recording spectrophotometer at 283 nm (a_m = 2.73 × 10^2 M^{-1} cm^{-1}).
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Proton Magnetic Resonance Spectra of the Heme Undecapeptide (Microperoxidase), its Reaction with Hydrogen Peroxide and its Cyanide Ligated Form

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Until recently, model systems of heme protein spectroscopy and function were primarily naked iron porphyrins of varying synthetic complexity [1–5]. The detailed use of heme–peptide complexes [6–10] such as the heme undecapeptide (microperoxidase, MP-11) and the heme octapeptide (OP) from the enzymic degradation of cytochrome *c* has lagged. The structure of these complexes includes the heme which is covalently bound to an 8–11 amino acid segment from the native protein *via* cys-14 and cys-17 in the enzyme's primary sequence [11–13]. NMR spectroscopy of OP has revealed interesting comparisons to cytochrome *c* [6]. This preliminary report represents an interest in the microperoxidase which originates with its peroxidase activity (hence its common name) and comparisons which might be made with cytochrome *c* peroxidase [14–17] and horseradish peroxidase [18, 19].

Experimental

Nuclear magnetic resonance (NMR) spectra of the heme undecapeptide were obtained at proton frequencies of 360 and 470 MHz. These experiments were carried out on Nicolet Magnetics spectrometers at the Purdue University Biochemical Magnetic Resonance Laboratory. Samples were purchased from Sigma and passed through a gel filtration column (Sephadex G-50) which was equilibrated with 1.0 *M* aqueous ammonia. This procedure takes advantage of MP-11 aggregation in nonligating bases [20]. The MP-11 band was collected, neutralized with 0.1 *M* HCl, extensively dialyzed against water and lyophilized. Isoelectric focusing using an LKB multiphor and pH 3–12 PAG plates produced a major and minor band as previously described [9]. NMR samples were prepared in 99.8% ^2H_2O (Merck). MP-11-CN was formed using KCN (Aldrich) and all pH s were adjusted to 7.0 ± 0.5 as reported in the figure captions.

Results and Discussion

The proton NMR spectra presented here reveal many resolved resonances outside the diamagnetic region of 0–10 ppm. These large shifts are caused by the paramagnetism which is characteristic of high, intermediate and low spin forms of ferric hemes. Current understanding of the theory and interpretation of such shifts for hemes, porphyrins and heme proteins has been presented elsewhere [4, 17, 21].

Figure 1A reveals the low and high field proton hyperfine shift region of the ferric heme undecapeptide. In 1A the low field spectrum shows a group of broad resonances, A–D, which are located in a position typical of heme methyl resonances in high spin ferric heme proteins (100–60 ppm) [4]. Simultaneously, a group of resonances (E–I) occur in a portion of the spectrum which is typical of low spin ferric heme proteins and heme models [4, 17, 21]. This spectrum, taken in aqueous solution without buffer, indicates that aggregation, which is common to water soluble natural hemins and porphyrins [22], including OP [6, 7] and MP-11 [8], may be characterized by a high spin ⇌ low spin equilibrium. The high spin form presumably reflects aqueo-MP-11, by analogy with native (aquo) cytochrome *c* peroxidase where ^2H_2O is the sixth ligand, in this case. The low spin resonances are most likely due to the aggregated species.

As reported earlier, MP-11 has peroxidase activity [23] and its NMR spectroscopic behavior upon reaction with H_2O_2 is of interest in comparison to that observed for the peroxidase enzymes [14, 15, 18, 19]. Figure 1B indicates that addition of a 25% mole excess of hydrogen peroxide to the aqueous MP-11 solution results in complete loss of the hyperfine resonances downfield (A–K) and nearly a complete bleaching of the upfield resonances (L–T). These spectral changes mimic those of cytochrome *c*

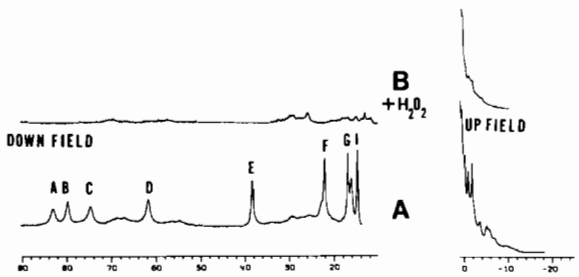

Fig. 1. (A) Proton NMR spectrum (360 MHz) of the hyperfine shift region of 8 × 10^{-4} *M* ferric heme undecapeptide (MP-11) in 99.8% ^2H_2O, pH′ 7.2. Peaks A–D are characteristic of the heme methyl groups of a high spin ferric heme protein. The pH′ is the observed meter reading. Peaks E–I are characteristic of a low spin ferric heme protein. (B) Spectrum which results from the addition of a 25% mole excess of hydrogen peroxide to the solution in (A). This bleached spectrum is characteristic of the cytochrome *c* peroxidase oxidized intermediates.

peroxidase in which spectral bleaching is observed [14, 15]. This phenomenon is associated with the presence of an amino acid based free radical, the result of H_2O_2 oxidation. This spectrum is dissimilar to that obtained for the oxidized intermediates of horseradish peroxidase. For horseradish peroxidase oxidation yields Compound I, which is a porphyrin centered cation free radical, with well resolved heme proton resonances 60–20 ppm downfield [18, 19].

The proclivity for aggregation demonstrated by both MP-11 and OP has led to their study in the presence of strongly coordinating ligands, such as CN^- because six-coordination tends to oppose aggregation [6]. Figure 2 presents the spectrum of CN-MP-11. Figure 3 details variable temperature data showing that resonances A–D and H–K are influenced by heme centered paramagnetism. The numbers in parentheses represent the extrapolation of this data to $T^{-1} = 0$, whereupon the Curie Law basis of the equations which govern the shift behavior [21] predict an intercept at the diamagnetic shift position (*i.e.* within 0–10 ppm).

Comparing Fig. 2 with published spectra of cytochrome *c*-cyanide and OP-CN (*cf.* Fig. 11, Ref. 6) it is obvious that, at 22 °C, the spectrum of CN-NP-11 is virtually identical with cytochrome *c*-CN. The decreasing linewidths of resonances A–C (A > B > C) which results in relative intensity increasing A < B < C, precisely mimic the published spectrum of cytochrome *c*-CN [6]. The published spectrum of OP-CN shows no similar variations; uniformly intense resonances are observed [6]. Moreover, the observed hyperfine shifts of A–C for CN-MP-11 are nearly identical to cytochrome *c*-CN. In contrast, OP-CN resonance positions are markedly different from cytochrome *c*-CN. One conclusion that can be drawn from this comparison with the heme octapeptide complex is that the heme methyl resonance assignment for CN-MP-11 is 5(A) > 8(B) > 1(C).

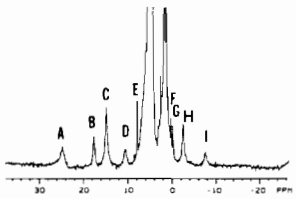

Fig. 2. 470 MHz proton NMR spectrum of CN-MP-11 (8×10^{-4} *M*) in 99.8% ^2H_2O, pH' 7.5, 22 °C.

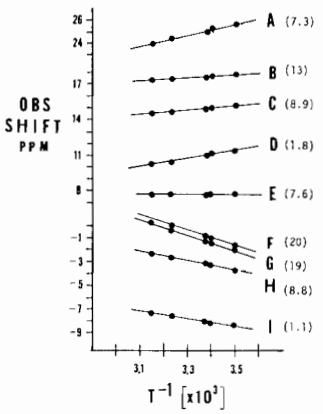

Fig. 3. Curie plot of the temperature dependence of the hyperfine shifts of a CN-MP-11 solution described in the Fig. 2 caption. Numbers in parentheses are the extrapolations of this data to $T^{-1} = 0$ using linear regression analysis.

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