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ASSIGNMENT OF EXCHANGEABLE PROXIMAL HISTIDINE RESONANCES IN HIGH-SPIN FERRIC HEMOPROTEINS: SUBSTRATE BINDING IN HORSERADISH PEROXIDASE*

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SUMMARY

Single-proton, exchangeable resonances have been detected in the high spin ferric hemoproteins, met-aquo myoglobin and horseradish peroxidase, which can be assigned to the proximal histidyl imidazole by virtue of their very large hyperfine shifts. While this proton is relatively labile in myoglobin, it is exchangeable in HRP only at extreme pH values, indicating a buried heme pocket. The insensitivity of the imidazole peak of HRP to substrate binding argues against direct interaction of imidazole and substrate.

Proton nmr spectroscopy of paramagnetic hemoproteins has provided a wealth of detailed structural information on their active site primarily due to the large hyperfine shifts which can be observed for the heme prosthetic group and any appended ligand such as the ubiquitous proximal histidine or an exogenous sixth ligand (1,2). The dominant use of heme resonances as structural probes can be attributed to their ready resolution and probable assignment based largely on model compound work (3), although recently several heme peak assignments based on selective deuteration have been reported (4-6).

Structural studies relying on the endogenous axial ligand resonances, (i.e. proximal histidyl imidazole), have been rare primarily because of the difficulty in making unambiguous assignment and, until recently, the absence of any characterized models (7). However, the need for assignments of such resonances is underscored by the proposed importance of ligand-iron interactions in modulating the reactivity of the iron center. The iron-histidine bond is central

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Abbreviations used: DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; HRP, horseradish peroxidase; metMb, met-myoglobin; IPA, indole propionic acid

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to the model for cooperativity in hemoglobins (8), and the variability of hydrogen bonding between the proximal histidyl imidazole and a peptide backbone carbonyl has recently been proposed as a general control mechanism in hemoproteins (9). In the case of horseradish peroxidase, the proximal histidyl imidazole has been proposed to act as a donor in hydrogen bonding to substrates (10).

At this time, unambiguous assignment of these imidazole resonances in hemoproteins is restricted to the pyrrolic N_1H because only this proton is both sufficiently strongly coupled to the unpaired iron spin to exhibit a large hyperfine shift (2,3) and is exchangeable with deuterium in ^2H_2O. Successful assignments have been reported for deoxy myoglobins and hemoglobins (7) and met-cyano myoglobins (11).

To date, however, relatively little nmr work has been reported on high-spin ferric hemoproteins due to a combination of the absence of any suitable models and the very substantial linewidths observed even for heme methyls (6, 12).

In view of the importance of this oxidation/spin state in heme peroxidases, catalases (13) and oxygenases (14), and the possible role of the histidine in governing their unique reactions, such assignments would be highly useful in investigating their structure-function relationships. We report here on the successful observation of exchangeable resonances in met-aquo myoglobin and horseradish peroxidase which can only arise from the proximal histidyl imidazole N_1H.

METHODS

Sperm whale myoglobin (Type II) and horseradish peroxidase, HRP, (Type VI, RZ = 3.1-3.2) were purchased from Sigma as salt-free, lyophilized powders. The nmr spectra were found insensitive to any further purification (15). Indole propionic acid, IPA, was obtained from Sigma and used without further purification.

Solutions were prepared in either ^2H_2O or 90% H_2O/10% ^2H_2O (2-3 mM) 0.2 M in NaCl. The pH was adjusted using 0.2 M NaO^2H and ^2HCl. pH readings (Beckman 3550 pH meter, Ingold micro-combination electrode) are uncorrected for isotope effect in ^2H_2O. 360 MHz nmr spectra were recorded on a Nicolet NT-360, utilizing 10K-100K transients with 8K points over 80 KHz bandwidth. The 90° pulse (8.7 μs) was centered on the solvent resonance which was suppressed by a saturating pre-pulse. Apodization introduced a negligible 20 Hz line broadening. Chemical shifts are given in ppm, referenced to 2,2-dimethyl-2-silapentane-5-sulfonate, DSS.

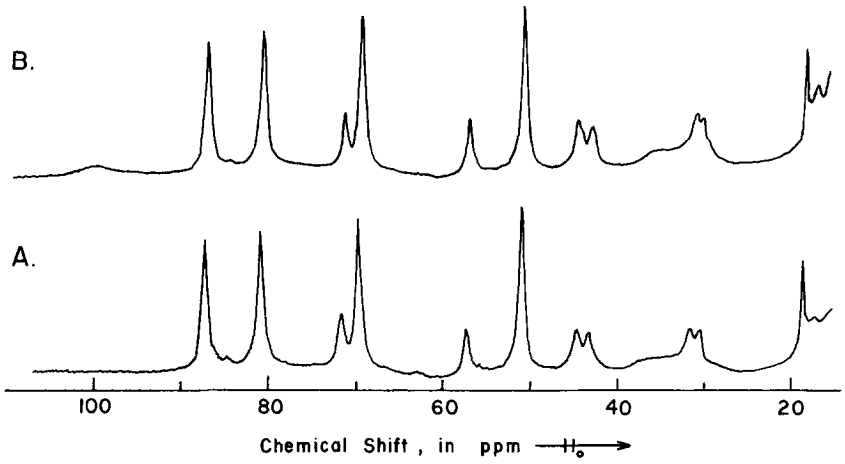

Fig. 1: Proton NMR Spectra of Sperm Whale Met-aquo Myoglobin

Low-field portion of the 360 MHz proton nmr traces of 2mM protein, pH 6.2, 35°C in A. 99.9% ^2H_2O; B. 90% H_2O, 10% ^2H_2O. The peaks common to A and B have been assigned by deuterium labeling (6).

RESULTS

The proton nmr spectra of the downfield region of sperm whale met-aquo myoglobin in ^2H_2O and H_2O at 35°C are compared in Figure 1. The only difference between the two traces is the appearance of a broad single proton resonance at 100 ppm in H_2O which is missing in the ^2H_2O solution.

The low-field proton nmr spectra of HRP in ^2H_2O and H_2O at 35°C are illustrated in A and B of Figure 2. The spectra are the same as reported earlier (16) except for much improved resolution in the 20-50 ppm region and the detection of a new peak of single-proton intensity at 96 ppm in both neutral H_2O and ^2H_2O. The same trace as in A run after the pH of the protein was first lowered to 3.1 and then returned to 7.0 is shown in C of Figure 2. A trace identical to that in C is also obtained (not shown) if the sample pH is first raised to 11.4 before readjusting to 7.0. Trace C differs from A in that the peak at 96 ppm has disappeared, clearly dictating that it is an exchangeable proton.

The effect of IPA binding on the nmr spectrum of HRP at 35°C is illustrated in D of Figure 2, which can be compared to that in A. While the four heme methyl peaks shift in a manner similar to that reported previously (16), the exchangeable peak at 96 ppm is unaffected.

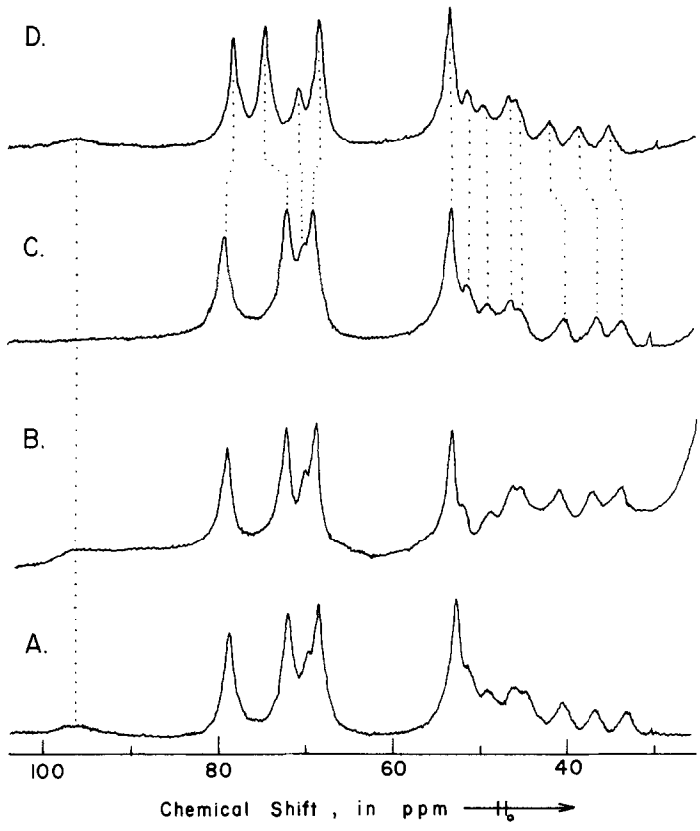

Fig. 2: Proton NMR Spectra of Horseradish Peroxidase

Low-field portion of the 360 MHz proton nmr traces of the resting state of HRP, pH 7.0, 35°C, in A. 99.9% ^2H_2O; B. 90% H_2O, 10% ^2H_2O; C. same as in A except after first lowering the pH to 3.1; D. in 99.9% H_2O after adding a 15-fold excess of indole propionic acid. The dotted lines indicate the shift change induced by IPA binding.

DISCUSSION

The appearance of the single proton peak at 100 ppm in H_2O but not in ^2H_2O solution of metMbH_2O (Figure 1) dictates that it is an exchangeable proton strongly coupled to the ferric ion but not belonging to the heme. Due to the σ spin delocalization which dominates the contact shifts in high spin ferric systems (3), it is highly improbable that the iron spin density can be transmitted to the proximal histidine peptide NH. Hence this peak must arise from the proximal histidyl imidazole N_1H. No suitable models for metMbH_2O have been characterized which could shed light on any unique role of the protein in influencing the iron-histidine bond. The fact that the peak at 100 ppm is ab-

sent immediately after dissolving protein in ^2H_2O is consistent with the ready access of water to the heme cavity and/or a relatively loose or exposed active site. NMR evidence for considerable motion of heme side chains has been presented recently (6).

In the case of HRP, a broad, single-proton peak is observed in both H_2O ^2H_2O, in the latter solvent even after several weeks at neutral pH, as shown in A and B of Figure 2. However, upon changing the pH to extreme values (3.1 or 11.4), where reversible denaturation occurs, this peak exchanges with ^2H_2O and disappears (C in Figure 2). If these pH changes are carried out in H_2O, the peak at 96 ppm remains unaffected. The very similar shift of this peak to that observed for metMbH_2O in Figure 1 strongly supports its assignment to the N_1H of a coordinated imidazole, and provides additional evidence for the presence of a proximal histidine in the resting state of HRP (17). The stability of this N_1H bond towards isotope exchange in ^2H_2O, however, is indicative of a heme pocket in HRP which is considerable more buried and less solvent accessible than that in myoglobin.

The similar N_1H shift in these two proteins also suggest that their stereochemistry must be similar. Although water is known to be coordinated to the iron in met-myoglobin (18), both nmr (19) and esr (20) evidence argue against a coordinated water in HRP. A five-coordinated structure is also found by resonance Raman spectroscopy (21). A more detailed analysis of the hyperfine shifts relevant to the degree of ligation of the iron in HRP must await assignment of all resonances of the heme in both proteins. Such a study is in progress in our laboratory.

Previous studies on the stereospecific HRP-induced paramagnetic relaxation of a proton-donor substrate has been interpreted (10) in terms of a structure where the binding is stabilized by formation of a hydrogen-bond between the proximal histidyl imidazole N_1H and the substrate heteroatom. Comparison of the traces B or C in Figure 2 with that of the HRP-IPA complex, given in D, reveals that, while substrate binding does influence the heme resonances, the

hyperfine shift of the exchangeable N_1H peak at 96 ppm is unchanged. This complete insensitivity of the proximal histidyl imidazole N_1H peak to IPA binding not only argues strongly against such hydrogen bonding (10), but suggests that the substrate binding is more likely on the distal side of the heme.

A more detailed description of the binding of the substrates to HRP based on extensive isotope labeling of heme resonances sensitive to IPA-binding will be presented elsewhere.

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