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High-Resolution Proton Nuclear Magnetic Resonance Spectroscopy of Chloride Peroxidase: Identification of New Forms of the Enzyme†

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ABSTRACT: Chloride peroxidase from the mold *Caldariomyces fumago* in the native high-spin iron(III) and low-spin cyanoiron(III) states has been subjected to high-field proton nuclear magnetic resonance spectroscopic measurements. Signals shifted well outside the diamagnetic envelope by the paramagnetic iron(III) center are surprisingly insensitive to pH changes over the range from pH 3 to pH 7. The previously identified major form of chloride peroxidase (form A) and the minor form (B) show very similar chemical shift patterns. Of greatest significance, however, is the discovery that each of the separable forms of the enzyme exhibits splitting of porphyrin ring methyl resonances. The appearance of two sets of signals in both native and cyanide-complexed enzyme is best explained by the existence of two additional forms of the A and B isoenzymes. Structural differences for the newly identified forms of chloride peroxidase must be located in the vicinity of the heme prosthetic group.

High-resolution nuclear magnetic resonance (NMR) spectroscopy constitutes an especially powerful technique for elucidation of hemoprotein electronic and molecular structures. The utility of proton NMR spectroscopy relies on the very large signal dispersion for nuclei within the proximity of the paramagnetic heme center. Key signals for the prosthetic group and heme-pocket amino acid residues are thus distinguished from the hundreds of protein backbone resonances. Major classes of hemoproteins including hemoglobins, myoglobins, cytochromes, and peroxidases have been subjected to detailed proton NMR studies. Among the peroxidases, horseradish peroxidase (HRP) (Williams et al., 1975; Morishima & Ogawa, 1978; Morishima et al., 1977, 1978; La Mar & de Ropp, 1979, 1980, 1982; La Mar et al., 1980a–c, 1981, 1982a,b, 1983a–d), turnip peroxidase (Williams et al., 1975), and yeast cytochrome *c* peroxidase (Satterlee & Erman, 1980, 1981a,b; Satterlee et al., 1983a,b; La Mar et al., 1982a) have been examined by high-resolution NMR spectroscopy. Commercial availability of HRP has stimulated numerous studies of the native, iron(II), heme-reconstituted [iron(III)], and high-valent forms of this enzyme.

Proton NMR spectral measurments for chloride peroxidase (CPO) are reported here for the first time. This enzyme (EC 1.11.1.10) with a molecular weight of 42 000 is produced by the mold *Caldariomyces fumago* for biosynthesis of the

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chlorinated antibiotic caldariomycin (Morris & Hager, 1966; Hager et al., 1966). Chloride peroxidase catalyzes the peroxidative halogenation of various substrates and is also capable of oxidative N-demethylation and H_2O_2 disproportionation reactions much as is the case for the respective cytochrome P-450 and catalase enzymes [for reviews of peroxidase chemistry, see Hewson & Hager (1979), Morrison & Schonbaum (1976), Dunford & Stillman (1976); and Dunford (1982)]. Rationale for characterization of the versatile CPO catalyst is in part based on common features in the catalytic function and presumed thiolate axial ligation for CPO and cytochrome P-450. The microbial enzyme may also serve as a valuable model for mammalian halide-activating hemoproteins such as myeloperoxidase and thyroid peroxidase.

The utility of CPO proton NMR measurements is demonstrated by confirmation of the existence of two separable forms (forms A and B) of the enzyme and recognition of two new components in each of these forms. The absence of major spectral changes with pH variation allows comment about acid–base equilibria for the heme and axial ligand residues. Comparison of hyperfine-shifted NMR resonances in the spectra of several peroxidase enzymes is now possible.

Experimental Procedures

Chloride peroxidase was obtained from *Caldariomyces fumago* (ATCC 16373) grown on the fructose–salt medium described by Pickard & Hashimoto (1982). Following removal of the mycelium by filtration, the culture medium was treated with ammonium sulfate to selectively remove the gelatinous dark pigment. CPO is obtained by addition of ammonium sulfate to saturation. Following dialysis and passage through a short DEAE-cellulose column, the A_{403}/A_{280} purity ratio is 1.34. Further purification by Whatman DE-53 chromatography yields material with a purity ratio of 1.44, and the separation of isoenzymes A and B is achieved. Details of this rapid, improved isolation scheme will be published elsewhere. The DE-53 column effluent was concentrated by ultrafiltration and exchanged with D_2O or D_2O containing 0.1 M phosphate buffer several times prior to NMR spectroscopic measurements. Uncorrected solution "pD" values were measured in the NMR tube with an Aldrich micro-NMR tube electrode.

Proton NMR spectra were recorded at 360 MHz with a Bruker WM-360 spectrometer. Sweep widths of 71 kHz were routinely employed, and from 3000 to 50 000 transients were recorded. The residual water signal and adjacent protein signals were suppressed by prepulse irradiation. Spectra were recorded at 25 °C unless otherwise indicated. Chemical shift values are referenced to internal 4,4-dimethyl-4-silapentanesulfonate (DSS); the 4.75 ppm residual HDO signal provided a convenient secondary reference.

Results and Discussion

Native Protein. The high-resolution proton NMR spectrum of native CPO in the 12 to −2 ppm (diamagnetic) region is provided in Figure 1. This spectrum is recorded for a D_2O solution, and hence many exchangeable proton signals are absent. A relatively slow acquisition rate was employed, and HDO saturation was avoided such that representative signal intensities might be measured. Signals in the 10–6 ppm region are clearly apparent for aromatic amino acids and for nonexchanged N–H residues. An intense signal in the 4 ppm region likely represents the CPO carbohydrate moieties and amino acid β-CH residues. Signals in the 3.0–0.0 ppm region must represent largely amino acid aliphatic side chains. On the basis of an earlier amino acid analysis (Morris & Hager, 1966) and known chemical shift values for amino acids, protein

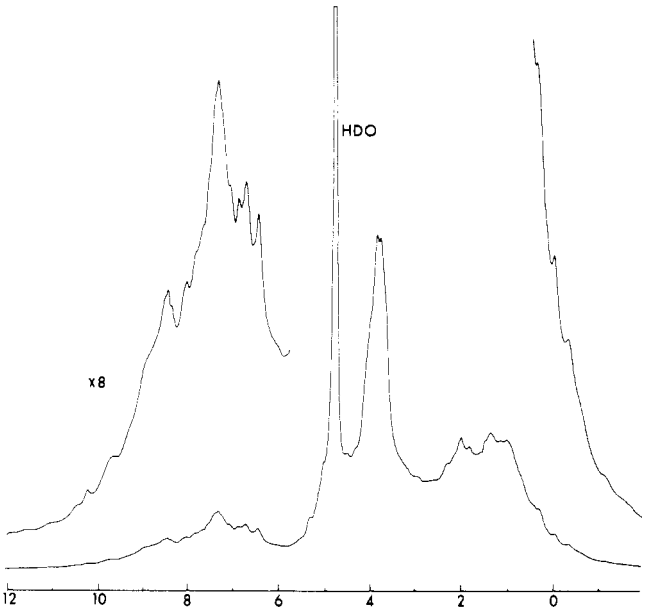

FIGURE 1: Proton NMR spectrum of the diamagnetic region of native CPO (mixture of A and B forms) with no suppression of the residual water signal; 25 °C, D_2O solvent, 1.0 mM enzyme, pD 5.8.

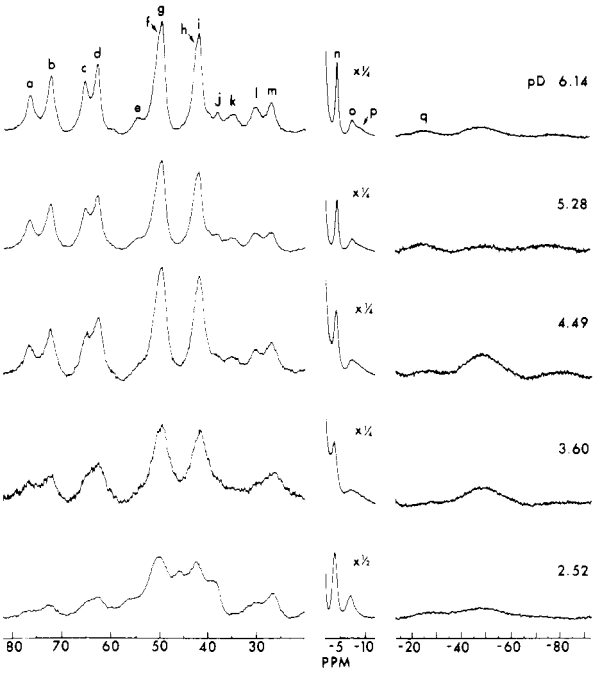

FIGURE 2: Proton NMR spectra of native CPO (mixture of A and B forms) at various pD values; 25 °C, D_2O solvent, 1.0 mM enzyme.

signals in the 3.0–0.0 ppm region are expected to result from a total of approximately 876 protons. The total intensity of overlapping signals in this spectral region can thus be compared with intensities of heme signals located far outside the diamagnetic region (vide infra).

Proton NMR spectra of native CPO resemble in large part those of other high-spin iron(III) hemoproteins. Signals in far-downfield and far-upfield positions are expected for heme and axial ligand protons. The four heme methyl signals in high-spin iron(III) porphyrins are often readily assignable on the basis of intensity and far-downfield position. Complexity appears, however, in the spectra of native CPO shown in Figure 2. At the higher pH values, more than four major downfield signals are clearly apparent. Intensities of signals a/b (combined), c/d (combined), and h/i (combined) correspond to an average of 2.3 protons each when compared with

Table I: Proton NMR Chemical Shift Values for Native Chloride Peroxidase[a]

peak	form A + B[b]	form A[c]	form B[d]
a	76.6	76.6 (A_2)	75.4 (B_2)
b	72.4	72.4 (A_1)	71.3 (B_1)
c	65.3	65.5 (A_2)	65.0 (B_2)
d	62.8	62.9 (A_1)	62.5 (B_1)
e	54.5	54.9	[e]
f	50.5	50.4 (A_2)	50.4 (B_2)
g	49.8	49.7 (A_1)	50.0 (B_1)
h	42.6	42.6 (A_2)	41.9 (B_2)
i	42.1	42.1 (A_1)	41.6 (B_1)
j	38.4	38.2	37.7
k	35.1	34.8	34.8
l	30.3	30.2	30.2
m	27.2	27.0	26.9
n	−4.18	−4.18	−4.18
o	−7.1	−7.2	[e]
p	−9.1	−8.7	[e]
q	−27	−27	[e]

[a] Peak listings match those shown in Figures 2 and 3; shifts referenced to DSS; D_2O solvent, 25 °C. [b] pD 5.84. [c] pD 6.43. [d] pD 5.80. [e] Signal/noise ratio insufficient to identify signal.

the 3.0–0.0 ppm region signals of Figure 1 (assumed to represent 876 aliphatic protons). The far-downfield signals may thus reasonably be assigned to heme methyl residues. Apparent splitting of methyl signals will be discussed shortly. It is likely that signals f/g (combined) also represent a methyl resonance and that increased intensity of this signal is a consequence of overlap with other signals. For example, high-spin iron(III) porphyrin vinyl protons are known to resonate in the 50 ppm region (La Mar et al., 1980c). Additional signals in the 38–27 ppm region likely represent porphyrin ring CH_2 moieties. A rather sharp upfield signal at −4.28 ppm corresponds in intensity to that of a methyl residue and may represent an amino acid side chain in close proximity to the heme center. Signals labeled o and p are in approximately the same upfield positions as vinyl CH_2 resonances assigned in other high-spin ferrihemoproteins (La Mar et al., 1980c). A very broad signal at −27 ppm is distinguished from broad base-line artifacts under all instrumental conditions (however, the intensity is distorted by our base-line-smoothing routine). Broad features at −47 and −80 ppm may also represent real signals, but apparent line widths of approximately 5 kHz are on the order of base-line "roll" artifacts, and an unequivocal statement about the features cannot be offered at this time.

Effects of pD changes on CPO spectra are summarized in Figure 2. The only dramatic effect occurs at pD values below 4.0, in which case the resonances show extensive broadening. For the pD 2.52 solution, new signals are seen at 46 and 39 ppm. In our hands, the protein precipitates and is irreversibly changed after a short period at this pD value. The protein is also unstable at neutral and higher pD values.

The previously recognized CPO forms A and B (Sae & Cunningham, 1979; Pickard & Hashimoto, 1982) were examined separately as shown in Figure 3. Spectra for the two identified forms are very similar, but measurable chemical shift differences are apparent in Table I. Furthermore, the spectrum for the A component in Figure 3 would appear to exhibit more favorable resolution than that of the mixture as a consequence of elimination of overlapping signals from the B form. Both A and B forms exhibit multiple porphyrin ring methyl signals identified as a/b, c/d, f/g, and h/i signal pairs. The newly identified pairs of signals are for convenience designated as components A_1, A_2, B_1, and B_2, where the major component of each form is assigned the "one" subscript. This

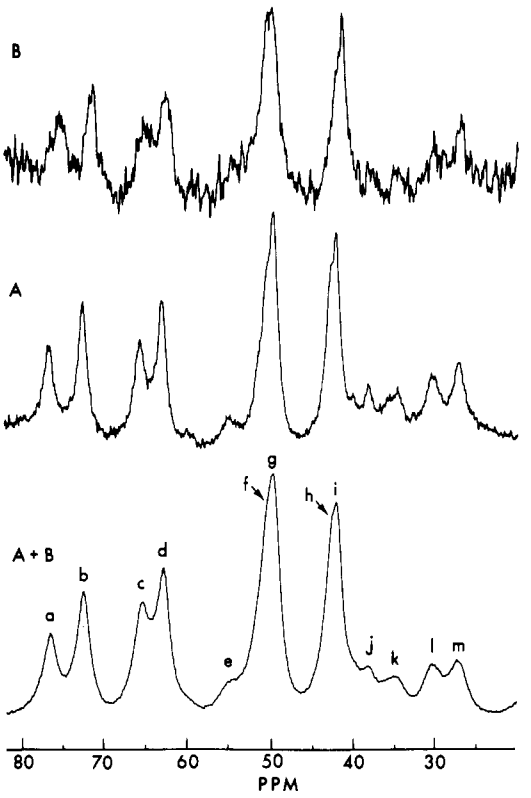

FIGURE 3: Proton NMR spectra of native forms of CPO; 25 °C, D_2O solvent. A + B mixture, pD 5.84, 1.0 mM enzyme; isolated A, pD 6.43, 0.6 mM enzyme; isolated B, pD 5.80, 0.1 mM enzyme.

additional heterogeneity of CPO preparations has not been noted by earlier workers, and indeed our cursory electrophoretic characterization did not serve to locate additional heterogeneity in the A and B forms. At first glance, it might appear illogical that readily separable forms (A and B forms) would yield very similar NMR spectra, whereas the heretofore unseparated A_1/A_2 or B_1/B_2 species show clearly inequivalent NMR spectra. This observation suggests that differences between components are intimately associated with the heme pocket. Whatever the nature of the perturbation, the absence of previously reported biphasic reactivity suggests that the different components have equivalent activity or that one component is essentially inactive.

Concerns about inadvertent generation of two CPO forms during our new isolation procedure were addressed through examination of enzyme prepared by two alternate routes. Native CPO isolated by the method of Hollenberg & Hager (1978) exhibited splitting of far downfield signals much as seen in Figure 2. Crude enzyme was also fractionated directly from the growth medium with a series of filtration steps. These included sequential passage through cheesecloth, coarse filter paper, Whatman no. 1 filter paper, cellulose powder, 5.0- and 1.0-μm membranes, and 0.45- and 0.25-μm membranes. The NMR sample was prepared by concentration of the clarified H_2O solution on an Amicon PM10 ultrafiltration membrane. The downfield signal pattern for this crude sample proved to be equivalent to that shown in Figure 2. Hence, we must conclude that detection of multiple CPO forms is not a consequence of a particular isolation methodology. It should be noted that H_2O solutions of both the crude enzyme and highly purified material did not show additional (exchangeable) signals in the 20–80 ppm region.

Variable-temperature spectra were recorded for D_2O solutions of native CPO at pD 5.8 over the temperature range 10–25 °C. All downfield signals moved further downfield as

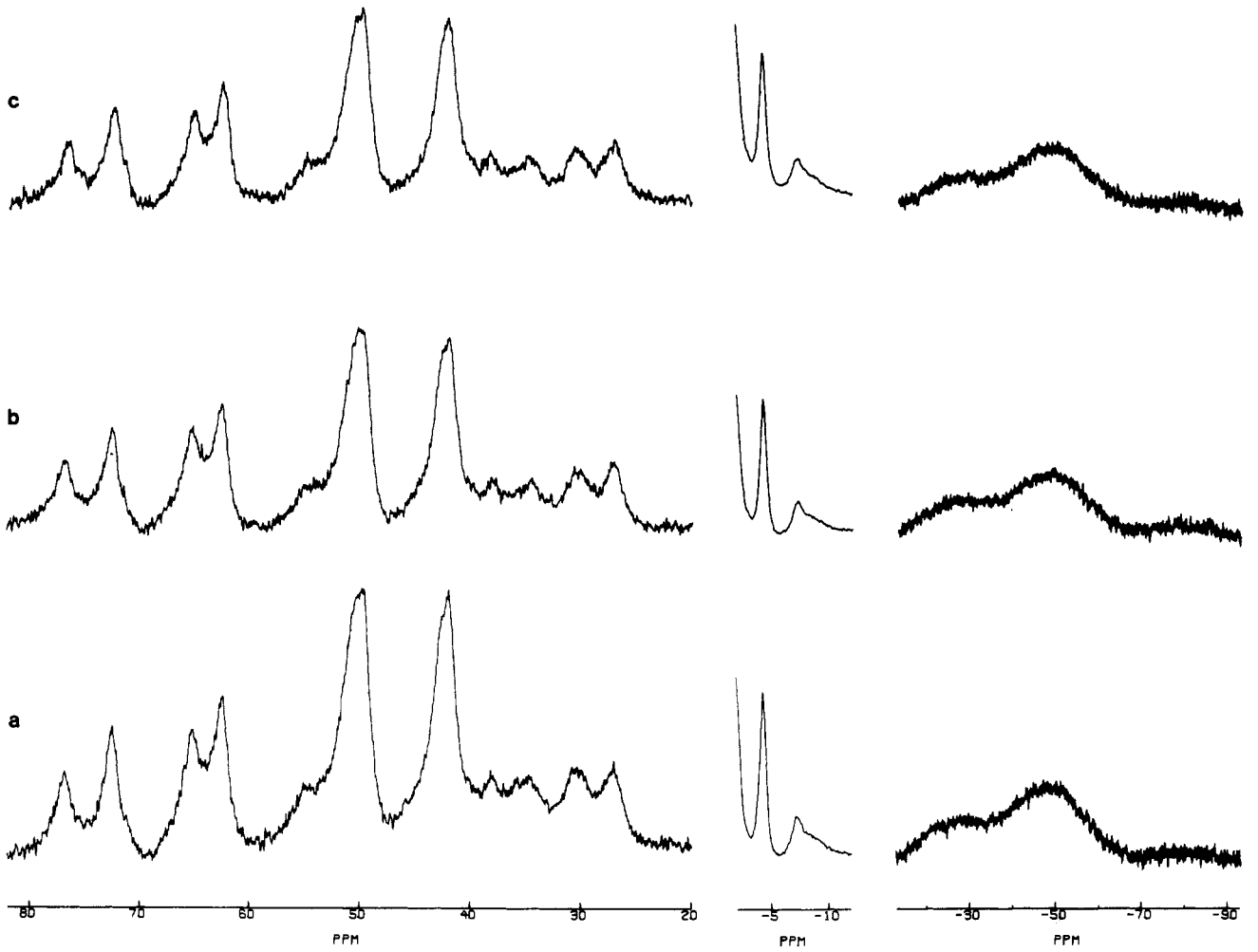

FIGURE 4: Proton NMR spectra of native CPO (mixture of A + B forms) at various chloride ion concentrations; 25 °C, D_2O solvent, 1.0 mM enzyme, pD 4.03. (a) Dialyzed solution; (b) 0.01 M NaCl; (c) 0.10 M NaCl.

the temperature was lowered, but the extent of shift was less than that expected for Curie law behavior. Signal broadening was significant at lower temperatures likely as a consequence of increased solution viscosity. No change in the relative intensities of far-downfield signals was noted over the narrow temperature range accessible for the D_2O solution (the protein is rapidly denatured at temperatures above 25 °C).

Changes in hydrogen ion concentration clearly do not change the relative fractions of one component over another, as may be seen in Figure 2. Effects of substrate chloride ion concentrations were also investigated, and results are presented in Figure 4. The sample was first "stripped" of salts by extensive ultrafiltration with D_2O. Proton NMR spectra recorded in the absence of chloride ion and in solutions containing 0.01 or 0.10 M sodium chloride are essentially identical. Although we have not demonstrated absolute removal of chloride ion from the stripped CPO, it is clear that partial substrate saturation is not responsible for generation of two NMR-detectable CPO components.

Cyanide Complex of CPO. Addition of a small excess of cyanide ion to native CPO results in conversion of the enzyme to the low-spin iron(III) complex. This process was carried out with stepwise titration such that any major CN^- affinity differences among the four forms of CPO might be detected. Results are presented in Figure 5. As the CN^- concentration is increased, the native high-spin iron(III) peaks diminish in intensity and several new downfield and upfield peaks grow in intensity. At pD 4.0, there are no apparent differences in the affinity of different forms of the enzyme for cyanide binding; the sharp methyl peaks labeled "b" through "g" exhibit the same chemical shift and splitting pattern throughout the titration (Table II). (The possibility exists, however, that CN^- binding rates of all forms are identical and that the system does not reach equilibrium during the typical 1 h of data

Table II: Proton NMR Chemical Shift Values for Ferric Cyanochloride Peroxidase[a]

peak	form A + B[b]	form A[c]	form B[d]
a	39.2	38.7	*e*
b	24.53 (B)		24.73 (B)
c	24.02 (A_1)	24.02 (A_1)	
d	23.93 (A_2)	23.93 (A_2)	
e	20.97 (B)		20.92 (B)
f	20.68 (A_1)	20.80 (A_1)	
g	20.46 (A_2)	20.61 (A_2)	
h	17.00	17.05	16.95
i	14.82	14.82	14.89
j	14.10	14.17	*e*
k	12.89	12.89	12.89
l	11.94	11.92	*e*
m	11.60	11.60	*e*
n	9.86	9.88	*e*
o	−2.57	−2.56	*e*
p	−3.94	−3.92	*e*
q	−4.52	−4.50	*e*
r	−5.10	−5.01	*e*
s	−21.2	−20.9	*e*

[a] Peak listings match those shown in Figures 5 and 6; chemical shifts referenced to DSS; D_2O solvent, 25 °C, 3-fold molar excess of KCN. [b] pD 5.8. [c] pD 6.4. [d] pD 5.8. [e] Signal/noise ratio insufficient to identify signal.

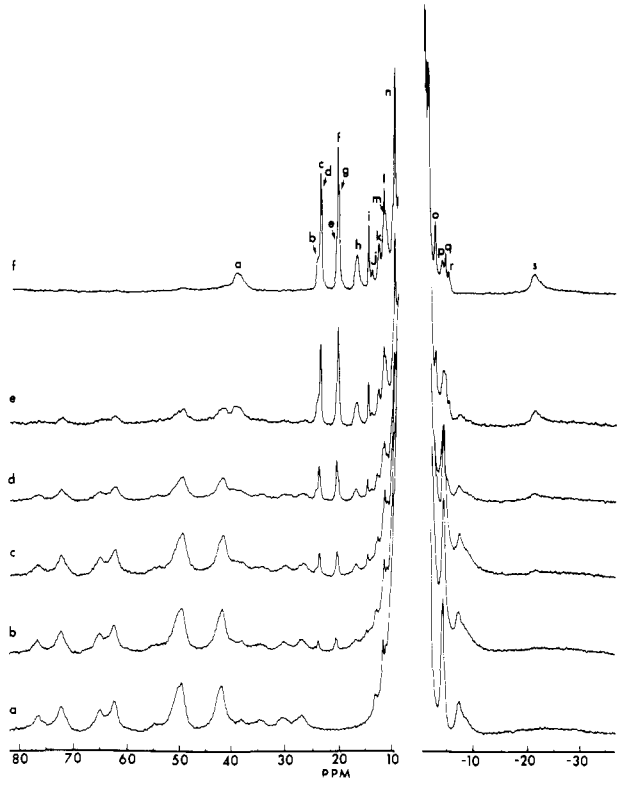

FIGURE 5: Proton NMR spectra for cyanide titration of CPO (mixture of A + B forms); 25 °C, D_2O solvent, pD 4.03, 0.95 mM enzyme, 0.10 M NaCl. (a) No KCN added; (b) 0.12 mM KCN; (c) 0.24 mM KCN; (d) 0.47 mM KCN; (e) 0.92 mM KCN; (f) 1.75 mM KCN.

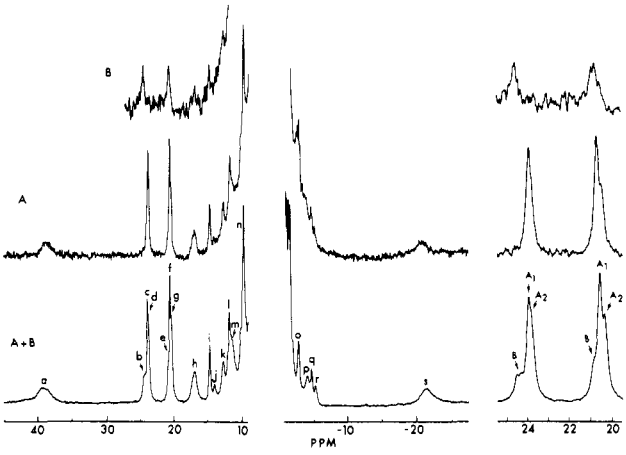

FIGURE 6: Proton NMR spectra of low-spin cyano forms of CPO; 25 °C, D_2O solvent, 5 mM KCN. The expanded region to the right illustrates components of the two sharp heme ring methyl signals. A + B mixture, pD 6.2, 1.0 mM enzyme; isolated A, pD 6.4, 0.6 mM enzyme; isolated B, pD 6.0, 0.1 mM enzyme.

acquisition following CN^- addition.) Obviously, the dynamics of the low-spin/high-spin conversion are slow on the (360 MHz) NMR time scale as judged by no change in the high-spin signal pattern with CN^- addition.

Among new signals observed in the low-spin CPO spectrum, the two groups of sharp peaks in the 20 and 24 ppm regions are tentatively associated with porphyrin ring methyl residues. Low-spin CPO spectra are shown in Figure 6 for the mixture of A and B forms and for the separated forms. The especially informative 20–25 ppm region is expanded on the right side of Figure 6. Signals for A and B components are clearly identified, and furthermore, splitting is readily apparent for the A form. Possible splitting of form B ring methyl signals is uncertain at the available signal/noise level. Observation

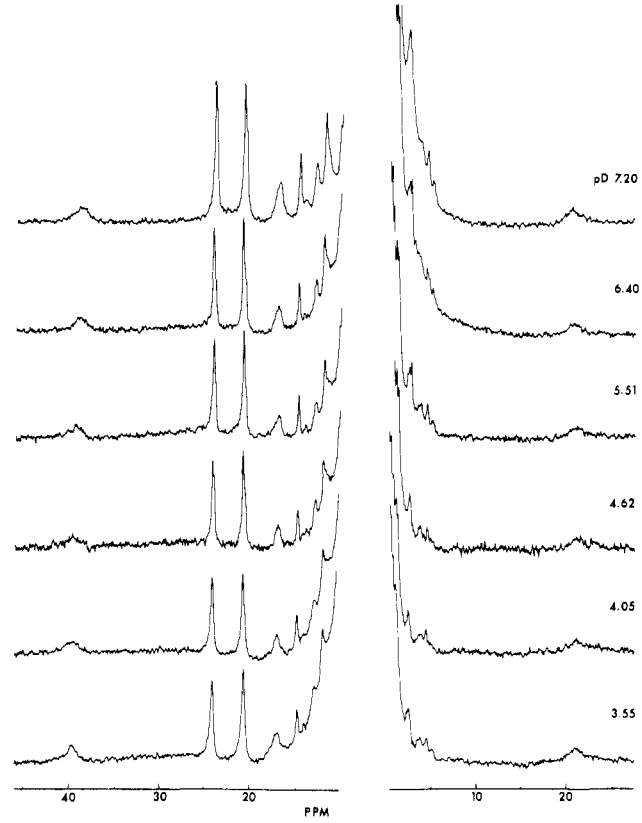

FIGURE 7: Proton NMR spectra of cyano-CPO form A at various pD values; 25 °C, D_2O solvent, 1.0 mM enzyme, 10 mM KCN.

of splitting in both high- and low-spin forms of CPO form A serves to demonstrate that native forms A_1 and A_2 (and likely forms B_1 and B_2) *do not* represent two separate electronic (spin) states or result from two different ligation states at the sixth iron position.

Proton NMR spectra of the cyanide complex in Figure 7 are surprisingly invariant with pH over the range 3.55–7.20. The only notable changes are in the region immediately upfield of 0 ppm. Resonaces in this region are reasonably associated with amino acid residues in the heme pocket. Thus, the pH-dependent chlorination activity of CPO is not reflected in electronic changes of the heme but is likely due to acid–base properties of a substrate binding site on the protein (Hewson & Hager, 1979).

Additional comment should be made concerning selected resonances in Figure 6 that are shifted well outside the diamagnetic region. Relatively sharp heme methyl signals are observed from the diamagnetic region to as far as 30 ppm downfield for other hemoprotein cyanide adducts. Thus, the intensity and relative positions for the 20.5 and 24.0 ppm signals in cyano-CPO leave little doubt as to their representing heme methyl resonances. Heme CH_2 and vinyl CH signals have also been assigned for other hemoproteins downfield of 10 ppm. Hence, signals h–m likely result from peripheral heme residues. The intensity of signal n matches that of a methyl residue. A broad signal at 39 ppm does not have precedent in spectra of other ferricyano hemoproteins. Multiple components are apparent in this signal likely representing forms A_1, A_2, and B. Integration of the far-downfield signal with respect to the methyl 20 and 24 ppm resonances reveals an intensity of 2.4 protons. Intensities of broad signals are likely to be underestimated when the signal/noise level is poor, and thus the 39 ppm resonance may be from either a CH_2 or a CH_3 residue. Assignment as a heme methyl signal is not unreasonable in view of the limited number of other sites

in the protein that could be so drastically perturbed by the paramagnetic iron center.

Several resonances slightly upfield of DSS are seen in Figure 6. Signals labeled "o" through "r" appear to have intensities of one proton or less on the basis of ring methyl intensities; less than integral intensity peaks must arise from the separate forms of cyano-CPO. Specific assignment of signals in the −2 to −5 ppm region is not possible at this time, but it should be noted that signals for both methine protons and amino acid residues located in the heme pocket have been detected in a near-upfield region for other cyanoferrihemoproteins.

The broad upfield signal located at −21 ppm in Figure 6 must represent a heme residue or an axial iron ligand. On the basis of comparison with ring methyl signals at 20 and 24 ppm, the far-upfield resonance has an integrated intensity of 2.1 protons for the A and B mixture. Reasonable assignments for this signal include (1) a heme ring CH_3 residue (distorted in intensity by the large line width and multiple enzyme forms), (2) a heme CH_2 residue, or (3) an amino acid CH_2 moiety such as the methylene group of cysteine. Cysteine mercaptide coordination in CPO is supported by a variety of spectroscopic methods (Cramer et al., 1978; Hewson et al., 1979). Hence, consideration should be given to assignment of the −21 ppm resonance to the coordinated cysteinyl residue. Model low-spin iron(III) porphyrin compounds bearing mercaptide and cyanide ligands are not available for investigation, but the coordination environment of native cytochrome *c* merits comparison. The coordinated methionine residue in cytochrome *c* exhibits a methyl proton resonance at −24.2 ppm and δ-CH_2 resonances at −25.1 and −28.1 ppm (Mc Donald & Phillips, 1973). Both thioether and mercaptide sulfur atoms are expected to interact with the partially occupied iron d_{xz}, d_{yz} set such that protons might receive similar paramagnetic shift contributions. However, a shortcoming of the tentative assignment of the −21 ppm cyano-CPO signal to a coordinated cysteinyl CH_2 groups is found in the expectation that such methylene protons should not be equivalent in a static protein environment.

Although the −21 ppm signal of cyano-CPO cannot be unambiguously assigned to a particular heme or amino acid site, certain possibilities may be eliminated on the basis of previously reported cyanohemoprotein spectra. Coordination of a methionine residue in CPO is unreasonable in that far-upfield CH_3 and CH_2 signals would be expected. Coordinated histidine is known to exhibit a far-upfield imidazole 2-H signal (La Mar et al., 1982a), but the intensity of the −21 ppm CPO peak eliminates this possibility.

Comments on Different Forms of CPO. Possible structural explanations for different CPO forms include the following: (1) amino acid substitutions; (2) carbohydrate substitutions; (3) modification of the polypeptide or polysaccharide by chlorination or enzymatic hydrolysis; (4) different enzyme conformations; (5) presence of a tightly bound substrate; or (6) heme rotational disorder effects. Different ligand combinations or electronic structure have been discounted earlier in this report. Options 4 and 5 appear to be unlikely in view of the widely differing conditions of preparation, pH, temperature, and cyanide ligation in which different forms are detected. Heme rotational disorder isomerism is now well recognized for both reconstituted and native hemoproteins (La Mar et al., 1980a, 1983c). This perturbation appears to induce much greater splitting of cyanoiron(III) porphyrin ring methyl signals than is observed for the CPO form A_1 and A_2 resonances. Accordingly, heme rotational disorder does not provide an attractive explanation. The ability to chromatographically separate CPO forms A and B suggests major charge differences on the surface of the proteins as would result from options 1, 2, and/or 3. A more subtle modification of the enzyme near the prosthetic group must be responsible for forms A_1, A_2 and B_1, B_2. Precedent certainly exists for NMR differences among the separable isozymes of turnip peroxidase (Williams et al., 1975), horseradish peroxidase (E. Gonzalez-Vergara, M. E. Meyer, and H. M. Goff, unpublished results), and monomeric insect larval hemoglobins (La Mar et al., 1983d). "Silent" mutations have also been detected by NMR spectroscopy in the later hemoprotein, even though separation of the two forms was not effected.

Comment should also be made about the previously reported proximity of the low-spin iron(III) state of native CPO. At low temperatures, transition from the $S = {}^5/_2$ to the $S = {}^1/_2$ state of CPO has been monitored by optical and EPR spectroscopy (Thomas et al., 1970; Hollenberg et al., 1980). Direct NMR evidence for such a spin-state transition is not apparent at ambient temperature over the pH range in which the protein is stable. However, deviations in Curie law behavior over the narrow temperature range investigated could result from a variety of contributions, among these being the spin equilibrium or mixing of excited states. Further NMR studies of CPO in various ligated complexes, in the iron(II) oxidation state, and in cryogenic solvents are in progress with objectives of assigning signals, structurally distinguishing different forms, and evaluating the role of low-spin states in the native structure.

Registry No. Chloride peroxidase, 9055-20-3.

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Binding Modes of Inhibitors to Ribonuclease T_1 As Studied by Nuclear Magnetic Resonance†

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ABSTRACT: The binding modes of inhibitors to ribonuclease T_1 (RNase T_1) were studied by the analyses of 270-MHz proton NMR spectra. The chemical shift changes upon binding of phosphate, guanosine, 2′-GMP, 3′-GMP, 5′-GMP, and guanosine 3′,5′-bis(phosphate) were observed as high field shifted methyl proton resonances of RNase T_1. One methyl resonance was shifted upon binding of phosphate and guanosine nucleotides but not upon binding of guanosine. Four other methyl resonances were shifted upon binding of guanosine and guanosine nucleotides but not upon binding of phosphate. From the analyses of nuclear Overhauser effects for the pair of H8 and H1′ protons, together with the vicinal coupling constants for the pair of H1′ and H2′ protons, the conformation of the guanosine moiety as bound to RNase T_1 is found to be C3′-endo-syn for 2′-GMP and 3′-GMP and C3′-endo-anti for 5′-GMP and guanosine 3′,5′-bis(phosphate). These observations suggest that RNase T_1 probably has specific binding sites for the guanine base and 3′-phosphate group (P1 site) but not for the 5′-phosphate group (P0 site) or the ribose ring. The weak binding of guanosine 3′,5′-bis(phosphate) and 5′-GMP to RNase T_1 is achieved by taking the anti form about the glycosyl bond. The productive binding to RNase T_1 probably requires the syn form of the guanosine moiety of RNA substrates.

Ribonuclease T_1 (RNase T_1)[1] (EC 3.1.27.3) is an acidic protein (104 amino acid residues) isolated from Takadiastase, a commercial product of *Aspergillus oryzae* (Sato & Egami, 1957; Egami et al., 1964). RNase T_1 specifically cleaves ribonucleic acid (RNA) chains at guanylic acid residues in contrast to the pyrimidine specificity of bovine pancreatic ribonuclease A. Rigorous recognition of guanine base by RNase T_1 is a typical example of RNA–protein interactions. A number of studies have been made to eludicate this specific

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[1] Abbreviations: 2′-GMP, guanosine 2′-phosphate; 3′-GMP, guanosine 3′-phosphate; 5′-GMP, guanosine 5′-phosphate; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; RNA, ribonucleic acid; RNase T_1, ribonuclease T_1.

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