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^{1}H-NMR Study of Temperature-Induced Structure Alteration at the Active Site of Horse Heart Cytochrome c[1]

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The molecular structure of the active site of horse heart met-cyano cytochrome c, as a function of temperature, has been investigated using ^{1}H-NMR. A temperature dependence study of the NMR spectra revealed that one heme methyl proton resonance exhibits anti-Curie behavior, *i.e.*, the hyperfine shift increases with increasing temperature. Analyses of the average heme methyl proton hyperfine shift and the proximal His imidazole proton resonances indicated that the iron-His bonding interaction in this protein is essentially independent of temperature. Since such an anomalous temperature dependence of the heme methyl proton resonance disappears in met-cyano complex of a heme peptide prepared by enzymatic degradation of the protein [Smith, M. and McLendon, G. (1981) *J. Am. Chem. Soc.* 103, 4912-4921], the anti-Curie behavior observed for the heme methyl proton resonance in met-cyano cytochrome c is attributed to a rotational displacement of the heme about the iron-His bond relative to the protein moiety due to a temperature-dependent conformational alteration of the heme-protein linkage. Such rotational mobility of heme at the active site of a protein may be responsible for the anomalous temperature dependence of heme methyl proton hyperfine shifts reported for many c-type ferri cytochromes.

Key words: Curie plot, cytochrome c, hemoprotein, hyperfine shift, NMR.

The hyperfine shifts exhibited by paramagnetic hemoproteins have provided a wealth of information on the electronic and molecular structural properties of the active site (*1-3*). Among the hyperfine shifted signals observed in NMR spectra of paramagnetic hemoproteins, heme methyl proton and carbon signals (see the structure of heme in Fig. 1) are of considerable importance because their shifts sharply and directly reflect the heme electronic structure (*1, 4-7*). The fact that the spread of the four heme methyl proton signals in the protein is always much larger than that for the corresponding model complex (*1*) indicates that asymmetry of the heme electronic structure in the protein is mainly due to an asymmetric heme-protein linkage. It has been clearly demonstrated that, in the case of a ferric low-spin complex, the heme methyl hyperfine shift pattern is well correlated with the axial imidazole orientation with respect to the heme (*5, 7*).

The temperature dependence of heme methyl proton or carbon hyperfine shifts has been used to investigate the electronic and molecular structures of the active site as a function of temperature (*8-11*). Since there is no angular dependence in the delocalization of an unpaired electron from the porphyrin π-system to heme methyl, heme methyl proton hyperfine shifts generally obey the Curie law. There, however, are two cases, so far reported, where heme methyl proton shifts exhibit anti-Curie behavior, *i.e.*, the hyperfine shift increases with increasing temperature. One is the case where an external ligand with an intermediate field strength, such as N_3^-, imidazole, or OH^-, binds to ferric heme iron. Such a complex exhibits a thermal spin equilibrium between high-spin ($S=5/2$) and low-spin ($S=1/2$) states (*12-15*), and the heme methyl hyperfine shift pattern at a given temperature is mainly determined by the equilibrium population of the two spin states (*16-20*). The other is the case where dynamic averaging over multiple structures with somewhat different heme methyl hyperfine shift patterns takes place and the populations change with temperature (*9-11*). The active site of hemoproteins exhibits remarkable flexibility, as reflected in various dynamic properties (*8, 10, 21-32*). Since X-ray crystallography does not reveal any pathways for ligand entry in myoglobin (Mb) (*33, 34*), such dynamic flexibility is thought to be essential for the accommodation of ligands in the binding site of Mb (*35*). NMR of paramagnetic hemoproteins has been utilized in detailed analyses of a variety of dynamic processes of the heme pocket such as reorientation of amino acid side-chains near the heme (*24*), rotation of the heme about one of its pseudo-C_2 axes (*25, 26*), and rotation of the heme about the iron-His bond (*8, 10, 27-32*). Although the last two processes directly modulate the asymmetry of the heme electronic structure in the protein, the rotation of the heme about the pseudo-C_2 axis passing through α,γ-meso protons (see Fig. 1) cannot be responsible for the anti-Curie behavior of the heme methyl proton shift because it proceeds much slower than the NMR time scale (*25, 26*).

It has been reported that two heme methyl proton resonances in horse heart ferric cytochrome c (cyt c) exhibit anti-Curie behavior, whereas its heme methyl

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Abbreviations: Mb, myoglobin; cyt c, cytochrome c.

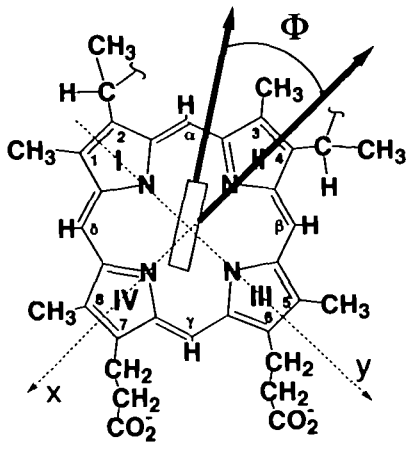

Fig. 1. **The dihedral angle, Φ is defined as the angle between the projection of the His18 imidazole plane onto the heme plane and the N_{III}-Fe-N_{IV} axis.** The rectangle represents the His18 imidazole plane. The *x* and *y* axes of an orthogonal coordination system are defined as shown in the figure and the *z* axis is perpendicular to the heme plane.

carbon resonances all obey the Curie law (*11*). Similar anti-Curie behavior of heme methyl proton resonances has been reported for other c-type hemoproteins (*11, 36-39*). It has been proposed that such anti-Curie behavior is due to the thermal mixing of two different electronic structures of the heme (*11*).

We examined in the present study the temperature dependence of heme methyl and the proximal His imidazole proton resonances in horse heart met-cyano cyt c in order to gain further insight into the temperature-induced structural alteration of the active site of this protein. The study revealed that anti-Curie behavior is retained by one heme methyl proton resonance in met-cyano cyt c. Analyses of the temperature dependence of both the average heme methyl proton hyperfine shift and the proximal His imidazole proton shifts have indicated that the iron-His bonding interaction in met-cyano cyt c is essentially independent of temperature. Since the ligation of CN^- to ferric heme iron provides a stable ferric low-spin complex, the anomalous temperature dependence of the heme methyl proton shift in met-cyano cyt c is interpreted in terms of a rotational displacement of the heme about the iron-His bond relative to the axial histidyl imidazole, *i.e.*, the dihedral angle, ϕ, defined in Fig. 1 changes with temperature. The proposed temperature-dependent structure alteration in met-cyano cyt c correlates well with the anomalous temperature dependence of the heme methyl proton shifts in ferric cyt c. The presence of rotational displacement of heme relative to the protein within the active site of c-type hemoproteins, together with rotation of the heme relative to the protein in various Mbs (*8, 27-32*), strongly suggests that the temperature dependence of the ϕ value is a general property of the active site of hemoproteins irrespective of the heme-protein interaction.

EXPERIMENTAL PROCEDURES

NMR sample preparation. Horse heart cyt c (type VI) was obtained from Sigma Chemical and used without further purification. Met-cyano cyt c was prepared in 50 mM phosphate buffer, pH = 7.22, in H_2O or ^2H_2O, with a 20-fold molar excess of KCN. The protein concentration was about 1 mM.

NMR measurements. ^{1}H-NMR spectra were recorded with a Bruker AC-400P FT-NMR spectrometer operating at a ^{1}H frequency of 400 MHz in the quadrature mode. A typical spectrum consisted of 2k transients with 8k data points over 25 kHz spectral width. The water resonance was suppressed with a 300 ms presaturation pulse. The signal-to-noise ratio was improved by apodization, which introduced 10 Hz line broadening. The spin-lattice relaxation time (T_1) was measured using the inversion-recovery method. The experimental errors in the obtained T_1 were estimated to be $< \pm 15\%$. The chemical shifts are given in ppm relative to sodium 2,2-dimethyl-2-silapentane-5-sulfonate with the residual H^2HO as an internal reference.

Temperature Dependence of the Hyperfine Shift for Heme Methyl Resonance—The observed shift (δ_{obs}) of a hyperfine shifted heme methyl proton or carbon resonance is expressed as in Eqs. 1 and 2,

$$\delta_{obs} = \delta_{dia} + \delta_{hf} \quad (1)$$

$$\delta_{hf} = \delta_c + \delta_{pc}^M + \delta_{pc}^L \quad (2)$$

where δ_{dia} is the diamagnetic shift, δ_{hf} the hyperfine shift, δ_c the contact shift, and δ_{pc}^M and δ_{pc}^L the metal- and ligand-centered pseudo-contact shifts due to the magnetic dipolar field arising from the unpaired electron at the heme iron and from the delocalized electron, respectively. In the case where a single spin level with an isotropic *g* tensor is populated and in the limit of validity of the Curie law, δ_c is given as in Eq. 3 (*40, 41*).

$$\delta_c = -(A/\hbar)(g\beta)S(S+1)/3\gamma_N kT \quad (3)$$

where *A* is the hyperfine coupling constant, $\hbar$ the Planck constant divided by 2π, *g* the electronic *g* factor, β the Bohr magneton, *S* the total electron spin quantum number, γ_N is the nuclear gyromagnetic ratio, *k* the Boltzmann constant, and *T* the absolute temperature. *A* is proportional to the unpaired electron density, ρ, at the carbon to which the heme methyl group is attached as follows,

$$A = Q\rho/2S \quad (4)$$

where *Q* is a constant, and its sign is negative for a heme methyl proton and positive for a heme methyl carbon because of the different mechanisms operating in delocalization of unpaired electron density from the porphyrin π-conjugated system to these nuclei (*42*).

δ_{pc}^M is expressed in terms of the magnetic susceptibility anisotropy (*40, 41*).

$$\delta_{pc}^M = (1/4\pi 2r^3)[(3\cos^2\theta - 1)(2\chi_{zz}/3 - \chi_{xx}/3 - \chi_{yy}/3) + \sin^2\theta\cos 2\Omega(\chi_{xx} - \chi_{yy})] \quad (5)$$

where χ_{ii}s are the principal components of the molecular susceptibility tensor, *r* the metal-nucleus distance, and θ and Ω the angles between the *r* and *z* axes and between the projection of *r* into the *xy* plane and the *x* axis, respectively. In the case of the $S = 1/2$ system, where zero-field splitting is absent (*41*), provided that the geometrical coordinates of the nucleus with respect to the molecular axes are independent of temperature, δ_{pc}^M is simply proportional to T^{-1}. Similarly, δ_{pc}^L is expressed in terms of the dipolar interaction between the nucleus and the delocalized unpaired electron, and, according to the first approximation, is

assumed to be directly proportional to ρ (*43, 44*). δ_{pc}^{M} and δ_{pc}^{L} are negative for both heme methyl proton and carbon resonances.

Since the temperature dependence of δ_{dia} can be quite small, compared with that of δ_{hf}, the slope of the Curie plot, *i.e.*, δ_{obs} *vs.* T^{-1}, is expressed as

$$\partial(\delta_{obs})/\partial(T^{-1}) = \partial(\delta_c)/\partial(T^{-1}) + \partial(\delta_{pc}^{M})/\partial(T^{-1}) + \partial(\delta_{pc}^{L})/\partial(T^{-1}) \quad (6)$$

As described above, for a heme methyl proton resonance, the first term of Eq. 6 is positive, whereas the last two terms are negative. Therefore, as long as $|\partial(\delta_{pc}^{M})/\partial(T^{-1}) + \partial(\delta_{pc}^{L})/\partial(T^{-1})| < |\partial(\delta_c)/\partial(T^{-1})|$, a Curie plot for a heme methyl proton resonance exhibits a positive slope. On the other hand, for a heme methyl carbon resonance, the Curie plot always exhibits a negative slope.

RESULTS

The 400 MHz ^{1}H-NMR spectrum of met-cyano cyt c in H_2O, pH = 7.22, at 40°C is illustrated in Fig. 2. Four heme methyl proton resonances are resolved in the downfield hyperfine shifted region and the assignments (*6*) are given with the spectrum. On the basis of model compound studies (*1, 45-47*), the broad signal at −10.60 ppm can be assigned to the proximal His 2-H proton resonance (*48*), and the similar broad signal in the downfield hyperfine shifted region most likely arises from the proximal His 4-H proton. The exchange of the solvent from H_2O to ^2H_2O leads to the disappearance of the single proton resonance at 14.89 ppm (see the spectrum of met-cyano cyt c in ^2H_2O, p^{2}H = 7.22, at 40°C illustrated in the inset). The T_1 value of this exchangeable proton resonance is 37 ms. Using the T_1 value of the heme methyl proton, 120-140 ms, and its known metal-proton distance, this exchangeable proton should be located at 0.49-0.50 nm from the heme iron. Hence this proton is assignable to the $N_\delta H$ proton of the proximal His.

Curie plots, δ_{obs} *vs.* reciprocal of absolute temperature, for heme methyl proton and proximal His imidazole proton resonances are shown in Fig. 3, A and B, respectively. The plots exhibit straight lines in the temperature range examined. Although the ligation of a CN^- ion to ferric hemoprotein provides a stable ferric low-spin complex, the heme 3-Me proton resonance exhibits anti-Curie behavior, *i.e.*, δ_{hf} increases with increasing temperature. The slopes and intercepts to $T^{-1} \rightarrow 0$ of the Curie plots in Fig. 3 are summarized in Table I. Provided that the heme electronic structure is independent of temperature, the slope of the Curie plot for the heme methyl proton resonance is roughly proportional to its δ_{hf}. The orders for not only the slope, *i.e.*, 3-Me < 0 < 8-Me < 1-Me < 5-Me, but also the intercept, *i.e.*,

TABLE I. **Slopes and intercepts to $T^{-1} \rightarrow 0$ for heme methyl proton and carbon, and His18 imidazole proton resonances in horse met-cyano cyt c and ferric cyt c.**

Met-cyano cyt c	^{1}H		
Heme methyl	Slope[b]	Intercept[c]	δ_{dia}[d]
1-Me	5.9	−3.2	3.46
3-Me	−2.0	17.9	3.84
5-Me	10.1	−10.6	3.58
8-Me	3.0	11.7	2.16
Average shift	4.2	4.0	3.26
His18			
$N_\delta H$	0.3	14.0	9.61
2-H	−11.8	27.2	0.41
4-H	18.9	−40.2	0.13

Ferric cyt c[a]	^{1}H			^{13}C		
Heme methyl	Slope[b]	Intercept[c]	δ_{dia}[d]	Slope[b]	Intercept[c]	δ_{dia}[d]
1-Me	−2.7	16.0	3.46[e]	−4.0	−4.2	13.86[g]
3-Me	8.3	4.4	3.84[f]	−21.0	15.7	13.74[g]
5-Me	−3.7	22.2	3.58[e]	−6.5	−2.7	13.51[g]
8-Me	10.7	−0.74	2.16[f]	−30.0	31.0	11.56[g]

[a]Taken from Ref. *58*. [b]Given in ppm × 10^3 K. [c]Intercept, in ppm, at infinite temperature in a Curie plot. [d]Diamagnetic shift in ppm: shifts in ferrous cyt c at pH 7.6 and 30°C. [e]Taken from Ref. *59*. [f]Taken from Ref. *60*. [g]Taken from Ref. *61*.

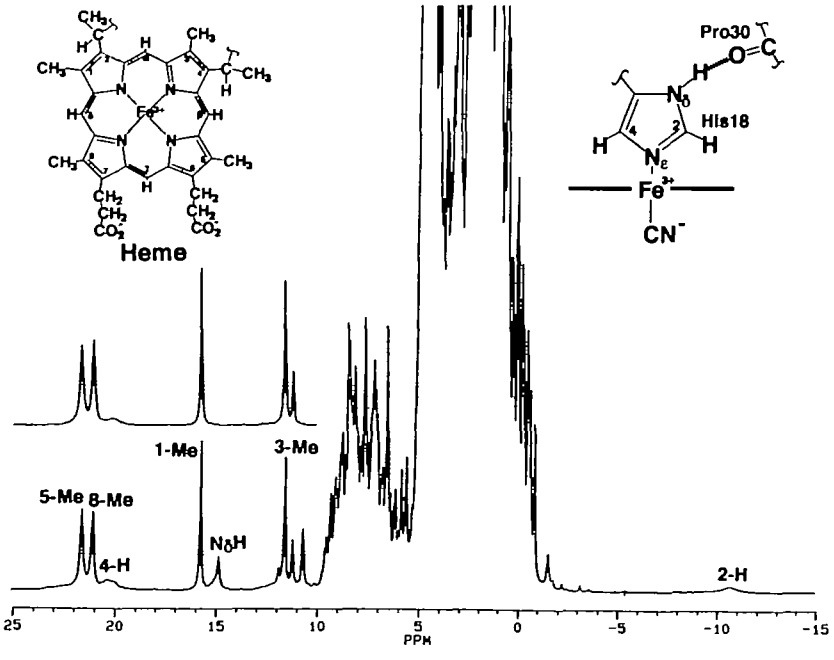

Fig. 2. **The 400 MHz ^{1}H-NMR spectrum of horse heart met-cyano cyt c in H_2O, pH 7.22, at 40°C.** The assignments of the heme methyl proton (*6*) and His18 imidazole proton resonances are indicated in the spectrum. The downfield hyperfine-shifted portion of met-cyano cyt c in ^2H_2O, p^{2}H 7.22, is illustrated in the inset. The exchangeable single-proton peak at 14.89 ppm was assigned on the basis of its T_1 value (see text). The T_1 value of another exchangeable proton resonance resolved at 10.7 ppm indicates that this proton is located > 0.6 nm from the heme iron. The structure and numbering system for heme (left) and His18 imidazole (right) are shown in the inset.

5-Me<1-Me<0<8-Me<3-Me, clearly indicate that 3-Me and 8-Me, and 1-Me and 5-Me resonances experience downfield- and upfield-shift biases, respectively, with increasing temperature. The Curie plot of the average shift for heme methyl proton resonances exhibits a straight line with an intercept of 4.0 ppm, this value being close to the δ_{dia} value for the heme methyl proton resonances in cyt c (*49*). Consequently, the unpaired spin density delocalized to the π-system of the porphyrin ring appears to be almost independent of temperature.

The proximal His imidazole proton resonances exhibit characteristic Curie plots. Their intercepts to $T^{-1}\rightarrow 0$ are not close to the δ_{dia} values for the corresponding resonances. The large negative slope and the intercept of 27.2 ppm for the 2-H proton indicate that this proton experiences a downfield-shift bias with increasing temperature. The small temperature dependence for the $N_\delta H$ proton also indicates a downfield-shift bias for this proton with increasing temperature. On the other hand, both the large positive slope and the intercept of -40.2 ppm for the 4-H proton demonstrate an upfield-shift bias for this proton with increasing temperature. Since analysis of the average shift for heme methyl proton resonances indicated that the unpaired electron density in the porphyrin π-system is essentially independent of temperature, the temperature dependence of the His18 imidazole proton shifts strongly suggests that the orientation of His18 imidazole with respect to the heme and/or the electronic structure of His18 imidazole changes with temperature. The former structural change induces alteration in both δ_c and δ_{pc}^M for the His18 imidazole proton resonances, and the latter directly modulates their δ_c values.

DISCUSSION

Temperature Dependence of Heme Methyl Proton Hyperfine Shifts in Met-cyano cyt c—Heme methyl proton resonances are resolved in the downfield hyperfine shifted region of a spectrum, and their shift pattern, *i.e.*, 5-, 8-, 1-, and 3-Me, in order of decreasing shift, is roughly consistent with the Φ value of 35° (*50, 51*), where one unpaired electron resides mainly in the d_{yz} orbital (*5*). Since the unpaired electron resides in the highest energy d_x orbital, the interaction with the p_z orbital of the axial imidazole plays an important role in determining the energy levels of the d orbitals (*5*). With the ferric low-spin state of the d^5 configuration, one unpaired electron resides in either the d_{xz} or d_{yz} orbital, whichever has the highest energy.

Equation 6 predicts that the slope of a Curie plot is proportional to δ_{hf} among heme methyl proton or carbon resonances. But, shown in Table I, heme methyl proton hyperfine shifts do not correlate with the slope of their Curie plots. Furthermore, the shift pattern is strongly influenced by temperature. The Curie plots in Fig. 3A show that d_{yz} is the highest d_x orbital at low temperature ($0.04<T^{-1}$), and d_{xz} is that at high temperature ($T^{-1}<0.02$). These results clearly indicate that the orientation of the axial histidyl imidazole with respect to the heme is influenced by temperature. Assuming a Φ value of 35° (*50, 51*) at ambient temperature, the temperature dependence of the heme methyl proton shift pattern dictates that the Φ value tends to increase, *i.e.*, the imidazole plane rotates counter clockwise about the iron-His bond, when the viewer is on the His18 side, with increasing temperature. Since the hydrogen-bond between $N_\delta H$ of His18 and C=O of Pro30 plays an important role in determining the Φ value (*50, 51*), the alteration of the Φ value would be induced through a conformational change of the protein moiety. Additionally, although the change in the Φ value observed in the present protein cannot be quantitated, it is expected to be quite small because of the presence of the covalent bond between the heme and the protein in c-type hemoproteins. Knowledge about the conformational stability of the heme-protein linkage is needed for detailed analysis of the temperature-induced structure alteration in the active site of met-cyano cyt c induced by temperature.

The heme methyl proton resonance for the met-cyano form of a hemoprotein generally follows the Curie law. But the 8-Me proton resonance of the mollusc *Dolabella* met-cyano Mb was found to exhibit anti-Curie behavior (*10*). This result was interpreted as reflecting a rotational displacement of the heme about the iron-His bond with respect to the protein. Since heme propionates are not bonded to the protein *via* salt bridges in this Mb, the presence of such rotational mobility of the heme in the heme pocket of *Dolabella* Mb was attributed to the lack of

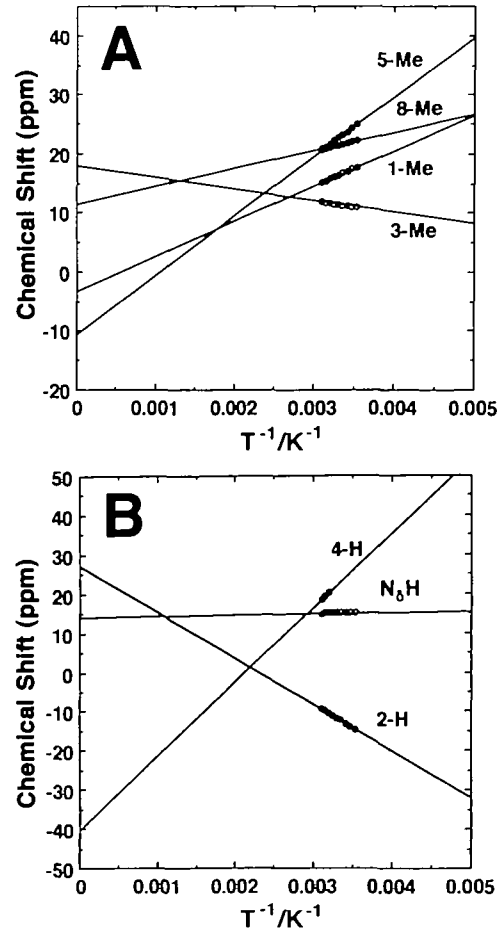

Fig. 3. **Curie plot, δ_{obs} *vs.* reciprocal of absolute temperature, for the heme methyl proton (A) and His18 imidazole proton (B) resonances of horse heart met-cyano cyt c in H_2O, pH 7.22.** Due to overlapping with other signals, the shift of the 4-H signal at <35°C cannot be obtained from the spectrum.

propionate-protein linkages. The dynamic properties of the heme with respect to rotation about the iron-His bond have been exhaustively investigated for Mb reconstituted with synthetic hemes that do not possess any propionate side-chains (*8, 27-29*). It has been proposed on the basis of studies on reconstituted Mbs with several hemes possessing a wide variety of peripheral substituents that such rotational mobility of heme is a general feature of the heme pocket, even when salt-bridges between propionates and the protein are present (*30-32*). Although the conformational state of the axial His side-chain as well as the Φ value in c-type hemoproteins is expected to be rather restricted, compared with in b-type hemoproteins, due to the presence of the covalent bonds between the heme and protein, the present study clearly demonstrated that the Φ value in met-cyano cyt c is influenced by temperature. Rotation of the heme, with respect to the protein, in the heme pocket of c-type hemoproteins would be induced by a conformational alteration of the heme-protein linkage rather than the torque induced about the iron-His bond, which is probably a major driving force for the heme rotation in b-type hemoproteins (*8, 27-32*). This conclusion is strongly supported by the fact that the removal of most of the protein moiety from cyt c abolishes the anti-Curie behavior of the 3-Me proton signal (*6*).

Temperature Dependence of the Axial His Proton Shifts in Met-cyano cyt c—The signal assignments of the axial imidazole 2-H and 4-H protons were obtained from their characteristic shifts and line widths, and that of the $N_\delta H$ proton was assigned from T_1 measurements. The Curie plots for these resonances indicate that the electronic structure and/or orientation of the axial imidazole with respect to the heme are influenced by temperature. In the absence of the zero-field splitting for the ferric low-spin complex (*41*), their anomalous Curie plots cannot arise from the inverse second power dependency of δ_{pc}^{M} on T. The hydrogen-bonding between His18 $N_\delta H$ and Pro30 C=O contributes significantly to δ_c of the $N_\delta H$ proton resonance, and some imidazolate character of the axial ligand has been confirmed by ^{15}N-NMR data (*52, 53*). The deprotonation of His18 $N_\delta H$ due to strong hydrogen-bonding with Pro30 C=O results in a decrease of δ_c for the $N_\delta H$ proton shift, with a concomitant increase of δ_c for the 2-H and 4-H proton shifts (*54*). The small temperature dependence and the intercept of 14.0 ppm for the Curie plot of the $N_\delta H$ proton, which are indicative of a downfield-shift bias with increasing temperature, indicate that the hydrogen-bond between His18 and Pro30 is retained over the temperature range examined. Furthermore, the fact that 2-H and 4-H protons exhibit opposite shift biases with changing temperature is not consistent with the alteration of the basicity of the axial imidazole through the His18-Pro30 hydrogen bonding. Hence the anomalous temperature dependence of the His18 imidazole proton resonance is likely to arise from the temperature-dependent His18 imidazole orientation with respect to the heme. As has been demonstrated for other ferric low-spin hemoproteins, the axial imidazole proton resonances experience significant δ_{pc}^{M} contributions (*55, 56*). The alteration of the His18 coordination structure would also lead to the modulation of the principal magnetic axes, which in turn largely alters δ_{pc}^{M} for the axial imidazole proton resonances. In spite of the presence of the covalent bonds between the heme and protein in c-type hemoproteins, the axial imidazole coordination structure was found to be altered by temperature. It is not clear at present whether this conformational alteration of the axial imidazole ligation results from a simple thermal fluctuation involving rapid motions within a given potential well or a jump between different potential wells.

Relevance to Ferric cyt c—It has been shown for horse ferric cyt c that the furthest upfield hyperfine shifted heme methyl proton resonance exhibits anti-Curie behavior (*36*), whereas all the four heme methyl carbon resonances obey the Curie law (*11*). Furthermore, anti-Curie behavior of the furthest upfield shifted heme methyl proton resonances has been reported for a variety of other c-type hemoproteins (*11, 36-39*). The anti-Curie behavior of the heme methyl proton resonances in ferric cyt c has been attributed to thermal mixing of the contributions from both d_{xz} and d_{yz} orbitals due to a relatively small energy gap between the two orbitals (*11*).

The heme methyl ^1H hyperfine shift pattern, *i.e.*, 8-, 3-, 5-, and 1-Me, in order of decreasing hyperfine shift in horse ferric cyt c (*49*), is completely different from that for the corresponding met-cyano complex, and is largely due to the lifting of the energy of d_{xz} orbital due to the interaction with lone pair electrons of the coordinated sulfur atom (*57*). Hence the heme electronic structure in ferric cyt c is predominantly determined by the coordination of Met80. The anti-Curie behavior of 1-Me and 5-Me proton resonances of horse ferric cyt c is apparent from their negative slopes (see Table I), indicating that the unpaired electron density for pyrroles I and III increases with temperature. Assuming that a similar conformational change in the Φ value takes place in both horse ferric cyt-c and its met-cyano derivative, clockwise rotational displacement of the heme, as we look down the heme from the His18 side, relative to the protein results in simultaneous rotation of the lone pair of sulfur atom about the Fe-S bond. This heme motion influences the delocalization of unpaired electron density in the porphyrin π system through the modulation of the interaction between the lone pair of sulfur atom and d orbitals of the heme iron, and hence leads to the anomalous temperature dependence on heme peripheral side-chain proton resonances in ferric cyt c.

The anti-Curie behavior observed for the heme methyl proton resonance stems from the competition in the temperature dependence between δ_c and $\delta_{pc}(=\delta_{pc}^{M}+\delta_{pc}^{L})$. Since both δ_c and δ_{pc} are negative for the heme methyl carbon resonance, the slope of its Curie plot is always negative. The temperature-dependent electronic structure of the heme in ferric cyt c, however, is also reflected in the slopes and intercepts of the Curie plots for the heme methyl carbon resonances. As shown in Table I, the slopes of the Curie plots for 1-Me and 5-Me carbon resonances are considerably smaller in magnitude than those for 3-Me and 8-Me carbon resonances. Additionally, the intercepts of 1-Me and 5-Me carbon resonances are negative, whereas those of 3-Me and 8-Me carbon resonances are positive. These results dictate that, with increasing temperature, the unpaired electron density in pyrroles I and III increases, and concomitantly that in pyrroles II and IV decreases. Such alteration in the heme electronic structure is completely consistent with the conclusion drawn from the results of analyses of the Curie plots of the heme methyl proton shifts.

Temperature Dependence of the Axial Coordination Structure in the Active Site of Hemoprotein—An X-ray crystallographic study provides the ground state structures of a variety of hemoproteins. But the effects of solution conditions such as temperature, pH and ionic strength on the molecular structure cannot be inferred from the X-ray coordinates. The present study clearly demonstrated that the orientation of the heme relative to the protein moiety in horse heart met-cyano cyt c is influenced by temperature. The anti-Curie behavior of heme methyl proton resonances observed for other c-type hemoproteins may also be due to similar temperature-dependent structure changes at the active site. The occurrence of the heme rotational displacement about the iron-His bond in b-type hemoproteins as well as c-type proteins, as found in the present study, strongly suggests that such heme mobility is common to all hemoproteins. Although the functional significance of such heme rotation is not clear, it is likely to be related to the dynamic nature of the active sites of these proteins.

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