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NATURAL ABUNDANCE ^{13}C-NMR STUDY OF PARAMAGNETIC HORSE HEART FERRICYTOCHROME c CYANIDE COMPLEX: ASSIGNMENT OF HYPERFINE SHIFTED HEME METHYL CARBON RESONANCES

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SUMMARY: Hyperfine shifted heme methyl carbon resonances of paramagnetic horse heart ferricytochrome c cyanide complex (Cyt-c(CN)) have been observed for the first time in the natural abundance ^{13}C-NMR spectrum and assigned using ^{1}H-^{13}C heteronuclear chemical shift correlated spectroscopy (^{1}H-^{13}C COSY). Individual heme methyl carbon NMR signal assignment permits a direct comparison between the hyperfine shifts of heme methyl carbon and attached methyl proton resonances which provides a useful information on the delocalization mechanism of the unpaired spin from the π-conjugated system of porphyrin ring into the peripheral methyl side chains.

NMR has played an important role in characterizing structure-function relationship in hemoproteins [1-3]. The interaction of nuclear spins in the heme pocket with the local magnetic field created by the heme ring current and/or the unpaired electron of heme iron significantly perturbs their NMR spectral parameters and provides a unique window on the active site of hemoproteins.

The heme peripheral ^{1}H NMR signals have been most effectively used as probes for the study of heme electronic/molecular structure in hemoproteins and relatively little work has been done using other NMR active nuclei, i.e., ^{13}C and ^{15}N, due to their low natural abundance and inherent low NMR sensitivity. Sankar et al. [4] have used the specifically ^{13}C-labeled hemins at vinyl groups to detect their resonances in various oxidation/ligation states of sperm whale myoglobin and we have recently demonstrated that the heme methyl carbon resonances

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of paramagnetic hemoprotein can be clearly observed even at the level of the natural abundance, therefore ^{13}C labeling at heme methyl carbons is not necessary to detect their resonances, in the well-resolved upfield hyperfine shifted spectral region [5]. The observation of heme carbon resonances in the natural abundance ^{13}C-NMR spectra is particularly important for c-type hemoproteins in which no chemical ^{13}C labeling on heme can be achieved due to the covalent bond between heme and polypeptide chain. We report herein on the natural abundance ^{13}C-NMR study of paramagnetic horse heart ferricytochrome c cyanide complex (Cyt-c(CN)) which demonstrates the first observation and assignment of all four heme methyl carbon resonances. The hyperfine shifts of individual heme methyl carbons of Cyt-c(CN) were compared with those of the attached protons and the results were also compared with those of iron protoporphyrin IX dicyano complex ($Hemin(CN)_2$), Fig. 1. The plots of the observed hyperfine shift of heme methyl proton (δ^{H}_{para}) vs. that of heme methyl carbon (δ^{C}_{para}) for Cyt-c(CN) and $Hemin(CN)_2$ revealed that the validity of McConnell-type equation for the analysis of heme methyl resonances in both model and hemoprotein.

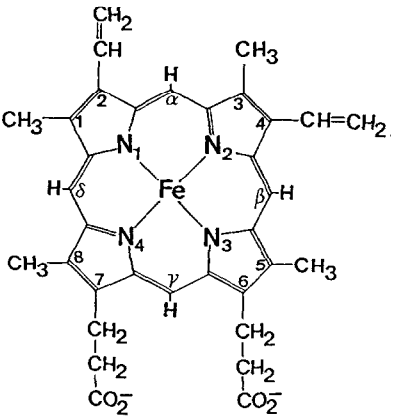

Fig. 1. The structure and numbering system of hemin. In the c-type hemoprotein, two sulfur atoms of cysteine residues of polypeptide chain are reacted with the heme vinyl groups to form thioether bondings.

MATERIALS AND METHODS: Horse heart cytochrome c (type VI) was obtained from Sigma Chemical Co. and used without further purification. Ferricytochrome c cyanide complex was prepared in 50 mM phosphate buffer, p^{2}H 7.0 in ^2H_2O with a 20 fold excess of KCN. The protein concentration was 20 mM.

NMR spectra were recorded using a JEOL GX-270 FT-NMR spectrometer, equipped with a 10 mm diameter tunable probe, operating at a carbon frequency of 67.8 MHz in the quadrature mode. The proton-decoupled ^{13}C-NMR spectrum of Cyt-c(CN) required 30 kHz spectral width, 16 μs 90 pulse, 32K data points, 1 s repetition time, and 20K transients. The spectrum was apodized with an exponential window function by which introduced 10 Hz line broadening. ^{1}H-^{13}C COSY spectrum was obtained using the standard pulse sequence [6] with $(2J)^{-1}$ = 3.6 ms. A total of 1K transients were accumulated per t_1 value with a pulse delay of 1 s. The initial data matrix was 2K (^{13}C-25000Hz) × 64 (^{1}H-15000Hz) in ω_2 and ω_1 dimensions, respectively, and was expanded to the final data matrix size 2K × 256 by zero-filling. The data matrix was apodized with an exponential window function in both dimensions and the absolute value mode is presented. Chemical shifts are given in parts per million, ppm, downfield from 2,2-dimethyl-2-silapentane-5-sulfonate (DSS).

RESULTS AND DISCUSSION: The natural abundance ^{13}C-NMR of Cyt-c(CN) at 35 C is shown in Fig. 2. ^{1}H-^{13}C COSY spectrum of Cyt-c(CN), together with its ^{1}H and ^{13}C-NMR spectra, is illustrated in Fig. 3. All four heme peripheral methyl proton signals [7], A(8-CH_3), B(5-CH_3), C(1-CH_3), and D(3-CH_3), are clearly observed in the downfield hyperfine shifted region in the ^{1}H spectrum. In the ^{1}H-^{13}C COSY spectrum, the cross-peaks connecting hyperfine shifted heme methyl proton resonances, 5-,8-,1-, and 3-methyl, to the upfield hyperfine shifted carbon resonance at -50.76, -46.22, -39.45, and -19.26 ppm, respectively, unambiguously establish the assignments of peaks a,b,c and e to heme 8-,5-,1-,3-methyl carbons, respectively. Therefore peaks d, f, and g arise from the carbon nuclei, other than heme methyl carbons, strongly coupled with the unpaired electron.

Line width of heme methyl carbon resonances increase with the magnitude of the hyperfine shift, as observed in the ^{13}C spectrum, indicating that the contact interaction plays an important role in the T_2 relaxation rate [8]. The hyperfine shifts of heme methyl carbon resonances and those of attached

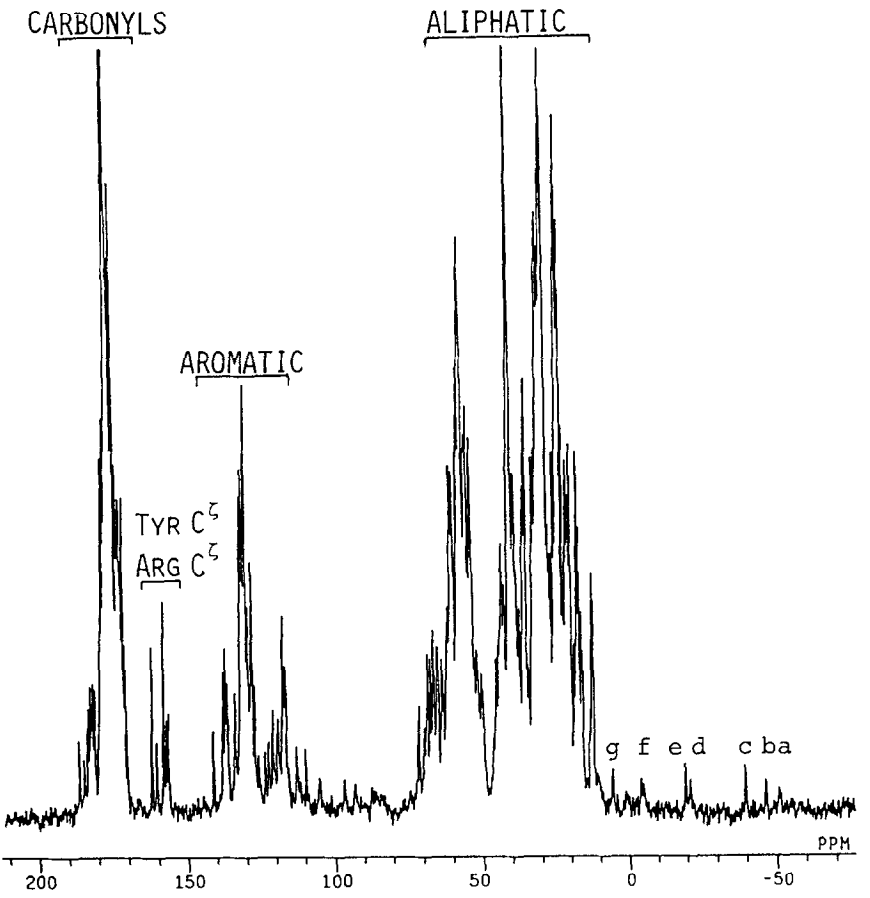

Fig. 2. The 67.8 MHz proton-decoupled natural abundance ^{13}C-NMR spectrum of Cyt-c(CN) in $^{2}H_2O$, p^{2}H 7.0, at 35 °C. The hyperfine shifted signals, peaks a - g, are clearly observed.

proton signals for Cyt-c(CN) and Hemin(CN)$_2$ are directly compared. Since the diamagnetic ^{13}C reference shift of heme methyl carbons for hemoproteins have not been reported, we used the reference shift of 13.85 ppm obtained for the diamagnetic zinc protoporphyrin IX [9]. The diamagnetic reference shifts of the individual heme methyl proton resonances for Cyt-c are obtained from the heme methyl proton resonance assignments of ferrocytochrome c [10].

The proton hyperfine coupling constant, A^H, of the heme methyl protons for the ferric low spin complexes can be approximated by the McConnell-type equation [11-13] as follows,

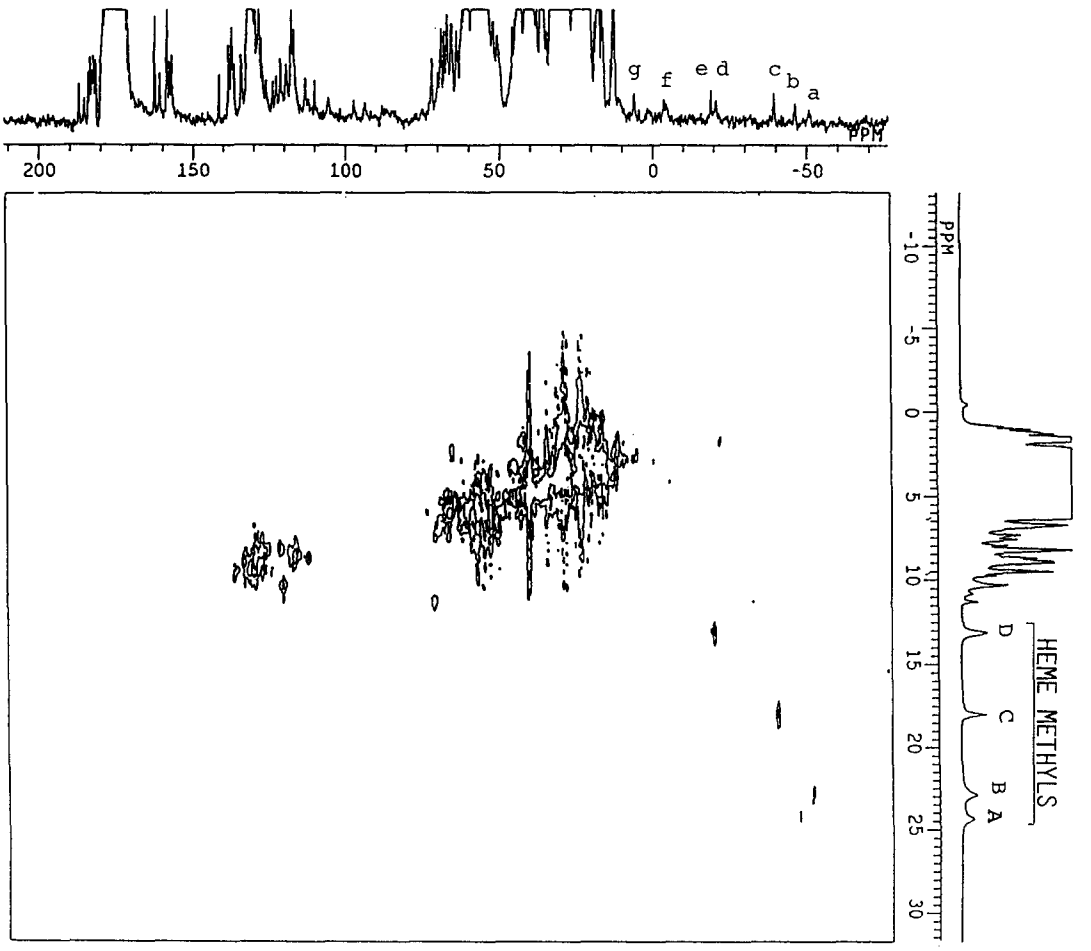

Fig. 3. ^{1}H-^{13}C COSY spectrum of 20 mM Cyt-c(CN) in 50 mM phosphate buffer, p^{2}H 7.0, at 35°C. The ^{1}H and ^{13}C spectra of Cyt-c(CN) are illustrated along F_1 and F_2 dimensions, respectively. The cross peaks connecting heme methyl proton resonances and the particular carbon signals provide the unambiguous assignments of the heme methyl carbon resonances.

$$A^{H} = Q^{H}_{CCH_3} \rho^{\pi}_{C} \qquad (1)$$

where $Q^{H}_{CCH_3}$ and ρ^{π}_{C} are the hyperconjugation parameter and the spin density on the aromatic carbon atom to which the methyl group is attached. For a case in which the Curie law is valid, the value A^{H} is defined by the contact shift (δ^{H}_{con}) according to

$$\delta^{H}_{con} = A^{H} \frac{|\gamma_e|}{|\gamma_H|} \frac{S(S+1)}{3kT} \qquad (2)$$

where γ_e and γ_H are the gyromagnetic ratios of the electron and proton, respectively, and S, k, and T are the total spin,

Boltzmann constant, and absolute temperature, respectively. Heme methyl carbon is not a part of the π system and to first order should have a contact shift term (δ^{C}_{con}) reflecting only polarization of its 2s electrons by π-spin density at the aromatic carbon to which it is attached. Thus, the following relation is generally assumed for the heme methyl carbon atom.

$$\delta^{C}_{con} = Q^{C}_{C'C} \rho^{\pi}_{C} \frac{|\gamma_e|}{|\gamma_C|} \frac{S(S+1)}{3kT} \qquad (3)$$

In the equation, $Q^{C}_{C'C}$ and γ_C are the spin polarization parameter and the gyromagnetic ratio of the carbon, respectively. Therefore a comparison between δ^{H}_{con} and δ^{C}_{con} yield the following relation,

$$\frac{\delta^{C}_{con}}{\delta^{H}_{con}} = \frac{Q^{C}_{C'C}}{Q^{H}_{CCH_3}} \frac{|\gamma_H|}{|\gamma_C|} \qquad (4)$$

and thus the plot of δ^{H}_{con} vs. δ^{C}_{con} for methyl resonances should give a straight line with a slope of the right hand side of Eqn.(4). Although the separation of the relative contributions to the observed hyperfine shifts for the heme methyl proton and carbon resonances is not so obvious in the present case, assuming a similar metal-centered dipolar contribution to their shift among the heme methyl proton or carbon atoms, the plots of δ^{H}_{para} vs. δ^{C}_{para} would be expected to give a straight line. Such plots are illustrated for the heme methyl resonances of Cyt-c(CN) and Hemin$(CN)_2$ in Fig. 4. The plots for Hemin$(CN)_2$ fall in a straight line. The temperature dependence studies for the heme methyl resonances of Hemin$(CN)_2$ indicate that the slope of this plot is independent of temperature [14]. The data for the heme methyl resonances of Cyt-c(CN) also fall on a straight line with a similar slope to that of Hemin$(CN)_2$ plot, revealing the validity of Eqns.(2) and (3) for hemoprotein.

In conclusion, heme methyl carbon resonances of Cyt-c(CN) have been detected and assigned for the first time. A direct

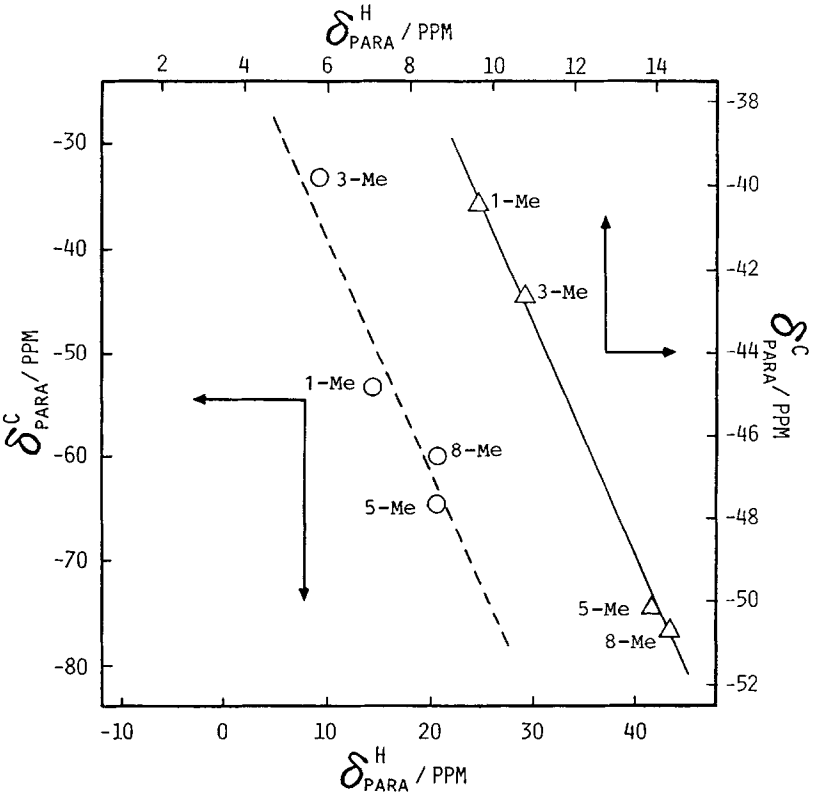

Fig. 4. Plots of observed hyperfine shift of heme methyl proton resonances (δ^{H}_{para}) vs. that of heme methyl carbon resonances (δ^{C}_{para}) for Cyt-c(CN)(o) and Hemin$(CN)_2$(Δ).

comparison of the shifts between heme methyl proton and carbon resonances of Cyt-c(CN) and Hemin$(CN)_2$ provided an useful information on the nature of the π spin delocalization towards the heme peripheral methyl groups. These signals are expect to serve as new probes for studying the structure-function relationship of Cyt-c.

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