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^{1}H NMR Study of mono- and di-cyanide ligated Hemin Complexes as Models of Hemoproteins

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Heme 단백질의 Model로서의 Hemin 착물에 관한 ^{1}H NMR 연구

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Abstract : ^{1}H NMR spectra for monocyanide ligated ferriprotoporphyrin(hemin) complex and dicyanide coordinated hemin complex in dimethylsulfoxide(DMSO-d_6) solution have been recorded and analyzed. NMR spectra of hemin-cyanide complexation in DMSO-d_6 exhibit that the cyanide ligation to hemin is temperature-dependent. Thermodynamic parameters for the monocyanide ligated hemin to dicyanide ligated hemin are consistent with endothermic process with $\Delta H^\circ = 736.6$ cal/mol and $\Delta S^\circ = 16.4$ eu. Detailed anlalysis of the anomalous deviation from Curie behavior for CN/DMSO coordinated hemin complex demonstrates the presence of a high spin character, and this weaker axial field relative to the purely low-spin dicyanide hemin complex is supposed to attribute to instantaneously ruptured iron-DMSO bond. This complex may serve as a useful model to characterize electronic/molecular structure of hemoproteins, which one of axial ligands is weak.

요약 : DMSO(dimethylsuloxide-d_6) 용액 속에 존재하는 CN/CN 리간드의 hemin 착물과 CN/DMSO의 hemin 착물이 ^{1}H NMR로 기록되어지고 분석되어졌다. Hemin으로의 CN 착물화 과정은 온도에 따라 변화함을 NMR 스펙트럼이 보여 주며, 한 개의 CN 리간드에서 두 개의 CN 리간드착물로 바뀌는 과정의 열역학함수는 $\Delta H^\circ = 736.6$cal/mol 및 $\Delta S^\circ = 16.4$ eu인 흡열과정을 나타낸다. CN/DMSO의 hemin 착물은 Curie behavior로부터의 벗어남은 high-spin 성격의 존재를 나타내고, 이는 Fe-DMSO 결합이 순간적으로 깨짐을 의미하며, 이러한 CN/DMSO hemin 착물이 한 개의 axial ligand가 약한 heme 단백질의 전자 및 분자구조의 model comlex로 작용할 수 있음을 보여 준다.

Key words : ^{1}H NMR, Hemin complex

Introduction

NMR spectra of paramagnetic transition-metal complexs yield valuable information on magnetic and electronic properties of the metal center, as well as on the nature and extent of metal-ligand co-

valency.[1,2] The iron complexes of the cyclic tetrapyrroles, the porphyrins are a particularly important group of complexes. All of the commonly accessible oxidation/spin states are paramagnetic, and the detailed patterns of the hyperfine shifts for the tetrapyrrole have been shown to be essentially diagnostic for a given oxidation/spin/ligation state of the iron.[3~5] The close parallel between hyperfine shift patterns of iron tetrapyrrole complexes and the intact hemoproteins with the same chromophore has made the simple complexes ideal models for elucidating protein-based spectral perturbations in the latter systems. Moreover, the asymmetric functionalization of the tetrapyrrole periphery leads to redistribution of the delocalized spin density that provide us with one of most sensitive methods for mapping ligand molecular orbitals.[7~9] The characterization of the in-plane asymmetry in paramagnetic heme complexes using NMR involves a detailed analysis of the origin of the isotropic shifts in terms of possible magnetic anisotropy and transferred unpaired spin density. The isotropic shift for a nucleus in a complex or protein is given by

$$(\Delta H/H)_{iso} = (\Delta H/H)_{con} + (\Delta H/H)_{dip} \quad (1)$$

where the contact shift is

$$(\Delta H/H)_{con} = -Ag\beta S(S+1)/(\gamma k\pi)3kT \quad (2)$$

and the dipolar shift is represented by

$$(\Delta H/H)_{dip} = 1/3N\{[\chi_z - 1/2(\chi_x+\chi_y)](3\cos^2\theta - 1)/r^3 + 3/2(\chi_x-\chi_y)\sin^2\theta\cos^2\Omega\} \quad (3)$$

where A is the Fermi contact coupling, χ_x, χ_y, χ_z are the principal components of the susceptibility tensor, θ is the angle between the metal-nucleus vector and the z axis, r is the length of this vector, and the Ω is the angle between the projection of r on the xy plane and the x axis.

In-plane asymmetry can arise from either differences in A(transferred spin to individual pyrroles) or rhombic magnetic anisotropy(i. e., the second term in eq. 3). Since low-spin ferric systems are known to be magnetically anisotropic, both cases must be considered.

The most serious barrier to the full utilization of the NMR spectral information in paramagnetic model complexes is the unambiguous assignment of the resonances, since the hyperfine shifts invalidate the functional group/shift correlation of diamagnetic systems and generally paramagnetic relaxation obscures multiplet structure. The assignment strategies have relied on ^1H-^1H COSY which detect the characteristic spin(scalar) connectivity of peripheral substituents of tetrapyrrole complexes.[10]

Heme model complex and ferric hemoproteins in a given spin ground state invariably exhibit Curie-like behavior for the majority of hyperfine-shifted resonances, and strong and systematic deviations from Curie behavior are generally taken as direct evidence for a spin equilibrium.[11]

Thus, the electronic structure for the mono- and di-cyanide hemins, temperature-dependent behavior for the NMR signals of mono- and di-cyanide hemins and the thermodynamic parameters for the equilibrium of cyanide ligand binding to the iron of hemin as models of hemoproteins will be documented in this report.

Experimental Section

Sample preparation : Protoporphyrin Ⅸ iron(Ⅲ) was used as purchased from Sigma without further purification. The low spin biscyano and monocyano complexes were prepared by dissolving the appropriate porphyrin iron chloride with 1:1.5 potassium cyanide in$(C^2H_3)_2SO$ in the 5mm NMR tube. Sample concentration was about 10mM.

NMR spectroscopy : ^1H NMR spectra were collected on Bruker AM-200 spectrometers operating in quadrature mode at 200-MHz. All 1D spectra were collected by using a standard one-pulse experiment, with a presaturation pulse when necessary to suppress the solvent signal, and with $5T_1$,

interpulse delay. Typical spectra resulted from collecting~256 on 16384 data points over 7000Hz bandwidth at a repetition rate of 0.4 sec^{-1}. To improve signal to noise ratio, the free induction decays were apodized, which introduced 2 Hz line broadening. Chemical shifts for all spectra are referenced to the residual solvent line. NMR spectra were recorded in 10℃(±0.1℃) intervals over the temperature range 24~55℃(±0.1℃). Nonselective T_1s were obtained from the initial slope of the semilogarithmic plots of the intensity data from an inversion-recovery experiment using a composite 180° pulse; for incompletely resolved lines, T_1s were calculated from the delay time which produced a null, τ_{null}, by the relation $T_1 = \tau_{null} / \ln 2$. For 2D spectra, an n-type magnitude COSY, M-COSY, sequence was used, with a presaturation pulse during the relaxation delay to suppress the solvent resonance when necessary. The 90° pulse for ^{1}H was 7~8μs. 2D ^{1}H-^{1}H COSY spectrum was collected with 1024 points in t_2 over the minimum bandwidth necessary to include all peaks with expected spin connectivities and centered about the solvent resonance when possible. The number of t_2 data points were chosen with the consideration of needing at least $2T_2$ ms for acquisition time length, where T_2 is the longest T_2 value for the complex as estimated from the linewidth for all peaks in the 1D reference spectrum. Resulting acquisition time for 2D spectrum shown was 10.2 ms. The repetition rate of the pulse sequences utilized was 0.65s^{-1} following the guideline of $1.5T_1$. Accordingly, pulse delays selected was 1.5 sec. 2D experiment includes 4 dummy scans prior collection of each block. $256t_1$ blocks were collected with 128 scans per block.

Results and Discussion

Equilibrium in heme-cyanide system : *Fig.* 1 shows the proton NMR spectra for the diverse ligand states of hemin at various potassium cyanide concentrations. In the absense of cyanide(*Fig* 1A), only the high spin hemin which has DMSO as its fifth and sixth ligands exists in the solution. The signals of high spin hemin complex exhibit a large paramagnetic shift to low field at 50~70 ppm (shown in inset of *Fig.* 1A). Upon addition of cyanide to the solution, the intensity of these resonances from high spin hemin decrease and finally disappear with the concomitant appearance of a new group of resonance peaks at 7~18 ppm. These new peaks which come from monocyanide hemin reach maximum intensity at a cyanide-hemin ratio of about 1 : 1 and then decrease thereafter, finally disappearing at about a 3 : 1 ratio, which gives only low spin dicyanide complex as shown in a series of spectra of *Fig.* 1. The paramagnetic shifts of methyls in monocyanide hemin complex(mixed ligand complexes) is smaller than those of dicyanide hemin complex and peaks in the former case are shifted upfield relative to those of later case. This could either indicate a larger magnetic anisotropy(larger positive dipolar shifts) or smaller contact contributions to the isotropic shifts of monocyanide hemin complex. It should also be pointed out a fact that the exchange rate of the mixed-ligand complexes is slow on the NMR time scale. This has been demonstrated clearly in a earlier investigation of the CN^- equilibria of monodicyanidehemin complex.[12]

Mono-and di-cyanide hemin complexes : *Fig.* 2 illustrates the 200-MHz ^{1}H NMR spectrum for the mixed ligand state hemins, dicyanidehemin-monocyanidehemin ratio of about 1 : 1. This state is reached when hemin-cyanide ratio is about 1: 1. 5. the resonances from dicyanidehemin and monocyanidehemin are labeled as capital letters and lower case letters, respectively. As shown previously, the chemical shifts of the signals from the dicyanidehemin complex in DMSO-d_6 solvent are very much the same to those of dicyanohemin complex in CD_3OD solvent. Although one of the ligand binding site is ligated by a weak ligand, DMSO in the case of monocyanohemin, the chemical shift dis-

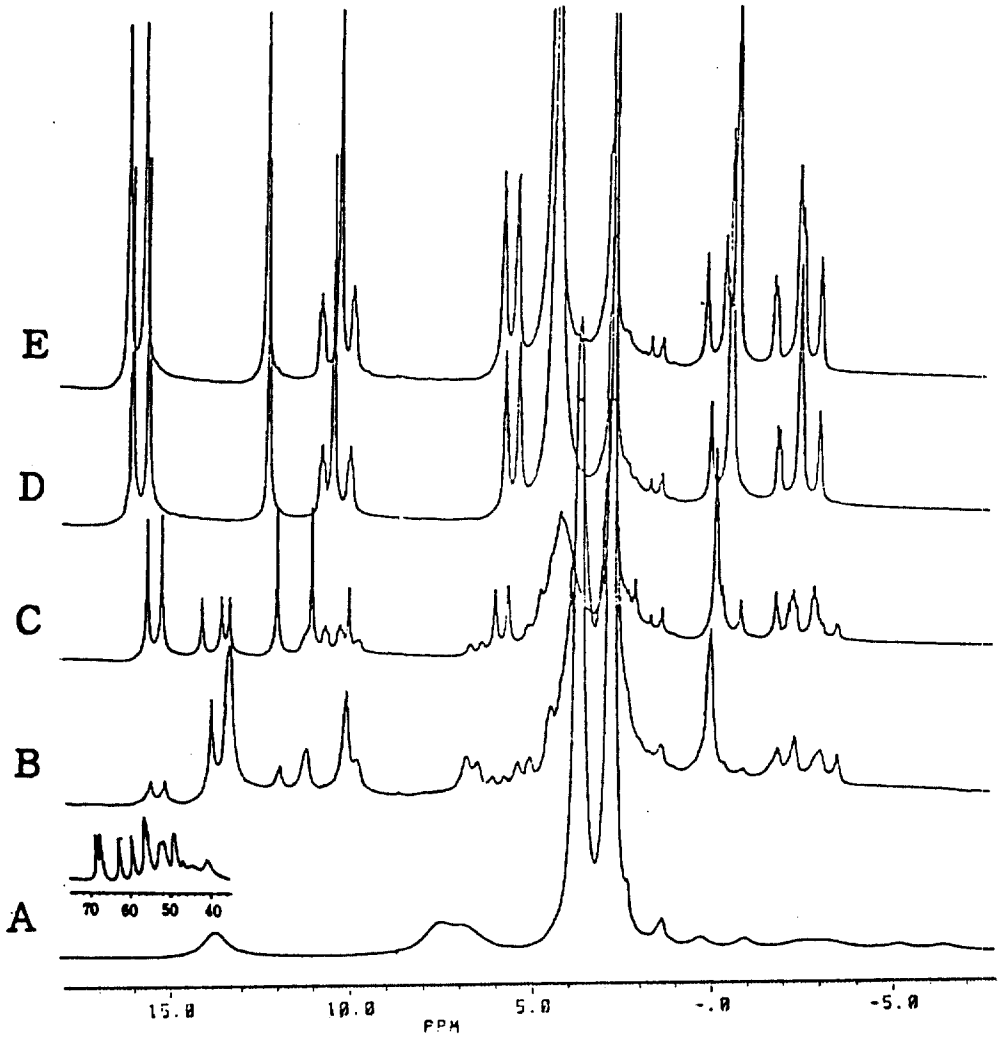

Fig. 1. 200 MHz ^{1}H NMR spectra of 10 mM ferriprotoporphyrin(hemin) in DMSO-d_6 solution at various potassium cyanide concentrations. The potassium cyanide concentrations are (A) 0.0 mM. The low field region of high spin hemin complex is given in the inset. (B) ~10 mM (C) ~20 mM (D) ~30 mM (E) ~40 mM

persion is very similar to that of dicyanohemin complex. The complete 200MHz M-COSY map recorded at ambient temperature and illustrated in *Fig.* 3. All expected cross peaks are observed between hyperfine-shifted scalar coupling protons, which are geminal and vicinal protons in heme vinyl and propionate groups(The structure and the labeling system of the heme are given in the inset of *Fig.* 3).

Cross peaks 1 and 2 indicates the scalar interactions in $2V_\alpha/2V_{\beta c}$(m, 1) and $2V_\alpha/2V_{\beta t}$(m, 2) in monocyanohemin complex, respectively. Continuing clockwise, we obtain $2V_\alpha/2V_{\beta t}$(d, 3), $4V_\alpha/4V_{\beta t}$ (d, 4), $4V_\alpha/4V_{\beta t}$(m, 5), $4V_\alpha/4V_{\beta c}$(m, 6), $4V_\alpha/4V_{\beta t}$ (d, 7), $2V_\alpha/2V_{\beta c}$(d, 8). m and d in parenthesis denote monocyanohemin and dicyanohemin complexes, respectively and numbers in parenthesis indicates the numbering from 2-D map of *Fig.* 3. In order to differentiate the signals between monocyanidehemin and dicyanidehemin complexes,

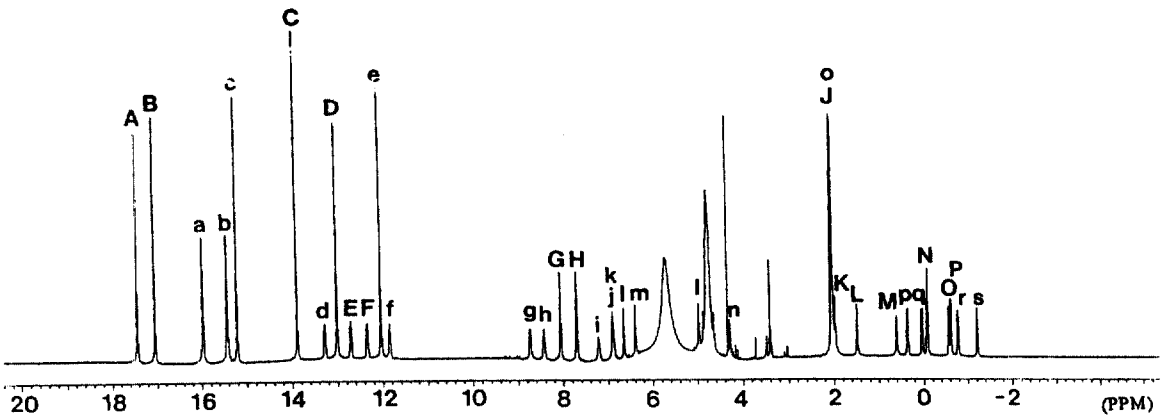

Fig. 2. Optimally resolved 200-MHz ^{1}H NMR trace for the 10 mM monocyanide and dicyanide hemin complex in DMSO-d_6 at 25℃. The signals from dicyanidehemin and monocyanidehemin are labeled as capital letters and lower case letters, respectively.

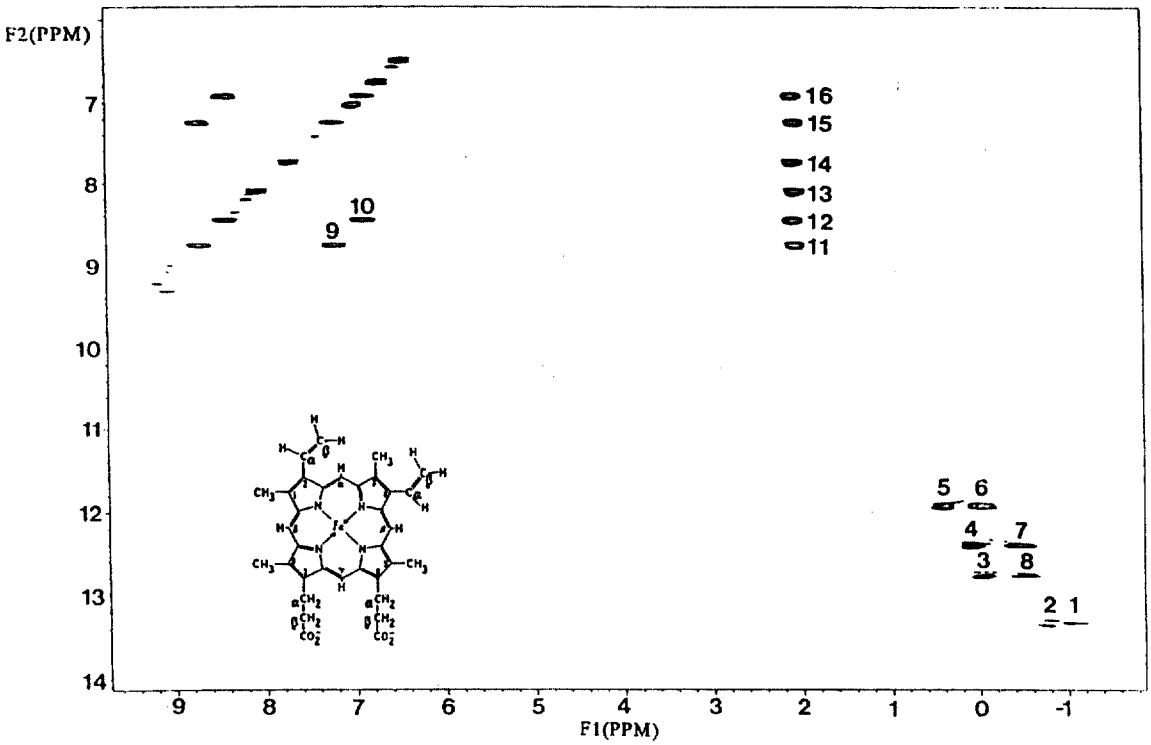

Fig. 3. 200-MHz M-COSY map of mono- and di-cyanide hemin complexes in DMSO-$_6$ at 25℃. Cross peaks connecting propionate and vinyl groups are illustrated. The collected data consist of 128 t_1 blocks of 2048 scans each with 1024 t_2 points over a 6000 Hz bandwidth for acquisition times in t_1 and t_2 of 10 ms and 20 ms, respectively. The optimal map resulted from apodizing over 1024×1024 points prior to zero-filling in t_1 for a final dizitization of 49 Hz/points.

cyanide titration method was utilized as shown in *Fig.* 1. and the assignment strategy for dicyanide-hemin complex was based on the chemical shift pattern of the equivalent complex in CD_3OD solvent as published earlier.[3] Cross peaks 9 and 10 are due to the scalar interactions of $7P_\alpha/7P_\alpha^1$(m, 9) and $6P_\alpha/6P_\alpha^1$(m, 10), respectively. The absence of strong cross peaks for $6P_\alpha/6P_\alpha^1$(d) and $7P_\alpha/7P_\alpha^1$(d) suggests that two peaks must be essentially degenerate between two geminal partner protons. Cross peaks 11~16 are from scalar couplings of $6P_\alpha/6P_\beta$(m, 11), $7P_\alpha/7P_\beta$(m, 12), $6P_\alpha/6P_\beta$(d, 13), $7P_\alpha/6P_\beta$(d, 14), $6P_\alpha^1/6P_\beta^1$(m, 15), and $7P_\alpha^1/7P_\beta^1$(d, 16), respectively. The M-COSY map data in *Fig.* 3 clearly show that coherence is detectable in paramagnetic iron complex, essentially all spin connectivities were detected that could be expected for an analogous diamagnetic system. The chemical shifts for monocyanohemin and dicyanohemin complexes are listed in *Table* Ⅰ and *Table* Ⅱ, respectively. Direct determiniation of nonselective T_1 values for resolved resonances by the inversion-recovery technique yielded the values in *Table* Ⅰ and *Table* Ⅱ.

The chemical shifts of mean meso protons at 25℃ for the dicyanohemin complex and monocyanohemin complex are 2.25 ppm and 6.05 ppm, respectively and this ~4 ppm chemical shift difference is con-

Table Ⅰ. ^1H NMR peak assignment and T_1 relaxation time of dicyandie ligated hemin in DMSO solution at 25℃.

Labels	Chemical Shift(ppm)	Peak Assignment	T1 relaxation time(ms)
A	17.43	8-CH_3	172.1
B	17.04	5-CH_3	178.7
C	13.88	3-CH_3	218.2
D	13.00	1-CH_3	214.2
E	12.70	2-V_α	228.5
F	12.34	4-V_α	228.5
G	8.05	6-P_α 6-P_α^1	140.5
H	7.69	7-P_α 7-P_α^1	142.4
I	4.97	γ-meso	56.6
J	2.03	6-P_β 6-P_β^1 7-P_β 7-P_β^1	126.1
K	1.97	β-meso	61.5
L	1.47	δ-meso	53.5
M	0.60	α-meso	49.0
N	−0.09	4-$V_{\beta t}$ 2-$V_{\beta t}$	215.7
O	−0.58	4-$V_{\beta c}$	245.3
P	−0.63	2-$V_{\beta c}$	242.6

Table Ⅱ. ^{1}H NMR peak assignment and T_1 relaxation time of monocyanide ligated hemin in DMSO solution at 25℃.

Labels	Chemical shift(ppm)	Peak Assignment	T1 relaxation time(ms)
a	15.96	8-CH_3	136.2
b	15.42	5-CH_3	140.9
c	15.20	3-CH_3	167.9
d	13.28	2-V_α	176.1
e	12.04	1-CH_3	171.1
f	11.85	4-V_α	165.5
g	8.73	6-P_α	96.8
h	8.42	7-P_α	97.2
i	7.21	6-P_α^1	93.0
j/k	6.89	7-P_α^1 γ-meso	60.6
l	6.64	β-meso	47.1
m	6.38	δ-meso	47.3
n	4.29	α-meso	63.6
o	2.03	6-P_β 6-P_β^1 7-P_β 7-P_β^1	126.1
p	0.36	4-$V_{\beta t}$	185.8
q	0.03	4-$V_{\beta c}$	201.1
r	−0.79	2-$V_{\beta t}$	183.2
s	−1.23	2-$V_{\beta c}$	216.0

sidered to be due to the minor portion of high spin character in monocyanohemin complex.

Temperature dependence : The hyperfine shifts of dicyanohemin complex in DMSO-d_6 solvent displayed very similar behavior when compared to the same complex in methanol solvent as shown in the Curie plot *Fig.* 4. As expected by the Curie law, the heme methyl signals in dicyanohemin complex displayed decreased shift at higher temperatures, which means that the electronic property of iron in dicyanohemin complex in DMSO-d_6 is the same with that of equivalent complex in CD_3OD solvent. The temperature dependence of the vinyl protons shows anti-Curie behavior, probably because there is a change in the average out-of-plane twisting of the vinyl group with temperature. Thus, low-spin myoglobin and cytochrome b_5 locked sterically with the adjacent CH_3 groups and protein matrix may cause the vinyls to be more perpendicular to the plane of the porphyrin ring, thus allowing them less spin delocalization in the low-spin heme protein which leads to Curie behavior. However, the

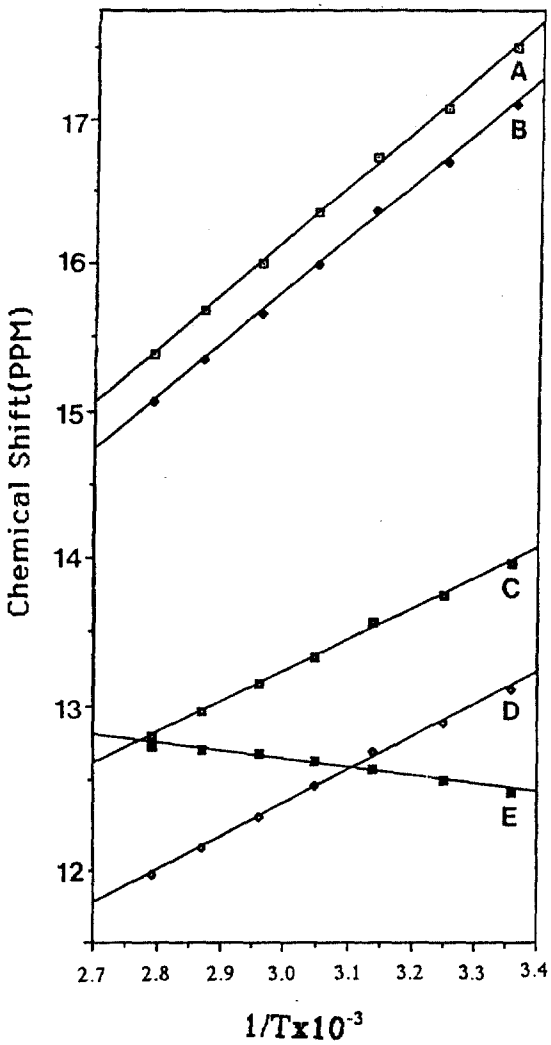

Fig. 4. Plot of observed chemical shifts versus reciprocal absolute temperature(Curie plot) of 10 mM dicyanidehemin complex in DMSO-d_6 at 25℃ for the selected signals(A, 8-CH_3; B, 5-CH_3; C, 3-CH_3; D, 1-CH_3; E, 2-V_α).

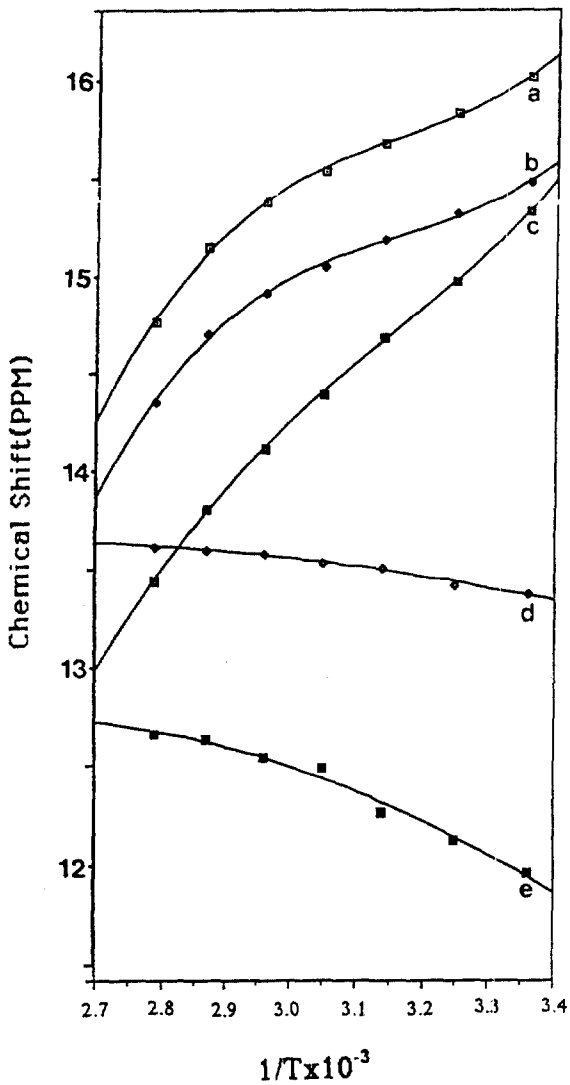

Fig. 5. Plot of observed chemical shifts versus reciprocal absolute temperature(Curie plot) of 10 mM monocyanidehemin complex in DMSO-d_6 at 25℃ for the selected signals(a, 8-CH_3; b, 5-CH_3; c, 3-CH_3; d, 1-CH_3; e, 2-V_α).

hyperfine shifts of monocyanohemin complex in DMSO-d_6 illustrated rather remarkablely anomalous behavior when compared to the well-characterized dicyanohemin complex as shown in *Fig.* 4. Although the hyperfine shifts of 8-CH_3, 5-CH_3 and 3-CH_3 decrease at elevated temperatures as expected by the Curie law, the temperature profiles of the various resonances in *Fig.* 5 illustrates the curvature as shown in structurally less characterized, spin-equilibrated cytochromes. The hyperfine shifts of $2V_\alpha$ and 1-CH_3 increase as temperature is raised, which leads to anti-Curie behavior in conjunction with the curvature. The unusual temperature dependence can be explained by the severe contribution from dipolar interaction between unpaired electron of iron and protons.

The detailed theory indicates that dipolar shifts arising from the zero-field splitting(ZFS) have a characteristic T^2 dependence for high spin d^5 ions, in contrast to the T^{-1} dependence of the contact contribution to the shift. Thus, the shift for high-spin(Ⅲ) can be written in the form

$$(\Delta H/H)^{iso} = \alpha/T + \varepsilon/T^2$$

where $\alpha = 35g\beta A/12kh\gamma_H$, $\varepsilon = (28g^2\beta^2 D/9k^2)(3\cos^2\theta - 1)/r^3$ and D is the ZFS parameter. The T^{-2} term represents the dipolar contribution. From relative geometric factors, the dipolar contribution at any other position in the complex can be determined.[1]

Ferric hemoprotein in a given ground state invariably exhibit Curie-like behavior for the majority of hyperfine-shifted resonances, and strong and systematic deviations from Curie behavior are generally taken as direct evidence for a spin-state equilibrium. The present obsevation of anti-Curie behavior for the heme methyls, together with their low temperature shifts, clearly dictates the presence of a low-spin(S=1/2) ground state in rapid equilibrium with a thermally accessible high-spin (S=5/2) state. The nature of the deviations from Curie behavior for different resonances in such an equilibrium depends on their relative shift differences in the low-spin, S=1/2, and high-spin, S=5/2, states. Protons with larger shifts of the same direction in high-spin than in low-spin form would exhibit anti-Curie behavior because of the larger effect of populating the high-spin state than the T^{-1} change of the low-spin state.

Two possibilitie for the high-spin state must be considered, a six-coordinate state where the spin equilibrium is within an invariant ligation state [such as for an intermediate ligand field observed for methydroxy-metazidomyoglobin] or a five-coordinate state where one ligand, DMSO-iron bond ruptured. The largely contact shift patterns for hemin is highly characteristic of the ligation state, differing primarily in the meso-H shift direction.

Monocyanidehemin-dicyanide hemins equilibrium

The observation of transition from monocyanidehemin to dicyanidehemin as a function of cyanide concentration was shown above. Likewise, observation of temperature-dependent monocyanide-dicyanidehemin complex equilibrium will yield data which can be used to determine the thermodynamic parameters of two entities in equilibrium. The rate of exchange among the two hemin species is slow on the NMR time scale

$$\text{Hemin CN} + CN^- = \text{Hemin}(CN)_2^-$$
$$K = [\text{Hemin.}(CN)_2] / [\text{Hemin. CN}][CN^-]$$

The observation of transition from monocyanide hemin to dicyanidehemin in DMSO-d_6 is

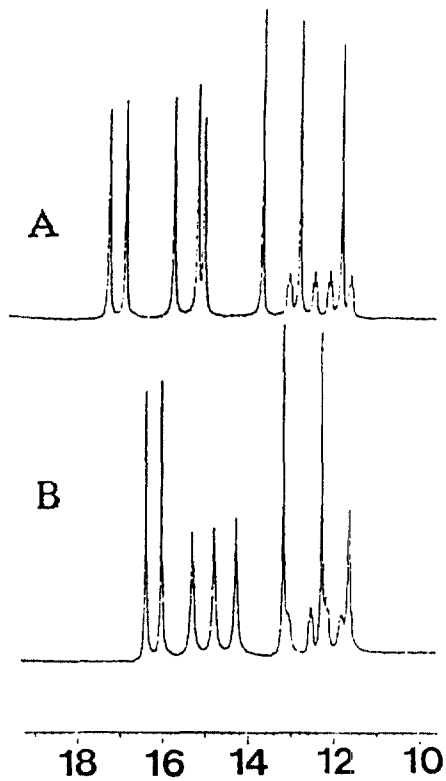

Fig. 6. 200 MHz ^{1}H NMR spectra of 10mM ferriprotoporphyrin(hemin) in DMSO-d_6 solution at(A) 25℃ and (B) 45℃.

illustrated in *Fig.* 6. *Fig.* 6 shows that the signal intensity for dicyanide hemin complex increase dramatically in compensation for decrease of the signal intensity of monocyanidehemin complex at elevated temperature. A plot of ln K. *vs.* T^{-1} yields the expected straight line, shown in *Fig.* 7. The ratio of $[Hemin.(CN)_2]/[Hemin.(CN)][CN^-]$ is calculated from the NMR signal intensity ratio of two different complexes and the concentration of free CN^{-1} was taken from the reference at 25℃. The plot yields the thermodynamic parameters $\Delta H^\circ = 736.6$cal/mol, $\Delta S^\circ = 16.4$ eu. The positive ΔH° shows that the monocyanidehemin complex goes to the dicyanohemin complex as temperature is raised, which is endothermic reaction and the positive ΔH° is consistent with the fact that the reaction above is a natural process.

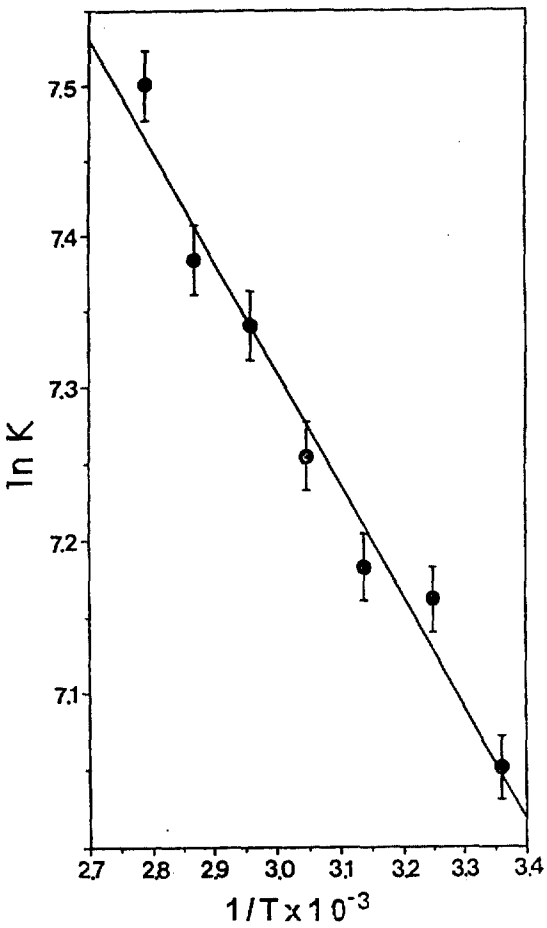

Fig. 7. Van't Hoff plot for the monocyanidehemin complex to dicyanidehemin complex equilibrium in DMSO-d_6. $\Delta H^\circ = 736.6$ cal/mol, $\Delta S^\circ = -4150.6$ cal/mol, $\Delta G^\circ = -4150.6$ cal/mol.

Biological implications : The thermally accessible six-coordinate spin equilibrium in monocyanohemin complex is in contrast to the behavior observed for the dicyanohemin complex, this low spin ⇌ high spin equilibria for ferrie hemoproteins are not uncommon, although only infrequently observed among other cytochromes with axial His/Met ligation and demands that the effective axial field(i. e. Fe-DMSO bond) is weakened in the present monocyanohemin complex. Previously reported NMR spectra of structurally characterized ferricytochromes with His/Met ligation exhibit anti-Curie behavior indicative of a low-spin ferric state.[13] Therefore, the elucidation for the electronic/molecular structure and ligation state of monocyanohemin complex will shed light on the characterization of those of hemoproteins, which one of axial ligands is weak. The next step will be to implement ^{13}C NMR studies to establish the relationship of the ^{13}C NMR parameters and spin equilibrated paramagnetism. These studies are in progress.

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