NMR STUDIES OF THE BINDING OF ^{15}N-LABELED LIGANDS TO HEMOPROTEINS

pH-Dependent features of heme-bound $C^{15}N^-$ resonances in cyanide complexes of myoglobin and cytochrome *c* and some implications for their heme environmental structures

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1. Introduction

For recent years proton NMR spectroscopy, among many other physical methods, has become a powerful tool for structural studies of hemoproteins, particularly in elucidating electronic states of the heme, characterizing the ligand binding situation and delineating the heme–apoprotein interaction [1]. Most of these studies have been dealt with the isotropic shift of peripheral porphyrin substituents in the hemoproteins or in model compounds. These studies have recently been complemented by ^{13}C NMR investigations of heme-bound ^{13}CO [2] and *R*–N^{13}C [3] in the diamagnetic low spin ferrous myoglobin, hemoglobin [3] and cytochrome *c* [4]. We are currently investigating the ^{15}N NMR isotropic shift of the ^{15}N-labeled cyanide bound to the paramagnetic heme iron in various hemoproteins and in model compounds [5,6]. Structural changes in the heme environment within proteins or ongoing from the model compound to the protein have been shown to be quite sensitively manifested in the large ^{15}N isotropic shift of the iron-bound $C^{15}N^-$ [5,6]. In order to gain an insight into possible presence of subtle interactions between the axial ligand and other groups around the heme pocket, we have studied here pH-dependent features of ^{15}N resonance of the hemebound $C^{15}N$ in myoglobin and cytochrome *c*. In this communication we illustrate utility of the heme-bound $C^{15}N^-$ resonance as a sensitive probe for structural change in the heme environment of these hemoproteins induced by pH variation.

Part three of a series [6]

2. Materials and methods

The ^{15}N NMR spectra were recorded in a pulse Fourier transform mode with Jeol FX-100 spectrometer operating at 10.15 MHz. The tunable (multinucleus) probe available for variable frequencies was used to measure the ^{15}N resonances. All the samples of horse myoglobin (Sigma, Type III) and horse heart cytochrome *c* (Sigma, Type VI) were made in 10 mm NMR tube in the presence of ^{15}N enriched-KCN (Prochem, 96.7% atom ^{15}N) in 0.1 M phosphate buffer (20–25 mM). Between 16 000 and 20 000 transients were collected using spectral width 20 kHz and 4 K data points. The pulse repetition time of 0.2 s was employed with 30 μs pulse width (90°C). Chemical shifts are reported in ppm downfield from internal $^{15}NO_3^-$.

3. Results and discussion

In fig.1 are shown the representative ^{15}N spectra of cytochrome *c* (at pH 9.0) and myoglobin (at pH 10.0). The heme-bound $C^{15}N^-$ resonances are observed at 848.0 ppm and 939.8 ppm far beyond normal diamagnetic region, together with the free $C^{15}N^-$ signal located at −117 ppm.

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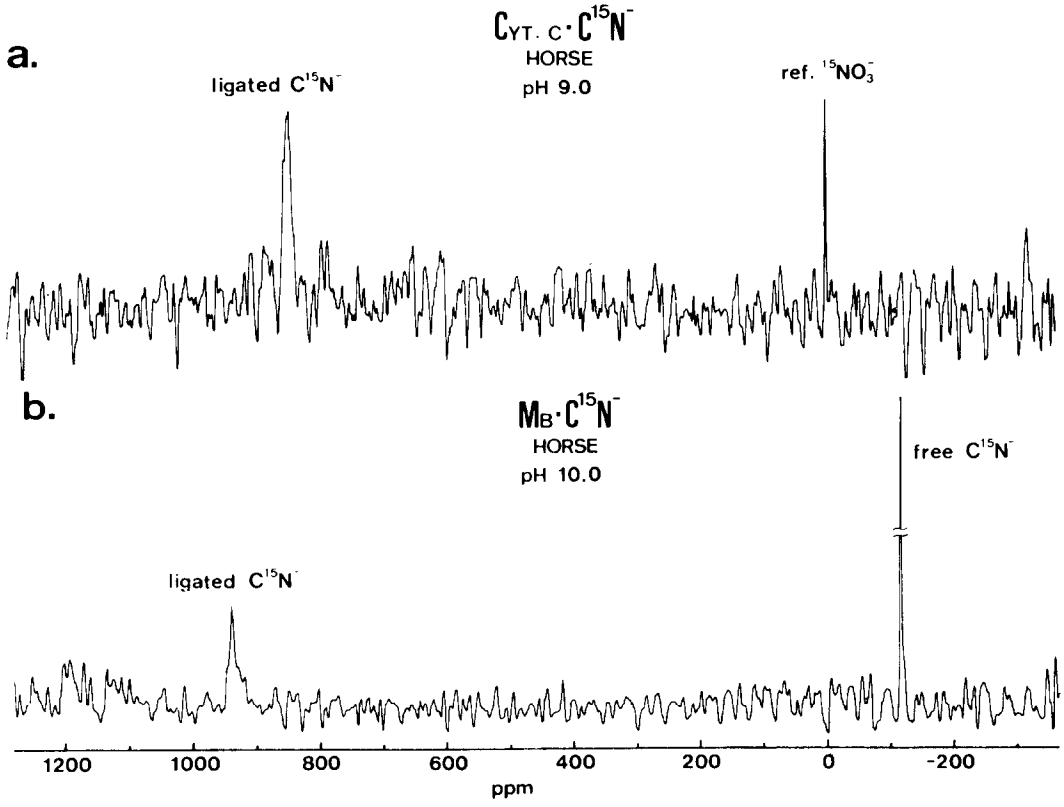

Fig.1. Cyanide resonance spectra of $C^{15}N$ complexes of myoglobin and cytochrome *c*. (a) Horse heart cytochrome *c*, at pH 9; (b) Horse myoglobin, at pH 10.

Figure 2 displays pH-dependent shift of these iron-bound $C^{15}N$ signals for cyano myoglobin and cytochrome *c*. It is evident in this figure that the $C^{15}N$ resonance of cyano myoglobin is very susceptible to pH variation from 5–10. Cyano cytochrome *c* also exhibits appreciable pH-dependent shift at pH 6 to pH 9 with behavior of pH titration curve. No ^{15}N signal was detected at pH>10 for cyano cytochrome *c*. In connection with these experiments, we have also examined pH-dependent features of hyperfine shifted proton resonances of heme peripheral substituents of the cyanide complexes for these hemoproteins [7]. In the acidic solution, no appreciable spectral shifts were encountered for cyano cytochrome *c*,[7] while slight spectral perturbations induced by pH variation were experienced for cyano myoglobin [8]. The ^{15}N resonance of cyano myoglobin experiences more complicated profile of pH-dependent shifts than does the proton resonances of heme peripheral groups. It is then likely that the heme-bound $C^{15}N$ resonance reflects quite sensitively some changes of heme environmental structure induced by pH variation, which is not accessible by proton resonances of heme side-groups. This is the case for cyano cytochrome *c* in which the heme-bound $C^{15}N$ resonance is sensitive to the pH-induced structural change in the heme environment, although this is small enough to be undetectable by the proton hyperfine shifted resonances. It has also been evident from the proton NMR companion studies that CN^- is still bound to the heme iron even at pH 12 for myoglobin [8] while in cytochrome *c* CN^- tends to be replaced by other ligand, possibly OH^-, at pH>10 [7] which is manifested in ^{15}N and ^1H NMR spectra. Namely, the $C^{15}N$ signal disappears at pH>10 and proton hyperfine shifted signals characteristic of cyano cytochrome *c* fade away, this follow-

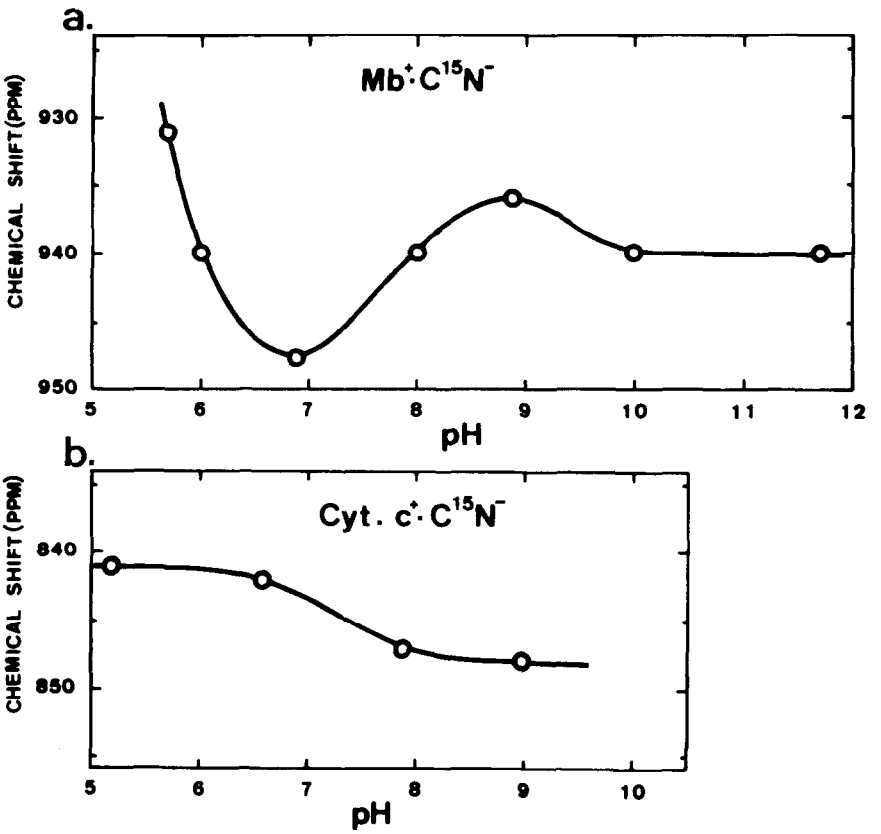

Fig.2. Nitrogen-15 chemical shift titration curves for $C^{15}N$ complexes of myoglobin and cytochrome *c*; ppm downfield from internal $^{15}NO_3^-$ as a function of solution pH. (a) Horse myoglobin; (b) Horse heart cytochrome *c*.

ed by growing of broad signals assignable to the OH^- bound cytochrome *c* [7].

The upfield bias of the $C^{15}N$ resonance of myoglobin cyanide induced by lowering pH from 7 to 5 is parallel with the same trend experienced for the heme-bound $C^{15}N$ resonance of the dicyano complex of hemin in going from DMSO ($\delta_{C^{15}N}$ 732 ppm) to methanol solution ($\delta_{C^{15}N}$ 506 ppm) as reported previously [6]. Increasing the H_2O content in DMSO–H_2O mixed solvent also resulted in the upfield shift of the heme-bound $C^{15}N$ resonance of dicyano–hemin complex [6]. These observations for model compounds may allow us to expect that substantial upfield shift of the iron-bound $C^{15}N$ resonance of cyano myoglobin with decreasing pH from 7 to 5 is associated with possible hydrogen bond effect between CN^- ligand and distal histidine. Since cyano myoglobin was not stable enough to be examined below pH 5, we could not follow the ^{15}N and ^1H NMR spectral shift till the end of the pH titration below pH 5 [8]. Nevertheless, this pH-dependent shift of the $C^{15}N$ resonance for cyano myoglobin appears to correspond to the ionization of the distal histidine, With lowering pH, protonation at the distal histidine may enhance this hydrogen bond, resulting in the $C^{15}N$ resonance shift to the upfield side. Such a marked spectral shift was not encountered for cytochrome *c* cyanide, probably because there is no such distal histidine around the heme pocket in cytochrome *c*.

The ^{15}N pH-dependent shift above pH 7 for cyano myoglobin is rather complicated and may be interpreted in terms of small perturbation of the conformation in the heme vicinity caused by ionization of some amino acid groups. The progressive upfield shift

of the bound $C^{15}N$ resonance with increasing pH from 7 to 9 may be attributed to this cause. Histidine-36 (C 1) is a possible candidate for the ionizable group which has been reported [9] to possess a high pK_a of 7.62 and 7.87 for horse metmyoglobin and myoglobin azide, respectively. The imidazole moiety of this residue is known to be stacked over the phenyl ring of phenylalanine-106 (G 7) with a distance of approx. 3.5 Å from the X-ray analysis [9]. If ionization of histidine-36 (C 1) would affect this interaction with phenylalanine-106 (G 7), the G-helix could more or less undergo movement from the normal position. Furthermore, this helix involves the important amino acid residues in van der Waals contact with the porphyrin ring of the heme group, such as leucine-104 (G 5) and isoleucine-107 (G 8) [10]. Conformational changes thus induced of polypeptide chain which influence the proximal histidine or the periphery of the porphyrin ring can ultimately manifest themselves as electronic effects at the heme iron and eventually as iron-bound $C^{15}N$ resonance shift. Moreover, the relatively small change of $C^{15}N$ shift above pH 9 may be attributed to similar conformational effect induced by ionization of tyrosine-103 (G 4) [11] at the interhelical turn next to leucine-104 (G 5) mentioned above. In the case of cyano cytochrome *c*, small but appreciable ^{15}N shift with varying pH from 6 to 9 may be also due to an alternation of the tertiary structure as a result of the ionization of some residue, probably histidine-33 [12].

In summary, the ^{15}N isotropic shift of the iron-bound $C^{15}N$ in myoglobin and cytochrome *c* serves as a quite sensitive probe for characterizing the ligand binding feature and/or delineating the environmental structure of the prosthetic group in hemoproteins, while the proton NMR shift of heme peripheral groups does not give any fruitful information concerning these effects. Applications of the present paramagnetic ^{15}N probe are currently being performed to other members of the hemoprotein and hemoenzyme family.

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