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Proton magnetic resonance of the bovine spleen green heme-protein

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The ferric spleen green heme-protein exhibits hyperfine-shifted proton resonances between 90 and 20 ppm for the high-spin resting form and the chloride complex, and between 46 and −9.4 ppm for the low-spin nitrite complex. The proton NMR spectral profile of the enzyme is similar to that of lactoperoxidase, but different from those of common heme-proteins. The appearance of a resonance at 76 ppm in the ferrous enzyme shows the presence of a proximal histidine residue linked to the iron. The proton relaxation rates of bulk water indicate that chloride binds to the sixth position of the iron in the chloride complex of the enzyme.

Heme-protein; Myeloperoxidase; NMR; (Bovine spleen)

1. INTRODUCTION

The spleen green heme-protein [1] exhibits many properties similar to those of granulocyte myeloperoxidase. Spectroscopic, ligand binding and enzymatic characterization has revealed that the chromophore (an iron chlorin) and its vicinity in the spleen green heme-protein are the same as those of granulocyte myeloperoxidase [2–4]. The spleen enzyme is a tetramer (M_r 1.5×10^5) with two heavy subunits (M_r 6×10^4 with a single prosthetic chromophore group per subunit) and two light subunits (M_r 1.5×10^4); these are the same as myeloperoxidase [5]. Together, these data indicate that the spleen green heme-protein can be used as a model for the study of the active center of myeloperoxidase [2–5]. Myeloperoxidase (EC 1.11.1.7), which catalyzes the formation of hypochlorous acid from hydrogen peroxide and Cl^- [6], is one of the essential components of the antimicrobial systems of polymorphonuclear neutrophils [7]. With its ability to cause rapid degradation of various biological compounds, hypochlorous acid is considered to be the pertinent bacteriocidal agent. Despite its importance, structural information on myeloperoxidase remains limited, perhaps because of the difficulty of isolating large enough quantities for such studies. We have undertaken a study of myeloperoxidase by use of the spleen enzyme, since large amounts of the highly purified enzyme could be easily obtained for structural studies [3].

Hyperfine-shifted ^1H NMR spectroscopy has allowed a detailed examination of the electronic and molecular structures of the hemes and their immediate environment in many paramagnetic heme-proteins, including several peroxidases [8–13]. Due to the paramagnetism of heme, signals for the heme group and its neighboring amino acids appear beyond the large bulk diamagnetic protein protons and aromatic proton regions. Thus, these signals serve as an excellent structural probe.

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^{1}H NMR spectral measurements for the spleen green heme-protein are reported here for the first time. We find the ferric enzyme to have anomalous spectra in both low- and high-spin states. In addition, we have measured the relaxation rates of bulk water in the resting and chloride complex of the enzyme to probe the interaction of chloride with the enzyme.

2. EXPERIMENTAL

The bovine spleen green heme-protein, prepared by a modification [4] of the procedure of Davis and Averill [1], had an $A_{428\ nm}/A_{280\ nm}$ value of 0.83. The catalytically active half-enzyme (M_r 7.5×10^4), which consists of one heavy (M_r 6×10^4) and one light subunit (M_r 1.5×10^4), was obtained as in [5].

The NMR spectra were recorded on either Bruker WH-360 or CXP-200 spectrometers. 8–64 blocks of 1024 free induction decays were accumulated with the pulse sequence of Inubushi and Becker [14] to suppress water and diamagnetic protein signals. The enzyme concentration was 2 mM iron chlorin. Chemical shifts are referred with respect to sodium 2,2-dimethyl-2-silapentane-5-sulfate.

The spin-lattice relaxation and spin-spin relaxation times of water protons (at 200 MHz) were obtained by the inversion recovery and Carr-Parcell-Meiboom-Gill methods, respectively, on a CXP-200 instrument. The total contribution of the enzyme to the proton relaxation rate was calculated by subtracting the relaxation rate observed with the buffer alone from that observed in the enzyme solution. The diamagnetic effect of the enzyme was estimated by measuring the relaxivity of the low-spin cyanide form where the paramagnetic contribution is expected to be vanishingly small. The diamagnetic effect was appropriately normalized and subtracted from the total observed effects to obtain the paramagnetic contributions of the enzyme and its chloride complex. The diamagnetic contribution of the enzyme was about 4% of the total observed effect. Linear relationships between the proton relaxation rates and enzyme concentration were observed in the range of the enzyme concentrations between 20 and 300 μM.

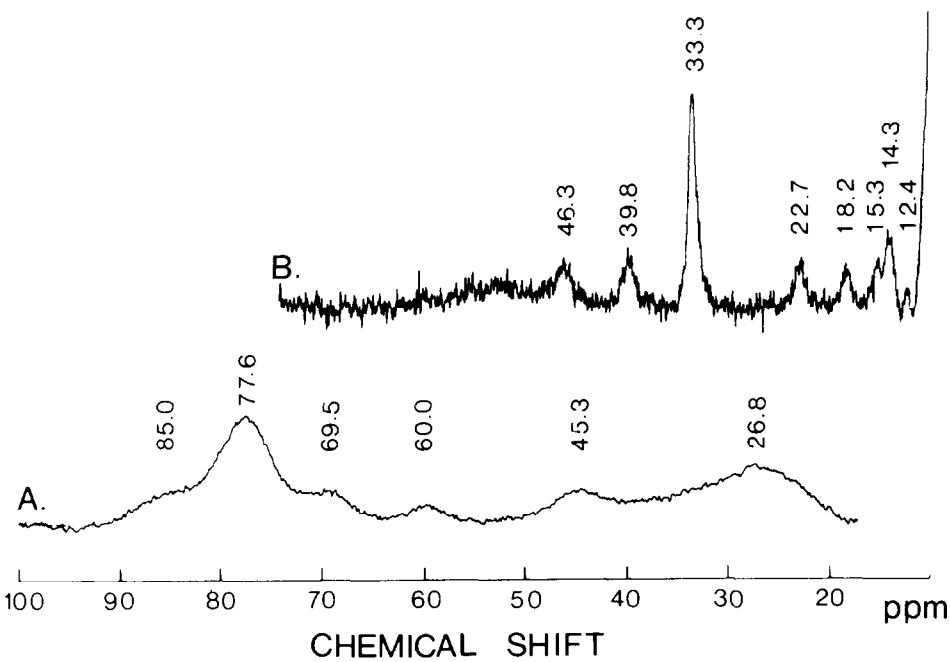

Fig.1. 360 MHz ^{1}H NMR spectrum of the ferric half-enzyme of spleen green heme-protein. (A) Resting enzyme (pH 6) at 5°C. (B) Nitrite complex at 20°C.

Buffers used were 0.2 M citrate phosphate (pH 3–6), and 0.2 M phosphate (pH 6–7).

3. RESULTS AND DISCUSSION

Fig.1 shows the downfield portion of the 360 MHz ^{1}H NMR spectra of the half-enzyme preparation of the resting ferric spleen green heme-protein (5°C) and its nitrite complex (20°C) at pH 6. The resting enzyme exhibits broad signals between 85 and 26.8 ppm, which most likely arise from the peripheral protons of the iron chlorin prosthetic group of the enzyme. The temperature dependence of these resonances obeyed the Curie law. We found no resonance in the upfield region as far as −50 ppm. The low-spin nitrite complex showed several small peaks between 46 and 13.8 ppm with only one large signal at 33.4 ppm in the downfield region and at −6.2 and −9.4 ppm in the upfield region (upfield spectrum not shown). The spectra of the spleen green heme-protein are distinctly different from those of common ferric heme model compounds and heme-proteins which show four well-resolved porphyrin methyl signals in the 50–90 ppm region in the high-spin state and 2–3 resolved methyl signals between 10 and 35 ppm in the low-spin state [8,9]. The major signals at 77.6 ppm for the high-spin resting state and at 33.4 ppm in the low-spin nitrite complex are in the ranges of chemical shift values of methyl signals commonly observed in low-spin hemes and heme-proteins [8,9], and likely represent the chlorin methyl group(s).

The salient features of the hyperfine-shifted ^{1}H NMR spectra of the ferric spleen green heme-protein, such as the extraordinarily broad linewidth of the high-spin signals, and the anomalous spectral pattern in both the low- and high-spin complexes, are remarkably similar to those of NMR spectra of lactoperoxidase [12,13]. Since the heme group in lactoperoxidase is considered to be a protoheme [15], the uncommon prosthetic group (an iron chlorin) of the spleen green heme-protein is not the sole origin of the anomalous spectral shape observed for the spleen green heme-protein. Goff et al. [12] ascribed the extraordinarily broad linewidth of the ferric high-spin spectrum to the heterogeneity of the lactoperoxidase preparation with subsequent overlap of heme signals from several different forms of the enzyme. However, this is not the case for the spleen green heme-protein, because the enzyme preparation is homogeneous [3,5] and because the EPR spectra of the resting form and chloride complex show the presence of a single paramagnetic component [3]. The sharp and well-separated signals for the low-spin nitrite complex also indicate the existence of only a single paramagnetic component in the preparation. A considerable improvement in the resolution was obtained neither on the 200 nor the 360 MHz spectrum by the use of the half-enzyme preparation which has a M_r of 7.5×10^4, indicating that the size of the macromolecule is not the major cause of the extraoridinarily wide linewidth of the resonances. This conclusion is also supported by the relatively sharp low-spin NMR signals. Resonance Raman results have indicated that the resting myeloperoxidase, the spleen green heme-protein and lactoperoxidase are six-coordinated high-spin species, different from horseradish peroxidase which exhibits a five-coordinated Raman spectrum [2,16–18]. Water coordination at the sixth position of the iron center was proposed in lacto- and myeloperoxidases [2,16–18] in their resting state. It is tempting to speculate that fluctuation of the weakly bound water (or bound chloride in the chloride complex) at the sixth coordination position of the iron centers of the enzymes might cause the unusually broad NMR spectrum of the enzymes in the ferric high-spin state. The anomalous hyperfine shift spectrum represents an unusually large asymmetry in the iron chlorin unpaired electron spin delocalization pattern, as stated by Goff et al. [12] for lactoperoxidase. Goff et al. [12] further suggested that the strong hydrophobic and/or steric interactions between the prosthetic group and surrounding protein are responsible for the anomalous hyperfine shift pattern observed in the lactoperoxidase NMR spectra. This may also be the case for the spleen green heme-protein, since the presence of such interactions has been inferred for lactoperoxidase [12,15] and the spleen green heme-protein [2].

By changing the pH from 7 to 4, the ^{1}H NMR spectrum of the ferric resting enzyme alters slightly as shown in fig.2. Measurement below pH 4 was hampered by the instability of the preparation. The major change induced by lowering the pH is the position of the largest peak; it moves from 75

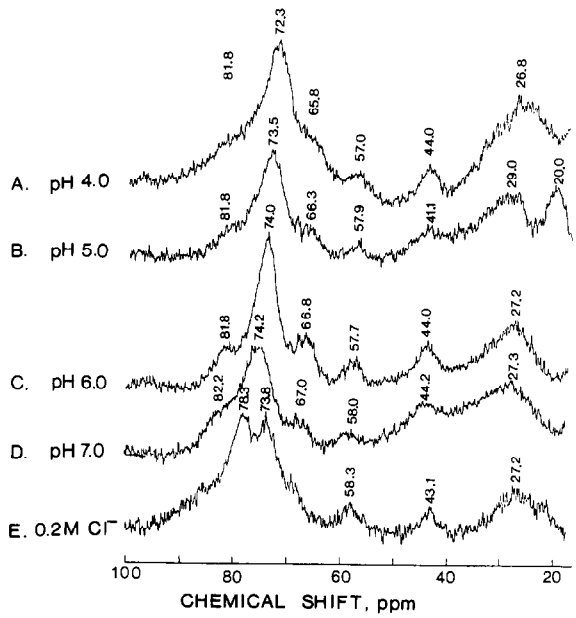

Fig.2. pH dependence of ^{1}H NMR spectra of the ferric resting half-enzyme (A–D), and the chloride complex of the enzyme (E) at pH 5 in the presence of 0.1 M NaCl at 20°C.

to 72 ppm. The presence of a proton dissociable group on the enzyme with a pK_a value of about 4 near the iron center was reported in ligand-binding studies [3,19]. The distal histidine residue, the presence of which was previously suggested [20], is a likely candidate for this ionizable group [3,19]. The relatively low pK_a for an imidazole group has been proposed to be due to the formation of a hydrogen bond with the iron-bound water [19]. The pH dependence of the proton spectrum of the resting enzyme can be related to the hydrogen bond between the bound water and distal group. As the pH is lowered from 7 to 4, the distal imidazole is protonated, which breaks or weakens the hydrogen bond [19]. This will increase the electron spin density on the iron-bound water, which reduces the electron spin density at the iron chlorin side chain protons. Thus, the upfield shifts in the hyperfine-shifted proton resonances of the iron chlorin side chains are observed at acidic pH.

Fig.2 also shows the ^{1}H NMR spectrum of the chloride complex of the spleen green heme-protein. The major peak of the resting enzyme at 74.5 ppm splits into two peaks at 78.3 and 73.8 ppm in the

Table 1

Paramagnetic contribution to water proton relaxation rates

Complex	Molar relaxivity ($M^{-1} \cdot s^{-1}$)	
	$(1/T_{1p})$	$(1/T_{2p})$
Enzyme	1930	6000
Enzyme + 0.3 M NaCl	760	2200

Determined at 200 MHz with an enzyme concentration of 0.3 mM in 0.2 M citrate-phosphate buffer (pH 5) at 20°C. $(1/T_{1p})$ and $(1/T_{2p})$ represent the respective paramagnetic contributions of the spin-lattice relaxation and spin-spin relaxation rates

chloride complex. Thus, the addition of chloride changes the electronic state of the iron center to another high-spin state, in accordance with previous observations [3,4,16,20]. We have also measured the effect of the enzyme upon the relaxation rates of water protons at 200 MHz. Table 1 lists the paramagnetic contribution to the water proton relaxation rates of the spleen green heme-protein and its chloride complex. The spin-lattice and spin-spin relaxation rates were reduced by about 60% upon formation of the chloride complex of the enzyme. These changes in relaxation rates can be qualitatively understood by considering the Solomon-Bloembergen equations adapted for high-spin heme-proteins [21]. Since both the resting enzyme and its chloride complex are ferric high-spin compounds ($S = 5/2$) with similar EPR g values, the paramagnetic component of the relaxation rate depends on the distance between the paramagnet and the proton, the correlation time for electron nuclear interaction, and the coordination number. The correlation time is approximately equal to the electron spin relaxation time in high-spin heme-proteins [21]. The electron spin relaxation time of ferric high-spin heme-proteins is of the order of 10^{-10}–10^{-11} s. Since the linewidth of the hyperfine-shifted proton resonances of the chloride complex are similar to those of the resting enzyme, it is unlikely that the electron spin relaxation time of the high-spin iron in the chloride complex differs considerably from that in the resting enzyme. Generally, the longer the electron spin relaxation rate for the paramagnetic system, the broader the line of the hyperfine-shifted NMR

signal [22]. Furthermore, the linewidth of the g_x signal of the EPR spectrum is changed from 3 to 2.5 mT upon chloride binding [3], which corresponds to only a 17% increase in the electron spin relaxation time [23]. It should be pointed out that even a 3.5-fold increase in the electron spin relaxation time from 2×10^{-10} to 7×10^{-10} s, which is expected to have a considerable effect on the linewidth of the hyperfine-shifted proton resonance and the EPR spectrum, decreases the proton relaxation rate by only about 20% at 200 MHz. The large decrease in paramagnetic contribution to the relaxation rate cannot be explained by the change in electron spin relaxation rate upon chloride binding. A slight increase in distance, the sixth power of which is inversely proportional to the relaxation rate, produces a large effect in the relaxation time. The coordination number affects the relaxation rate linearly. Therefore, the likely interpretation of the smaller relaxation rate in the chloride complex would be that the accessibility of water protons to the paramagnetic iron is reduced due to the replacement of the iron-bound water by chloride. Thus, the present NMR data coincide with previous observations which support the notion that chloride binds directly to the iron center to form a six-coordinated high-spin complex [3,16,19,20]. The presence of a sizable paramagnetic effect in the chloride complex suggests the existence of another water molecule or of an exchangeable proton of an amino acid residue, such as the distal imidazole, located close to, but not directly bound to, the iron in the chloride complex.

The 200 MHz ^{1}H NMR spectra of the ferric and ferrous holoenzyme at 25°C are shown in fig.3. The ferric holoenzyme shows essentially the same spectrum as the half-enzyme. The ferrous enzyme exhibits proton resonances at 76, 35, 28 and 18 ppm. Ferrous high-spin heme-proteins and heme-imidazole compounds commonly show porphyrin methyl resonances in the 25–10 ppm range, and the proximal imidazole N_1H proton in the 80–60 ppm range [10,24]. The peak at 18 ppm is likely to be due to the ring methyl protons. The detection of a single proton resonance at 76 ppm for the ferrous enzyme with a hyperfine shift comparable to that found for the proximal imidazole N_1H in ferrous forms of myoglobin, hemoglobin, horseradish peroxidase and model iron-porphyrin complexes [10,24] supports the previous proposal of the presence of a proximal histidine residue in the spleen green heme-protein and myeloperoxidase [2,20,25].

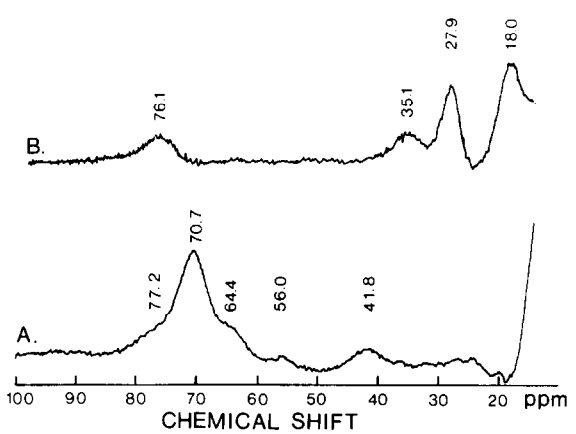

Fig.3. 200 MHz ^{1}H NMR spectra of the resting spleen green heme-protein (A) and its reduced form (B); pH 5.0, 25°C.

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