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Quantitative Mapping of Metal-Centered Dipolar Field in Hemin Dicyano Complex by Solution NMR

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The metal-centered pseudo-contact shift contribution in the hyperfine shift of NMR resonance of paramagnetic hemin dicyano complex ([$\mathrm{Fe^{III}(proto)(CN)_2}$]) in solution was quantitatively determined from the combined use of ^{1}H and ^{13}C NMR data. Taking advantage of the axially symmetric magnetic property for [$\mathrm{Fe^{III}(proto)(CN)_2}$] in solution, the paramagnetic metal-centered dipolar field was quantitatively mapped. The highly solvent-dependent magnetic anisotropy, ($g^2_{/\!/}-g^2_{\perp}$), of this complex in $\mathrm{CD_3OD}$ was estimated to be 7.2 and the obtained value is placed between the values in $\mathrm{D_2O}$ (4.4) and in $\mathrm{CDCl_3}$ (9 .7).

Heme occupies the active sites of various hemoproteins and its electronic structure is crucially related to the functional properties of these proteins.[1] NMR presents potentially one of the most powerful spectroscopic techniques to investigate the structure-function relationship of hemoproteins in solution.[2–8] In the case of paramagnetic hemoproteins and hemin complexes, the hyperfine field due to the unpaired electron(s) of the heme iron provides a unique probe to characterize the electronic/molecular structure of the heme, because the hyperfine shifted NMR resonances arising from the heme and/or the surrounding amino acid residues in paramagnetic hemoproteins are extremely sensitive to the change in the heme molecular environments. The observed chemical shift of a hyperfine shifted resonance (δ_{obs}) is composed of three terms;[9,10] $\delta_{\mathrm{obs}}=\delta_{\mathrm{dia}}+\delta_{\mathrm{c}}+\delta_{\mathrm{pc}}(\delta_{\mathrm{c}}+\delta_{\mathrm{pc}}$ is called the hyperfine shift (δ_{hf})), where δ_{dia} is the chemical shift of a suitable diamagnetic complex and δ_{c} and δ_{pc} are the contact and the metal-centered pseudo-contact shifts, respectively, which stem from the interaction of the nucleus with the unpaired electron(s). The δ_{c} value reflects the unpaired electron density delocalized into the s orbital of that nucleus and the δ_{pc} value can be interpreted in terms of the spatial relationship between that nucleus and the unpaired electron localized at the heme iron. Therefore the relative contributions of δ_{c} and δ_{pc} to δ_{hf} have to be determined in order to quantitatively analyze the δ_{c} value in terms of the unpaired spin delocalization, i.e., electronic structure of the heme, and the heme molecular structure.

Several procedures for separating δ_{hf} to δ_{c} and δ_{pc} have been proposed.[9,11–13] Those procedures usually start with the determination of magnetic anisotropy and the location of magnetic axes and, therefore, generally rely on the *g*-tensor measurements of either single crystal or frozen sample.[14–16] Even for ideal magnetic behavior, questions must remain about the application of those solid data to interpret solution NMR results. Goff[13,17] has demonstrated an approach to estimate the metal-centered pseudo-contact shift of fourfold symmetric iron(III) tetraphenylporphyrin derivative-imidazole complexes using solution NMR measurements with the assumption that the resonances from the peripheral phenyl groups experience predominantly metal-centered pseudo-contact shifts and, hence, their contact shifts are neglected. Since cyanide ion is the most frequently used as an external ligand for iron(III) hemoproteins to ensure a stable low-spin complex and the NMR spectral properties of iron(III) hemoprotein-cyano (met-cyano) complex are considerably different from those of iron(III) hemoprotein–imidazole (met-imidazole) complex, it is of interest to investigate the metal-centered dipolar field in met-cyano complex and to compare it with that in met-imidazole complex.

We present herein a novel method to quantitatively characterize the paramagnetic metal-centered dipolar field in paramagnetic low-spin hemin dicyano complex by making use of the difference in the δ_{pc} value between heme methyl carbon and attached proton nuclei. Since it has been proved that in-plane magnetic anisotropy of hemin dicyano complex in solution is averaged to zero,[18] the assumption of axially symmetric dipolar field can be confidently applied to analyze the metal-centered dipolar field of this complex. The proposed method can be useful to map the metal-centered dipolar field in paramagnetic low-spin hemin complexes and hemoproteins with insignificant in-plane magnetic anisotropy.

Experimental

Material; Hemin chloride and (protoporphyrinato IX) zinc(II) were purchased from Sigma Chemical Co. and Aldrich Chemical Co., respectively, and were dissolved in methanol-d_4($\mathrm{CD_3OD}$) with 5-fold excess of KCN and pyridine-d_5($\mathrm{C_5D_5N}$), respectively, for the NMR measurements. Concentration of both samples are ca. 20 mM.

Instrumentation; NMR spectra were recorded on a JEOL GX-270 FT NMR spectrometer operating in the quadrature mode (^{1}H and ^{13}C frequencies are 270 and 67.9 MHz, respectively). Typical ^{1}H spectra consisted of 256 transients with 32K data points over 5000 and 10000 Hz for

Zn and Fe complexes, respectively. Proton noise decoupled ^{13}C NMR spectra were recorded using a 10 mm diameter tunable probe, about 5000 transients, 16K data points over 30 kHz. The obtained free induction decay was apodized at 1—10 Hz. ^{1}H-^{13}C heteronuclear chemical shift correlated spectrum (^{1}H-^{13}C COSY spectrum) of (protoporphyrinato IX)zinc(II) ([Zn^{II}(proto)]) in C_5D_5N was recorded using the standard pulse sequences.[19] The time domain data matrix, 2K(^{13}C-15000 Hz)X128(^{1}H-4000 Hz) was expanded to the final matrix size 2KX512 by zero filling. The data matrix was apodized with a sine bell function in both dimensions and the spectrum is presented in the absolute value mode. Chemical shifts are given in parts per million (ppm) downfield from 2,2-dimethyl-2-silapentane-5-sulfonate (DSS).

Results and Discussion

Resonance Assignment in ^{1}H and ^{13}C NMR Spectra of [Zn^{II}(proto)]. ^{1}H-^{13}C COSY spectrum of diamagnetic [Zn^{II}(proto)] in C_5D_5N at 29 °C, together with the ^{1}H and ^{13}C NMR spectra attached along the F_1 and F_2 axes, respectively, is illustrated in Fig. 1. The individual ^{1}H signal assignment is obtained by a combined use of ^{1}H-^{1}H chemical shift correlated and nuclear Overhauser effect correlated spectroscopies (results not shown) and is shown with the ^{1}H spectrum in the figure. From the known ^{1}H assignment, the ^{13}C assignment is clearly established by the ^{1}H-^{13}C connectivities in the ^{1}H-^{13}C COSY spectrum. The resonances from the quaternary β-pyrrole carbon nuclei were assigned using the long-range ^{1}H-^{13}C COSY experiment (results not shown) and no attempt was made for assigning the quaternary α-pyrrole carbon resonances. The present assignments on both ^{1}H and ^{13}C spectra confirm the partial assignment previously reported[2,20] and are summarized in Table 1. Because of the presence of a pseudo-C_2 symmetry in [Zn^{II}(proto)] molecular structure, C_2 symmetric nature of its electronic structure with respect to the α,γ-meso axis is clearly reflected in the NMR spectra. The chemical shift values of [Zn^{II}(proto)] are used as the diamagnetic reference shift (δ_{dia}) in the analysis on the shifts of the paramagnetic hemin dicyano complex([Fe^{III}(proto)(CN)$_2$]) (see below).

Temperature Dependence Study of [Fe^{III}(proto)(CN)$_2$] NMR Spectra. ^{13}C NMR spectra of [Fe^{III}(proto)(CN)$_2$] measured at various temperatures are given in Fig. 2. The individual ^{13}C signal assignment of this

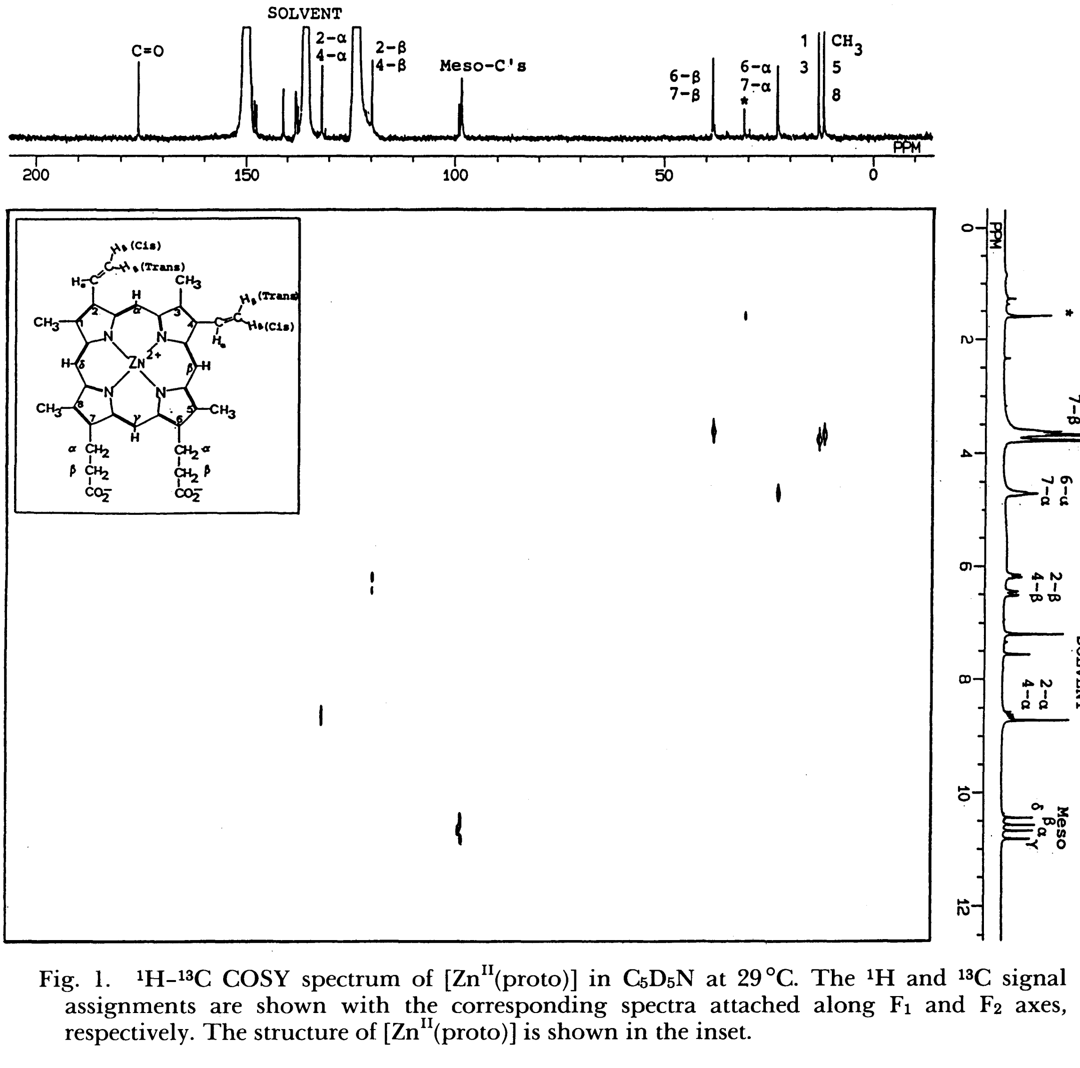

Fig. 1. ^{1}H-^{13}C COSY spectrum of [Zn^{II}(proto)] in C_5D_5N at 29 °C. The ^{1}H and ^{13}C signal assignments are shown with the corresponding spectra attached along F_1 and F_2 axes, respectively. The structure of [Zn^{II}(proto)] is shown in the inset.

Table 1. Chemical Shifts[a)] of Heme ^{1}H and ^{13}C Resonances

^{1}H	$[\mathrm{Fe^{III}(proto)(CN)_2}]^{b)}$	Intercept$^{c)}$ at $T^{-1}=0$	$[\mathrm{Zn^{II}(proto)}]^{d)}$	^{13}C	$[\mathrm{Fe^{III}(proto)(CN)_2}]^{b)}$	Intercept$^{c)}$ at $T^{-1}=0$	$[\mathrm{Zn^{II}(proto)}]^{d,e)}$
1-CH_3	12.80	5.9	3.79	1-CH_3	−24.4	11.6	13.0
3-CH_3	13.88	7.3	3.77	3-CH_3	−26.7	8.6	13.0
5-CH_3	16.82	7.2	3.67	5-CH_3	−33.7	9.8	11.7
8-CH_3	17.22	7.0	3.70	8-CH_3	−34.3	9.2	11.7
2-α	13.21	12.6	8.64	2-α	71.7	134.1	131.5
4-α	12.67	12.7	8.64	4-α	73.3	135.6	131.5
2-β(Trans)	−0.82	8.1	6.48	2-β	ca.165$^{f)}$	119.2	119.6
2-β(Cis)	−1.37	8.6	6.17	4-β	162.6	118.4	119.6
4-β(Trans)	−0.61	8.2	6.48	6-α	−14.9	23.1	22.8
4-β(Cis)	−1.22	8.9	6.17	7-α	−13.5	24.4	22.8
6-α	8.10	9.1	4.71	6-β	93.6	41.9	38.3
7-α	7.70	8.7	4.71	7-β	91.1	39.7	38.3
6-β	0.99	4.8	3.62	α	83.2	76.7	98.9
7-β	0.99	4.8	3.62	β	66.1	127.6	98.4
α	0.60	12.5	10.67	γ	55.0	n.d.	98.2
β	1.69	13.0	10.57	δ	73.3	82.7	98.4
γ	3.43	11.9	10.82	C=O	180.2	180.2	175.6
δ	1.21	12.2	10.44				

a) Chemical shifts are given in ppm and referenced to DSS through the residual solvent signal. b) Measured in CD_3OD at 40 °C. c) Linear least-square fitting procedure on the Curie plots were used. d) Measured in C_5D_5N at 29 °C. e) The values were found to be 1.5 ppm smaller than the previous results.[20)] f) Resonating under free CN^- signal. n.d.; Not determined.

complex has been reported previously.[21)] The observed shifts for the heme methyl, meso, and quaternary β-pyrrole carbon resonances are plotted against reciprocal of absolute temperature in Fig. 3. Curvatures of the plots, especially in the plots of β-pyrrole carbon resonances, are apparently like the Curie plots for the ^{13}C resonances of iron(III) tetraphenylporphyrin derivative-imidazole complexes,[13,17)] and, as reported previously,[2)] ^{1}H data also exhibit some curvature in their Curie plots (results not shown). Non-linear temperature dependence of the pseudo-contact shift[17)] does not simply account for the degree of the curvature in their Curie plots because the curvature of the β-pyrrole carbon resonances is greater than that of the meso carbon resonances although the latter carbon nuclei are closer to the heme iron. Therefore the effects of aggregation[22)] at low temperature might be responsible for the non-linear Curie plot. But the observed shifts of $[\mathrm{Fe^{III}(proto)(CN)_2}]$ NMR resonances at 40 °C were essentially independent of concentration from 5 to 50 mmol dm^{-3}. The intercepts (at $T^{-1}=0$) of the Curie plots for all the resonances were calculated from the least-square fitting of the plots and are given in Table 1. The difference between the chemical shift value in $[\mathrm{Zn^{II}(proto)}]$ and the intercept of the Curie plot, for the corresponding resonances, is relatively small for the heme side-chain carbon resonances and is large for the ^{1}H resonances, although the intercepts are close with each other among the resonances for the ^{1}H nuclei of same type.

Quantitative Mapping of Paramagnetic Metal-Centered Pseudo-Contact Field in $[\mathrm{Fe^{III}(proto)(CN)_2}]$. The observed shifts of the heme methyl carbon resonances are plotted against those of the attached proton

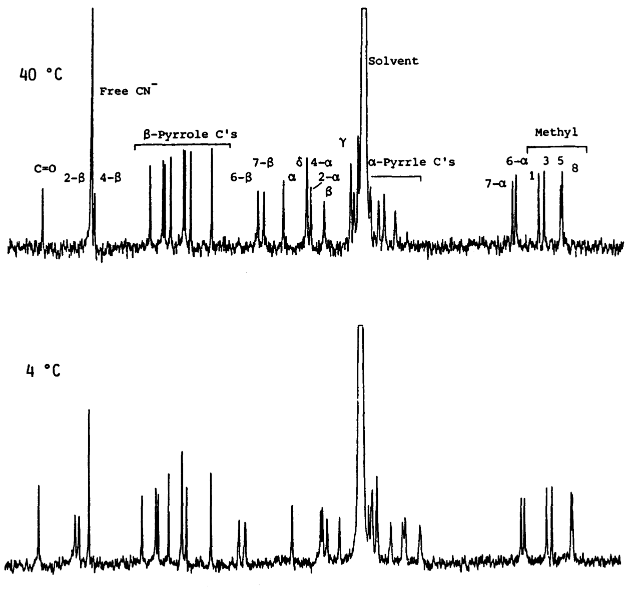

Fig. 2. Proton-noise decoupled ^{13}C NMR spectra of $[\mathrm{Fe^{III}(proto)(CN)_2}]$ in CD_3OD at the indicated temperatures. The assignment of the resonances has been reported previously.[21)]

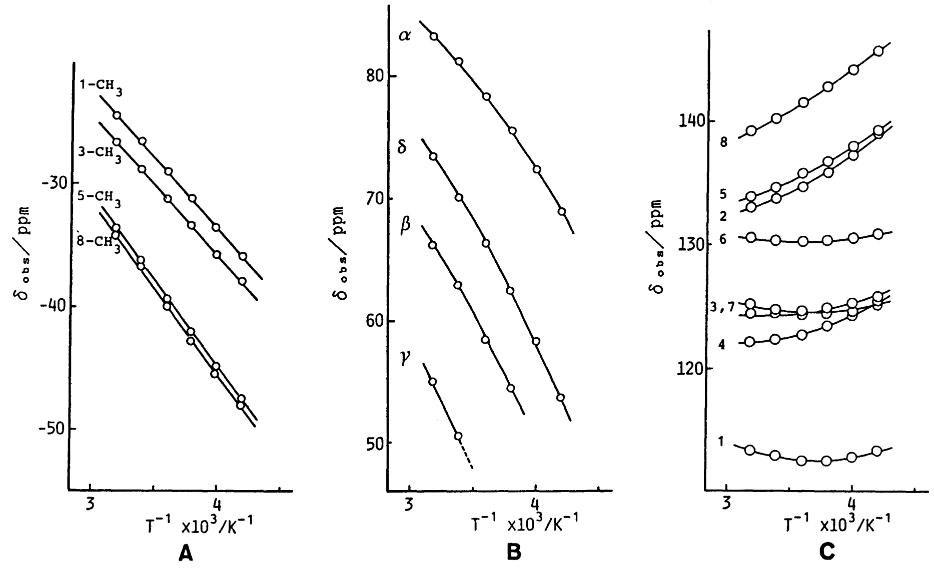

Fig. 3. Curie plot, the observed shift (δ_{obs}) vs. reciprocal of absolute temperature, for the heme methyl (A), meso (B), and β-pyrrole (C) carbon resonances. The curvature of the plots is most significant for the β-pyrrole resonances.

resonances, at various temperatures, in Fig. 4. As previously reported,[23] those plots give straight lines with a slope of $Q^{C}_{C'C}\gamma_H/Q^{H}_{CCH_3}\gamma_C$ for the system of axial symmetry ($Q^{C}_{C'C}$ and $Q^{H}_{CCH_3}$ are the parameters that account for the transfer of the unpaired spin density in the π orbital of the pyrrole carbon to directly-bonded carbon nucleus via the spin polarization and to the heme methyl proton nuclei through the hyperconjugation, respectively, and γ_H and γ_C are the gyromagnetic ratios of the proton and the carbon nuclei, respectively). The plots in Fig. 4 are straight and exhibit almost same slope over the temperature range examined, indicating $Q^{C}_{C'C}/Q^{H}_{CCH_3}$ is independent of temperature from −35—40 °C. The value of $Q^{C}_{C'C}=-39$ MHz reported for π-radical system[24] combined with the slope of the plots(=1.90) yield 82 MHz for $Q^{H}_{CCH_3}$.

For the limiting case of axial symmetry, δ_{pc} is expressed by Eq. 1,[10]

$$\delta_{pc}=\frac{(\chi_{/\!/}-\chi_{\perp})}{3N}\,\frac{(3\cos^2\theta-1)}{r^3} \tag{1}$$

where N is Avogadro's number. $\chi_{/\!/}$ and $\chi_{\perp}$ are the components of the molecular susceptibility parallel and perpendicular to z axis of the hemin molecule (z axis is defined along the normal to the porphyrin plane). The quantity, $(3\cos^2\theta-1)/r^3$, is called axial geometric factor which defines the spatial position of the observed nucleus with respect to z axis and iron center of the hemin. Our procedure for mapping the paramagnetic metal-centered dipolar field relies on the assumption of axial symmetry in the magnetic property of [Fe^{III}(proto)(CN)$_2$]. But it has been verified for [Fe^{III}(proto)(CN)$_2$] that the in-plane anisotropy, which is present in the frozen glass state, is averaged to zero in solution.[18] The linear plots shown in Fig. 4 also prove the insignificant in-plane anisotropy of this complex.

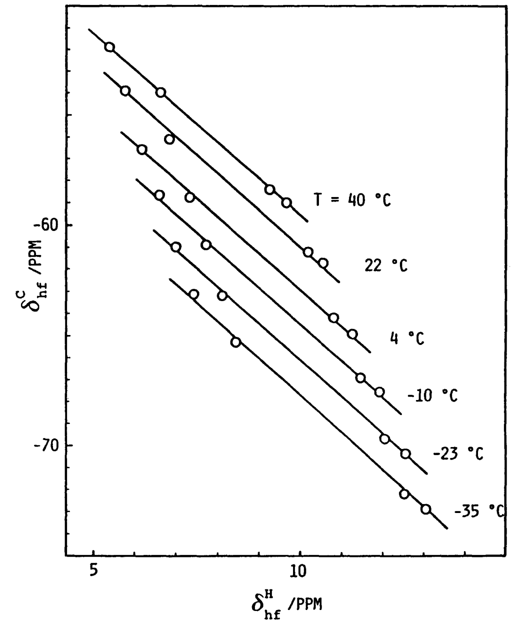

Fig. 4. The plots of heme methyl carbon hyperfine shift against the attached proton hyperfine shift at various temperatures. The plots strongly support that the in-plane magnetic anisotropy of [Fe^{III}(proto)(CN)$_2$] in solution is not significant and the $Q^{C}_{C'C}/Q^{H}_{CCH_3}$ is independent of temperature.

Since the δ_c values of the heme methyl carbon and the attached proton resonances are proportional to the unpaired electron density in the π orbital of the pyrrole carbon to which the methyl group is attached, they vanish simultaneously as the density becomes

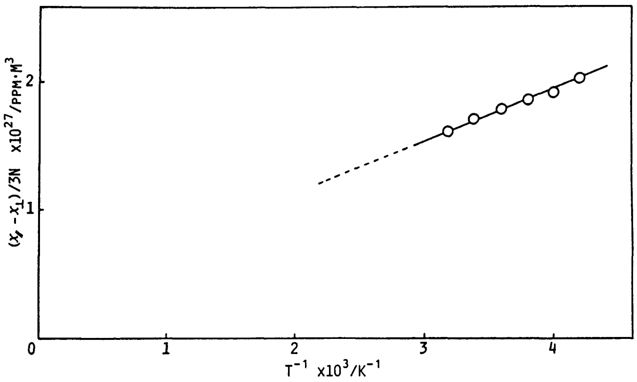

Fig. 5. The plots of $(\chi_{/\!/}-\chi_\perp)/3N$ against T^{-1}. The linear relationship indicates that the magnetic anisotropy is independent of temperature over the temperature range examined. Non-zero intercept would arise from unknown temperature dependence of $(\chi_{/\!/}-\chi_\perp)\,(3\cos^2\theta-1)/r^3$.

0. Therefore the slope and intercept of the plots in Fig. 4 combined with the axial geometric factors[25] of heme methyl carbon and proton nuclei yield the coefficient of Eq. 1, $(\chi_{/\!/}-\chi_\perp)/3N$, and hence the axial metal-centered dipolar field given in Eq. 1 can be determined. δ_{pc} value of a given nucleus can be calculated from its axial geometric factor using the obtained equation.

The $(\chi_{/\!/}-\chi_\perp)/3N$ values are calculated at different temperatures and are plotted against T^{-1} in Fig. 5. The plot exhibits a straight line over the temperature range studied, supporting the validity of the present method for estimating δ_{pc}. The magnetic anisotropy appears to be essentially independent of temperature over the temperature range examined. If we assume that there is only a single spin state with insignificant second-order Zeeman contribution, $(\chi_{/\!/}-\chi_\perp)/3N$ is rewritten in terms of the g-factor as $\beta^2S(S+1)(g_{/\!/}^2-g_\perp^2)/9kT$ (β, S, and k are the Bohr magneton, total spin and Boltzmann constant, respectively).[10] The slope of the plot in Fig. 5 yields the value of 7.2 for the quantity, $g_{/\!/}^2-g_\perp^2$. It has been demonstrated that the magnetic anisotropy, $g_{/\!/}^2-g_\perp^2$, for [Fe$^{\mathrm{III}}$(proto)(CN)$_2$] is highly solvent sensitive and is 4.4($g_{/\!/}\approx 2.9$, $g_\perp\approx 2.0$) in D_2O[26] and 9.7($g_{/\!/}\approx 3.6$, $g_\perp\approx 1.6$) in $CDCl_3$.[27] The value of 7.2 for CD_3OD solvent, obtained in the present study, is between those values and yields the δ_{pc} value of −14 ppm for the meso protons at 40 °C that is relatively large compared with the value previously estimated (−10 ppm) for met-imidazole complex,[13] but is comparable to the earlier estimation for [Fe$^{\mathrm{III}}$(proto)(C_5H_5N)(CN)] complex.[11] Using −14 ppm for the δ_{pc} of meso proton resonances, the π-electron density(in percent) of −0.25, −0.33, −0.42, and −0.33(minus sign indicates negative spin density.) are calculated for the α, β, γ, and δ meso carbon nuclei, respectively, and +0.83, +0.88, +1.03, and +1.04 for 1, 3, 5, and 8-pyrrole carbons, respectively. Non-zero intercept of the plot shown in Fig. 5 would arise from unknown temperature dependence of $(\chi_{/\!/}-\chi_\perp)(3\cos^2\theta-1)/r^3$.

The present method can be applied to any paramagnetic low-spin iron(III) porphyrin complexes including hemoproteins for mapping the paramagnetic metal-centered dipolar field as long as their in-plane magnetic anisotropy is not so significantly large. The heme methyl carbon resonances have been observed in various paramagnetic low-spin hemoproteins.[23,28]

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