M phosphate buffer (H_2O) monitored by proton NMR. A 30-fold excess of cyanide ion was present in each case.

Examination of reduced *Coprinus* peroxidase by NMR spectroscopy reveals that the protein is five-coordinate in the reduced state. Resonances are observed at 67.0, 22.7, 21.7, 17.7, −2.8, and −6.6 ppm. The proton NMR spectrum is very similar to that reported for Fe(II) HRP and myoglobin with the notable exception that the exchangeable proton resonance assigned to the N1 proton of the histidine proximal ligand is observed at 67 ppm. For HRP and myoglobin, this resonance is observed at 79 ppm (La Mar & de Ropp, 1982) and 77.8 ppm (La Mar et al., 1977), respectively.

When the cyanide ion titration of the native ferric enzyme is monitored by proton NMR, signals for both the native and cyanide-bound forms of the enzyme are observed simultaneously, indicating that the cyanide is in slow exchange on the 300-MHz NMR time scale. The cyanide complex does not follow strict Curie law behavior as noted by the $T^{-1} = 0$ intercepts indicated on the Curie plots for the hyperfine-shifted resonances of the complex shown in Figure 6B. There are two signals (32.6 and −22.6 ppm) that exhibit a marked pH dependence in the proton NMR spectrum of *Coprinus* peroxidase/cyanide (see Figure 7). The furthest downfield signal seen at 32.6 ppm is observed below pH 7.9 but not at higher pH values. The relative intensity of this signal increases slightly as the pH decreases. Examination of the proton NMR spectra of *Coprinus* peroxidase/cyanide with deuterated methyl groups indicates that this 32.6 ppm peak is not due to a methyl group and that it is exchanged when the sample is placed in D_2O. A downfield shift is noted for the broad resonance at −22.6 ppm with increasing pH. None of the other resonances show a discernible trend with increasing pH.

The proton NMR spectra of native *Coprinus* peroxidase titrated with azide show that azide is in slow exchange on the NMR time scale. The temperature dependence of the *Coprinus* peroxidase/azide resonances does not follow Curie law behavior, perhaps due to a spin equilibrium between $S = {}^1/_2$ and $S = {}^5/_2$ states. Formation of the anion complex is highly dependent on pH. In the presence of a 10-fold excess of azide,

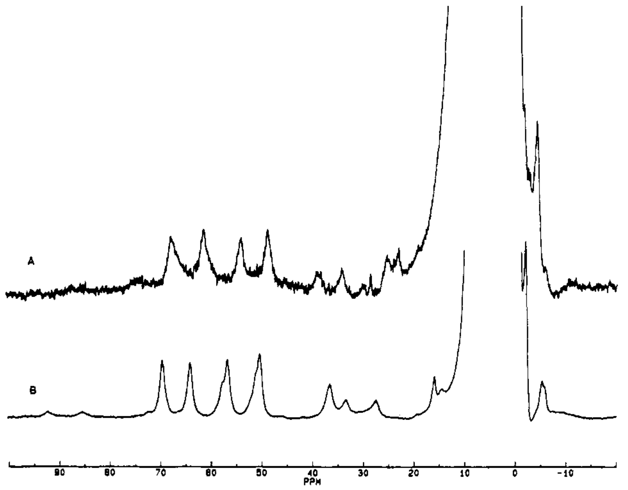

FIGURE 8: 300-MHz proton NMR spectra of (A) 0.6 mM *Coprinus* peroxidase/thiocyanate in 0.1 M phosphate (D_2O), "pH" 5.8 with a 10-fold excess of sodium thiocyanate and (B) 2.5 mM myoglobin/thiocyanate in 0.1 M phosphate buffer (D_2O) "pH" 7.0, with a 14-fold excess of sodium thiocyanate.

the complex is completely formed at "pH" 6.6 (49.0, 40.7, 38.9, 35.4, 33.7, 30.0, 28.6, 22.0, and 20.9 ppm). When the "pH" is raised to 7.0, peaks due to the native protein are observed in the NMR spectrum. Resonances due to the *Coprinus* peroxidase/azide complex do not, however, shift over the "pH" range 4.8–7.6.

The thiocyanate complex of *Coprinus* peroxidase differs from the cyanide and azide complexes in that it is predominantly high spin at room temperature. The enzyme anion complex resonances display non-Curie law behavior. Interconversion of native and thiocyanate complexes is slow on the NMR time scale. The proton NMR spectrum shows four strong downfield resonances presumed to be associated with heme methyl groups (67.8, 61.3, 53.9, and 48.6 ppm) (see Figure 8). Formation of the thiocyanate complex is also favored at low pH values. At a "pH" value higher than 6.6, resonances due to native enzyme are observed in the proton NMR spectrum. The downfield proton resonances of the *Coprinus* peroxidase/thiocyanate complex do not shift with "pH" over the range of 4.6–6.6 and completely disappear at and above "pH" 7.6.

The designation of the green complex generated upon addition of 1 equiv of H_2O_2 to *Coprinus* peroxidase as compound I is further substantiated by its hyperfine-shifted proton NMR spectrum. Figure 9 compares the proton NMR spectra of native *Coprinus* peroxidase and its compound I at 10 °C. By comparison to the hyperfine-shifted resonances in the HRP compound I proton NMR spectrum (Thanabal et al., 1986), the resonances at 85.1, 71.7, 61.5, and 48.4 ppm are tentatively assigned to the four heme methyls. The peaks at −22.8, −24.4, and −27.0 ppm are tentatively assigned to vinyl protons. Under conditions required for obtaining a proton NMR spectrum (0.3 mM *Coprinus* peroxidase; 1 equiv of H_2O_2; 10 °C), *Coprinus* peroxidase compound I has a half-life of approximately 12 min. Conversion back to the native enzyme is complete within 1.5 h.

DISCUSSION

Comparison of the optical spectroscopic data and enzyme activity data suggests some similarity between the *Coprinus* peroxidase and HRP heme sites. However, further examination of the electronic nature of the heme site of *Coprinus* peroxidase indicates that there are a number of significant differences. The EPR spectrum of the native *Coprinus* peroxidase (g values of 5.59 and 1.98) reflects an axial symmetry which is in contrast to the rhombic nature of the EPR spectrum for HRP (g values of 6.35, 5.65, and 2.0) (Blumberg et al., 1968), cytochrome *c* peroxidase (g values of 6.4, 5.4, and 1.97) (Yonetani & Anni, 1987), LPO (g values of 6.43, 5.43, and 1.99) (Lukat et al., 1987), and for most other peroxidases for which the EPR spectrum is known.

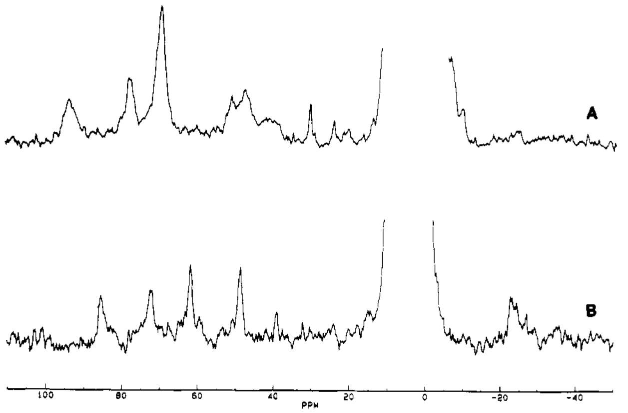

FIGURE 9: 300-MHz proton NMR spectrum of (A) native *Coprinus* peroxidase and (B) *Coprinus* peroxidase plus 1 equiv of hydrogen peroxide. Both spectra were acquired at 10 °C.

The low-spin EPR spectrum observed for the *Coprinus* peroxidase/thiocyanate complex confirms the presence of the temperature-dependent spin equilibrium indicated by the non-Curie law behavior of the proton resonances in the NMR spectrum. The lack of a well-defined third component in the EPR spectra for the cyanide and thiocyanate complexes makes calculation of rhombic and tetragonal factors impossible. However, hand integration of the EPR spectrum of the cyanide complex reveals intensity in the region of the spectrum where the third component of the spectrum is expected (see Figure 3). For the azide case, the g values 2.96, 2.15, and 1.59 yield $\Delta/\lambda = 4.38$ and $V/\Delta = 0.54$ (Palmer, 1983). These values are similar to those obtained for LPO/azide (Bolscher et al., 1984); the similarity in azide complexes is interesting considering the differences in the native EPR spectra of these two enzymes.

The three-line hyperfine splitting observed in the EPR spectrum of the ferrous *Coprinus* peroxidase/NO adduct is in contrast to the nine-line hyperfine splitting pattern (indicative of a proximal nitrogen atom) reported for ferrous HRP/NO (Yonetani et al., 1972) and LPO/NO (Lukat et al., 1987). It has been demonstrated that LPO must be completely reduced in order to observe a nine-line splitting pattern (Lukat et al., 1987). To ensure complete reduction of the *Coprinus* peroxidase sample, an NO adduct sample was titrated with increasing amounts of a stock sodium dithionite solution. Even at a 1000-fold excess of dithionite ion, the three-line spectrum is observed.

Appearance of only three lines from NO hyperfine coupling in the ferrous *Coprinus* peroxidase/NO can be interpreted in several ways. The first possibility is that the proximal amino acid ligand is bound through some atom other than nitrogen (such as sulfur or oxygen). However, optical spectral data and carbon-13 NMR data for ferrous *Coprinus* peroxidase/CO are not indicative of a proximal cysteinyl sulfur ligand (Sono et al., 1986). Second, the proximal ligand may dissociate from the heme upon iron reduction. This would result in a four-coordinate Fe(II) heme prior to NO binding. The proton NMR of reduced *Coprinus* peroxidase, however, is indicative of a five-coordinate species. A resonance is also seen at 67 ppm which is tentatively assigned to the labile ring proton of the proximal histidine on the basis of comparison to reduced myoglobins (La Mar et al., 1977), hemoglobins (La Mar et al., 1977; Nagai et al., 1982), and HRP (La Mar & de Ropp, 1982). Thus, dissociation upon reduction seems unlikely.

The third possibility is that the histidine Fe–N bond is significantly weakened or broken as a consequence of tenacious NO coordination to the Fe(II) (Maxwell & Caughey, 1976). EPR studies of model compounds have shown that the six-coordinate nitrosylpipyridyltetraphenylporphinatoiron(II) complex exhibits a rhombic EPR spectrum that lacks detailed hyperfine structure. The five-coordinate nitrosyltetraphenylporphinatoironn(II), however, shows a strong three-line hyperfine splitting pattern at g_z and weak splitting at g_x and g_y (Szabo & Perutz, 1976). It has been demonstrated that upon addition of inositol phosphate the EPR spectrum of hemoglobin changes from a spectrum similar to that of the six-coordinate nitrosylpipyridyltetraphenylporphinatoiron(II) complex to one representative of a five-coordinate system [like nitrosyltetraphenylporphinatoiron(II)]. Furthermore, it has been shown that the changes observed in the EPR spectrum are due solely to the α subunits (Nagai et al., 1978). Infrared studies also support the weakening or total loss of the Fe–N proximal bond (Maxwell & Caughey, 1976) of the α subunits. The lack of characteristic electron spin-echo envelope modulation frequencies further supports the absence of a six-coordinate NO species and disruption of the proximal imidazole bond to iron in α subunits of low-affinity hemoglobin (Hille et al., 1979). It is interesting to note that the proton NMR shifts for the labile ring proton of the proximal histidine of the α and β subunits of hemoglobin are 63.6 and 76.7 ppm, respectively (Nagai et al., 1982). The shift observed for the α subunits is very similar to that seen for ferrous *Coprinus* peroxidase. This suggests that the environment of the proximal ligand in reduced *Coprinus* peroxidase and the α chain of hemoglobin A may be similar with both proteins favoring a five-coordinate ferrous/NO complex.

Finally, on the basis of the temperature dependence of the EPR spectrum of single-crystal MbNO, it has been reported that the conformation of the bonding NO is drastically altered upon freezing (Hori et al., 1981). A temperature-dependent equilibria between two forms of MbNO at low temperatures and similar effects noted for iron(II) protoporphyrin IX/imidazole have also been observed (Morse & Chan, 1980). In addition to the three possible interpretations listed above, these observation make it difficult to come to any unambiguous structural conclusions about *Coprinus* peroxidase based solely on the EPR spectrum of its ferrous NO adduct.

Although a nine-line hyperfine splitting pattern is not observed for ferrous *Coprinus* peroxidase/NO, other spectral results support the presence of an axial histidine residue in the native and various other states. The carbon-13 chemical shift of the ferrous *Coprinus* peroxidase/CO adduct is supportive of axial histidine ligation. The resonance for a single, exchangeable proton in the NMR spectra of native myoglobin (100 ppm) and HRP (96 ppm) has been detected and assigned to the proximal histidyl imidazole on the basis of its large hyperfine shift (La Mar & de Ropp, 1979). The presence of an exchangeable proton resonance at 87.0 ppm in the NMR spectrum of native *Coprinus* peroxidase analogous, although not as obvious because of peak overlap, to the N1–H resonances of myoglobin and HRP is supportive of a proximal histidine ligand.

Assignment of the proximal heme bound histidyl 2-H imidazole signal in the −27 to −30 ppm region has been offered for horseradish peroxidase/cyanide isoenzymes (La Mar et al., 1982; Gonzalez-Vergara et al., 1985). The presence of a similar broad signal at −22 ppm in the *Coprinus* peroxidase/cyanide spectrum provides further evidence for a proximal histidine residue in this enzyme. Model compound studies indicate that the magnitude of the upfield shift for the proximal 2-H imidazole signal is quantitatively correlated with the degree of proximal imidazole hydrogen bonding (La Mar et al., 1982). Thus, a downfield shift of the histidyl 2-H signal in *Coprinus* peroxidase/cyanide relative to HRP/cyanide suggests that the hydrogen bonding for the proximal ligand of *Coprinus* peroxidase is measurably diminished (compared to the HRP case).

Although the proton NMR spectra of native *Coprinus* peroxidase and native HRP and their cyanide complexes have several features in common, there are significant differences between the NMR spectra of these two peroxidases. Examination of the room temperature proton NMR spectrum of ferric *Coprinus* peroxidase shows that two of the methyl resonances are not resolved from one another. In the HRP room temperature proton NMR spectrum, all four methyl resonances are resolved from one another (Gonzalez-Vergara et al., 1985). The ordering of the methyl group hyperfine shifts also appears to be different for the two enzymes: (going upfield) 3, 8, and (1 + 5) for *Coprinus* peroxidase and 5, 1, 8, and 3 for HRP (La Mar et al., 1980b).

Two structural features for *Coprinus* peroxidase reported by Morita et al. (1988) merit comment with regard to the magnetic resonance studies. Although the enzyme prepared by these authors appears to be generally equivalent to the commercial enzyme, the presence of a non-heme iron atom reported by Morita et al. (1988) is not evident in our EPR measurements. A rhombic $g = 4$ signal is associated with a non-heme protein-bound ferric center. The absence of a strong rhombic iron(III) signal in our EPR spectra could reflect an unusually well spin-relaxed iron(III) center or retention of a non-heme iron(II) oxidation state. Alternately, the commercial enzyme may simply lack the non-heme iron atom.

There are sufficient differences between the electronic and molecular structure of *Coprinus* peroxidase and HRP that further characterization is necessary to determine the effect of the different heme environments on the reactivity and specificity of *Coprinus* peroxidase relative to HRP.

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