^{15}N MAGNETIC RESONANCE STUDIES OF THE BINDING OF ^{15}N-LABELED CYANIDE TO VARIOUS HEMOPROTEINS

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SUMMARY

The ^{15}N paramagnetic shifts of iron-bound $C^{15}N^-$ were studied for myoglobin, hemoglobin, cytochrome c and other modified hemoproteins. Two characteristic ^{15}N resonances at 977 and 1045 ppm(with respect to $^{15}NO_3^-$ as an internal standard) were found for human adult hemoglobin cyanide, while only single resonances were observed for other cyano hemoproteins. These two resonances are assigned to iron-bound $C^{15}N$ of α and β subunits of hemoglobin. The substantial difference in the $C^{15}N$ isotropic shifts in various hemoproteins is discussed in relation to iron-proximal histidine binding and heme-apoprotein interactions.

A number of reports have appeared that utilize ^{13}C-enriched carbon monoxide, strongly bound to hemoproteins, to investigate the structure and reactivity of various hemoglobins and myoglobins(1). Recently ^{13}C-enriched isocyanide(2) has also been used as an alternative to ^{13}CO to probe microenvironmental structure of the heme in ferrous state hemoglobin and myoglobin. Although ^{13}C resonances of bound ^{13}CO and $R\text{-}N^{13}C$ in hemoproteins were found to differentiate heme moieties of α and β subunits of hemoglobin, these ^{13}C shifts were not so sensitive to a variety of species from which hemoglobins are taken and to pH variation(1), possibly because these ^{13}C shifts are of diamagnetic origin. We are currently interested in the NMR paramagnetic shifts of axial ligands bound to heme iron in ferric low spin hemoproteins and hemin complexes(3), in which these shifts are expected to become powerful tool in

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elucidating electronic state of the heme, characterizing the ligand binding and delineating the ligand-apoprotein interaction in the heme environments(3). We wish to report here ^{15}N NMR data of ^{15}N-enriched cyanide($C^{15}N^-$) bound to the heme in hemoglobin, myoglobin, cytochrome c and its carboxymethylated derivative and to show that ^{15}N isotropic shift of iron-bound cyanide serves as a quite sensitive probe for heme environmental structure of hemoproteins.

MATERIALS AND METHODS

Human adult hemoglobin(Hb) was prepared from blood freshly drawn from one of the authors by standard procedures and was oxidized with $K_3[Fe(CN)_6]$(4). Methemoglobin purified by CM52 column (0.01 M phosphate buffer, pH 6.0) was concentrated by ultrafiltration. Myoglobins of sperm whale(Sigma, Type II) and of horse(Sigma, Type III) and cytochrome c(Sigma, Type VI) were purchased from the commercial sources and used without further purification.

Dicarboxymethylated cytochrome c was prepared after the method of Schejter and Aviram(5). Mesohemin was synthesized from protohemin by reaction with PdO(6). Recombination of globin(sperm whale) with mesohemin was performed as previously described(7) and purified through CM52 column. Samples of Hb, Mb and Cyt. c were prepared in D_2O with 10~20 mM.

The ^{15}N NMR spectra were recorded in a pulse Fourier transform mode with Jeol PFT-100 spectrometer operating at 10.15 MHz, using the deuterium resonance of internal D_2O as a lock. 50,000 to 200,000 transients were collected using spectral width 10 KHz and 8K data points. The pulse repetition time of 0.42 sec was employed. Chemical shifts are reported in ppm from internal $^{15}NO_3^-$.

RESULTS

Addition of $KC^{15}N$ to a solution of horse metmyoglobin(pH 8.3) afforded an ^{15}N NMR spectrum with a broad signal at 943 ppm far downfield from internal reference $^{15}NO_3^-$ and sharp signal of free $C^{15}N^-$ at -117 ppm(Fig. 1). Use of $KC^{14}N$ did not yield both signals under the same condition. This broadened signal located far beyond normal diamagnetic spectral region(-96~-112 ppm for cyano complexes) exhibited temperature dependence obeying the Curie's law. Thus this signal is unambiguously assigned to the hyperfine shifted resonance of heme iron-bound $C^{15}N^-$ of cyano metmyoglobin.

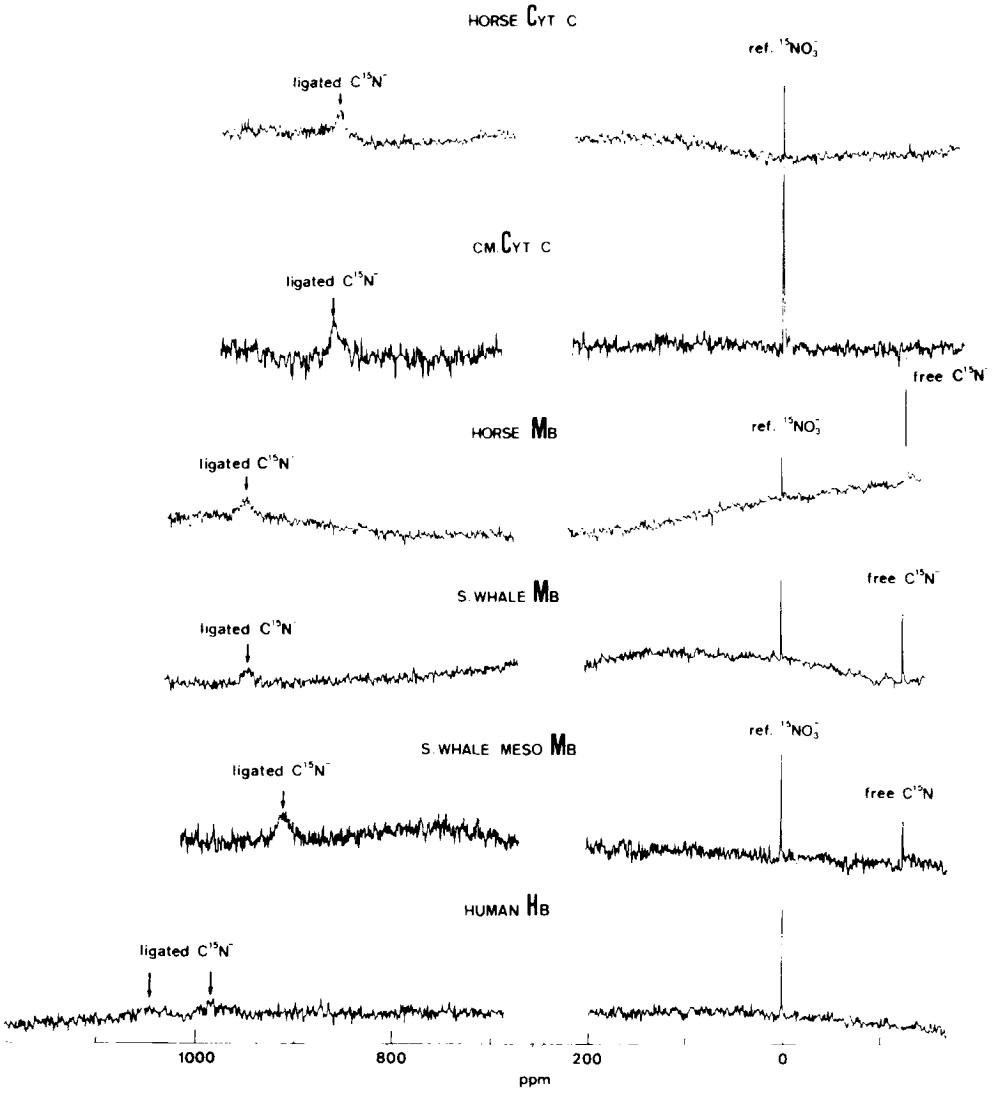

Figure 1: ^{15}N NMR spectra of various $C^{15}N^-$ bound hemoproteins. Chemical shifts are represented in ppm from $^{15}NO_3^-$ as an internal standard. From the top, horse heart cytochrome c, dicarboxymethylated cytochrome c, horse myoglobin, sperm whale myoglobin, sperm whale myoglobin reconstituted with mesohemin and human adult hemoglobin, respectively.

To further confirm this, we have also studied ^{15}N NMR of biscyano complex of protoporphyrin IX in dimethyl sulfoxide and in methanol. A broad ^{15}N signal was also found at +732 ppm and fairly sharp

Table I. Chemical shifts of heme-bound $C^{15}N^-$, proximal histidyl NH proton and heme peripheral methyl protons for various hemoproteins

Protein	pH	Iron-bound $C^{15}N$ shift (at 25°C)[a]	Proximal histidyl NH proton shift[b]	Heme peripheral methyl proton shift[b]
Mb(horse)	6.9 8.0 9.0	+948 +939 +936	21.5[c]	
Mb(sperm whale)	8.8	+945	21.5[c]	26.9, 18.2, 13.2[f]
meso Mb(sperm whale)	8.6	+910		
Hb(human adult)	7.3	+975(α) +1047(β)	21.9[d] 22.8[d]	22.7, 16.6[d] 21.7, 15.7[d]
Cyt. c(horse)	9.0 7.8 6.6	+848 +847 +842	14.0[e]	24.0, 22.2, 17.1, 11.3[f]
CM Cyt. c(horse)	9.0	+852		24.7, 22.3, 17.0, 11.5[f]

a) Shifts are given in ppm with respect to an internal standard of $^{15}NO_3^-$.
b) Shifts are given in ppm with respect to an internal standard of 2,2-dimethyl-2-pentasilane-5-sulfonate.
c) Sheard, B., Yamane, T. and Shulman, R.G. (1970) J. Mol. Biol., 53, 35-48.
d) The shifts obtained in α and β isolated chains. Ogawa, S., Shulman, R.G. and Yamane, T. (1972) J. Mol. Biol., 70, 291-300.
e) Morishima, I. and Masuda, H., unpublished observation.
f) Morishima, I., Ogawa, S., Yonezawa, T. and Iizuka, T. (1977) Biochim. Biophys. Acta, in press.

signal at +506 ppm, respectively, in addition to free $C^{15}N^-$ signal at -100 ppm(3). Similar results were obtained for sperm whale myoglobin, cytochrome c and human adult hemoglobin(Hb) at various pH. Cyano Hb exhibited two broad signals at +975 and +1047 ppm at pH 7.3. The concentration ratio of $C^{15}N^-$ and metHb was 1:1, in an insufficient saturation condition. When pH was raised from 7.3 to 7.6, the relative intensity of the lower field peak with respect to that of upfield one was decreased, accompanied by substantial resonance shifts to the lower field side (at +984 and +1067 ppm).

There is small but appreciable difference(9 ppm) in the iron-bound $C^{15}N$ shifts between horse and sperm whale myoglobins at the same pH 8.8. Cyano cytochrome c also exhibited a single resonance at +848 ppm, about 90 ppm upfield from the Mb.$C^{15}N$ resonance. We have also performed similar experiments for cyanide complexes of carboxymethylated cytochrome c and mesohemin-reconstituted sperm whale myoglobin and their ^{15}N spectra are included in Fig. 1. All the results are summarized in Table I.

DISCUSSION

Inspection of Table I reveals that there are substantial changes of the heme-bound $C^{15}N$ resonance shifts in various hemoproteins. These shifts are also pH-dependent and susceptible to the modifications of the peptide chain in the heme vicinity(CM Cyt. c) and of heme peripheral groups(mesoMb).

Like ^{13}C NMR spectra of heme-bound ^{13}CO(1) and $C_2H_5N^{13}C$(2) in ferrous hemoglobin, ^{15}N NMR spectra of Hb.$C^{15}N$ show two hyperfine shifted ^{15}N resonances while single peak was encountered for Mb and Cyt. c derivatives. These two ^{15}N resonances in Hb should correspond to the $C^{15}N^-$ bound to the Fe(III) of the α and β subunits. At the insufficient concentration of $C^{15}N^-$, the differential ligand affinities to the ferric heme-iron of α and β subunits of Hb are experienced at pH 7.6, as evidenced by observation of unequal $C^{15}N$ signal intensities. On the basis of the assumption that the ligand CN^- binding to the α heme is slightly more favorable than to the β heme, which has been shown in the isolated α and β chains(8),and is in contrast with N_3^- binding to the ferric heme-iron and O_2 and CO binding to the ferrous heme-iron, the $C^{15}N$ signals located at +975 and +1047 ppm at pH 7.3 are expected to be assigned tentatively to the heme-bound $C^{15}N$ in α and β subunits, respectively. Present finding that $C^{15}N$ ligand binding affinity tends to be decreased with

raising pH appears to be in accord with well established results of pH dependence of exogenous ligand binding constants(8). The signals corresponding to $C^{15}N^-$ bound to the α and β subunits in Hb are separated by ca. 60 ppm, which is in marked contrast to the corresponding ^{13}C shift of 0.56 ppm for heme-bound ^{13}CO and 0.92 ppm for $C_2H_5N^{13}C$ in Hb in ferrous low spin state(2).

In Table I are also presented proton NMR signal positions of the proximal histidine NH and the heme peripheral methyl groups of cyano Mb, Hb and Cyt. c. It is generally seen that the larger the hyperfine shifts of proximal histidine NH proton, the larger the heme-bound $C^{15}N$ resonance shifts and the smaller the heme-peripheral methyl proton shifts, when these data are compared between Mb and Hb. The trans and cis effects on the hyperfine shifts of axial and equatorial ligand appear to be operative. The trans effect which is manifested in a parallel relation between heme-bound $C^{15}N^-$ and proximal histidyl NH proton shifts also holds for cyano Cyt. c. The small difference in the bound $C^{15}N^-$ shifts between horse and sperm whale Mb cyanides may be due to this effect. It is reasonably considered that the hyperfine shifts of the axial ligand reflect more sensitively the electronic structures of the heme-iron than those of the heme peripheral groups which is likely more susceptible to subtle interaction between heme and apoprotein. Inspection of the Table I also reveals that the ^{15}N isotropic shift of the heme-bound $C^{15}N^-$ is about 100 times as sensitive as the proximal histidyl NH proton shift toward the heme electronic structure.

The effect of chemical modification in the heme vicinity on the heme-bound $C^{15}N$ resonance is found to be small as shown in Table I. The shift difference of 4 ppm between Cyt. c and CM Cyt. c cyanides at pH 9 is comparable with pH dependent shift change of 6 ppm at pH 6.6 and 9.0 for Cyt. c, which possibly arises from slight

conformational change caused by ionization of some amino acid residue. In Mb and Hb, the presence of distal histidine located in a favorable position to form hydrogen bond with sixth ligand(9) appears to be responsible for substantial pH dependent shift of the iron-bound $C^{15}N$ at pH 5~7, arising from pH-induced modulation of this possible hydrogen bonding. It is not the case for Cyt. c in which there is no such distal ionizable amino acid residue(10) with pK<7.0. It is therefore likely that substantially large ^{15}N shift changes for cyanide complexes of Mb, Hb(α), Hb(β) and Cyt. c may be attributed to changes in the iron-histidine binding nature, not to the cyanide-protein nonbonded interaction. The considerable difference in bound $C^{15}N^{-}$ shifts(~30 ppm) was also found between natural and heme-reconstituted (meso) Mb. Whether the difference is due to changes in the bond between the iron and cyanide, or to any other subtler interactions between the ligands and other groups around the heme pocket is presently unknown. It is likely that these two effects may be synchronously caused and can not be separated.

In order to gain further insight into the structural details of heme environments in various hemoproteins, it seems desirable to investigate in details the iron-bound $C^{15}N$ resonance of cyanide hemin complexes with various mixed ligands as model compounds and their medium effects. This is now under way in our laboratory.

REFERENCES

1. Moon, R.E., Dill, K. and Richards, J.H. (1977) Biochemistry, 16, 221-228, and references cited therein.
2. Mansuy, D., Lallemand, J.-Y., Chottard, J.-C., Cendrier, B., Gacon, G. and Wajcman, H. (1976) Biochem. Biophys. Res. Comm., 70, 595-599.
3. Morishima, I. and Inubushi, T., J. Am. Chem. Soc., submitted for publication.
4. Geyer, D. and Lemberg, R. (1971) Biochim. Biophys. Acta, 229, 284-285.
5. Schejter, A. and Aviram, I. (1970) J. Biol. Chem., 245, 1552-1557.
6. Yonetani, T. and Asakura, T. (1968) J. Biol. Chem., 243, 4715-4721.

7. Antonini, E., Burunori, M., Caputo, A., Chiancone, E., Rossi-Fanelli, A. and Wyman, J. (1964) Biochim. Biophys. Acta, 79, 284-300.
8. Gibson, Q.H., Parkhurst, J.P. and Geraci, G. (1969) J. Biol. Chem., 244, 4668-4676.
9. Stryer, L., Kendrew, J.C., and Watson, H.C. (1964) J. Mol. Biol., 8, 96-104.
10. Dickerson, R.E., Takano, T., Eisenberg, D., Kallai, O.B., Samson, L., Cooper, A. and Margoriash, E. (1971) J. Biol. Chem., 246, 1511-1535.