^{19}F NMR Study of the Heme Orientation and Electronic Structure in a Myoglobin Reconstituted with a Ring-Fluorinated Heme

Yueki Hirai, Yasuhiko Yamamoto,* and Akihiro Suzuki†

Department of Chemistry, University of Tsukuba, Tsukuba 305-8571

†Department of Materials Engineering, Nagaoka National College of Technology, Nagaoka, Niigata 940-8532

(Received May 2, 2000)

13,17-Bis(2-carboxylatoethyl)-3,8-diethyl-2-fluoro-7,12,18-trimethylporphyrinatoiron(III) was reconstituted with sperm-whale apomyoglobin to investigate the molecular and electronic structures of the heme-active site by ^{19}F NMR spectroscopy. The detection of the nuclear Overhauser effect between the substituted fluorine atom for a heme side-chain and an amino acid side-chain proton in the reconstituted myoglobin unequivocally determines the heme orientation relative to the protein matrix. The orientation of this fluorinated hemin in the protein was found to differ by a 180° rotation of the heme plane about its C_2-axis from that of structurally analogous mesohemin in the active site of myoglobin. Thus, the difference between the methyl group and fluorine atom as the peripheral side-chain at the 2-position of the porphyrin is a critical determinant for the thermodynamical stability of the heme orientational isomers. The effects of the heme orientation on the electronic structure of the active site of myoglobin in a variety of iron oxidation, spin, and ligation states were characterized by ^{19}F NMR spectroscopy. The present study demonstrated that the ^{19}F NMR signals observed for hemoprotein reconstituted with fluorinated hemin provides useful information on the relationship between the heme orientation relative to the protein and its electronic structure in all accessible oxidation/spin states of the protein, that can be hardly obtained by ^{1}H NMR spectroscopy.

Oxygen-binding hemoproteins, such as myoglobin (Mb) and hemoglobin (Hb), are probably the best understood protein in terms of their biosynthesis, structure, and function.[1] Mb from skeletal muscle of sperm-whale (*Physter catodon*) is a monomeric protein with a molecular weight of about 17 kDa (1 Da = 1.66054×10^{-27} kg); also, a single iron-protoporphyrin IX prosthetic group (see Fig. 1) is embedded within its protein moiety, which consists of eight helices (labeled A through H). The heme iron is bound to the protein matrix through the proximal His residue (His F8), located at the 8th residue in the F helix. The nature of the molecular recognition between the heme and proteins in *b*-type hemoproteins is not sufficiently specific to force a single orientation of the prosthetic group within its protein matrix.[2,3] Although their crystal structures have invariably shown that the heme possesses a unique orientation within the protein, a solution NMR study has clearly demonstrated that the heme in these proteins is seated in two different orientations that differ by 180° rotation of the heme plane, about the 5,15-H *meso*-proton axis, relative to the protein matrix (A and B in Fig. 1), and that the heme orientational disorder is present in almost all native *b*-type hemoproteins.[2–6] The dominant component in Mb possesses the same heme orientation as found in the crystal structure (A in Fig. 1), called the normal form; a protein with a reversed heme orientation (B in Fig. 1) is called the reverse form.

The functional consequence of the heme orientation appears to be dependent on the particular protein. The influence of the heme orientation on the O_2 affinity is negligible in sperm-whale Mb,[7] but is relatively significant in insect (*Chironomus thummi thummi*) Hbs.[8] Similarly, the Bohr effect of the insect Hb,[8] the autoxiadtion rate of oxy-Mb[9] and tetrameric human adult oxy-Hb,[10] the redox potential of ferricytochrome b_5,[11] and the thermal spin-equilibrium for both

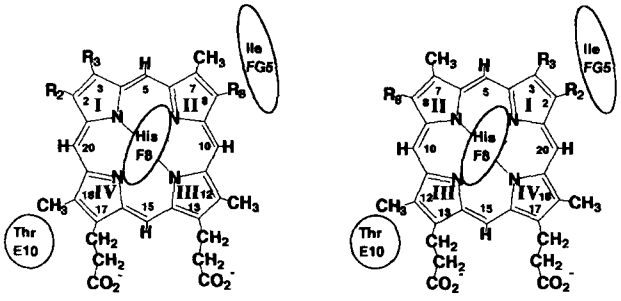

Fig. 1. The structure and numbering system of 2-MF, mesohemin, and protohemin and two possible orientations of hemin relative to Thr E10, His F8, and Ile FG5; (A) that found in the crystal structure of sperm whale Mb and (B) that with the heme rotated 180° about the 5,15-H *meso* axis from that of (A).

met-azido insect Hb[12] and ferricytochrome b_{562}[13] have been reported to be influenced by the heme orientation. Thus, an understanding of the molecular mechanism underlying the modulation of functional properties for *b*-type hemoproteins through the interaction of the prosthetic heme periphery with the protein matrix demands detailed scrutiny of the influence of the heme orientation on the molecular and electronic structures of the heme-active site.

Although the heme orientational disorder in *b*-type hemoproteins is most readily detected by ^{1}H NMR spectroscopy, the characterization of the effect of the heme orientation on the electronic structure of the active site in physiologically relevant forms of the protein is often hampered by the resolution of the signals.[10,14] Taking advantage of the utility of ^{19}F NMR spectroscopy,[15,16] we recently demonstrated that the introduction of a ring-fluorinated heme into apomyoglobin (apoMb) leads to an observation of the ^{19}F signals in various oxidation, spin, and ligation states of the Mb, which are expected to serve as potential spectroscopic probes for characterizing the heme-active site.[17] In order to gain insight into the influence of the heme orientation on the electronic structure of Mb, we carried out a ^{19}F NMR study on Mb reconstituted with 13,17-Bis(2-carboxylatoethyl)-3,8-diethyl-2-fluoro-7,12,18-trimethylporphyrinatoiron(III) (2-MF) (see Fig. 1) in its functional and non-functional forms.

We report herein on the ^{19}F NMR spectra for various forms of the reconstituted Mb, which sharply reflect the electronic structure of their heme active sites. The influence of fluorine substitution on the heme electronic structure was inferred from a ^{1}H NMR spectral comparison between the reconstituted Mbs with 2-MF and mesohemin (see Fig. 1). The presence of a heme orientational disorder results in the observation of two signals in the spectra, and the observed shift difference is reasonably interpreted in terms of the in-plane asymmetry of the heme electronic structure exerted predominantly by an asymmetric bonding interaction between the heme iron and the axial His side-chain. The observation of nuclear Overhauser effect (NOE) between the substituted fluorine atom of the heme and nearby amino acid side-chain proton in the Mb reconstituted with 2-MF clearly demonstrated that the reverse form is thermodynamically more stable than the normal form.

Experimental

Preparation of Apomyoglobin and Reconstitution of Myoglobin. Sperm-whale (*Physter catodon*) Mb was purchased as a lyophilized powder from Biozyme and used without further purification. Apoprotein was prepared at 4 °C according to the procedure of Teale.[18] Myoglobin (50 mg) was dissolved in 50 ml water, acidified to pH 2.6 with 0.1 M HCl (1 M = 1 mol dm^{-3}) and treated four times with 2-butanone to extract the released heme. The apoMb solution was dialyzed exhaustively against water to remove 2-butanone, then against 50 mmol dm^{-3} Bis–Tris buffer (pH 6.8) and finally against water to remove any salts. Any precipitate was removed by centrifugation. 13,17-Bis(2-carboxylatoethyl)-3,8-diethyl-2-fluoro-7,12,18-trimethylporphyrinatoiron (2-MF) was synthesized as previously described.[19] Mesoporphyrin IX dichloride was purchased from Sigma Chemical Co. and iron was inserted into the porphyrin using a reported method.[20] Protohemin was purchased from Sigma Chemical Co. The reconstitution of apoMb with the hemins was carried out by the standard procedure.[9] The hemin was dissolved in a minimal amount of 0.2 M NaOH and the hemin solution was diluted to a hemin concentration of ca. 1 mg cm^{-3}. The apoMb solution was titrated with the hemin solution using optical spectroscopy. ApoMb was reconstituted with stoichiometric amounts of hemin while monitoring the pH value.

NMR Sample Preparation. 2-MF, mesohemin and protohemin were dissolved in ^2H_2O in the presence of a large excess of potassium cyanide to prepare their bis-cyano complexes. A dimethylester complex of iron-free ring-fluorinated porphyrin, i.e., 13,17-bis(2-carboxylatoethyl)-3,8-diethyl-2-fluoro-7,12,18-trimethylporphyrin, was dissolved in C^2HCl_3. The Mb reconstituted with 2-MF was concentrated to ca. 1 mmol dm^{-3} in an ultra-filtration cell (Amicon) and, for a ^2H_2O sample, the solvent was exchanged to ^2H_2O. A 10-fold molar excess of potassium cyanide or sodium azide was added to met-aquo Mb to prepare met-cyano Mb or met-azido Mb, respectively. Carbonmonoxy Mb was prepared by the injection of CO gas and the addition of sodium dithionite. Deoxy Mb was prepared from met-aquo Mb, which had been evacuated and flushed with N_2 gas several times, by the addition of sodium dithionite. The p^{2}H of the sample was measured using a Horiba F-22 pH meter equipped with a Horiba type 6069-10c electrode. The p^{2}H of the sample was adjusted using 0.2 M NaO^{2}H or ^{2}HCl. The isotope effect was not considered to correct the p^{2}H value.

NMR Spectroscopy. ^{1}H NMR spectra were recorded on a Bruker AC-400P FT-NMR spectrometer operating at a ^{1}H frequency of 400 MHz. A typical spectrum consisted of 2 K transients, a 20 kHz spectral width, 8 K data points, and a 9.5 μs 90° pulse. The water signal was suppressed by a decoupler pulse. The signal-to-noise ratio of the spectrum was improved by apodization, which introduced a 10—50 Hz line-broadening. Two-dimensional shift-correlated spectroscopy (COSY) and NOE-correlated spectroscopy (NOESY) were recorded with the standard pulse sequences. The 512 free induction decays were acquired with 2 K data points and a spectral width of 25 kHz. The time-domain data matrix was expanded to 1 K×2 K by zero-filling. A phase-shifted sine bell function was used to apodize the spectrum in both dimensions. The chemical shifts of ^{1}H NMR spectra are given in parts per million (ppm) relative to 4,4-dimethyl-4-silapentane-1-sulfonate, with H^{2}HO used as an internal reference.

^{19}F NMR spectra were recorded on a Bruker AC-400P FT-NMR spectrometer operating at a ^{19}F frequency of 376 MHz. A typical spectrum consisted of 20 K transients with a 100 kHz spectral width and 8 K data points. The signal-to-noise ratio of the spectrum was improved by apodization, which introduced 20—100 Hz line broadening. ^{19}F–^{1}H heteronuclear nuclear Overhauser effect (NOE) measurements were carried out by selectively saturating a desired ^{1}H peak for 100 ms; the results are presented in the form of NOE difference spectra. The chemical shifts are given in ppm downfield from trifluoroacetic acid as an external reference.

Results

^{1}H and ^{19}F NMR Spectra of Bis-Cyano 2-MF Complex. The 400 MHz ^{1}H spectrum of bis-cyano 2-MF complex, 3 mmol dm^{-3} in ^2H_2O, at 25 °C is illustrated in trace A of Fig. 2, and is compared with those of bis-cyano mesohemin and protohemin complexes in traces 2B and 2C, respectively. The spectrum in trace 2A shows that the purity of the 2-MF is $>98\%$. The signal assignments of the spectra in traces

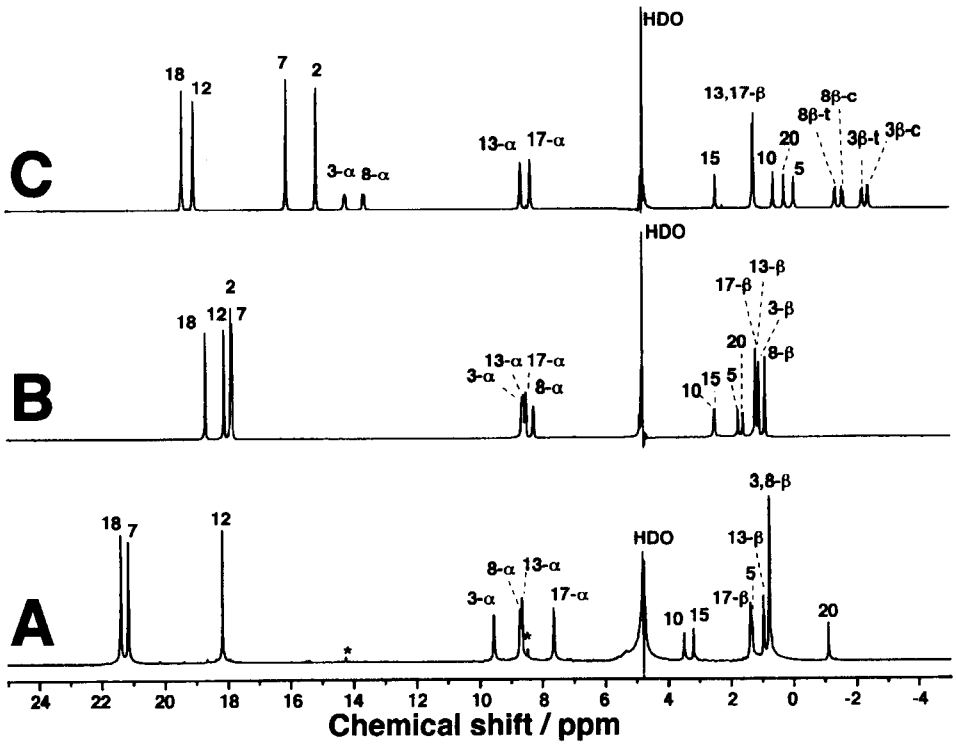

Fig. 2. The 400 MHz ^{1}H NMR spectra of (A) bis-cyano 2-MF, (B) bis-cyano mesohemin, and (C) bis-cyano protohemin in $^{2}H_2O$ at 25 °C. The signal assignments are indicated with the spectra. Concentration of the hemins is ca. 3 mmol dm^{-3} and the signals exhibit concentration-dependent shifts. The peak indicated by an asterisk in (A) arises from an impurity.

2A and 2B were obtained based on the 2D NMR scalar and dipolar connectivities (results not shown); that of the bis-cyano protohemin complex has been reported previously.[21] The spread of the heme methyl proton signals in the spectrum of trace B is smaller than that of trace 2C, i.e., ca. 1 and ca. 4 ppm for the mesohemin and protohemin complexes, respectively. This result indicates that the substitution of ethyl groups for the vinyl groups of protohemin enhances a C_4 symmetric nature of the heme electronic structure. The C_4 symmetry of the heme electronic structure for bis-cyano mesohemin complex is also confirmed from the smaller spread of its *meso*-proton signals, i.e., ca. 1 and ca. 2.5 ppm for the mesohemin and protohemin complexes, respectively. The substitution of fluorine atom for the methyl group at the 2-position of mesohemin introduces a sizable rhombic perturbation on the π-system of the porphyrin ring. The comparison of the corresponding signals between traces 2A and 2B shows that the shifts of the signals arising from the protons attached to pyrroles I, II, and IV are influenced by the fluorine substitution, whereas those for the protons of pyrrole III are essentially unaltered. The similarity in the shifts of 12-CH_3 and 13-CH_2CH_2 proton signals between traces 2A and 2B indicates that the magnetic anisotropy as well as the unpaired electron density delocalized to pyrrole III are not influenced by the ring-fluorination. Additionally, the signal for *meso* 20-H proton, which is located closest to the fluorine atom among the *meso* protons, exhibits the largest fluorine-induced shift among the *meso*-proton signals.

The 376 MHz ^{19}F NMR spectrum of bis-cyano 2-MF complex, 3 mmol dm^{-3} in $^{2}H_2O$, at 25 °C is illustrated in trace C of Fig. 3 and the signal for 2-F is observed at −27.98 ppm. The Curie plot, observed shift vs. reciprocal of absolute temperature, for this signal exhibit straight line with a positive slope and the intercept of −123.3 ppm in the temperature range between 5—35 °C (Table 1).

^{19}F NMR Spectra of the Reconstituted Mb Derivatives. The 376 MHz ^{19}F NMR spectra of reconstituted Mb with 2-MF in a variety of ligation states are illustrated in Fig. 3. The reconstitution of apoMb with 2-MF results in the splitting

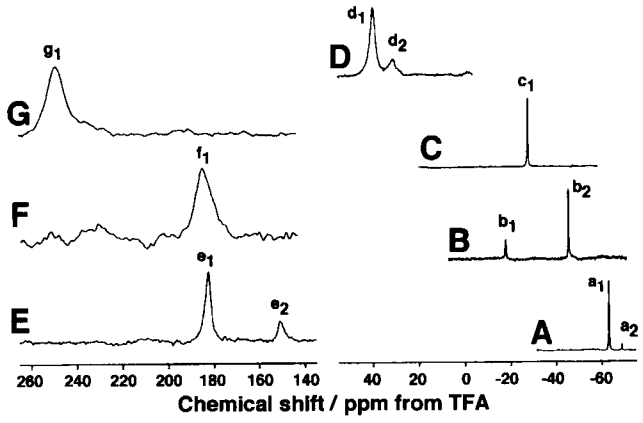

Fig. 3. The 376 MHz ^{19}F NMR spectra of (A) the reconstituted carbonmonoxy Mb, pH 6.46, (B) reconstituted met-cyano Mb, pH 7.57, (C) bis-cyano 2-MF, (D) the reconstituted met-azido Mb, pH 7.30, (E) the reconstituted deoxy Mb, pH 5.85, and the reconstituted met-aquo Mb, (F) pH 11.00, and (G) pH 7.03. All the spectra were recorded at 25 °C, except (F) and (G) measured at 35 °C.

Table 1. ^{19}F Chemical Shifts, Curie Slopes, and Curie Intercepts for 2-MF in Some Mb Derivatives

Mb	pH	Peak label[a)]	Assignment	Shift[b)]	Curie slope[c)]	Curie intercept[d)]
Carbonmonoxy Mb	6.46	a_1	Reverse[e)]	−63.28(73)[f)]	n.d.[g)]	n.d.
		a_2	Normal[e)]	−68.97(79)	n.d.	n.d.
Met-cyano Mb	7.57	b_1	Normal	−18.07(168)	41.0	−122.5
		b_2	Reverse	−45.68(60)	10.9	−82.2
Met-azido Mb	7.30	d_1	Reverse[e)]	39.40(987)	−59.0	237.8
		d_2	Normal[e)]	30.80(806)	−25.3	116.1
Deoxy Mb	5.85	e_1	Reverse[e)]	182.48(918)	124.6	−236.7
		e_2	Normal[e)]	149.66(976)	94.3	−167.0
Met-aquo Mb[h)]	11.00	f_1	Reverse[e)]	185.03(ca. 2800)	n.d.	n.d.
	7.03	g_1	Reverse[e)]	248.78(ca. 3000)	n.d.	n.d.
Bis-cyano 2-MF		c_1		−27.98(66)	28.5	−123.3
Bis-cyano 3,7-DF[i)]				−18.04(96)	31.0	−122.2

a) As labeled in Fig. 1. b) In ppm, referenced to trifluoroacetic acid, at 25 °C. c) Slope in plot of observed shift against reciprocal of absolute temperature, in ppm×K×10^3. d) Intercept at $T^{-1}\rightarrow 0$, in ppm, for plot of observed shift against reciprocal of absolute temperature. e) Tentative assignment. f) The numbers in parentheses are line width in Hz. g) n.d.: Not determined. h) At 35 °C. i) Obtained from Ref. 17.

of the ^{19}F signal into two peaks due to the presence of two possible orientations of 2-MF,[2,3] i.e., A and B of Fig. 1, relative to the surrounding protein, in the active site of Mb. The spectrum of met-cyano Mb freshly reconstituted with 2-MF is shown in trace 3B; this spectrum is essentially identical to that reported previously,[19] except that the ratio of the intensity between the two signals in trace 3B is slightly different from that in the previous one. The signals, peaks b_1 and b_2, in trace 3B have been tentatively assigned to 2-F signals of the normal and reverse forms, respectively, from an analysis of the heme methyl proton hyperfine shift pattern.[19] This assignment can be confirmed by measuring the ^{19}F—^{1}H NOE connectivity. ^{1}H NMR spectrum of met-cyano Mb reconstituted with 2-MF, at 25 °C, is illustrated in trace A of Fig. 4. An upfield-shifted methyl proton signal at −3.34 ppm has been assigned to Ile FG5 δ-CH_3 proton of the major form using measurements of the 2D scalar and dipolar connectivities (results not shown) and the corresponding signal for met-cyano Mb, reconstituted with mesohemin, is observed at −3.71 ppm (result not shown). The shift for the corresponding proton signal for native met-cyano Mb has been reported to be −3.46 ppm (pH 8.0, 25 °C).[22] The similarity in its shift between the reconstituted and native met-cyano Mbs indicates that the heme-protein interaction is not significantly altered by modifying of the heme peripheral substituents. Assuming that the heme-protein interaction in the Mb reconstituted with 2-MF is similar to that in the crystal structure of native Mb,[23] fluorine atoms in the normal and reverse forms are expected to be located at ca. 1.18 and ca. 0.40 nm, respectively, from the Ile FG5 δ-CH_3 proton. As shown in trace 4C, the irradiation of the Ile FG5 δ-CH_3 proton signal exhibits a negative NOE only to peak b_2 (trace 4C). The observed NOE connectivity unambiguously confirms the assignments of signals b_1 and b_2 to the 2-F signals of the normal and reverse forms, respectively. With the unambiguous signal assignment, it is now concluded that the reverse orientation of 2-MF is indeed thermodynamically more stable than the normal form. The ratio of the intensity between the two signals in the spectrum of the freshly reconstituted Mb is approximately 2.3 in trace 3B. The signal for the reverse form increases at the expense of that of the normal form. The splitting between peaks b_1 and b_2, due to the heme orientational disorder, is 27.61 ppm at 25 °C; this value is similar to the shift difference, 30.63 ppm at 25 °C, between the two ^{19}F signals observed in the spectrum of met-cyano Mb reconstituted with 13,17-bis(2-carboxylatoethyl)-3,7-difluoro-2,8,12,18-tetramethylporphyrinatoiron(III) (3,7-DF),[17] which possesses fluorine atoms at the 3- and 7-positions and methyl groups at the 2- and 8-positions as side-chains (see the numbering system in Fig. 1). The Curie plots for the signals in trace 3B exhibit straight lines with positive slopes in the temperature range examined, 5—35 °C (Table 1).

Similarly, two peaks, a_1 and a_2, are observed in the spectrum of the reconstituted carbonmonoxy Mb, illustrated in trace 3A. Their shifts are close to the value of −70.53 ppm for the ^{19}F signal of the iron-free porphyrin dimethylester in C^2HCl_3 at 25 °C (spectrum not shown). With an assumption that the preference in the heme orientation relative to the surrounding protein for carbonmonoxy Mb is similar to that for met-cyano Mb, peaks a_1 and a_2 in the spectrum are attributed to the reversed and normal forms, respectively. The difference in the relative intensity between the two signals in traces 3A and 3B probably results from equilibration of the heme reorientation reaction.

The value at $T^{-1}\rightarrow 0$ of the Curie plot for a signal of ferric low-spin met-cyano Mb is expected to represent the shift of the corresponding signal of diamagnetic Mb. Although there is a large difference between the intercept of the Curie plot for the signal of the reconstituted met-cyano Mb and the observed shift of the corresponding signal of the reconstituted carbonmonoxy Mb, the shift pattern, such that the signal for the reverse form is downfield-shifted relative to that of the normal form, is predicted from the intercepts, i.e., the shift, −82.2 ppm, for the reverse form is larger than that for the

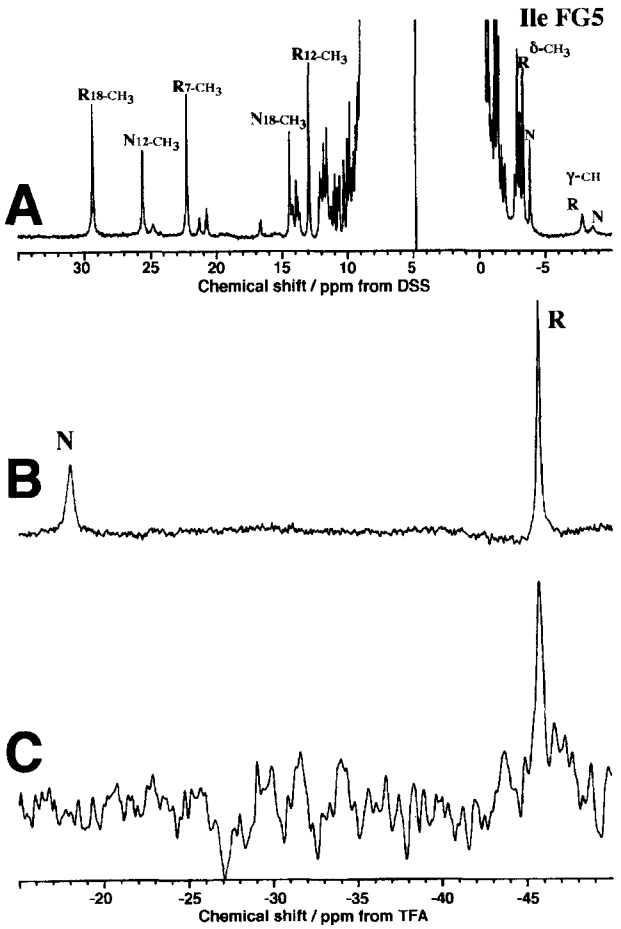

Fig. 4. The 400 MHz ^{1}H NMR (A) and the 376 MHz ^{19}F NMR (B) spectra of the met-cyano Mb reconstituted with 2-MF, in ^2H_2O, pH 8.03, at 25 °C and the NOE difference spectrum (C) resulted from the saturation of the Ile FG5 δ-CH_3 proton signal of the major form for 100 ms. The assignments of the Ile FG5 γ-CH and δ-CH_3 proton and heme methyl proton signals are shown in trace A and N and R indicate the signals for the normal and reverse forms, respectively. The observation of the NOE only to the ^{19}F signal at −45.68 ppm indicates that the heme in the reconstituted Mb is predominantly in the reverse from (B in Fig. 1).

normal form, −122.5 ppm.

Two broad signals, d_1 and d_2, are observed in the spectrum of the reconstituted met-azido Mb in trace 3D. Assuming that the reverse heme orientation is predominant in this Mb complex, peaks d_1 and d_2 are tentatively assigned to 2-F of the reversed and normal forms, respectively. The partial high-spin character in the met-azido Mb[24—26] and the contribution of Curie spin relaxation[27,28] render the line width of these signals to be much larger than those for the low-spin met-cyano form. The thermal spin equilibrium[24—26] in met-azido Mb is clearly manifested in negative slopes for their Curie plots (Table 1).

The spectrum of the reconstituted deoxy Mb is illustrated in trace 3E. Similarly to the cases for the other complexes, peaks e_1 and e_2 in trace 3E are tentatively assigned to 2-F of the reversed and normal forms, respectively. The shift difference of 32.82 ppm at 25 °C between the signals of the two forms in trace 3E indicates a large rhombic asymmetry of the heme electronic structure in the deoxy Mb.

The spectra of the reconstituted met-aquo Mb at 35 °C and pH values of 11.00 and 7.03 are shown in traces 3F and 3G, respectively, and an extremely broad signal is observed in each spectrum. The pK_a value of 8.5 obtained for the equilibrium between its acidic and basic forms of the reconstituted met-aquo Mb, obtained from a pH dependence study of its optical spectra, is slightly smaller than that of native Mb,[29] i.e., 9.0. Therefore, signals f_1 and g_1 are attributed to 2-F of the major component, i.e., the reverse form. Furthermore, signal f_1 shifts towards downfield with decreasing pH value (result not shown); the progressive pH-dependent shift of the signal between the shifts of f_1 and g_1 dictates that the rate of the transition between the acidic and basic forms in this Mb is $\gg 2.4\times10^4$ s^{-1} at 35 °C.

Discussion

Electronic Structure of Bis-Cyano 2-MF Complex. The difference between the ^{1}H NMR spectra of three bis-cyano hemin complexes can be interpreted in terms of the effect of the heme peripheral substituents on the heme electronic structure. The observed shift (δ_{obs}) of a paramagnetically shifted signal is given by the sum of the diamagnetic shift (δ_{dia}) and the paramagnetic shift (δ_{para}). Since the δ_{dia} values of the corresponding proton signals for the hemins used in the present study are expected to be similar to each other, the shift difference between the corresponding signals in the ^{1}H NMR spectra of bis-cyano complexes is largely attributed to δ_{para}, and can be interpreted in terms of the interaction of its proton with an unpaired electron. Generally, a contact shift (δ_c), due to a delocalized unpaired electron, and a pseudo-contact shift (δ_{pc}), due to a magnetic dipolar field arising from the unpaired electron, contribute to δ_{para} of the heme peripheral side-chain proton signals.[30] Despite the difference in the overall spectral pattern, the shift differences of the 13- and 17-CβH_2 proton signals are less than 0.2 ppm among the spectra of the hemin complexes. Since the δ_{pc} contribution is predominant to the δ_{para} values for these C$_\beta$H$_2$ proton signals, the similarity in their observed shifts indicates that the magnetic anisotropy is essentially unaltered by a modification of the heme peripheral side-chains. Furthermore, the smaller spread of the heme methyl and *meso* proton signals of a bis-cyano mesohemin complex than that of a bis-cyano protohemin complex reflects that the substitution of ethyl groups for the vinyl groups of the latter hemin enhances the C_4 symmetry of the heme electronic structure. Additionally, the substitution of a fluorine atom for the methyl group at the 2-position of mesohemin distorts the C_4 symmetry of the porphyrin π-system, as manifested in the difference in the shift pattern for the heme methyl and *meso* proton signals between traces 2A and 2B.

Although the in-plane asymmetry of the heme electronic structure of the 2-MF complex cannot be analyzed from its ^{19}F NMR spectrum, because of only a single substituted

fluorine atom, the effect of fluorine substitution on the delocalization of the unpaired electron from the iron to the porphyrin π-system is inferred from an analysis of the ^{19}F shift. The shift of −27.98 ppm at 25 °C for 2-F signal of the bis-cyano 2-MF complex is compared with the value of −18.15 ppm at 25 °C for the signal of the bis-cyano 3,7-DF complex.[17] Since the spectral parameters for the signals of the bis-cyano complexes depends on the sample concentration, because of a possible aggregation of the complexes, the observed shifts cannot be solely interpreted in terms of the electronic structure of the molecule. However, the similarity between the Curie intercepts for the bis-cyano 2-MF and 3,7-DF complexes (see Table 1) indicates that their δ_{dia} values are similar to each other. This conclusion is supported by the observation that the shifts for the ^{19}F signals of iron-free dimethylester of 2-MF and 3,7-DF are almost identical to each other, i.e., −70.53 and −71.22 ppm for the former and the latter, respectively (spectra not shown). Since the δ_{pc} value is not influenced by the heme peripheral side-chains, the shift difference of ca. 10 ppm between these signals is attributed to the difference in the δ_c contribution to the shift. For the shift of a signal arising from a fluorine atom bound to an aromatic carbon, the shifts due to spin polarization through the σ bond (δ_{sp}) and direct delocalization through π orbitals (δ_{dd}) contribute to its δ_c.[30,31] Both δ_{sp} and δ_{dd} are proportional to the unpaired electron density at the aromatic carbon, to which a fluorine atom is bound, although they are different in sign as well as in magnitude. The positive δ_{para} value for the observed ^{19}F signals indicates that the δ_{dd} contribution is predominant to the shift of the signal arising from the substituted fluorine atoms. The fact that the larger δ_{dd} shift for the signal of the bis-cyano 3,7-DF complex than that of the bis-cyano 2-MF complex indicates that the unpaired electron density delocalized from the iron orbital to the porphyrin π-system is larger for the former complex. This could be attributed to the difference in the number of the substituted fluorine atoms. Although the electron-withdrawing ability of the fluorine atom is expected to be diminished by a strong repulsive interaction between the electrons in the p orbitals of the fluorine atom and in the π orbital of the carbon atom, when a fluorine atom is bound to an sp^2 carbon,[32] the present result suggests that the delocalization of the unpaired electron from the heme iron to the π-system of porphyrin increases with the number of substituted fluorine atoms.

Molecular Structure of the Heme-Active Site in Reconstituted Mb. The van der Waals radius of the fluorine atom, 0.135 nm, is only slightly larger than that of the hydrogen atom, 0.11 nm. Therefore, the steric hindrance between the heme peripheral side-chains and the surrounding protein in the active site of the Mb reconstituted with 2-MF is expected to be smaller than that of the Mb reconstituted with mesohemin. Since δ_{para} of Ile FG5 δ-CH_3 proton signal is determined solely by δ_{pc}, the similarity in its shift for the three met-cyano Mbs investigated in the present study indicates not only that the spatial relationship between the Ile FG5 residue and the heme in the reconstituted Mbs is similar to that in native Mb, but also that the magnetic anisotropy of the unpaired electron is not influenced by the side-chain substitution of the heme. These results suggest that the active site structures, except for the difference in the orientation of the heme plane relative to the protein (see below), of the reconstituted and native Mbs are highly alike.

Heme Orientation in the Reconstituted Mb. The present study unequivocally demonstrated that the reverse orientation of the heme, B of Fig. 1, is predominant in the Mb reconstituted with 2-MF. The Mb reconstituted with mesohemin is favored in the normal form with the equilibrium ratio of 5.5±0.6 between the normal and the reverse forms.[33] Consequently, differences in the physicochemical properties between the methyl group and the fluorine atom at the 2-position appear to be a critical determinant for the major heme orientation in these reconstituted Mbs. The X-ray structure revealed that the heme side-chains at the 2-, 3-,7-, and 8-positions in the normal form are in close contact with (Val E10, Ala E14, Leu F4), (Leu E15, Leu G5, Ile G8, Ile G12, Phe H15), (Leu B13, Ile FG5, Tyr G4, Ile G8), and (Leu B13, Phe CD1, Thr C4, Lys C7, Ile FG5, Tyr G4), respectively.[23] All of the possible orientations of 2-MF and mesohemin at the active site of Mb are illustrated in Fig. 5. The experimental results revealed both that the energy level of 5B is lower than that of 5A and that 5C is energetically more stable than 5D. Considering the steric hindrance between the heme peripheral side-chains and the surrounding protein, the energy levels of 5A and 5B are expected to be lower than those of 5C and 5D, respectively, because of the difference in the size of the substituents at

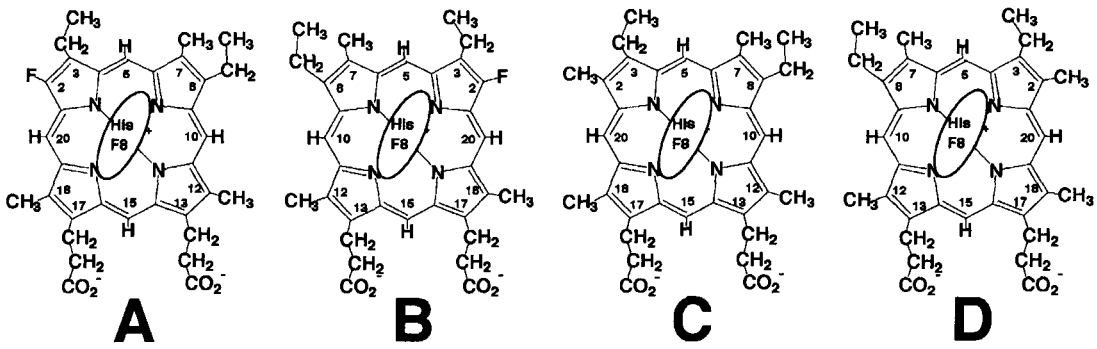

Fig. 5. Possible orientations of 2-MF and mesohemin relative to His F8. (A) Normal and (B) reverse forms of the Mb reconstituted with 2-MF and (C) normal and (D) reverse forms of the Mb reconstituted with mesohemin.

the 2-position. Consequently, these orientational states are aligned as 5B < 5A < 5C < 5D in order of increasing energy level. The small difference in their energy levels can be understood by the fact that the energy difference between 5B and 5D is solely accounted for by the difference in the steric hindrance between the protein and the fluorine atom or the methyl group at the heme 2-position in the reversed form. Furthermore, in the normal form, the protein-heme contacts at the heme 8- and 3-position exerts a larger steric hindrance than those at the heme 2- and 7-positions, respectively. Similar conclusions were derived from the results reported by Miki et al.,[34,35] which revealed that 8-isopropyl-3-vinyldeuterohemin (R_2 = –CH_3, R_3 = –CH=CH2, R_8 = –$CH(CH_3)_2$ in the heme structure of Fig. 1) possesses the reverse orientation in the active site of Mb, whereas 3,8-diisopropyldeuterohemin (R_2 = –CH_3, R_3 = R_8 = –$CH(CH_3)_2$) and 3-isopropyl-8-vinyldeuterohemin (R_2 = –CH_3, R_3 = –$CH(CH_3)_2$, R_8 = –CH=CH_2) are predominantly in the normal form.[35] The fact that 8-ethyldeuterohemin (R_2 = –CH_3, R_3 = –H, R_8 = –CH_2CH_3) is preferentially in the reverse form, whereas mesohemin and 3-ethyldeuterohemin (R_2 = –CH_3, R_3 = –CH_2CH_3, R_8 = –H) are in the normal form,[34] is also consistent with the present conclusion.

Electronic Structure of 2-MF in the Active Site of Mb. The presence of the two heme orientations of 2-MF, together with a fixed orientation of the axial ligands, and/or asymmetry of protein environment, results in the observation of two ^{19}F signals in the spectra of the reconstituted Mb. Since, according to the X-ray structure,[23] the 2-F atom of 2-MF in both the normal and reverse orientations is likely to be located at least 0.5 nm away from the center of gravity for nearby benzene rings of Phe side-chains, the separation of the ^{19}F signals observed in the spectrum of the carbonmonoxy Mb (trace 3A) is attributed to the intrinsic in-plane asymmetry of the electronic structure of 2-MF and an asymmetric bonding interaction between the heme iron and the axial His imidazole.[17,36–38] The shift difference of 5.69 ppm between the two signals of the carbonmonoxy Mb reconstituted with 2-MF (trace 3A) is twice as large as that between the ^{19}F signals observed for the carbonmonoxy Mb reconstituted with C_2-symmetric 3,7-DF, i.e., 2.39 ppm.[17] Hence, for the in-plane asymmetry of the electronic structure of 2-MF in the carbonmonoxy Mb, the contribution of the asymmetric Fe–His bonding would be almost comparable to that of the intrinsic asymmetry of the electronic structure.

Since studies on reconstituted met-cyano Mbs with a wide variety of chemically modified hemins have demonstrated that δ_{pc} is essentially independent of the heme side-chain substituents,[39] the value of −6 ppm for δ_{pc} of the 2-F signal can be calculated from the equation reported for native met-cyano Mb.[40] Consequently, using δ_{obs} of the corresponding signals in the spectrum of carbonmonoxy Mb (trace 3A) as δ_{dia}, the δ_c values of +24 and +57 ppm for the 2-F signals of the normal and the reverse forms, respectively, of met-cyano Mb are obtained from their δ_{obs} and the estimated δ_{pc} values. Thus, the shift difference of 27.61 ppm at 25 °C between the signals in trace 3B is largely attributed to the difference in their δ_c values. The estimated δ_c values indicate that the unpaired electron density at the pyrrole carbon bonded to the 2-F atom in the normal form is larger by more than two-fold compared with that in the reverse form. The large difference in line width between the two signals in trace 3B can be interpreted in terms of a ligand-centered dipolar contribution, which accounts for the modulation of the dipole–dipole interaction between the nucleus and the delocalized unpaired electron spins in both the fluorine and the pyrrole carbon to which fluorine is bonded, and the contact hyperfine coupling interaction.[41]

As has been shown from ^1H NMR studies of ferric high-spin model complex, σ spin delocalization contributes predominantly to δ_c, although the contribution from π spin delocalization cannot be neglected.[42,43] The fact that the shift difference between the 2-F signals for the normal and the reverse forms is smaller for met-azido form than for met-cyano form is interpreted as an indicative of predominance of σ spin delocalization to δ_c in the former Mb. Furthermore, the larger high-spin contents in met-azido Mb reconstituted with 2-MF than in the Mb reconstituted with 3,7-DF is manifested in the larger downfield shifts, the greater line widths, and the smaller separation of the two observed ^{19}F signals for the former Mb. Since the spin equilibrium in met-azido Mb is influenced by the Fe–His bonding interaction,[44,45] the larger high-spin contents in the Mb reconstituted with 2-MF than in the Mb reconstituted with 3,7-DF indicates that the coordination of the axial His side-chain to Fe is weaker in the former Mb. The electron density at the heme iron is modulated by the electron-withdrawing fluorine substituents, and hence the differential Fe–His bonding interaction between the two reconstituted Mbs might be attributed to the difference of electron density at the heme iron between the two ring-fluorinated hemins, which would arise from the difference in the number of the substituted fluorine atoms to the porphyrin ring.

The paramagnetic shift of > 200 ppm for the ^{19}F signals of the reconstituted deoxy Mb is predominantly attributed to δ_c, although a small δ_{pc} contribution exists due to magnetic anisotropy arising from zero-field splitting.[46] The fact that the shift difference between the ^{19}F signals in deoxy Mb is only slightly larger than that in met-cyano Mb, despite the much larger downfield shift in the former, is interpreted as indicative of the predominance of the σ-delocalization mechanism over a π-mechanism for delocalization of the unpaired electron, as predicted by model studies.[43] The values of 149.66 and 182.48 ppm for 2-F signals of the normal and the reverse forms, respectively, of the deoxy Mb are close to the shifts of 154.47 a nd 173.76 ppm observed for 3- and 7-F signals of the deoxy Mb reconstituted with C_2-symmetric 3,7-DF.[17] This result strongly suggests that the in-plane asymmetry of the heme electronic structure in the deoxy form stems from asymmetric Fe–His bonding. Further, the ^{19}F NMR characterization of the heme electronic structure is expected to provide direct information on the orbital ground state and Fe–ligand bonding in this physiologically relevant form of Mb.

References

1 R. E. Dickerson and I. Geis, "Hemoglobin: Structure, Function, Evolution, and Pathology," The Benjamin/Cummings Publishing Co., Menlo Park, California (1983).

2 Y. Yamamoto, *Annu. Rep. NMR Spectrosc.*, **36**, 1 (1998).

3 G. N. La Mar, J. D. Satterlee, and J. S. De Ropp, "The Porphyrin Handbook," ed by K. M. Kadish, K. M. Smith, and R. Guilard, Academic Press, New York (2000), Vol. 5, p. 185.

4 G. N. La Mar, D. L. Budd, D. B. Viscio, K. M. Smith, and K. C. Langry, *Proc. Natl. Acad. Sci. U. S. A.*, **75**, 5755 (1978).

5 G. N. La Mar, Y. Yamamoto, T. Jue, K. M. Smith, and R. K. Pandey, *Biochemistry*, **24**, 3826 (1985).

6 K. B. Lee, G. N. La Mar, L. A. Kehres, E. M. Fujinari, K. M. Smith, T. C. Pochapsky, and S. G. Sligar, *Biochemistry*, **29**, 9623 (1990).

7 W. R. Light, R. J. Rholfs, G. Palmer, and J. S. Olson, *J. Biol. Chem.*, **262**, 46 (1987).

8 K. Gersonde, H. Sick, M. Overkamp, K. M. Smith, and D. W. Parish, *Eur. J. Biochem.*, **157**, 393 (1986).

9 Y. Yamamoto, T. Nakashima, E. Kawano, and R. Chûjô, *Biochim. Biophys. Acta*, **1388**, 349 (1998).

10 Y. Yamamoto and G. N. La Mar, *Biochemistry*, **25**, 5288 (1986).

11 F. A. Walker, D. Emrich, J. E. Rivera, B. J. Hanquet, and D. H. Buttlaire, *J. Am. Chem. Soc.*, **110**, 6234 (1988).

12 G. N. La Mar, P. D. Burns, J. T. Jackson, K. M. Smith, K. C. Langry, and P. Strittmatter, *J. Biol. Chem.*, **256**, 237 (1981).

13 J. Z. Wu, G. N. La Mar, L. P. Yu, K. B. Lee, F. A. Walker, M. L. Chiu, and S. G. Sligar, *Biochemistry*, **30**, 2156 (1991).

14 G. N. La Mar, N. L. Davis, D. W. Parish, and K. M. Smith, *J. Mol. Biol.*, **168**, 887 (1983).

15 B. D. Sykes and W. E. Hull, *Meth. Enzymol.*, **49**, 270 (1978).

16 J. T. Gerig, *Prog. NMR Spectrosc.*, **26**, 293 (1994).

17 Y. Yamamoto, Y. Hirai, and A. Suzuki, *J. Biol. Inorg. Chem.*, **5**, 455 (2000).

18 F. W. J. Teale, *Biochim. Biophys. Acta*, **35**, 543 (1959).

19 A. Suzuki, T. Tomizawa, T. Hayashi, T. Mizutani, and H. Ogoshi, *Bull. Chem. Soc. Jpn.*, **69**, 2923 (1996).

20 J. W. Buchler, "Porphyrins and Metalloporphyrins," ed by K. M. Smith, Elsevier Scientific Publishing Co., New York (1975), p. 181.

21 C. Yu, S. W. Unger, and G. N. La Mar, *J. Magn. Reson.*, **67**, 346 (1986).

22 S. D. Emerson and G. N. La Mar, *Biochemistry*, **29**, 1545 (1990).

23 G. S. Kachalova, A. N. Popov, and H. D. Bartunik, *Science*, **284**, 473 (1999).

24 T. Iizuka and M. Kotani, *Biochim. Biophys. Acta*, **167**, 257 (1969)

25 T. Iizuka and M. Kotani, *Biochim. Biophys. Acta*, **181**, 275 (1969).

26 T. Iizuka and M. Kotani, *Biochim. Biophys. Acta*, **194**, 351 (1969).

27 M. Gueron, *J. Magn. Reson.*, **19**, 58 (1975).

28 A. J. Vega and D. Fiat, *Mol. Phys.*, **31**, 347 (1976).

29 T. Iizuka and I. Morishma, *Biochim. Biophys. Acta*, **400**, 143 (1975).

30 I. Bertini and C. Luchinat, "NMR of Paramagnetic Molecules in Biological Systems," The Benjamin/Cummings Publishing Co., Menlo Park, California (1986), p. 19.

31 G. N. La Mar, "NMR of Paramagnetic Molecules, Principles and Applications," ed by G. N. La Mar, W. D. Horrocks, Jr., and R. H. Holm, Academic Press, New York (1973), p. 85.

32 R. D. Chambers, "Fluorine in Organic Chemistry," John Wiley & Sons, New York (1973).

33 G. N. La Mar, H. Toi, and R. Krishnamoorthi, *J. Am. Chem. Soc.*, **106**, 6395 (1984).

34 K. Miki, Y. Ii, M. Yukawa, A. Owatari, Y. Hato, S. Harada, Y. Kai, N. Kasai, Y. Hata, N. Tanaka, M. Kakudo, Y. Katsube, K. Kawabe, Z. Yoshida, and H. Ogoshi, *J. Biochem.*, **100**, 269 (1986).

35 K. Miki, S. Harada, Y. Hato, S. Iba, Y. Kai, N. Kasai, Y. Katsube, K. Kawabe, Z. Yoshida, and H. Ogoshi, *J. Biochem.*, **100**, 227 (1986).

36 R. G. Shulman, S. H. Glarum, and M. Karplus, *J. Mol. Biol.*, **57**, 93 (1971).

37 T. C. Traylor and A. P. Berzinis, *J. Am. Chem. Soc.*, **102**, 2844 (1980).

38 Y. Yamamoto, N. Nanai, R. Chûjô, and T. Suzuki, *FEBS Lett.*, **264**, 113 (1990).

39 U. Kolczak, J. B. Hauksson, N. L. Davis, U. Pande, J. S. de Ropp, K. C. Langry, K. M. Smith, and G. N. La Mar, *J. Am. Chem. Soc.*, **121**, 835 (1999).

40 S. D Emerson and G. N. La Mar, *Biochemistry*, **29**, 1556 (1990).

41 T. J. Swift, "NMR of Paramagnetic Molecules, Principles and Applications," ed by G. N. La Mar, W. D. Horrocks, Jr., and R. H. Holm, Academic Press, New York (1973), p. 53.

42 G. N. La Mar, "Biological Applications of Magnetic Resonance," ed by R. G. Shulman, Academic Press, New York (1979), p. 305.

43 H. M. Goff, "Iron Porphyrin," ed by A. B. P. Lever and H. B. Gray HB, Addison-Wesley Publishing Co., London (1983), Part I, p. 237.

44 Y. Yamamoto and G. N. La Mar, *Biochim. Biophys. Acta*, **996**, 187 (1989).

45 Y. Yamamoto, R. Chûjô, and T. Suzuki, *Eur. J. Biochem.*, **198**, 285 (1991).

46 C. M. Bougault, Y. Dou, M. Ikeda-Saito, K. C. Langry, K. M. Smith, and G. N. La Mar, *J. Am. Chem. Soc.*, **120**, 2113 (1998).