Iron–Histidine Bonding Interaction in Deoxymyoglobin and Deoxyhaemoglobin

Yasuhiko Yamamoto* and Riichirô Chûjô

Department of Biomolecular Engineering, Tokyo Institute of Technology, 12-1, 2-Chome, O-okayama, Meguro-ku, Tokyo 152, Japan

Natural abundance ^{13}C NMR resonances of the proximal histidyl imidazole carbons in the deoxy form of O_2-binding haemoproteins have been observed and these resonances are found to serve as sensitive probes for the electronic structure of this ubiquitous histidine, which is thought to control the iron reactivity.

The iron–histidine bonding interaction in the deoxy form of O_2-binding haemoprotein has been of particular interest, because the electronic control of the haem iron reactivity is proposed to be exerted primarily through this bond.[1] There are two spectroscopic parameters so far available for characterising the iron–histidine bonding interaction in the deoxy form of haemoprotein. One is the Fe–N-3 stretching frequency, ν_{Fe-N},[2] and the other is the NMR resonance of the proximal histidyl imidazole N(1)H proton[3] (see the structure in the inset of Fig. 1). The combined analysis of these two parameters has been shown to allow semiquantitative characterisation of the nature of the Fe–N-3 bond.[4] Complications in interpreting the labile N(1)H proton resonance, however, arise from the fact that its shift is modulated by hydrogen bonding in addition to the nature of the iron–histidine bonding interaction. Ideally, NMR characterisation of the iron–histidine bonding interaction should be carried out on the basis of the proximal histidyl imidazole resonances other than the labile N(1)H proton resonance. Unfortunately, no such resonances in deoxy O_2-binding haemoprotein have been reported as yet.

In this communication, we present the first observation of

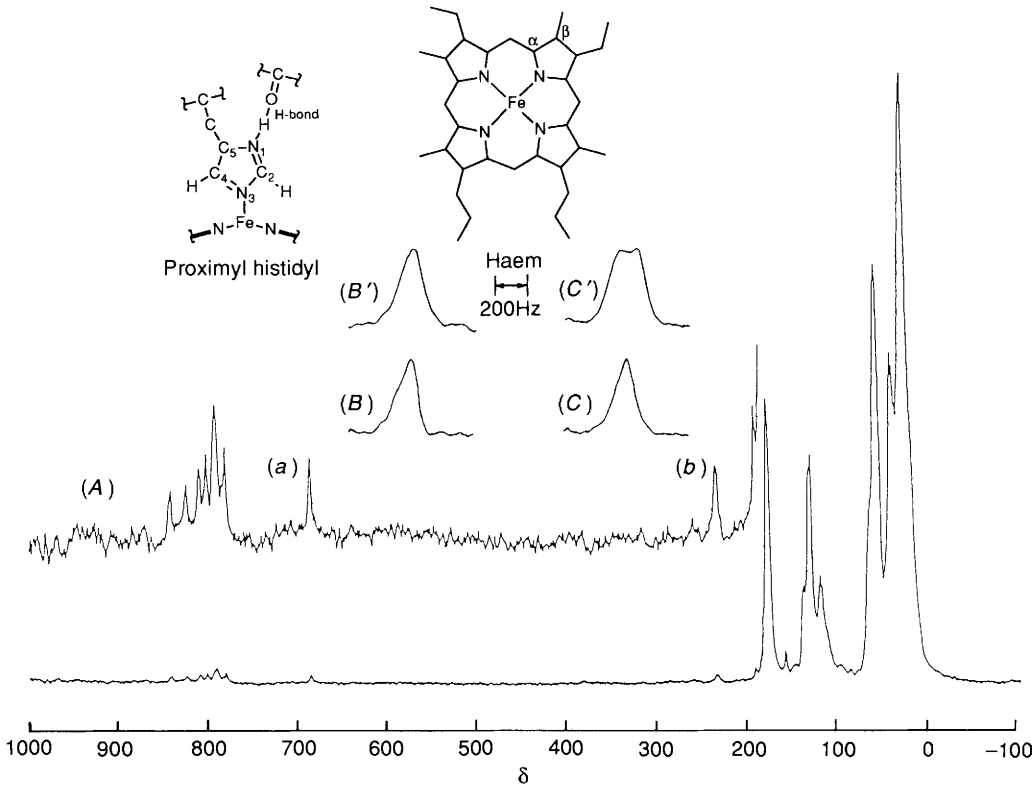

Fig. 1 The 67.8 MHz coupled ^{13}C NMR spectrum of 3 mmol dm^{-3} horse deoxymyoglobin in D_2O at 35 °C. In order to prepare the deoxy form of Mb, metMb was reduced by the addition of $Na_2S_2O_4$ under an O_2 free atmosphere. 150 000 transients and 0.3 s repetition time were used to record this spectrum. Optical spectroscopy indicated that the autoxidation of the sample during the measurement was not significant. The chemical shifts are referenced to 2,2-dimethyl-2-silapentane-5-sulfonate. (*A*) The *Y*-scale is expanded by a factor of 10. The signals arising from the C-2 and C-4 carbons are labelled (*a*) and (*b*). (*B*), (*C*) and (*B'*), (*C'*) are the C-2(C-4) resonances with and without ^1H decoupling, respectively. The numbering system of the imidazole and porphyrin rings is indicated in the inset.

the proximal histidyl imidazole ring ^{13}C resonances in horse deoxymyoglobin (Mb) and deoxyhaemoglobin A (Hb A) and also demonstrate their sensitivity to the Fe–N-3 bond. The 67.8 MHz ^{13}C NMR spectrum of horse deoxymyoglobin in D_2O, pD 6.5, at 35 °C is illustrated in Fig. 1. There are several signals resolved in the downfield hyperfine shifted region and their shifts clearly indicate that these resonances strongly interact with unpaired electrons at the haem iron. The downfield hyperfine shifted resonances in Fig. 1 may be assigned on the basis of the model compound study reported by Shirazi *et al.*,[5] which are tabulated in Table 1. From the comparison of the shift between the resonances of the model compound and Mb, peaks (*a*) and (*b*) are assignable to the imidazole C-2 and C-4 resonances, respectively, and the signals at δ 780–840 are attributed to the pyrrole-α and -β carbons. The effect of ^1H decoupling on peaks (*a*) and (*b*) are examined in (*B*), (*B'*) and (*C*), (*C'*), respectively. Peak (*b*) exhibits a linewidth of 160 Hz with ^1H decoupling and splits into two without decoupling. On the other hand, with the removal of decoupling field, the linewidth of peak (*a*) changed from 200 to 240 Hz. These results are consistent with the $^1J_{CH}$ values of 190 and 208 Hz for the corresponding C–H bonds in imidazole, respectively.[6] Signal overlap around δ 800 which consists of 16 peaks in total, prevents the discrimination between the two types of the pyrrole carbon resonances, while the two furthest downfield shifted signals may be assigned to the pyrrole-α carbons owing to their larger linewidth. Although Shirazi *et al.*[5] have suggested the potential of observing other ^{13}C resonances arising from the axial ligand and haem in the diamagnetic chemical shift region, they were not unambiguously identified in the present spectrum.

There are two signals observed at δ ~210 in the ^{13}C NMR spectrum of Hb A (not shown) and probably arise from the C-4 carbon in the α and β subunits. Their assignments to the individual subunits were tentatively made, as shown in Table 1, from the inositol hexaphosphate (IHP)-induced shift. Both peaks exhibit downfield shift in the presence of IHP; at 28 °C and pD 6.3, the upfield peak shifts +3.3 ppm with the addition of a three-fold molar excess of IHP and the downfield peak exhibits a +0.9 ppm shift. Binding of IHP to tetrameric Hb converts its quaternary structure from the T (tensed) state to the R (relaxed) state.[7] In this R → T conversion, it has been shown that larger structural alteration is induced on the α subunit than the β subunit.[8,9] In spite of the fact that stripped Hb A is already in the T state[10] and that the effect of IHP addition on the ν_{Fe-N} is quite small,[2] the IHP-induced shift changes observed on the C-4 resonance indicate not only that the electronic structure of the proximal histidyl imidazole in the T state of Hb A is influenced by the binding of IHP, but also the sensitivity of the C-4 resonances to structural alteration of Hb A. The other downfield hyperfine shifted resonances of Hb A could not be observed probably due to extensive line-broadening.

The Curie plots, observed shift *vs.* reciprocal of tempera-

Table 1 ^{13}C NMR shifts of resolved resonances in Mb and Hb A at 35 °C[a]

		Hb A		
Carbon	Mb	α	β	Model[b]
C-2	686.3			890
C-4	235.3	208.4	214.7	390
Pyrrole-α	790–840			1029
Pyrrole-β	780–815			834

[a] All numbers are given in ppm. [b] Taken from ref. 5. 0.03 mol dm^{-3} [(*m*-Me)TPP]Fe and 0.06 mol dm^{-3} 1,2-dimethylimidazole in CH_2Cl_2 at 26 °C. Due to the near fast-exchange limit, the chemical shifts were calculated from their ligand concentration dependence.

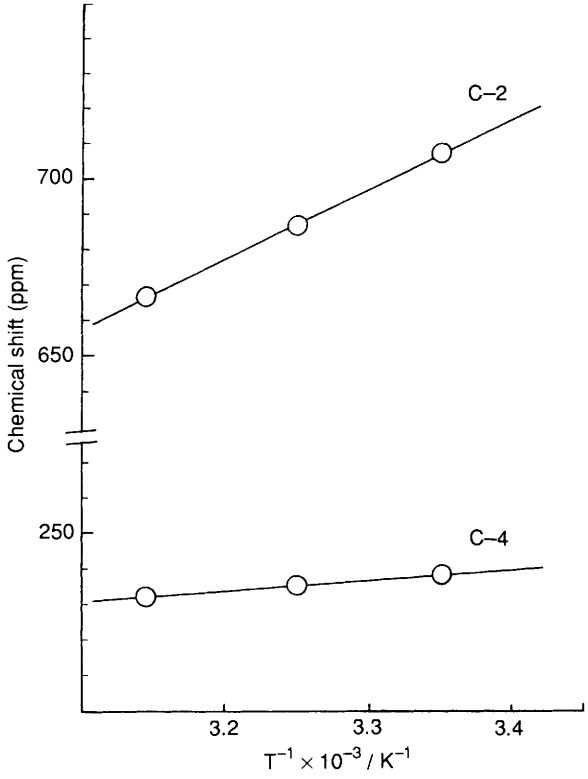

Fig. 2 The observed shift *vs.* reciprocal of temperature for the C-2 and C-4 resonances in Mb

ture, for the C-2 and C-4 resonances of Mb are shown in Fig. 2. Linear extrapolation was done in order to obtain the chemical shift at $T^{-1} \rightarrow 0$. Using the shift to $T^{-1} \rightarrow 0$ as tentative diamagnetic shift (δ_{dia}), the observed shifts (δ_{obs}) of the C-2 and C-4 resonances at 35 °C are separated into individual contributions in Table 2. The pseudo-contact shift (δ_{pc}) was calculated using the relationship obtained on high-spin iron(II) porphyrin complexes[11] and the X-ray coordinates of the model compound, iron(III) tetraphenylporphyrin-bis(imidazole) complex.[12] The remarkable difference in the contact shift (δ_c) between the C-2 and C-4 resonances indicates that the unpaired electron density on the former atom is much larger than that on the latter in the proximal histidyl imidazole of Mb.

Similar analysis on the C-4 resonance shifts of Hb A using the δ_{dia} of 142 yields the δ_c's of 40 and 47 for the resonances of the α and β subunits, respectively. Larger δ_c for the resonance of Mb than those of Hb A is explained in terms of the greater Fe–N-3 covalency in the former protein and these results are completely consistent with their ν_{Fe-N} values, *i.e.* 220 and 216 cm^{-1} for Mb and Hb A, respectively.[2,9]

Table 2 Separation of hyperfine shifts (δ_{hf}) into pseudo-contact (δ_{pc}) and contact (δ_c) contributions for the proximal histidyl imidazole ^{13}C resonances in Mb at 35 °C

Resonance	δ_{obs}[a]	δ_{dia}[a,b]	δ_{hf}[a]	G.f.[c]	δ_{pc}[a]	δ_c[a]
C-2	686.3	51	635	62.5	28.2	607
C-4	235.43	142	93	58.8	26.2	67

[a] Shifts in ppm. [b] The shift at $T^{-1} \rightarrow 0$ obtained from the Curie plot of Fig. 2. [c] Geometric factor $(3\cos^2\theta - 1)/r^3$, in units of 10^{-3} Å^3. The crystal coordinates of the iron(III) tetraphenylporphyrin-bis(imidazole) complex were used to calculate these factors with the average Fe–N-3 distance of 1.974 Å and the *Z*-axis is aligned along the Fe–N-3 vector.

The present study indicates the feasibility of studying the proximal histidyl imidazole in high-spin iron(II) haemoproteins using natural abundance ^{13}C NMR and the sensitivity of the imidazole ring ^{13}C resonances to the iron–histidine bonding interaction. It has been shown that, for imidazole derivatives, the electron density of the C-2 carbon is much more influenced by the hydrogen bond of the N(1)H proton than that of the C-4 carbon.[13] Therefore, the nature of both the iron–histidine bonding interaction and the hydrogen bond between the N(1)H proton and the protein acceptor in the active site of high-spin iron(II) haemoprotein could be characterised simultaneously from the analysis of the shifts of the C-2 and C-4 resonances.

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