Histidine Residues of Myoglobin studied by ^{1}H Nuclear Magnetic Resonance Spectroscopy

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Summary Titration curves of all the H-2 and H-4 resonances of the eleven titrating histidine residues in ferrous carbon monoxide sperm whale myoglobin are given; the H-4 resonance of the distal histidine has been observed at *ca.* 2·2 p.p.m. upfield of its normal position and on titration, the distal histidine resonances give a pK' of 5·2 at 40 °C.

IN ferrous carbon monoxide leghaemoglobin (a diamagnetic complex of a monomeric haem protein) the H-2 resonance of the distal histidine was observed at 2·6 p.p.m. upfield of its normal unperturbed position at high pH and moved downfield to its normal position on protonation of the histidine.[1] This unusual behaviour at high pH was due to the proximity of the uncharged form of the distal histidine to the porphyrin ring current.[2] When the pH was lowered by protonation of the histidine, it moved away from the porphyrin ring into an aqueous environment.

Histidine residues of the ferrous carbon monoxide complexes of human haemoglobin and sperm whale myoglobin have also been observed in perturbed magnetic environments. In particular, we report the occurrence of the distal histidine H-4 resonance of ferrous carbon monoxide sperm whale myoglobin ($Fe^{II}COMb$) and the titration curves of the H-2 and H-4 resonances of the eleven titrating histidine residues. The twelfth histidine residue, the proximal, does not titrate.

Sperm whale ferric myoglobin (Sigma) was converted into $Fe^{II}COMb$ in D_2O,[1] and ^{1}H n.m.r. spectra were collected at 270 MHz and 40 °C at various pH values measured at 40 °C and not corrected for deuterium isotope effects. The $Fe^{II}COMb$ was deuteriated by incubation in D_2O at pH 9 and 35 °C for 42 days under CO. Apomyoglobin was prepared by a standard procedure[3] and deuteriated in 6 M guanidine deuteriochloride (guDCl) in D_2O.

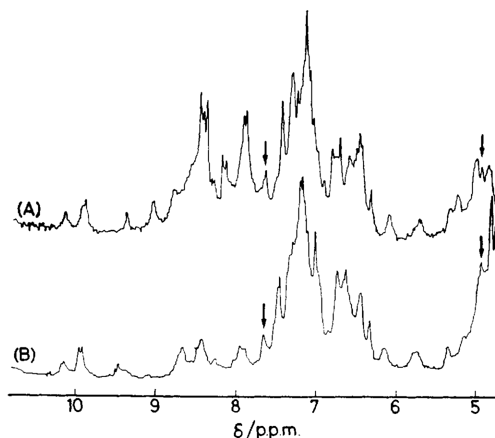

FIGURE 1. ^{1}H N.m.r. spectra at 270 MHz of the aromatic region of $Fe^{II}COMb$, 3 mM in D_2O at pH meter reading 5·88 and 40 °C; (A) normal spectrum, (B) spectrum after deuteriation (see text).

The group of sharp resonances downfield from the arrowed resonance at δ 7·7 in Figure 1(A) are from C-2 histidine protons, because they shift with change of pH and are removed by deuteriation [see Figure 1(B)]. The singlet arrowed peak at δ *ca.* 5·0 shifts with change of pH (see Figure 3, curve 22) and its upfield position strongly indicates that it originates from the distal histidine residue.

The resonance was not removed either by deuteriation of $Fe^{II}COMb$ or by deuteriation of apomyoglobin in 6 M guDCl in D_2O followed by reconstitution of $Fe^{II}COMb$. It is therefore assigned to the H-4 distal histidine.

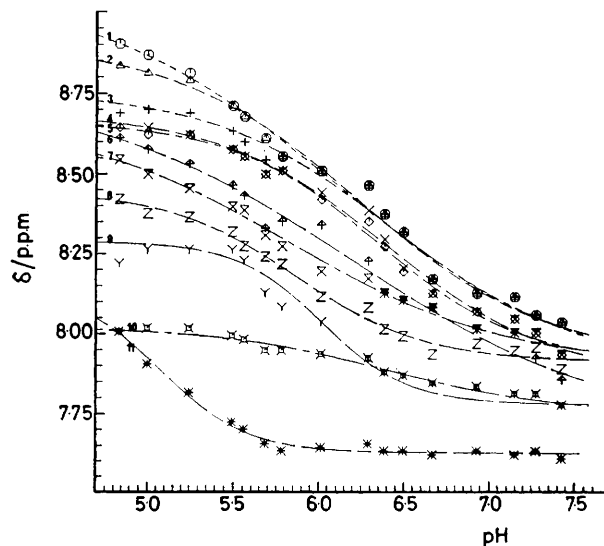

FIGURE 2. Graph of δ p.p.m. *vs.* pH meter reading at 40 °C for the H-2 histidine resonances of $Fe^{II}COMb$.

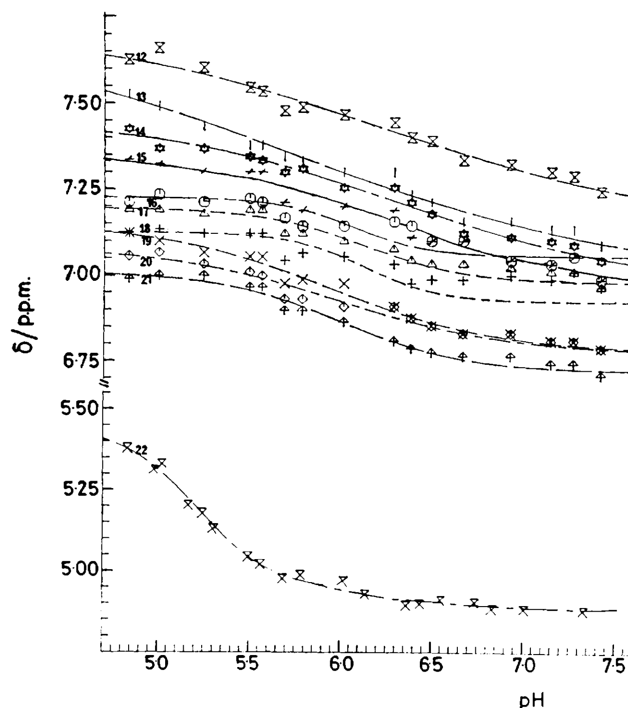

FIGURE 3. Graph of δ p.p.m. *vs.* pH meter reading at 40 °C for the H-4 histidine resonances of $Fe^{II}COMb$.

The arrowed resonance at δ 7·7 (corresponding to curve **11**, Figure 2) remained after deuteriation of $Fe^{II}COMb$, but was removed by deuteriation of apomyoglobin in 6 M guDCl followed by reconstitution of $Fe^{II}COMb$. This arises from a H-2 resonance of a histidine in $Fe^{II}COMb$ that has a low pK and is inaccessible to solvent, as in the case of the H-2 distal histidine of ferrous carbon monoxide leghaemoglobin.[1] Thus it is likely that this resonance is the H-2 distal histidine resonance and the reasonable agreement between the pK' values for the titration curves **11** and **22** in Figures 2 and 3 (5·1 and 5·3 respectively), is consistent with this explanation. The error in these pK' values is probably ±0·2, because only partial titration curves are available. However, the agreement is only fair with the pK' value of 4·6 found at 2 °C in an i.r. study of $Fe^{II}COMb$.[4]

The titration curves in Figures 2 and 3, determined by n.m.r. difference spectroscopy[5] of convolution difference spectra[6] have been plotted using a linear least squares computer curve fitting procedure. Continuities of curves are uncertain in regions of severe overlap. These curves from diamagnetic $Fe^{II}COMb$ (which will be interpreted more fully in a subsequent publication), arise from all the histidine residues (except the proximal), in contrast with studies of paramagnetic myoglobins[7] where some residues are not observed because of broadening and shifting of resonances of histidine protons that are near the paramagnetic iron atom.

The distal histidine H-4 resonance is *ca.* 2·2 p.p.m. upfield of its normal unperturbed position and the likely distal H-2 resonance is about normal. Using a computer program,[2] it has been possible to determine the likely orientation of the distal histidine ring relative to the porphyrin ring; a comparison of the n.m.r. result with a neutron diffraction study[8] is currently in progress.

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