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^{13}C NMR Signal Detection of Iron-Bound Cyanide Ions in Ferric Cyanide Complexes of Heme Proteins

Hiroshi Fujii*

Institute for Molecular Science and Center for Integrative Bioscience, Okazaki National Research Institutes, Myodaiji, Okazaki 444-8585, Japan

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Small-molecule axial ligands potentially can serve as useful NMR probes for characterization of the environment and electronic structure of a prosthetic group in heme proteins.[1] In this regard, the diamagnetic ferrous states have been examined thoroughly because of easy signal detection from iron-bound small molecule.[2] The ^{13}C NMR signal of ^{13}CO form of heme protein proves to be sensitive to the nature of the trans amino acid ligand. For the paramagnetic ferric state, cyanide ion would appear to have the greatest potential because of its extremely high affinity to ferric heme iron center.[1] ^{15}N NMR signals of the iron-bound C^{15}N have been detected in a far-downfield region for both iron(III) porphyrin model complexes and heme proteins.[3,4] However, the ^{15}N NMR spectroscopy remains ambiguous as a NMR probe since the ^{15}N NMR shift reflects the nature of both the hydrogen bond in the distal side and amino acid ligand in the proximal side. On the other hand, ^{13}C NMR spectroscopy of the iron-bound ^{13}CN has been investigated in less detail.[5,6] Although ^{13}C NMR signals of the iron-bound ^{13}CN are detectable in a far-upfield region (∼−2500 ppm from TMS) for bis-cyanide iron(III) porphyrin model complexes, extreme line-broadening of the signal seemed to preclude the signal detection in heme proteins, and a resonance of the iron-bound ^{13}CN for ferric heme protein has not yet been located. During a more extensive ^{13}C NMR study, we found the ^{13}C NMR signals of the iron-bound ^{13}CN of ferric cyanide complexes of heme proteins and its model complexes at an unexpectedly large upfield region (∼−4000 ppm from TMS). Here, we report the first detection of the ^{13}C NMR signal of the iron-bound ^{13}CN in heme proteins such as sperum whale myoglobin (Mb), human hemoglobin (Hb), horse heart cytochrome *c* (Cyt-c), and horseradish peroxidase (HRP). This study shows that the ^{13}C NMR spectroscopy of the iron-bound ^{13}CN provides a probe for studying the nature of the proximal ligand in ferric heme protein.

Figure 1 shows ^{13}C NMR spectra of bis-cyanide and cyanide−imidazole complexes of iron(III) protoporphyrinIX dimethyl ester (PPDME) in CD_2Cl_2 at 297 K. As in the previous report,[5] the ^{13}C NMR signal of the iron-bound ^{13}CN for the bis-cyanide complex is observed far upfield at −2516 ppm from TMS (Figure 1a). Alternatively, the ^{13}C NMR signal of the iron-bound ^{13}CN for the cyanide−imidazole complex, which is a model for cyanide complexes of heme proteins with a histidine proximal ligand, is not observed in this region.[7] Surprisingly, the ^{13}C NMR signal of the iron-bound ^{13}CN of the cyanide−imidazole complex of iron(III) PPDME is observed in much more upfield region at −3926 ppm from TMS (Figure 1b). The ^{13}C NMR signal of the iron-bound ^{13}CN shifts upfield extremely with changing the proximal trans ligand from cyanide to imidazole. The ^{13}C NMR signal for the cyanide−imidazole complex ($\Delta\nu_{1/2}$ = ∼3000 Hz) is much broader than that for the bis-cyanide complex ($\Delta\nu_{1/2}$ = ∼500 Hz). Convincing evidence for assignment of the signal to the iron-bound ^{13}CN is found in the temperature dependence. The Curie plot for the ^{13}C NMR signal is linear, but with a nonzero intercept, over the temperature range from 233 to 303 K. In contrast to the previous ^{15}N NMR spectroscopy, the ^{13}C NMR signal for the cyanide−imidazole complex does not show a significant solvent-dependent NMR shift, indicating that the hydrogen bond to the iron-bound ^{13}CN does not affect the ^{13}C NMR shift.[3,8] Since the dipolar shifts estimated from EPR *g* values for the bis-cyanide and the cyanide−imidazole complexes are ∼+400 ppm, the extremely large upfield shifts of the ^{13}C NMR signals would be due to Fermi contact shift resulting from a negative σ-orbital spin polarized at the ^{13}C atom by the iron $d_{xz}(d_{yz})$ orbital spin.[1,9] The estimated Fermi contact shift (∼−4100 ppm) for the cyanide−imidazole complex is larger than that (∼−2700 ppm) for the bis-cyanide complex. Changing of the proximal trans ligand from anionic cyanide to neutral imidazole would strengthen the binding of ^{13}CN ion to the iron as a result of the trans effect and enhance the spin polarization to the iron-bound ^{13}CN. This idea is further confirmed by the ^{13}C NMR spectrum of the cyanide−imidazolate complex of iron(III) PPDME.[10,11] The ^{13}C NMR spectrum of the cyanide−imidazolate complex shows the ^{13}C NMR signal of the iron-bound ^{13}CN at −3507 ppm from TMS. The ^{13}C NMR paramagnetic shift is drastically decreased with changing the axial ligand from neutral imidazole to anionic imidazolate. All of these results indicate that the ^{13}C NMR shift of the iron-bound ^{13}CN is a sensitive probe for the nature of the proximal trans ligand.

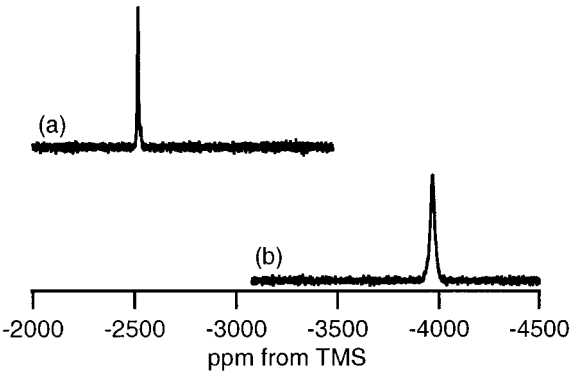

Figure 1. ^{13}C NMR spectra of iron-bound ^{13}CN of iron(III) protoporphyrinIX dimethyl ester in CD_2Cl_2 at 296 K. (a) Bis-cyanide complex. (b) Cyanide−imidazole complex.

We further examined the ^{13}C NMR signal of iron-bound ^{13}CN of ferric cyanide complexes of heme proteins and succeeded in the first detection of ^{13}C NMR signals for heme proteins. Figure 2 shows ^{13}C NMR spectra of ferric cyanide complexes of Mb, Hb,

* To whom correspondence should be addressed. E-mail: hiro@ims.ac.jp.

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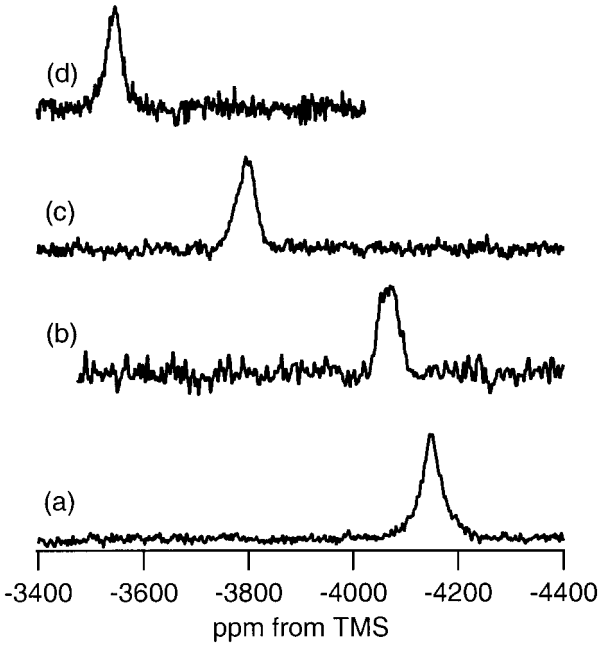
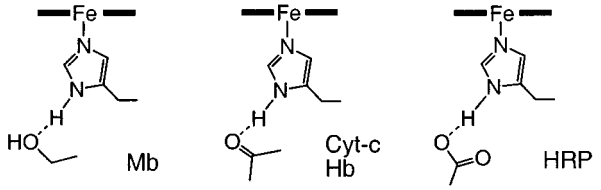


Figure 2. ^{13}C NMR spectra of iron-bound ^{13}CN of cyanide complexes of ferric heme proteins at 296 K. (a) Sperum whale myoglobin in 0.1 M phosphate buffer, pH = 7.0. (b) Human hemoglobin in 0.1 M Tris-HCl buffer, pH = 7.0. (c) Horse heart cytochrome *c* in 0.1 M phosphate buffer, pH = 7.0. (d) Horseradish peroxidase in 0.1 M phosphate buffer, pH = 7.0.

Cyt-c, and HRP at 297 K. The ^{13}C NMR signal of the iron-bound ^{13}CN is observed at −4145, −4074, −3761, and −3543 ppm from TMS for Mb, Hb, Cyt-c, and HRP, respectively.[12,13] The paramagnetic upfield shifts of the ^{13}C NMR signals for these heme proteins are similar to those for the cyanide−imidazole model complex, but the line widths ($\Delta\nu_{1/2} = \sim 8000$ Hz) of the ^{13}C NMR signals for heme proteins are much broader than those for the cyanide−imidazole model complex. In the pH range examined here between 7 and 9, we did not observe substantial ^{13}C NMR shift of the iron-bound ^{13}CN for these heme proteins.

Interestingly, the ^{13}C NMR signal of the iron-bound ^{13}CN changes the resonance position in the range of 600 ppm although all of these heme proteins have a histidine−imidazole as a proximal ligand. As shown above, the ^{13}C NMR shift of the iron-bound ^{13}CN is sensitive to the nature of the proximal ligand, but not to the hydrogen bond in the distal side. Therefore, the present ^{13}C NMR shifts for these heme proteins would reflect the nature of the proximal histidine−imidazole. Although we compared the X-ray crystal structures for these heme proteins, we could not observe a good correlation between the ^{13}C NMR shift and the binding nature of the histidine−imidazole, such as the bond length of Fe−N(His) and the bend and tilt angles of the histidine−imidazole.[14−17] However, we found a good correlation with the nature of the hydrogen bond of the proximal hisitidine−imidazole. The NH proton of the proximal histidine−imidazole interacts with the hydroxyl group of serine in Mb, the carbonyl oxygen of main chain in Hb and Cyt-c, and the carboxyl group of aspartate in HRP (see Scheme 1).[14−17] This means that the imidazolate character of the proximal histidine increases in the order of Mb, Hb, Cyt-c, and HRP,[18] which conforms to the order of the ^{13}C NMR paramagnetic shifts. In fact, the proximal histidine in HRP has been thought to have strong imidazolate character, and the ^{13}C NMR shift for HRP is close to that for the cyanide−imidazolate model complex.[17] All of present results indicate that the ^{13}C NMR signal of the iron-bound ^{13}CN is a useful probe to study the nature of the proximal ligand, such as the imidazolate character, in ferric heme proteins.



In conclusion, we first show ^{13}C NMR signals for heme proteins and their model complexes in an extremely large upfield region. This study demonstrates that the ^{13}C NMR signal of the iron-bound ^{13}CN is a sensitive probe to study the nature of the proximal ligand in ferric heme protein, in much the same way that CO serves as a powerful probe for ferrous heme proteins. A detailed study including further application of the ^{13}C NMR spectroscopy to other heme proteins is under investigation in our group.

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(7) The ^{13}C-labeled cyanide−imidazole complex of iron(III) PPDME was prepared by addition of 1 equiv of ^{13}C-labeled tetrabutylammonium cyanide to the bis-imidazole complex of iron(III) PPDME in CD_2Cl_2.
(8) While ^{15}N NMR isotropic shift of the iron-bound C^{15}N for the cyanide−imidazole complex was decreased ∼10% with addition of water into DMSO-d_6 solvent, the ^{13}C NMR isotropic shift was decreased only ∼0.2%.
(9) The following parameters were used for calculations of the dipolar shifts, $g_1 \approx 1.0$, $g_2 \approx 2.3$, $g_3 \approx 3.6$, $r \approx 1.98$ Å for the bis-cyanide complex and $g_1 \approx 0.74$, $g_2 \approx 1.89$, $g_3 \approx 3.4$, $r \approx 1.98$ Å for the cyanide−imidazole complex (ref 1b).
(10) The cyanide−imidazolate complex was prepared by addition of sodium methoxide to the cyanide−imidazole complex in CD_2Cl_2 and confirmed its formation by ^{1}H NMR spectrum (ref 11).
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(12) As the case of the resonance Raman bands of the ν(Fe−CN), the ^{13}C NMR signals for the α and β subunits of Hb could not be separated completely. These suggest that the Fe−CN bond character is not significantly different between the α and β subunits of the cyanide complex of Hb.
(13) The isotropic shifts of the iron-bound ^{13}CN were proportional to those of the iron-bound C^{15}N for these heme proteins except HRP. The strong hydrogen bond of the iron-bound CN with amino acid residues in the distal side would change the ratio of ^{13}C/^{15}N NMR shifts for HRP.
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(18) The distance between the imidazole nitrogen and carbonyl oxygen was 2.78 Å for Cyt-c and 2.97 Å (average) for Hb.

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