ASSIGNMENT OF PROXIMAL HISTIDINE PROTON NMR PEAKS IN MYOGLOBIN AND HEMOGLOBIN*

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SUMMARY

The proton nmr spectra of model compounds of deoxy myoglobin and hemoglobin have yielded the assignment of the axial imidazole resonances. The information leads directly to an assignment of all three proximal histidyl imidazole proton resonances in sperm whale deoxy myoglobin, and the two nonequivalent proximal histidyl imidazole NH signals of the α and β chains in deoxy hemoglobin A. The NH peaks are expected to serve as valuable probes for the T $\rightleftharpoons$ R transition in hemoglobins.

Proton nmr spectroscopy has played an important role in the elucidation of structure-function relationships in hemoproteins. Particularly useful have been found the hyperfine-shifted resonances due to heme substituents in the paramagnetic low-spin met and high-spin deoxy hemoglobins (1,2). Although hyperfine-shifted resonances have been reported which provide an empirical index of changes near the heme periphery accompanying ligation, as well as pH or organic phosphate induced T $\rightleftharpoons$ R interconversion, it has not been possible to interpret the shift changes in terms of structural changes in the heme cavity. In large part, this is due to the fact that all resonances monitored to date arise from heme substituents (1,2), while the control of ligand affinity at the iron has been proposed to arise from tension in the proximal histidine-iron bond (3).

An idealized probe for this axial tension would be the hyperfine-shifted resonances for the proximal histidyl imidazole, whose magnitudes could reflect the degree of covalency, and hence stretching in this bond (4). Such resonances, however, have been conspicuous by their absence in deoxy hemoglobin proton nmr spectra. We report here on a proton nmr study of model compounds (5,6) of

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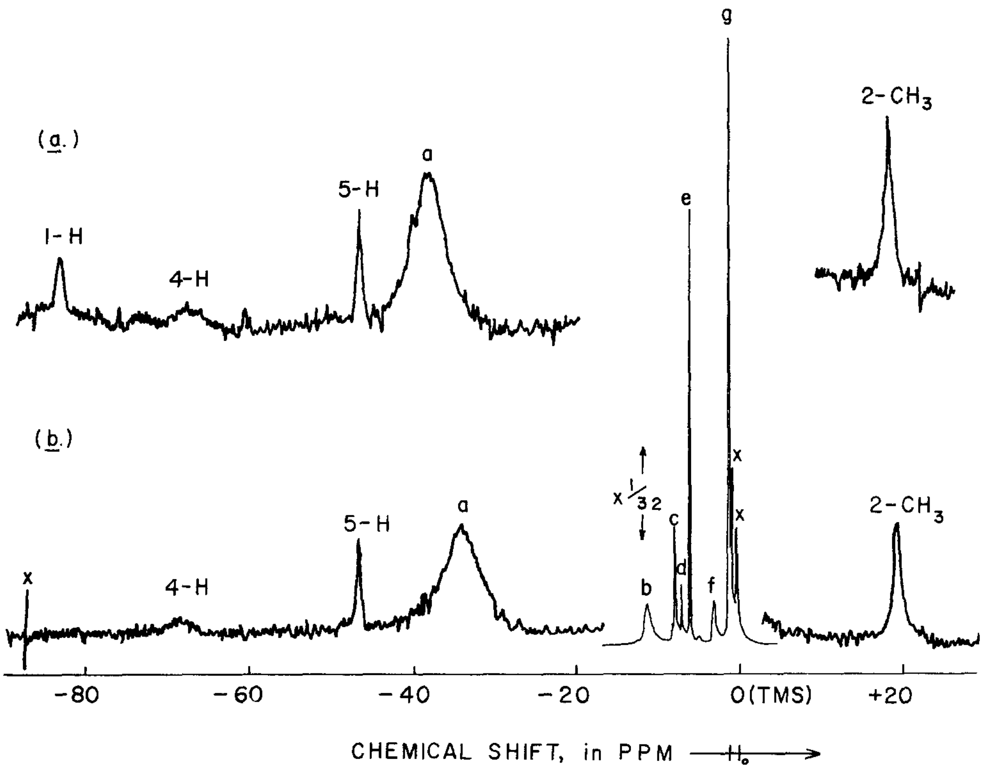

Fig. 1: Proton NMR Spectra of a Deoxy Model Compound

Proton nmr trace of coordinated 2-MeIm of PFe(2-MeIm) in slow exchange in benzene-d_6 at 25°; a) 0.010 M PFe and 0.003 M 2-MeIm, and b) 0.010 M PFe and 0.003 M 1-d,2-MeIm. The numbering of the imidazole is conventional, with the 3 position coordinated to the iron; peaks a-d and g are the average porphyrin peaks; e is the residual solvent resonance.

deoxymyoglobin and hemoglobin which provides information on the location of the axial imidazole resonances which is subsequently utilized to both locate and unambiguously assign at least one proximal histidyl resonance in each of myoglobin and hemoglobin.

METHODS

Model Compounds: The model compounds (5,6) used are the mono-aducts of ferrous meso-tetra-p-isopropylphenylporphyrin, PFe, with methyl substituted imidazoles. PFe, reacting with either L = 2-methylimidazole, 2-MeIm, or L = 4,5-dimethylimidazole, 4,5-Me_2Im, is described by the equilibrium: $PFe(S=1) + L \rightarrow PFeL(S=2)$, where the equilibrium constant $\sim 3 \times 10^4\ M^{-1}$ for L = 2-MeIm. Hence, it is necessary to have a large excess of L to ensure complete for-

Table 1: Chemical Shifts and Linewidths of Imidazole Resonances in Deoxy Model Compounds and Hemoproteins[a]

	1-H	2-H	4-H	5-H
PFe(2-MeIM)	-85(∼100)[b]	---	-71(∼300)	-48(∼30)
PFe(4,5-Me_2Im)	---	-53(∼400)	---	---
Sperm whale Mb in 0.2 M NaCl D_2O, 'pH' = 7.0	---	[-46(∼1000)]		---
Sperm whale Mb in 0.2 M NaCl H_2O pH = 6.8	-77.8(∼100)	c	c	---
Hb A, stripped, in 0.2 M NaCl H_2O pH = 6.0	-75.9 -63.9 (∼300)	c	c	---

a) Shifts in ppm, at 25°, from internal DSS or TMS; Uncertainties in NH shifts in Mb, ± 0.2 ppm, in Hb, ± 0.4 ppm. b) Linewidths, in Hz, are given in parentheses. c) Not resolved in H_2O solution.

mation of desired product, PFeL. However, exchange of L is very rapid on the nmr time scale, even at low temperatures, so that only the average ligand shifts for L and PFeL are observed in the presence of excess L. The only method which allows the direct observation of the coordinated L hyperfine shifts in slow exchange is to minimize the amount of free L. Thus when only about one-third of the PFe in a 10 mM solution is converted to PFeL, the L resonances of PFeL are observed in slow exchange. However, rapid hopping of L from PFeL to PFe results in fast exchange of the P environments (7). The proton nmr spectra were recorded on a JEOL-PFT-100 spectrometer operating at 99.5 MHz; 100 to 500 transients were collected using 16 K points over a 6.25 KHz bandwidth.

Hemoproteins: Sperm whale met-Mb (Sigma) was dissolved (15 mM) in 0.2 M NaCl H_2O and D_2O, reduced to the deoxy form by the addition of an excess of $Na_2S_2O_4$, and the pH adjusted to 6-7 with 0.1 M HCl or NaOH; pH values in D_2O solutions are uncorrected and hence referred to as 'pH'. Human adult Hb-O_2 was prepared in a standard manner (8) from fresh blood drawn from one of us (DLB), stripped of phosphates by dialysis, and 4 mM solutions prepared in 0.2 M NaCl H_2O and D_2O. Deoxy Hb A was prepared by passing purified N_2 over the stirred solution; the pH was adjusted to ∼6. Typically 10-100 K pulses were required using 2 K data points over a 25 KHz bandwidth. The very intense H_2O signal was suppressed using a rapid pulse repetition rate, 20 sec^{-1}.

RESULTS

The spectrum of the coordinated 2-MeIm in PFe(2-MeIm) in slow exchange is illustrated in a of Figure 1. Repetition of the experiment using 2-MeIm,

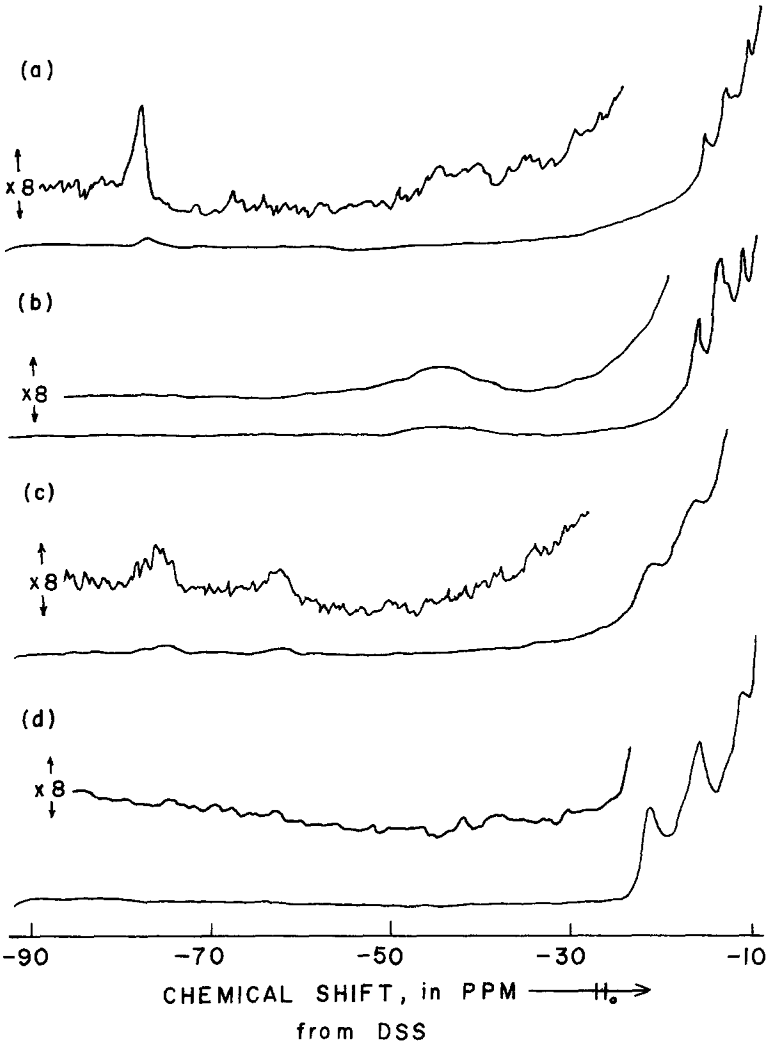

Fig. 2: Proton NMR Spectra of Deoxy Myoglobin and Hemoglobin

The low field nmr traces of: (a) 14 mM sperm whale deoxymyoglobin in 0.2 M NaCl H_2O, pH = 6.8, 40 K transients; (b) 9 mM sperm whale deoxymyoglobin in 0.2 M NaCl, 99.8% D_2O, 'pH' = 7.0, 10 K transients; (c) 4 mM deoxyhemoglobin A in 0.2 M NaCl H_2O, pH = 6.0, 50 K transients; (d) 4 mM deoxyhemoglobin A in 0.2 M NaCl, 99.8% D_2O, 'pH' = 6.3, 50 K transients. Chemical shifts are referenced against internal DSS; probe temperature is 25°C.

where the labile 1-H was pre-exchanged with deuterium, *i.e.* 1-*d*,2-MeIm, resulted in the trace depicted in *b* of Figure 1. The observed shifts from tetramethylsilane, TMS, for the imidazole protons in the model compounds are listed in Table I.

The proton nmr trace of sperm whale deoxy Mb and H_2O is illustrated in a of Figure 2. In addition to the previously reported (2,9) methyl peaks at -8 to -16 ppm from 2,2-dimethyl-2-silapentane-5-sulfonate, DSS, we see two new absorptions at -78 (narrow) and -46 (broad, asymmetric). The trace of the sample in D_2O, shown in b of Figure 2, retains the broad peak at -46 ppm, while the -78 ppm peak is missing. Integration indicates that the downfield peak has unit intensity compared to the presumed heme methyl peak at -16 ppm, while the broad peak is consistent with containing two protons. The intensity and position of the low-field peak was found to be independent of pH from 6.5 to 9.0. The proton traces of stripped Hb A in H_2O and D_2O are illustrated in c and d of Figure 2, respectively. In addition to the heme methyls (1,2), -23 and -12 ppm from DSS, we resolve a pair of equally intense, broad (300 Hz) signals which are absent in the D_2O solution. The shifts for both proteins are given in Table I.

DISCUSSION

Comparison of a and b in Figure 1 clearly identifies the furthest downfield peak as 1-H of the coordinated 2-MeIm in the model compound. The 4-H and 5-H peaks are readily assigned based on the relative linewidth and their proximity to the iron (10). The 2-H shift was determined using L = 4,5-Me_2Im. The wide spread and large downfield bias for all of the imidazole proton peaks relative to the heme signals in the high-spin ferrous complexes (7) suggest that unambiguous assignment of at least the proximal histidyl NH resonances in all the deoxy hemoproteins should be possible. We show subsequently that the excellent correlation between the imidazole NH shifts in the complexes and the deoxy hemoproteins emphasizes the validity of these complexes as models of the active site of Mb and Hb (5).

The absence of the furthest downfield peak in D_2O in sperm whale Mb and the similarity of the shift to that of 1-H in the model compound allows us to assign it unambiguously to the NH of the proximal histidyl imidazole. Varying the pH of the solution from 6.5 to 9.0 had no effect on either the integrated intensity or position of the NH peak. This is consistent with the inability to protonate the iron-coordinated imidazole and the absence of the alkaline Bohr effect in this protein. The linewidth and position of the slightly asymmetric resonance at -46 ppm is consistent with it containing both the 2-H and 4-H peaks of the histidyl imidazole. A variable temperature plot of the hyperfine shifts for this peak yields a large non-zero intercept similar to that found for the 2,4-H in model compounds (7). The assigned NH signal should provide us with a valuable probe for tension in characterizing the tertiary structural changes responsible for the well-characterized alkaline Bohr effect in the monomeric Chironomus hemoglobins (11).

The two downfield peaks at -77 and -63 ppm in Hb A in H_2O disappear in the D_2O solution. The similarity of the shifts to the NH in the model compound and Mb indicates that they originate from exchangeable NH peaks for a proximal histidyl imidazole. The 2,4-H imidazole peaks, which are expected to be more than three times broader than the NH (7,10) are not resolved. The observation of a pair of hyperfine-shifted NH peaks in stripped Hb A suggests that they originate, one each, from the non-equivalent α and β chains. This chain non-equivalence is much more dramatic for the proximal histidine resonances than for the previously studied heme methyl signals (1,2,12). The clear resolution of the two signals will permit less ambiguous determinations of the preference of ligands for the individual subunits (12). The sizable difference in the NH shifts for the two subunits also attests to their sensitivity to local structure, and establishes the single proton resonances in the region -60 to -80 ppm from DSS as valuable new probes for the environment around the proximal histidine.

The similarity of one of the proximal histidyl NH shifts in Hb A and in sperm whale Mb suggests that their environments do not differ significantly. Thus a tentative interpretation suggests less of a difference in NH environments in the two proteins than expected, even though the oxygen afinity under this condition in Hb is reduced compared to that of Mb by a factor of 70.

Current work is directed towards assigning the pair of NH peaks to individual chains using the valency hybrids and separated chains, and towards testing the sensitivity of the NH resonances to the T ⇌ R transition in mutant and modified hemoglobins (13,14).

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