^{15}N Nuclear Magnetic Resonance Studies of Iron-Bound $C^{15}N^-$ in Ferric Low-Spin Cyanide Complexes of Various Porphyrin Derivatives and Various Hemoproteins[1]

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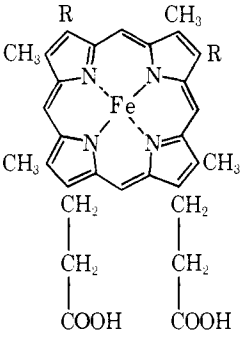
Abstract: Studies on ^{15}N paramagnetic shifts of the iron-bound ^{15}N-labeled cyanide in low-spin ferric cyanide complexes of various porphyrin derivatives and hemoproteins are reported. The ^{15}N resonance of $C^{15}N$ coordinated to the heme-iron atom can be separately detected from the bulk resonance of $C^{15}N^-$, and the ^{15}N chemical shifts for various hemin and hemoproteins range from 448 to 1070 ppm with respect to internal $^{15}NO_3^-$. The cis and trans ligand effects on the ^{15}N shifts for the mono- and dicyano complexes of proto-, deutero-, meso-, hemato-, and octaethylporphyrins are also investigated. The trans effect induced by a change in the axial ligand is much more pronounced than the cis effect caused by substitution of the porphyrin peripheral groups. A substantial difference in the iron-bound cyanide ^{15}N shifts in the cyanide complexes of cytochrome *c*, myoglobin, and hemoglobin was primarily interpreted in terms of this trans effect, the effect of subtle changes in the proximal histidine-Fe binding nature. The ^{15}N isotropic shifts for cyanohemin complexes are also shown to be very sensitive to solvent. Proton donor solvents were most effective in causing upfield bias of the $C^{15}N^-$ shift. The sizable ^{15}N shift induced by pH variation for cyanomyoglobin was interpreted in relation to possible involvement of hydrogen bonding between distal histidine and heme-bound cyanide.

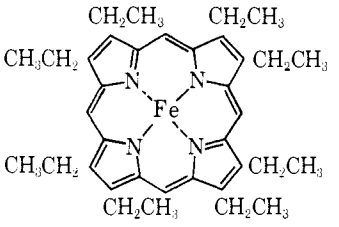
The environmental structure of the prosthetic group in hemoproteins has long been a problem of keen and continuous interest to biochemists and chemists alike. In recent years proton NMR spectroscopy, among many physical methods, has become a powerful tool for structural studies of hemoproteins, particularly in elucidating electronic states of the heme, characterizing the ligand binding situation, and delineating the heme-apoprotein interaction.[2] Most of these studies have dealt with the proton 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[3] and C_2H_5-$N^{13}C$[4] in the diamagnetic low-spin ferrous myoglobin. The use of isotopically enriched ligand molecules bound to the heme iron in hemoproteins can be a convenient and effective tool in the NMR spectroscopic study of hemoproteins to probe heme environmental structure and ligand-heme interactions. Information concerning molecular structure and/or electronic effects at the heme iron binding site, as well as details concerning kinetic affinities of ligands for heme iron, can be obtained from such studies. Although ^{13}C resonances of heme-bound ^{13}CO and C_2H_5-$N^{13}C$ 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,[3a] possibly because these ^{13}C shifts are of diamagnetic origin.

We are currently investigating the NMR paramagnetic shifts of axial ligands such as imidazole and pyridine bound to heme iron in ferric low-spin hemoproteins and hemin complexes,[5] in which these shifts have been shown to become a powerful tool in elucidating the electronic state of the heme, characterizing the ligand binding, and delineating the ligand-apoprotein interaction in heme environments. Studies of the ^{13}C or ^{15}N hyperfine-shifted resonances of heme-iron-bound cyanide in hemoproteins or in model compounds have long been awaited.[6] This is probably because cyano derivatives, which are readily formed due to strong binding of cyanide to oxidized or ferric hemoproteins, can be of great interest in the inhibition or poisoning of metabolism. Indeed, one of the first things done to new hemoproteins has been to add cyanide. Effects of cyanide on properties of hemoproteins have been well studied by visible, Soret, EPR, IR, resonance Raman and Mössbauer spectroscopies as well as proton NMR. We have recently succeeded in the observation of the ^{15}N resonance of heme-iron-bound $C^{15}N^-$ in cyanide complexes of hemin and hemoproteins.[1,7] We wish to report here some details of our systematic investigation of the ^{15}N paramagnetic shifts of iron-bound $C^{15}N$ in ferric cyanide complexes of porphyrin derivatives (**1–5**) and of various hemoproteins.



1, protohemin (R = $CH{=}CH_2$)
2, deuterohemin (R = H)
3, mesohemin (R = CH_2CH_3)
4, hematohemin (R = $C(OH)HCH_3$)



5, octaethylporphyriniron(III)

Here we are particularly concerned with cis and trans effects on the iron-bound ^{15}N isotropic shift of dicyano, pyridine-cyano, and imidazole-cyano complexes of hemins, with the aim of analyzing the factors that influence the $C^{15}N$ shift in various cyano hemoproteins. It is revealed that structural changes in the heme environment within the proteins or on going from the

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model compound to the protein are quite sensitively manifested in the ^{15}N isotropic shifts of the heme-bound $C^{15}N$. We have also found that the iron-bound $C^{15}N$ isotropic shifts for the heme model compounds are sensitive to the solvent, which was interpreted in terms of the effect of hydrogen bonding on the ligand binding situation in ferric porphyrin complexes, with relevance to axial ligand–apoprotein interaction in some hemoproteins.

Experimental Section

Preparation of Hemins. Deutero- and mesoporphyrin IX were synthesized from protohemin chloride (Sigma, Type III) following the literature methods.[8,9] Hematoporphyrin IX (Wako, Osaka, Japan) and octaethylporphyrin (Strem) were commercially available. Incorporation of iron into porphyrins was performed in glacial acetic acid with $FeSO_4$. Chloride adducts of ferric porphyrin complexes were converted with saturated NaCl solution.[10] All the porphyrin iron chloride derivatives were purified chromatographically.[11]

Preparation of Hemoproteins. Human adult hemoglobin (Hb) was prepared from blood freshly drawn from one of the authors by standard procedures and was oxidized with $K_3[Fe(CN)_6]$.[12] Methemoglobin purified by a CM52 column (0.01 M phosphate buffer, pH 6.0) was concentrated by ultrafiltration. Myoglobins of sperm whale (Sigma, Type II), horse (Sigma, Type III), and cytochrome *c* (Sigma, Type IV) were purchased from commercial sources and used without further purification. The purity of these hemins and hemoproteins was checked optically and by proton NMR. The samples used were 99% pure.

NMR Measurements. The ^{15}N NMR spectra were recorded in a pulse Fourier transform mode with a Jeol PFT-100 (partly with FX-100) spectrometer operating at 10.15 MHz. All the samples of cyanohemins and hemoproteins were made in 10-mm NMR tubes in the presence of ^{15}N-enriched KCN (Prochem, 96.7 atom % ^{15}N) in appropriate deuterated solvents for the purpose of ^2D lock in the NMR measurements. However, the buffer solutions of hemoproteins were not deuterated enough (presence of ca. 20% H_2O) to define the pD values, so that the uncorrected pH values (direct readings of pH meter) were used throughout this work. Experimental conditions, especially the concentration ratio of hemin, cyanide, and pyridine (solvent) to make a pyridine–cyano complex of hemin, were referenced to the literature.[13] The presence of a stoichiometric amount (1 equiv) of $C^{15}N^-$ in pyridine (or pyridine derivative)–D_2O or *N*-methylimidazole–Me_2SO mixture afforded both monocyano and a small portion of unreacted species, so that only the $C^{15}N^-$ resonances of monocyano species were detectable in ^{15}N NMR measurements. These situations were confirmed by our porton NMR studies.[14] When a slight excess (1.2 equiv) of $C^{15}N^-$ was added to these solutions, the $C^{15}N$ resonances due to both mono- and dicyano species could be detected concurrently. Dicyanohemin complexes and cyanohemoproteins were prepared by the addition of more than a tenfold excess of $C^{15}N^-$. Typically, 5000 (for dicyanohemin complexes) to 100 000 transients (for monocyanohemin complexes and for cyanohemoproteins) were collected using a spectral width of 10 kHz and 8000 data points. The pulse repetition time of 0.42 s was employed with 25-μs (90°) pulses. Details of the experimental conditions are given in the footnotes of Tables I–III and the captions of Figures 1–7. Chemical shifts are reported in parts per million from an internal $^{15}NO_3^-$, with a positive sign for downfield shifts. The accuracy of the ^{15}N chemical shift for iron-bound $C^{15}N^-$ is ±1 ppm due to the paramagnetically broadened resonance line.

Results

Iron-Bound $C^{15}N$ Resonances of Cyanide Complexes of Various Hemin Derivatives. As Figure 1 shows, biscyanide complexes of various ferric porphyrin derivatives in dimethyl sulfoxide (Me_2SO) using ^{15}N-enriched KCN gave a broad ^{15}N signal at a resonance position far beyond the diamagnetic region.[15] This arises from the bound $C^{15}N^-$ to the heme iron, separately observed from the free $C^{15}N^-$ signal in Me_2SO. Use of the usual $C^{14}N^-$ ligand did not afford these two signals under the same conditions. Similar observations were obtained for the protohemin–dicyanide complex in various solvents such as pyridine, methanol, methanol–water mixed solvent (1:1),

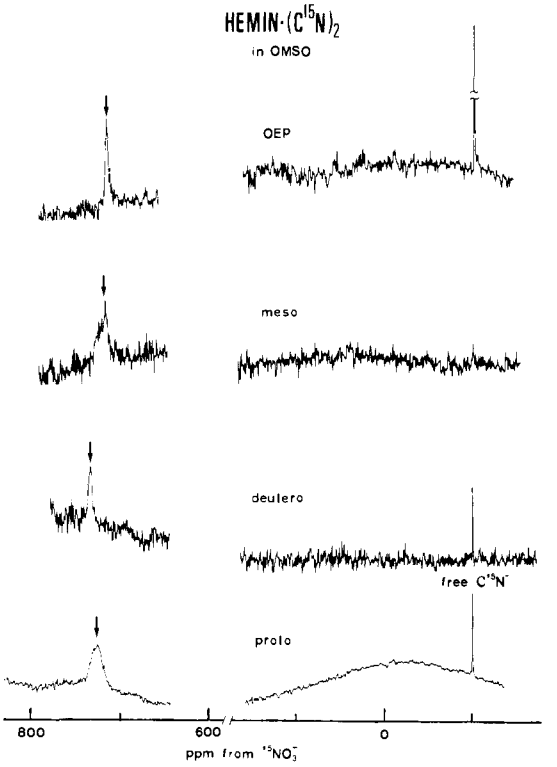


Figure 1. ^{15}N NMR spectra of various hemin–dicyanide complexes in Me_2SO; from the top, OEP, meso-, deutero-, and protohemins, respectively. The concentrations of dicyanohemin complexes were 20–30 mM, and 5000 to 10 000 transients were accumulated to obtain these ^{15}N NMR spectra.

and pure water. In the methanol solvent, the ^{15}N resonance is located far upfield with a small line width, compared with that in the Me_2SO solvent. This upfield bias of the ^{15}N hyperfine shift and signal sharpening were also experienced in methanol–water mixed solvent and in water solvent.

We have also examined ^{15}N NMR of monocyano derivatives of protohemin in pyridine–water mixed solvent with appropriate ratios following the method of Caughey et al.[13] The ^{15}N signal of bound $C^{15}N$ in pyridine–cyanoprotohemin species with mixed ligands was successfully observed separately from dicyano species. For example, the iron-bound $C^{15}N$ resonance is found at +1000 ppm for pyridine–cyanoprotohemin species and at +657 ppm for the dicyano species in pyridine–water mixed solvent (5:1) (Table II). The $C^{15}N$ resonances of monocyano adducts containing various pyridine derivatives as the fifth ligand were studied in order to confirm the effect of basicity of pyridine derivatives on the $C^{15}N$ shifts. These resonances also depend profoundly on the amount of water in the mixed solvent (see Figure 5), so that the values listed in Table I are obtained under conditions of $[D_2O]/[\text{hemin}] = 500$, to compare with each other. We have also obtained the $C^{15}N$ signal arising from the monocyano species of *N*-methylimidazole–cyanoprotohemin complex, with the appropriate ratio of *N*-methylimidazole–Me_2SO mixed solvent (see the footnotes to Table I).

The iron-bound $C^{15}N$ resonances of dicyano complexes were also observed for meso-, deutero-, hemato-, and octaethylporphyrins in Me_2SO and methanol solvents. Figure 2 represents the ^{15}N NMR spectra for various dicyanohemin complexes in methanol (CH_3OD). However, we could not observe the iron-bound $C^{15}N$ signals for dicyano complexes of hematoporphyrin in Me_2SO and of mesotetraphenylporphyrin (TPP) in Me_2SO or methanol, probably because the solubility of TPP iron(III) chloride is limited in these solvents or because the cyanide binding affinities are too small to obtain a sufficient

Table I. Observed Chemical Shifts of Axially Coordinated $C^{15}N^-$ to Various Hemin Derivatives (at 25 °C)

Hemin cyanide complex	Solvent condition	Chem shift,[a] ppm from $^{15}NO_3^-$
Protohemin $(C^{15}N^-)_2$	Me_2SO	+732
	Py	+696[b]
	CH_3OD	+506
	$CH_3OD + D_2O$ (1:1)	+480
	H_2O (pH 9.4)	+451
	H_2O (pH 9.2)	+448
Deuterohemin $(C^{15}N^-)_2$	Me_2SO	+738
	$Me_2SO + D_2O$ (20:1)	+716
	CH_3OD	+509
Mesohemin $(C^{15}N^-)_2$	Me_2SO	+720
	CH_3OD	+496
Hematohemin $(C^{15}N^-)_2$	CH_3OD	+495
OEP $(C^{15}N^-)_2$	Me_2SO	+718
	CH_3OD	+474
Protohemin (4-AcPy) ($C^{15}N^-$)	4-AcPy + D_2O[c]	+945
Protohemin (Py) ($C^{15}N^-$)	Py + D_2O[c]	+989
	Py + D_2O[d]	+1034
Protohemin (3,5-Lu) ($C^{15}N^-$)	3,5-Lu + D_2O[c]	+1070
Mesohemin (Py) ($C^{15}N^-$)	Py + D_2O[c]	+984
Deuterohemin (Py) ($C^{15}N^-$)	Py + D_2O[d]	+1040
Hematohemin (Py) ($C^{15}N^-$)	Py + D_2O[c]	+982
Protohemin (*N*-MeIm) ($C^{15}N^-$)	*N*-MeIm + Me_2SO	+926

[a] A plus sign designates downfield shift from an internal standard, $^{15}NO_3^-$. Solutions (20–32 mM) of mono- and dicyano complexes were prepared; 5000 to 10 000 transients for dicyano complexes and 50 000 to 100 000 transients for monocyano complexes were accumulated to read the ^{15}N chemical shift of iron-bound $C^{15}N^-$ signal. For preparations of mono- and dicyanohemin complexes see the text. Abbreviations used are: Me_2SO, dimethyl sulfoxide; Py, pyridine; 4-AcPy, 4-acetylpyridine; 3,5-Lu; 3,5-lutidine; N-MeIm, *N*-methylimidazole. [b] The value obtained by extrapolating the data in the D_2O titration experiment (see Table II). [c] $[D_2O]$:[hemin] = 500:1. [d] $[D_2O]$:[hemin] = 230:1. [e] [*N*-MeIm]:$[C^{15}N^-]$:[hemin] = 100:1:1.

Table II. The Effect of Water Content on the Chemical Shifts of Bound $C^{15}N^-$ for Dicyano- and Monocyanoprotohemins (at 25 °C)

Hemin-cyanide complex	Solvent conditions	Chem shift,[a] ppm from $^{15}NO_3^-$
Protohemin $(C^{15}N^-)_2$	$Me_2SO + D_2O$ (18:1)	+716
	$Me_2SO + D_2O$ (18:2)	+699
	$Me_2SO + D_2O$ (18:4)	+671
	Py + D_2O (20:4)	+657
	Py + D_2O (20:5)	+652
	Py + D_2O (20:7)	+637
Protohemin (Py) ($C^{15}N^-$)	Py + H_2O (5:1)	+1000
	Py + H_2O (2:1)	+941
	Py + H_2O (3:2)	+931

[a] A plus sign designates downfield shift from an internal standard, $^{15}NO_3^-$. For the experimental conditions and abbreviations used here see the footnotes to Table I.

amount of dicyano complexes for the ^{15}N NMR experiment. The autoreduction of these ferric complexes may be also responsible for this difficulty. It is generally seen that the iron-bound $C^{15}N$ signals of cyano ferric porphyrin complexes appear to be substantially narrower than those of cyanohemoproteins (vide infra), except for the cyanide complex of hematoporphyrin. Unusual broadening of the $C^{15}N$ resonance

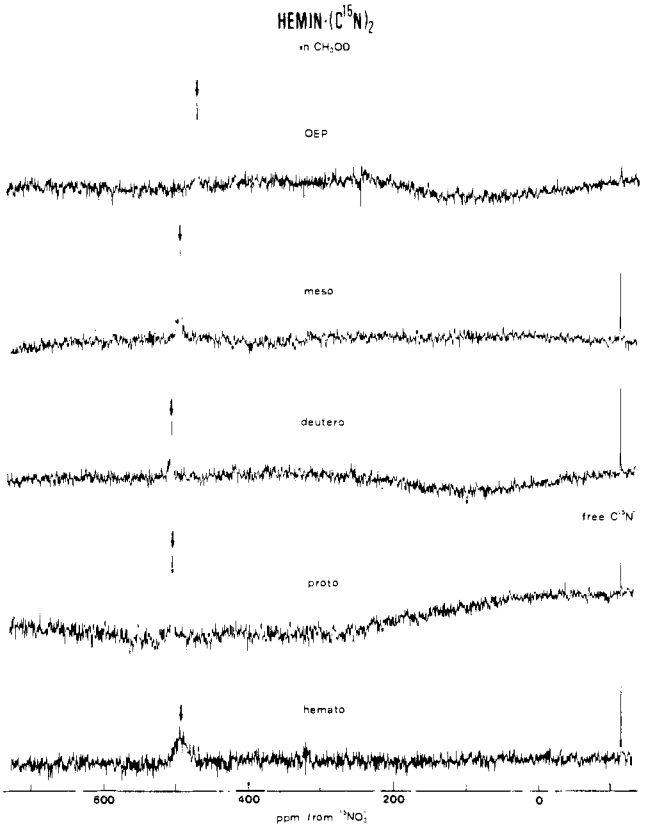


Figure 2. ^{15}N NMR spectra of various hemin-dicyanide complexes in methanol; from the top, OEP, meso-, deutero-, proto-, and hematohemins, respectively. Solutions (20–30 mM) were prepared and 10 000 transients were accumulated.

for hematoporphyrin is demonstrated and compared with other hemin derivatives in Figure 2, even though the resonance shift is almost the same as those of other hemin complexes. We have also examined the effect of porphyrin peripheral substituents on the iron-bound $C^{15}N$ shift for monocyanopyridine complexes, such as meso-, deutero-, proto-, and hematoprophyrins, and the data are also listed in Table I.

Since the large downfield shift of the bound cyanide ^{15}N resonance ranges from +448 to +1070 ppm, far beyond the normal diamagnetic region of ^{15}N resonances for nitrogen-containing compounds (0 to −400 ppm),[15] these shifts are unequivocally of paramagnetic origins. This is further supported by the Curie law behavior of the temperature dependence of the ^{15}N chemical shift of bound $C^{15}N^-$ in hemin cyanide complex, as is shown in Figure 3. The ^{15}N chemical shifts of bound $C^{15}N^-$ in hemin complexes, together with the experimental conditions, are summarized in Table I.

In order to further examine the solvent effect on the iron-bound $C^{15}N$ resonance shift, we have measured ^{15}N NMR of hemin cyanide complexes under various solvent conditions. Figure 4 displays representative spectral changes of the $C^{15}N$ resonance of the dicyanoprotohemin complex with varying amounts of D_2O in Me_2SO solution. The iron-bound $C^{15}N$ signal, shifted downfield far beyond the diamagnetic spectral region, exhibited a large upfield shift with increasing D_2O content. A similar spectral shift was also encountered for iron-bound $C^{15}N$ resonances for dicyano and pyridine-cyano complexes of hemin in pyridine-water mixed solvent. The influences of D_2O content on the $C^{15}N$ shift of various monocyanopyridine complexes are demonstrated in Figure 5, where the $C^{15}N^-$ shifts depend characteristically on the amount of D_2O. The results are given in Table II. The ^{15}N shift change experienced by varying the D_2O fraction in pyridine-water

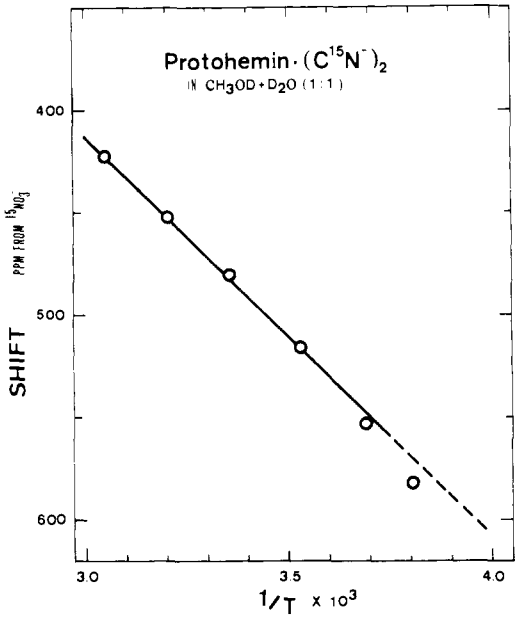


Figure 3. Plot of the ^{15}N chemical shift of axially coordinated $C^{15}N^-$ vs. T^{-1} for protohemin-dicyanide in $CH_3OD + D_2O$ (1:1). Sample conditions are: [hemin] = 22 mM, $[C^{15}N^-]$ = 180 mM. 5000 transients were collected.

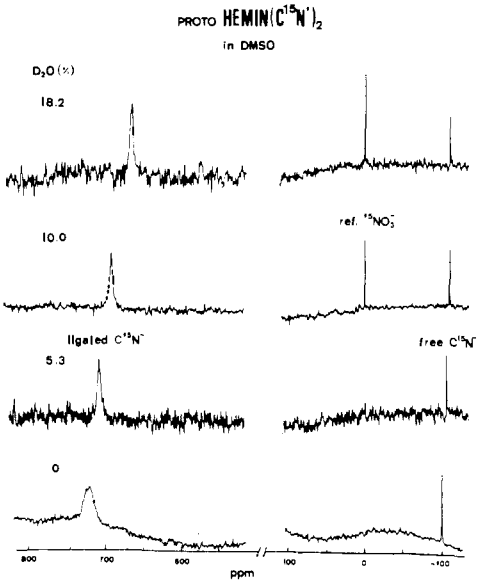


Figure 4. ^{15}N NMR spectral perturbation for dicyano-protohemin complex in Me_2SO induced by the addition of D_2O. Concentrations of hemin (21 mM) and $C^{15}N^-$ (200 mM) were held throughout the experiment.

mixed solvent allowed us to estimate the $C^{15}N$ shift of the dicyanohemin complex in pure pyridine solvent which is given in Table I. It is generally seen that there is a striking difference in the bound $C^{15}N$ isotropic shifts between the aprotic and protic solvents, with an upfield bias in the latter solvent. This is also the case for the monocyano complex.

Iron-Bound $C^{15}N$ Resonances of Cyanohemoproteins. In the presence of a sufficient amount of $C^{15}N^-$ (tenfold excess to the heme concentration), horse myoglobin and cytochrome *c* also afforded the very broad ^{15}N signals at +936 ppm (pH 8.0) and at +847 ppm (pH 7.8), respectively and, concurrently, a sharp free $C^{15}N^-$ signal at −117 ppm. The temperature dependence of this ^{15}N resonance was also encountered for cyanomyoglobin in which the bound $C^{15}N$ resonance experienced a 30-ppm upfield shift upon raising the temperature from 25

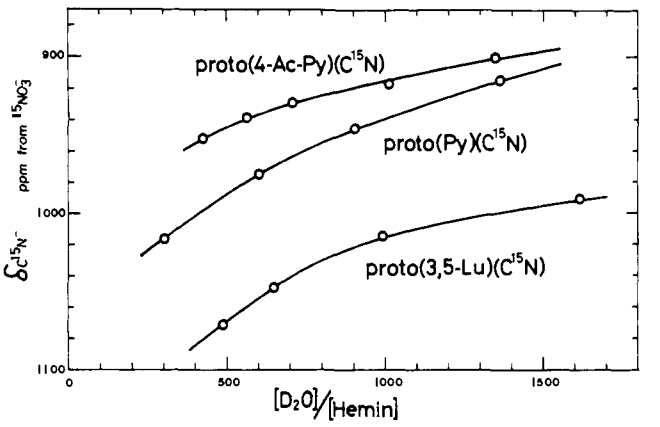


Figure 5. The effect of D_2O content on the iron-bound $C^{15}N$ shifts for various pyridine-cyanoprotohemin complexes. For experimental details and abbreviations, see the footnotes to Table I.

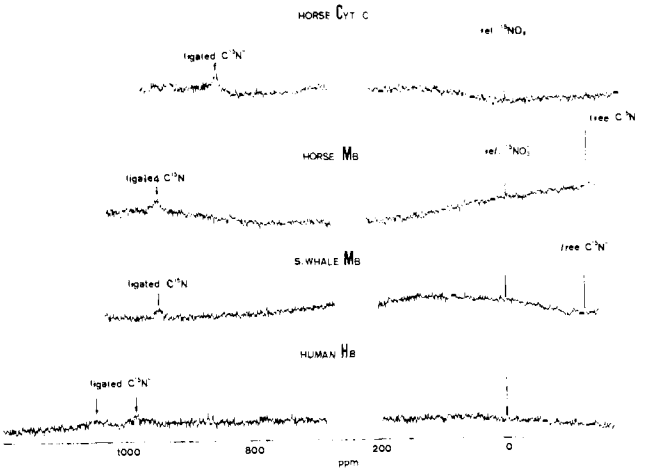


Figure 6. ^{15}N NMR spectra of various $C^{15}N^-$-bound hemoproteins. Chemical shifts are represented in parts per million from $^{15}NO_3^-$ as an internal standards; from the top, horse heart cytochrome *c*, horse myoglobin, sperm whale myoglobin, and human adult hemoglobin, respectively. Concentrations of myoglobin (horse), myoglobin (sperm whale), cytochrome *c* and hemoglobin are 20, 16, 25, and 4 mM, respectively; 100 000 transients were accumulated for myoglobin and cytochrome *c*, and 200 000 transients for hemoglobin.

to 33 °C. We have performed similar experiments at room temperature for cyanide complexes of sperm whale myoglobin and of human hemoglobin. For human adult hemoglobin, we obtained two broad ^{15}N signals at +1047 and +975 ppm (pH 7.3), substantially more downfield compared with those for other hemoproteins. With insufficient saturation of $C^{15}N^-$, the intensity of the lower field signal is slightly smaller than the remaining one (Figure 6). These two signals should be assigned to the $C^{15}N^-$ bound to the heme-iron in the α and β subunits of hemoglobin. The studies on the differential ligand binding affinity of cyanide to split α and β forms of hemoglobin may allow us to tentatively assign the signal at high field to the $C^{15}N$ bound to the α subunit and the lower field one to the β subunit.[16] This is based on the assumption that preferential cyanide binding to the α-split subunit (k_{on} = 170 M^{-1} s^{-1} for α and k_{on} = 160 M^{-1} s^{-1} for β)[17] is held for tetrameric hemoglobin. ^{15}N assignments by using hemoglobin split forms are currently being performed in our laboratory. The ^{15}N chemical shifts of bound $C^{15}N$ in hemoproteins at various pHs are summarized in Table III.

Discussion

General Features of Iron-Bound $C^{15}N$ Isotropic Shifts. The simultaneous presence of the ^{15}N signals of free and heme-

Table III. Observed Chemical Shifts of Axially Coordinated $C^{15}N^-$ to Various Hemoproteins (at 25 °C)

Cyanohemoprotein	pH	Chem shift,[a] ppm from $^{15}NO_3^-$
Myoglobin ($C^{15}N^-$)[b] (horse)	5.7	+931
	6.0	+939
	6.9	+948
	8.0	+936
	10.0	+940
	11.7	+940
Myoglobin ($C^{15}N^-$)[b] (sperm whale)	8.8	+945
Cytochrome *c* ($C^{15}N^-$)[b]	5.2	+841
	6.6	+842
	7.8	+847
	9.0	+848
Hemoglobin ($C^{15}N^-$)[c] (human)	7.3	+975 (α)
		+1047 (β)
	7.7	+985 (α)
		+1055 (β)

[a] A plus sign designates downfield shift from an internal standard, $^{15}NO_3^-$. For the experimental conditions see the caption to Figure 6. [b] 0.1 M phosphate buffer. [c] 0.5 M phosphate buffer.

iron-bound $C^{15}N^-$ implies a slow exchange on the NMR time scale, between the two species under the conditions examined here. The large downfield ^{15}N shift of the bound $C^{15}N^-$ of about +448 to +1070 ppm for hemin and hemoproteins appears to be comparable to that for ferricyanide, $[Fe(C^{15}N)_6]^{3-}$, of about +660 ppm at 25 °C.[18] The fact that the iron-bound $C^{15}N$ shift is of paramagnetic origin is clearly demonstrated in Curie's law behavior for the resonance of the dicyanoprotohemin complex as is shown in Figure 3. The intercept to the infinite temperature in this plot gave the value of −172 ppm. Although the corresponding diamagnetic ^{15}N shift is not known at the present, the value of the intercept (−172 ppm) is slightly larger than the ^{15}N chemical shift of diamagnetic cyano-metal complex (−96 to −112 ppm).[19] This is probably due to the second-order Zeeman effect which is sometimes encountered in the proton paramagnetic shift of ferric low-spin porphyrin complex.[20] In this plot (Figure 3) the deviation from linearity experienced at low temperatures may be due to self-aggregation of protohemin-cyanide complex[21] or to the change in solvent conditions. However, the linearity of the plot of ^{15}N chemical shift vs. T^{-1} is held well enough at high temperatures (above 0 °C) to estimate the intercept of the infinite temperature.

The large upfield ^{13}C shift (3000 ppm) of the iron-bound ^{13}CN in $[Fe(^{13}CN)_6]^{3-}$ has been reported,[22] which may allow us to expect negative spin density on the cyanide carbon nucleus in cyanohemin and hemoprotein complexes. It is thus likely that these opposite signs of the spin densities induced on the cyanide ligand result from the spin polarization mechanism of spin transmission from ferric iron atom through σ and/or π bonds.

Before discussing the ^{15}N isotropic shift data, we estimate the dipolar shift contribution to the apparent downfield shift of the bound $C^{15}N^-$. The ^{15}N dipolar shift for the $C^{15}N^-$ bound to the heme-iron is given by $-[\chi_{zz} - (\chi_{xx} + \chi_{yy})/2](3\cos^2\theta - 1)/2r^3$, where χ_{ii}'s are the susceptibilities along the principal axes, θ is the angle between nitrogen-metal vector and the z axis (viz. 0° in this case), and r is the length of this vector.[23] Available magnetic susceptibility data for $Mb^+ \cdot CN^-$ [24] allow us to estimate this dipolar shift as ca. +100 ppm when r is taken as 3.0 Å.[25] Accordingly, the difference between the observed shift and this dipolar shift consists of contact and

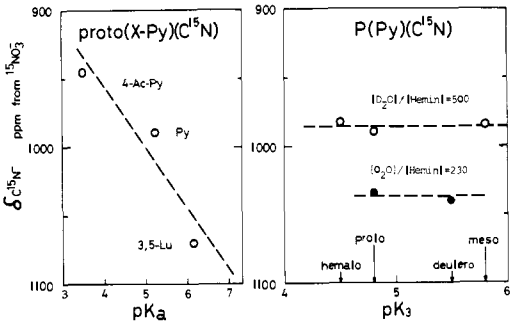


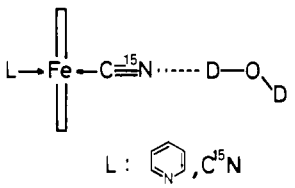
Figure 7. Trans (a, left) and cis (b, right) effects on the iron-bound $C^{15}N$ shifts for pyridine-cyanohemin complexes. The pK_a values are taken from ref 27, and the pK_3 values from the literature (R. Makino and I. Yamazaki, *J. Biochem. (Tokyo)*, **72**, 655 (1972)). For abbreviations see the footnotes to Table I.

diamagnetic coordination shifts including ring current effects. The latter contribution may be evaluated as ca. +100 ppm for the diamagnetic coordination shift and +10 ppm for the ring current effect.[26] It then follows that the contact contribution to the observed ^{15}N shift of the bound $C^{15}N^-$ for $Mb^+ \cdot CN^-$, for example, amounts to ca. 950 ppm, a substantially large value of the downfield shift characteristic of positive spin density induced on the nitrogen. Detailed discussions on the mechanism of electron spin transmission onto the iron-bound cyanide will appear in a spearate paper.

Cis Ligand Effect in the ^{15}N Isotropic Shifts of Iron-Bound $C^{15}N^-$ in Hemin- and Hemoprotein-Cyanide Complexes. The ^{15}N resonance data for dicyano complexes of proto-, deutero-, meso-, and hematoporphyrins and OEP compiled in Table I allow us to examine the cis ligand effects, viz., the effect of heme peripheral substituents, on the ^{15}N isotropic shift of the axial $C^{15}N$ ligand. There is no marked difference in these ^{15}N shifts observed in Me_2SO or in methanol. In the Me_2SO solution, the ^{15}N shift ranged from +718 (OEP) to +738 ppm (deuterohemin), while in the methanol solution it ranged from +492 (mesohemin) to +511 ppm (deuterohemin). These ^{15}N shift changes appear to be negligibly small compared with a large solvent-induced shift of ca. 200 ppm. Similar situations were also encountered for the monocyanopyridine complexes, where the $C^{15}N$ shifts ranged from +982 (hematohemin) to +989 ppm (protohemin) with varying porphyrin substituents. These results are demonstrated in the plot of $C^{15}N$ chemical shifts vs. the pK_3 values of porphyrins, which reflects the basicities of porphyrin core nitrogen (Figure 7b). The ^{15}N shift does not reflect a consistently electron-donating substituent (mesoporphyrin) or an electron-withdrawing substituent (hematoporphyrin) which may serve as a model of heme c involved in cytochrome *c*. It is therefore likely that the substantial ^{15}N shift difference between cyanide complexes of myoglobin (+936 ppm) and cytochrome *c* (+847 ppm) may not be attributed to this cis effect but rather to the trans effect arising from different binding modes of proximal histidine or to changes in subtle interactions between the iron-bound $C^{15}N$ and apoprotein in the heme pocket. Whether this apoprotein effect is due to heme-apoprotein interaction or to changes in the Fe-CN binding nature is presently unknown. However, this apoprotein effect, if any, does not seem to exceed the $C^{15}N$ shift change between cyanide complexes of cytochrome *c* and myoglobin, indicating that it may be primarily attributable to the different profile of the Fe-histidine binding in these hemoproteins, not to the cis effect or the ligand-apoprotein interaction. In this sense, the heme-bound $C^{15}N$ shift appears to serve as quite a sensitive probe of the binding nature of proximal histidine to the heme-iron.

Trans Ligand Effect in the ^{15}N Isotropic Shifts of Iron-Bound $C^{15}N^-$ in Hemin Complexes. Replacement of one cyanide li-

gand with pyridine causes a trans effect to give a large paramagnetic shift (+951 to +1000 ppm) of the bound $C^{15}N^-$, which is rather close to the value for cyanomyoglobin (+936 ppm). This designates the pyridine–cyano species as a close structural model for these cyanohemoproteins, in which the proximal histidine (N base) is present as a trans ligand to the iron to the protohemin prosthetic group in these cyanohemoproteins. This has also been suggested by Caughey et al.[13] on the basis of the close similarity of IR and visible data and proton hyperfine shifts of heme peripheral groups between the pyridine–cyano complex of hemin and cyanomyoglobin. The iron-bound $C^{15}N$ shifts increase in the order of 4-acetylpyridine (+945 ppm) < pyridine (+989 ppm) < 3,5-lutidine (+1070 ppm), which appears to reflect the ligand binding strength of pyridine derivatives, their pK_as being 3.51, 5.45, and 6.15, respectively.[27] As the basicity of ligand increases, the downfield ^{15}N hyperfine shift of iron-bound C^{15} becomes more pronounced. This profound dependence of the trans ligand basicity is demonstrated in Figure 7a, which is in marked contrast to the cis effect examined for the corresponding monocyanopyridine complexes (Figure 7b). This striking effect in the model compounds may allow us to expect that the substantial difference in the $C^{15}N$ shifts between myoglobin and

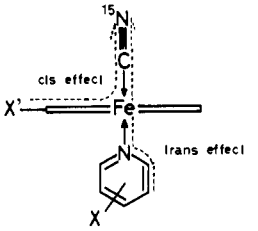


cytochrome *c* cyanides may be resulting from the change in the effective strength of proximal histidine binding to the heme-iron, rather than from the cis effect due to structural change of the peripheral groups of heme b and heme c involved in these hemoproteins. It is also to be noted that the protohemin complex with mixed ligands of *N*-methylimidazole and cyanide, which is structurally more relevant for the hemoprotein model, exhibits more similarity to the iron-bound $C^{15}N$ hyperfine shift (+926 ppm) than to that of cyanomyoglobin.

Characteristics of Heme–Ligand Binding Properties in Various Hemoproteins. Inspection of Figure 6 reveals that there are substantial differences in the iron-bound ^{15}N shifts within various hemoproteins, ranging from +840 to +1055 ppm in the order of cytochrome *c* < myoglobin < hemoglobin. On the basis of the general trend that the iron-bound $C^{15}N$ shift is most effectively influenced by the trans effect rather than the cis effect or heme–apoprotein interaction, the ^{15}N shift can be a sensitive probe for illuminating the iron–proximal histidine binding nature of various hemoproteins. Proximal histidine in hemoglobin is thus considered to be most strongly bound to the heme-iron, compared with myoglobin and cytochrome *c*. The sizable ^{15}N shift difference (~70 ppm) between the cyanides bound to α and β subunits in cyanohemoglobin may be interpreted along with this line.[7] On the other hand, proton hyperfine shifted signals of heme peripheral groups are not appropriate for this purpose, because these are influenced by the heme–apoprotein and/or the axial ligand–apoprotein interactions resulting from a subtle structural change in the apoprotein.[28] It is therefore likely that the $C^{15}N$ resonance can serve as a sensitive probe in delineating the binding nature between heme-iron atom and the fifth ligand (proximal histidine) in various hemoproteins.

Solvent Effect. It is particularly worth noting in Tables I and II that the bound $C^{15}N$ resonance positions in hemin–cyanide complexes are strikingly sensitive to the solvent for both dicyano- and monocyanohemin derivatives. The observed solvent-induced $C^{15}N$ shift of as much as 200 ppm appears to be too large to be attributable to changes in the electronic structure of the heme-iron caused by a specific interaction such as hydrogen bonding between coordinated $C^{15}N^-$ and protic solvent.

Such a solvent effect has been found by Frye and La Mar[29] for proton isotropic shifts of the porphyrin peripheral groups in dicyanohemin complexes, with a downfield bias of at most 2–3 ppm on going from aprotic (Me_2SO) to protic (methanol) solvent. This proton shift markedly contrasts with the present result of the large $C^{15}N$ shift (as much as 200 ppm) for the corresponding solvent variation. The solvent-induced proton shift has been reasonably interpreted[29] primarily in terms of the variation in magnetic anisotropy responsible for dipolar (or pseudocontact) shift of porphyrin peripheral groups. The upfield bias of the iron-bound $C^{15}N$ resonance with proton donor solvent is consistent with a substantial change in *g* value anisotropy. Available data[29] of $g_\parallel \cong 1.8$ and $g_\perp \cong 3.6$ in $CDCl_3$ and $g_\parallel \cong 2.0$ and $g_\perp \cong 2.9$ in D_2O allow us to expect the dipolar ^{15}N downfield shift of 130 ppm in $CDCl_3$ and 57 ppm in D_2O for the iron-bound $C^{15}N$, resulting in the upfield shift of 73 ppm on going from weaker proton donor to stronger proton donor solvent. However, the present result of the ^{15}N solvent shift of 200 ppm appears to be too large to be attributed to this anisotropy effect only. The hydrogen bonding to the coordinated cyanide which weakens the axial ligand field reduces the magnetic anisotropy[29] and its σ basicity toward the metal ion, decreasing the spin transfer from iron to cyanide. The upfield bias of the $C^{15}N$ shift in CH_3OD compared with that in Me_2SO and the upfield shift of bound $C^{15}N^-$ in mono-



and dicyano complexes upon increasing the amount of D_2O are interpretable along with this line. This solvent-induced change in the ligand binding situation is consistent with the proton shift study of Frye and La Mar.[29] Thus, it is reasonably considered that the hyperfine shifts of the axial ligand reflect more sensitively the solvent effect on the heme–iron electronic structure than those of the heme peripheral groups, which are probably more susceptible to subtle interactions between heme and apoprotein.

Interaction between Iron-Bound $C^{15}N$ and Apoprotein in the Heme Pocket. The striking solvent effect on the heme-bound $C^{15}N$ resonance in the hemin–cyanide complexes prompted us to explore such an effect in the cyanohemoproteins. In the heme pocket, a water molecule may be accessible to the heme-bound cyanide to form a hydrogen bond and the distal histidine is presumably associated with a hydrogen bond with this cyanide. This may also affect the ^{15}N resonance of cyanohemoproteins. We have performed a study on pH-dependent features of the ^{15}N resonance of heme-bound $c^{15}N$ in cytochrome *c* and myoglobin cyanide complexes together with proton NMR. The ^{15}N NMR results are also shown in Table III. In the pH range examined here between 5 and 10 for cytochrome *c* cyanide and between 6 and 12 for myoglobin cyanide, there was no substantial ^{15}N shift for cytochrome *c* ~7 ppm), while we obtained a sizable pH-dependent shift for myoglobin cyanide. The sizable shift (17 ppm) for myoglobin with pH variation from 5.7 to 6.9 could be reasonably interpreted in terms of a possible hydrogen-bond effect between distal histidine and heme-bound axial cyanide.[30] With a lowering of pH, protonation at the distal histidine may enhance this hydrogen bond, resulting in the $C^{15}N$ resonance shift to

the upfield side.[31] Such a large shift was not encountered for cytochrome *c* cyanide, probably because there is no such distal histidine in the proximity of the heme in cytochrome *c*.[32]

The rather complex profiles of the ^{15}N shift above pH 7 for myoglobin cyanide and cyano cytochrome *c*[33] appear to reflect a structural change of heme environment caused by ionization of some ionizable amino acid residues in the heme vicinity. 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[34] to possess a high pK_a of 7.62 and 7.87 for horse metmyoglobin and its azide derivative, respectively. The imidazole moiety of this residue is known to be stacked over the phenyl ring of phenylalanine-106 (G7) with a distance of ca. 3.5 Å from the x-ray analysis.[34] If ionization of histidine-36 (C1) would affect this interaction with phenylalanine-106 (G7), the G helix could more or less undergo movement from the normal position. Furthermore, this helix involves the important amino acid residue in van der Waals contact with the porphyrin ring of the heme group, such as leucine-104 (G5) and isoleucine-107 (G8).[25] Conformational changes thus induced of the 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 an iron-bound $C^{15}N$ resonance shift. Moreover, the relatively small change of the $C^{15}N$ shift above pH 9 may also be attributed to a similar conformational effect induced by ionization of tyrosine-103 (G4)[35] at the interhelical turn next to leucine-104 (G5) mentioned above. In the case of cyanocytochrome *c*, a small appreciable ^{15}N shift with varying pH values from 6 to 9 may also be due to an alteration of the tertiary structure as a result of the ionization of some residue, probably histidine-33.[36]

In summary, the ^{15}N paramagnetic shift of the iron-bound $C^{15}N$ in ferric porphyrin and hemoprotein–cyanide complexes serves as a quite sensitive probe for delineating the heme iron–ligand binding nature, the solvent–axial ligand interaction in hemin complexes, and the heme ligand–apoprotein interaction in hemoproteins.

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