Analysis of the Temperature Dependence of the ¹H and ¹³C Isotropic Shifts of Horse Heart Ferricytochrome *c*: Explanation of Curie and Anti-Curie Temperature Dependence and Nonlinear Pseudocontact Shifts in a Common Two-Level Framework

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Abstract: The ¹H and ¹³C hyperfine shifts of the heme methyls of horse heart ferricytochrome *c* have been measured over the temperature range 278−328 K in order to interpret the "anomalous" temperature-dependence of the hyperfine shifts in terms of their pseudocontact and contact shifts. By taking advantage of the available pseudocontact shifts for protein nuclei measured at 303 and 323 K (Santos, H.; Turner, D. L. *Eur. J. Biochem.* **1992**, *206*, 721−728), the metal-centered pseudocontact shifts have been analyzed in terms of a thermally accessible excited state lying 355−590 cm⁻¹ to higher energy which has a magnetic susceptibility tensor with the rhombic anisotropy, $\Delta\chi_{rh}$, which is rotated by 90° to that of the ground state. The metal-centered pseudocontact shifts have been evaluated at all temperatures at which the chemical shifts were measured, and these calculated values were used to evaluate the contact shifts of each heme methyl for the two nuclei. The temperature dependence of the heme methyl contact shifts for both ¹H and ¹³C, assuming a thermally accessible excited state, was then used to evaluate the spin density for the four β-pyrrole heme carbons to which the methyls are attached. The ligand-centered pseudocontact shifts have been estimated and found to give a modest contribution to the experimental behavior. The ¹H and ¹³C data are highly self-consistent. The present analysis provides deep insight into the electron distribution on the porphyrin ring in low-spin Fe(III) hemes.

Introduction

Since Kowalsky's pioneering report of the 56.4-MHz proton NMR spectrum of horse heart cytochrome *c* in 1962[1] there have been many extensive studies of various aspects of the NMR spectra of a number of cytochromes *c*. These studies have included assignments of the ¹H[1−11] and ¹³C[12−16] heme resonances of both the reduced and oxidized forms of the proteins, assignment of the ⁵⁷Fe resonance of the reduced protein,[17] assignment of the majority of the protein ¹H resonances and determination of three-dimensional protein structure of cytochromes *c* from several different organisms,[18−22] and studies of protein dynamics, especially protein folding/unfolding.[23−25]

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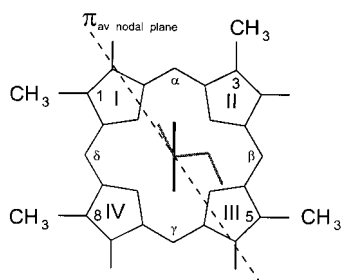(19) Qi, P. X.; Beckman, R. A.; Wand, A. J. *Biochemistry* **1996**, *35*, 12275−12286.

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Chart 1

Figure 1. Schematic drawing reporting the possible cases of temperature dependences of a hyperfine shifted signal. The sign, the slope, and the sign of the intercept univocally identify a Curie, hypo-Curie, hyper-Curie, and non-Curie type temperature dependences (see text).

Wüthrich and co-workers first showed, using ^{1}H NMR spectroscopy, that there are two types of methionine-bound type I cytochromes *c* from bacterial sources which differ in the chirality of the bound methionine and thus produce large differences in the shifts of the heme substituents.[2-9] The imidazole and methionine axial ligands remove the pseudoaxial symmetry of the Fe−heme moiety, which is shown in Chart 1; the d_{xz} and d_{yz} orbitals, which are degenerate in axial symmetry, are split by the combined effect of the p_π orbitals of imidazole and methionine. The average nodal plane of these ligands determines the molecular *x* and *y* directions by giving rise to linear combinations of the porphyrin π orbitals and metal d_π orbitals of the type[26]

$$\phi_1 = \cos\beta(|d_{xz}\rangle + |\pi_x\rangle) + \sin\beta(|d_{yz}\rangle + |\pi_y\rangle)$$

$$\phi_2 = \cos\beta(|d_{yz}\rangle + |\pi_y\rangle) - \sin\beta(|d_{xz}\rangle + |\pi_x\rangle) \qquad (1)$$

where β is the angle between the mean axial ligand nodal plane and the reference axis system of the heme. The heme macrocycle tends to fix the *x* and *y* axes along the Fe-pyrrole nitrogen axes, whereas the p_π orbitals of the axial ligands tend to move them toward directions which depend on the β angle which describes the linear combination of the d_{xz} and d_{yz} orbitals that is aligned with the nodal planes along (and perpendicular to) the average plane orientation of the axial ligands. Karplus,[27] and, more recently, Turner[16,28] have shown that the spin density distribution on the heme carbons depends on this angle and on the energy separation ΔE_π between ϕ_1 and ϕ_2. These very same parameters also determine, with a counterrotation rule, the molecular axes of the magnetic susceptibility tensor.[28,29] In turn, magnetic susceptibility anisotropy is the origin of the pseudo-contact shifts. Therefore, the origin of the hyperfine shifts (the chemical shifts of the paramagnetic oxidized species minus the chemical shifts of the diamagnetic reduced species), both contact and metal-centered pseudocontact, in low-spin Fe(III) cytochromes, is in principle well understood. This description holds as long as the molecular *z* axis is perpendicular to the heme plane, which has been found to be an acceptable approximation in many cases.[22]

As expected, the signs of the hyperfine shifts of protons and carbons of methyl heme substituents are different: proton methyl hyperfine shifts are downfield and carbon methyl hyperfine shifts are upfield. Indeed, (i) the contact shifts mainly arise
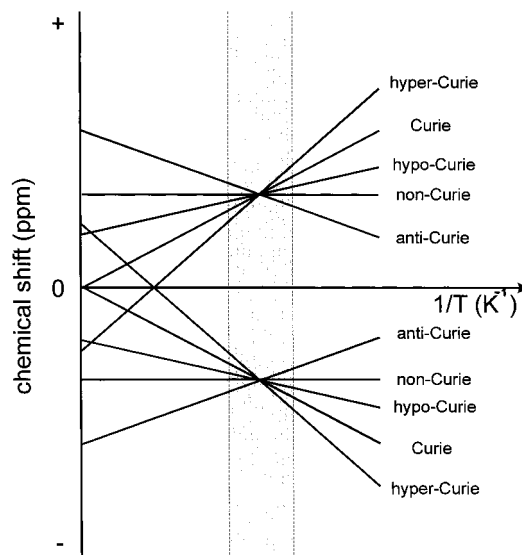
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from unpaired spin density in p_z orbitals of pyrrole carbons, and thus, for carbons are of opposite sign to attached protons, and (ii) the contact shifts dominate over the metal-centered pseudocontact shifts, and the latter are negative for both nuclei.[22] A peculiarity of the hyperfine shifts of ^{13}C and ^{1}H nuclei of the four methyl groups of the heme moiety is that the slope of the temperature dependence of carbons and of their attached protons is different: Most of the hyperfine shifts of the heme substituent nuclei do not intercept zero in linear $1/T$ plots, as would be expected from the Curie law. Some of them have larger slopes and, therefore, intercepts of opposite sign to the shifts; some others have smaller slopes (intercepts of the same sign) or even slopes of opposite sign to the shifts (so-called anti-Curie behavior). Because the use of terms such as Curie, non-Curie, and anti-Curie temperature dependence is not unequivocal in the literature, the observed behaviors and the terminology used in this paper are summarized in Figure 1. According to this terminology, in low-spin Fe(III) porphyrin centers, particularly the cytochromes *c*, methyl carbons 8 and 3 are hyper-Curie, methyl carbons 5 and 1 are hypo-Curie, methyl protons 8 and 3 are approximately Curie, and methyl protons 5 and 1 are anti-Curie (see below, Figure 2). All of these definitions are based upon the extrapolated (linear) temperature dependences, as are apparent from the measurements made over ambient temperatures, i.e., those over which heme proteins are stable. We feel that it is worth addressing these fine details, which may provide a deeper insight into the unpaired electron distribution in the heme ring. Our approach is based on the following assumptions: (i) there are two states described by wave functions ϕ_1 and ϕ_2, which are separated by an energy ΔE_π; (ii) there are two magnetic tensors associated with ϕ_1 and ϕ_2; (iii) the proton and carbon shifts of heme methyl substituents depend on the spin density in the p_z orbital of the pyrrole carbon to which the methyl is attached. The experimental data to be fit are the hyperfine shifts of the four methyl carbons and protons at 11 temperatures between 278 and 328 K for ^{1}H and six temperatures in the same range for ^{13}C. The experimental magnetic susceptibility anisotropies are available at 303 and 323 K.[14] The results of this analysis are satisfactory in terms of the fitting of the experimental data and provide information on the product
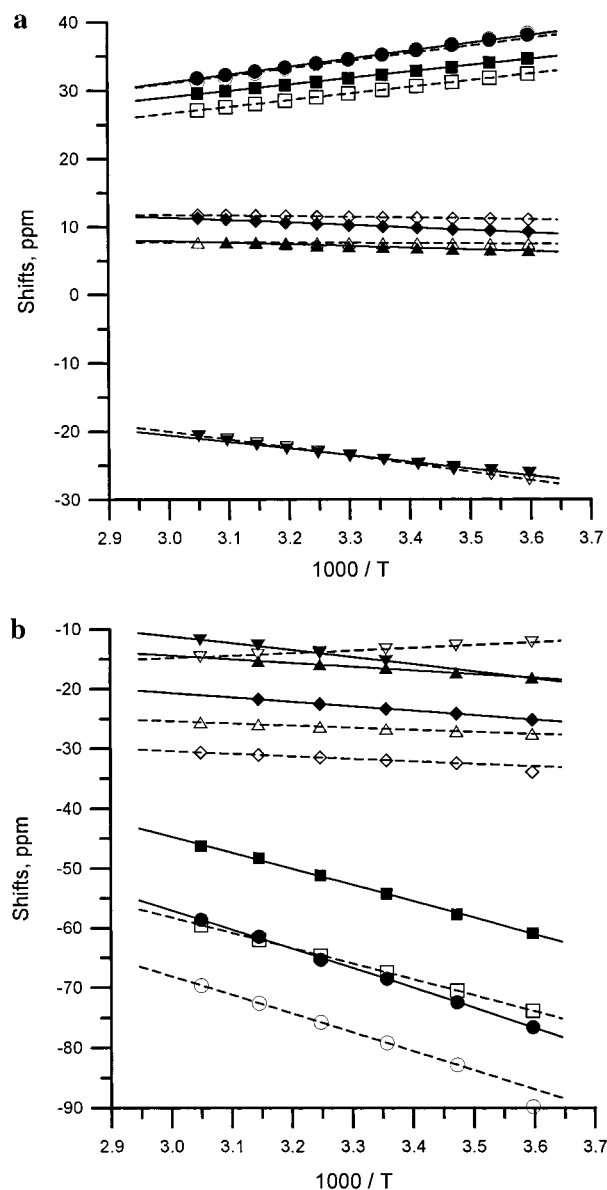
a

b

Figure 2. Curie plot of the experimental (closed symbols) and contact (open symbols) shifts of (a) the ^1H and (b) the ^{13}C resonances of the heme methyls: ▲, 1-Me; ■, 3-Me; ◆, 5-Me; ●, 8-Me; ▼, Met-Me.

chrome b_5[33] have been satisfactorily analyzed with a two-level model and partial estimate of the pseudocontact contribution.[34] The energy differences ΔE_π calculated by this fitting procedure for these two ferriheme proteins and several model ferriheme systems are eminently reasonable in terms of d-orbital energy differences calculated from EPR g values.[34] However, the issue of the apparently different behavior of ^1H and ^{13}C resonances in the same compound, and in particular the different temperature dependence, has not been addressed in detail or understood.

In this work, we have found that ^1H and ^{13}C hyperfine shifts are indeed consistent with each other and that a part of the reason for the anti-Curie temperature dependence of the ^1H shifts of two methyl groups as compared to the hypo-Curie temperature dependence of the ^{13}C shifts of the same methyl groups is due to the opposite signs of the metal-centered pseudocontact and contact shifts for ^1H. If properly analyzed, hyperfine shifts of either nucleus can be used to obtain the electron densities at the pyrrole carbons. A modest contribution of the ligand-centered dipolar term to the ^{13}C hyperfine shifts is found to be consistent with the data.

Experimental Section

Horse heart cytochrome c was purchased from Sigma Chemical Co. as a lyophilized powder and used without further purification. The NMR samples were prepared in D_2O, in 100 mM phosphate buffer, pH 7.0 (uncorrected for the isotope effect). The sample of the reduced form of the protein was obtained adding solid dithionite directly into the NMR tube under Ar atmosphere.

All NMR experiments were performed on a Bruker DRX-500 spectrometer operating at 500.13 MHz proton Larmor frequency. The $^1H-^{13}C$ heteronuclear multiple quantum coherence experiments (HMQC)[35] were recorded using a 5-mm inverse detection probe without decoupling during acquisition to avoid sample overheating. A recycle time of 300 ms and a refocusing time of 2.5 ms were used. All spectra were acquired in the phase-sensitive mode using standard pulse sequences and processed using XWINNMR Bruker software. Proton chemical shifts were referenced to the solvent, which in turn had been calibrated to TMS. Carbon chemical shifts were referenced to external DSS. Due to the digital resolution of the 2D spectra, the ^{13}C chemical shifts are estimated to be accurate to ± 0.3 ppm.

Results and Discussion

The ^1H chemical shifts of the heme and Met-80 methyls of horse heart cytochrome c have been obtained from 1D NMR spectra at 11 temperatures; part of these data are listed in Table 1, and the complete data set is presented in Table S1 (Supporting Information). The ^{13}C chemical shifts of the same groups were obtained from 2D HMQC spectra at six temperatures, and the data for 298 K are also listed in Table 1, again with the complete data set reported in Table S1. After subtraction of the diamagnetic shifts (listed in Table 1), the experimental hyperfine shifts of the proton and the carbon nuclei of the heme methyl substituents and of the axial ligand Met-80 methyl are obtained for each of the temperatures. In Figure 2, the temperature dependences of the proton and carbon hyperfine shifts of the four heme methyl groups is reported. As already reported in the literature,[16] the ^1H and ^{13}C resonances, respectively, of the same methyl group, have different sign and different temperature dependence. In particular, the proton shifts of all the CH_3 groups are positive while the carbon shifts are negative. As anticipated, the ^1H shifts of the 8- and 3-CH_3 groups have

of the McConnell constant Q_N[30] and the spin densities at the p_z orbitals of the pyrrole carbons to which the methyls are attached. The hyperfine shift of the Met-80 CH_3 has been monitored to rule out the possibility of methionine detachment over this temperature range, as may occur in some His-Met-coordinated cytochromes, or with a $S = \frac{1}{2}-\frac{5}{2}$ spin equilibrium, as occurs in the case of *Escherichia coli* periplasmic cytochrome b_{562}.[31]

Previous to this work, two different temperature dependences of the hyperfine shifts within a given set of nuclei (e.g., the four methyl protons or the four methyl carbons) were accounted for by a two-level treatment.[16,28,32−34] ^1H temperature-dependence data on *Aplysia* cyanometmyoglobin [32] and cyto-

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Table 1. Experimental Chemical Shifts (ppm) of Selected Resonances of the Heme−Axial Ligand Moiety in Horse Heart Cytochrome c^a

T (K)	δ_{exp}	δ_{dia}	$\delta_{pc\ calc}{}^b$	$\delta_{pc\Delta E=355\ cm^{-1}}{}^c$	$\delta_{lc\Delta E=355\ cm^{-1}}$	$\delta_{fc\Delta E=355\ cm^{-1}}$	$\delta_{fc\Delta E=590\ cm^{-1}}$
			HM1				
298	7.04	3.46		−4.02	−0.09	7.62	7.59
303	7.16	3.46	−3.92	−3.90	−0.09	7.65	7.62
323	7.75	3.46	−3.45	−3.48	−0.10	7.71	7.75
			HM3				
298	32.29	3.84		−1.74	−0.53	30.10	30.15
303	31.76	3.84	−1.50	−1.68	−0.53	29.56	29.57
323	29.96	3.84	−1.50	−1.47	−0.49	27.59	27.45
			HM5				
298	10.03	3.58		−4.90	−0.22	11.43	11.37
303	10.23	3.58	−4.80	−4.76	−0.23	11.49	11.44
323	11.11	3.58	−4.19	−4.24	−0.23	11.69	11.72
			HM8				
298	35.23	2.16		−2.10	−0.84	35.22	35.25
303	34.58	2.16	−1.96	−2.04	−0.83	34.51	34.53
323	32.30	2.16	−1.90	−1.79	−0.77	31.90	31.88
			CM1				
298	−16.5	15.4		−5.32	−0.36	−26.67	−26.67
303		15.4	−5.19	−5.16	−0.36		
323		15.4	−4.57	−4.60	−0.34		
			CM3				
298	−54.2	15.2		−1.99	−0.61	−67.44	−67.49
303		15.2	−2.82	−1.92	−0.60		
323		15.2	−1.84	−1.68	−0.54		
			CM5				
298	−23.3	15.0		−6.36	−0.71	−31.93	−31.94
303		15.0	−6.22	−6.18	−0.70		
323		15.0	−5.44	−5.51	−0.67		
			CM8				
298	−68.5	13.1		−2.50	−1.80	−79.15	−79.16
303		13.1	−2.32	−2.42	−1.76		
323		13.1	−2.27	−2.12	−1.62		
			HE3				
298	−24.20	−3.30		3.46		−24.19	−24.25
303	−23.69	−3.30	3.02	3.35		−23.54	−23.59
323	−21.39	−3.30	3.44	2.93		−21.15	−21.10
			CE				
298	15.3	15.4		13.19		−13.31	−13.39
303		15.4	12.31	12.78			
323		15.4	12.03	11.29			

^{a} For each resonance, the experimental data obtained at various temperatures are reported (δ_{exp}), together with the shift of the corresponding resonances in the reduced form (δ_{dia}) determined at 298 K and considered invariant with temperature. The metal-centered pseudocontact shift contribution at the shift (δ_{pc}) for each resonance is reported. The latter values were calculated as reported in the text with the magnetic anisotropy parameter reported in Table 2 using the X-ray[40] data as structural model. The ligand-centered pseudocontact shifts (δ_{lc}) and the Fermi contact shifts (δ_{fc}) were calculated as described in the text. ^{b} Independently at 303 and 323K using eq 2. ^{c} Using eq 5.

normal Curie dependence with temperature, while the corresponding ¹³C resonances exhibit a hyper-Curie dependence with large downfield intercepts at infinite temperature. Also, the resonances of the 5- and 1-CH₃ groups have different behavior for the two nuclei: The protons exhibit anti-Curie behavior with downfield intercept, while the carbon resonances exhibit hypo-Curie dependence with intercepts at upfield values. Furthermore, the dependence of the shifts with respect to $1/T$ is not perfectly linear, but shows a small curvature, which has also been observed in other cytochromes.[36−38]

Analysis of the Data. (a) Metal-Centered Pseudocontact Shifts. To proceed with the analysis of the hyperfine shifts and their temperature dependence, separation of the metal-centered pseudocontact and contact contributions to the hyper-

fine shifts is necessary. For this purpose, the metal-centered pseudocontact shift of the methyl nuclei at all the temperatures of the experimental measurements should be evaluated. The metal-centered pseudocontact shifts of nuclei can be calculated at different temperatures with the Kurland and McGarvey equation[39] once the magnetic anisotropies and the principal directions of the tensors at the various temperatures are known. The latter parameters, in turn, can be determined by fitting the experimental shifts of those nuclei which are separated from the metal ion by several bonds so as not to experience any contact contribution to the shift, nor any ligand-centered contribution. A number of pseudocontact shifts of protein nuclei can be obtained from the literature data at 303 and 323 K.[14] They allow the determination at these temperatures of the axial and rhombic anisotropies of the magnetic susceptibility tensor χ, $\Delta\chi_{ax}$, and $\Delta\chi_{rh}$ and of the three principal directions of the tensor from the following equation:

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$$\delta_{pc} = (1/12\pi r^3)[\Delta\chi_{ax}(3\cos^2\theta - 1) +$$
$$(^3/_2)\Delta\chi_{rh}\sin^2\theta\cos 2\Omega]$$

$$= K^{pc}[\Delta\chi_{ax}G_{ax} + (^3/_2)\Delta\chi_{rh}G_{rh}] \qquad (2)$$

where $K^{pc} = 1/12\pi r^3$, and r, θ, and Ω are the polar coordinates of the nuclei, $G_{ax} = (3\cos^2\theta - 1)$, $G_{rh} = \sin^2\theta\cos 2\Omega$, and the magnetic axes determined by the principal directions of the χ tensor; $\Delta\chi_{ax}$ and $\Delta\chi_{rh}$ are defined as

$$\Delta\chi_{ax} = \chi_{zz} - (^1/_2)(\chi_{xx} + \chi_{yy})$$

$$\Delta\chi_{rh} = \chi_{xx} - \chi_{yy}$$

where χ_{ii} are the principal components of the magnetic susceptibility tensor. The five parameters ($\Delta\chi_{ax}$, $\Delta\chi_{rh}$, and the three Euler angles that are conventionally used to define the orientation of the tensor in the molecular frame) for each of the two experimental tensors are reported in Table 2. The calculations have been performed using both the X-ray[40] and solution[22] structural data to give an idea of the possible uncertainties in the fitting procedure, that critically depend on the availability of a structural model.

From the knowledge of the tensor parameters at 303 and 323 K, the metal-centered pseudocontact shifts for the heme protons and carbons at these two temperatures can be evaluated using eq 2.[41] The calculated values are reported in Table 1. The temperature dependence of the metal-centered pseudocontact shifts at all other temperatures can be inter-/extrapolated from the values at these two temperatures. However, if two electronic levels separated by ΔE_π are populated in the temperature range investigated, as in the present case, the metal-centered pseudocontact shift for any nucleus can also be expressed as a sum of two terms arising from each electronic function ϕ_1 and ϕ_2 for each level, weighted by their Boltzmann populations f_1 and f_2:[26,28,34]

$$f_1 = 1/(1 + e^{-\Delta E_\pi/kT}); \quad f_2 = e^{-\Delta E_\pi/kT}/(1 + e^{-\Delta E_\pi/kT}) \quad (3)$$

Therefore, the total metal-centered pseudocontact shift for each nucleus can be expressed as

$$\delta_{pc} = K^{pc}[(\Delta\chi_{1ax}G_{1ax} + (^3/_2)\Delta\chi_{1rh}G_{1rh})f_1 +$$
$$(\Delta\chi_{2ax}G_{2ax} + (^3/_2)\Delta\chi_{2rh}G_{2rh})f_2] \quad (4)$$

where $\Delta\chi_{1ax}$, $\Delta\chi_{1rh}$ and $\Delta\chi_{2ax}$, $\Delta\chi_{2rh}$ are the components of two limiting tensors, $\Delta\chi_1$ and $\Delta\chi_2$ for the ϕ_1 and ϕ_2 levels.[42]

In eq 4, the effect of temperature on the metal-centered pseudocontact shifts arises both from a change in population f_1 and f_2 with temperature and from the temperature dependence implicitly associated with each χ tensor.[42] At this point three assumptions are introduced, which are validated a posteriori.

(1) The rhombic components of χ_1 and χ_2 differ by a 90° rotation. This assumption arises from the fact that ϕ_1 and ϕ_2 must be orthogonal and approximately related by a 90° rotation about the heme z axis which is taken as normal to the heme

Table 2. Parameters Characterizing the Magnetic Susceptibility Tensor as Obtained (a) from an Independent Fit of Experimental Pseudocontact Shifts at Two Temperatures and (b, c) from a Simultaneous Fit of Both Data Sets[a]

struct data	temp (K)	$\Delta\chi_{ax}$ (m³ × 10³²)	$\Delta\chi_{rh}$ (m³ × 10³²)	α (deg)	β (deg)	γ (deg)
(a) Independent Fit at Each Temperature						
X-ray	303	2.94	−1.22	22.9	14.6	−20.9
	323	2.65	−0.99	25.0	14.1	−22.6
REM	303	2.98	−1.36	16.2	15.3	−21.7
	323	2.68	−1.15	15.9	15.1	−21.5

struct data	temp (K)	$\Delta\chi_{1ax}$ (m³ × 10³²)	$\Delta\chi_{rh}$ (m³ × 10³²)	$\Delta\chi_{2ax}$ (m³ × 10³²)	α (deg)	β (deg)	γ (deg)
(b) Fit of Both Temperature Sets with Eq 5 ($\Delta E = 355$ cm⁻¹)							
X-ray	303, 323	4.79	−1.74	−6.61	23.5	14.4	−21.5
REM	303, 323	4.84	−1.94	−6.58	16.0	15.2	−21.6
(c) Fit of Both Temperature Sets with Eq 5 ($\Delta E = 590$ cm⁻¹)							
X-ray	303, 323	3.94	−1.34	−12.31	23.5	14.4	−21.4
REM	303, 323	4.02	−1.49	−12.74	16.0	15.2	−21.6

[a] Separate fits have been performed using the X-ray[40] and the restrained energy minimized (REM)[22] solution structure coordinates.

plane. This assumption is more realistic the more the d_{xy} orbital is separated from the other two.

(2) The temperature dependence of the χ components is linear with $1/T$.[42] This assumption is not so severe, as a different temperature dependence would not alter significantly the actual values nor change the conclusions.

(3) ΔE_π is in the range between 355 and 590 cm⁻¹ (from independent estimates[45]).

In this way, the data at the two temperatures (303 and 323 K) for the nuclei of residues not directly bound to the heme can be fit simultaneously to eq 5, which derives from eq 4 with the conditions $\Delta\chi_{1rh} = -\Delta\chi_{2rh} = \Delta\chi_{rh}$, $G_{1ax} = G_{2ax} = G_{ax}$ and $G_{1rh} = G_{2rh} = G_{rh}$.

$$\delta_{pc}(T) = K^{pc}(298/T)([\Delta\chi_{1ax}{}^{298}G_{ax} + (^3/_2)\Delta\chi_{rh}{}^{298}G_{rh}]f_1 +$$
$$[\Delta\chi_{2ax}{}^{298}G_{ax} - (^3/_2)\Delta\chi_{rh}{}^{298}G_{rh}]f_2) \quad (5)$$

The two magnetic tensors are therefore obtained (Table 2). Note that $\Delta\chi_{1ax}{}^{298}$ and $\Delta\chi_{2ax}{}^{298}$ turn out to have different signs and similar magnitude.[50] The above approach permits not only the evaluation of the metal-centered pseudocontact shifts of the nuclei of interest at all temperatures where the experimental data are available (in this case the heme methyls and methionine methyl) but also their decomposition into the two contributions

(43) Horrocks, W. D., Jr.; Greenberg, E. S. *Biochim. Biophys. Acta* **1973**, *322*, 38−44.

(44) Horrocks, W. D., Jr.; Greenberg, E. S. *Mol. Phys.* **1974**, *27*, 993−999.

(45) The range of ΔE_π values spans those calculated from the EPR g values. These have been reported by Mailer and Taylor:[46] $g_{zz} = 3.06$, $g_{yy} = 2.25$, and $g_{xx} = 1.25$, and using the three-orbital spin−orbit mixing treatment of Griffith[47] and Taylor,[48] these g values yield a splitting between the d_{xz} and d_{yz} orbitals, V/λ, of 1.478. Depending on the value chosen for λ (400 cm⁻¹ as we have used previously[49] or 240 cm⁻¹ as Turner has used[16,28]), this means that $355 < V < 590$ cm⁻¹. If we then assume that $V \approx \Delta E_\pi$, this yields a reasonable range of values.

(46) Mailer, C.; Taylor, C. P. S. *Can. J. Biochem.* **1972**, *85*, 1048−1055.

(47) Griffith, J. S. *Proc. R. Soc. London* **1956**, *235*, 23−36.

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(49) Walker, F. A.; Huynh, B. H.; Scheidt, W. R.; Osvath, S. R. *J. Am. Chem. Soc.* **1986**, *108*, 5288−5297.

(50) This finding is consistent with the theoretical expectation that ϕ_1 and ϕ_2 of eq 1 should have $g_z > g_y > g_x$ and $g_z < g_y < g_x$, respectively (see, e.g., ref 29), and with angular overlap calculations performed by us (unpublished results) using the CAMMAG program.[51,52]

(40) Bushnell, G. W.; Louie, G. V.; Brayer, G. D. *J. Mol. Biol.* **1990**, *214*, 585−595.

(41) For the present calculations, the ¹H and ¹³C resonances for which a firm assignment was reported[14] were selected.

(42) Indeed, we cannot rigorously talk of a χ tensor of a level, as the **g** tensor would be more appropriate. However, for homogeneity of notation, we leave χ, which is related to **g** by the relationship $\chi = \mu_0(g^2\mu_B^2S(S + 1)/3kT$, if second-order Zeeman contributions are small.[43,44]

from the ground and excited states. The best-fit parameters are shown in Table 2.

The calculated metal-centered pseudocontact shift values for the methyl nuclei of the heme moiety and the methionine ligand are reported in Table 1. It is worth noting that the metal-centered pseudocontact shifts for the 1 and 5 methyl nuclei are large for the ground state and small for the excited state, whereas the reverse is true for the pair of methyls 3 and 8. This indicates that, as already noted, the *x* and *y* axes of the two tensors are not far from the pyrrole nitrogen directions, so that a methyl pair from two opposite pyrrole rings is close to experiencing the largest in-plane metal-centered pseudocontact shift in the ground state, and the other pair is close to experiencing the smallest metal-centered pseudocontact shift.

(b) Contact Shifts. The Fermi contact contribution to the methyl proton and carbon chemical shifts can be related to the spin density for each electronic level in the corresponding ϕ_i function of the p_z orbital of a β-pyrrole carbon to which the methyl is attached, again weighed by its Boltzmann population.[26,28] In general, the Fermi contact shift for each nucleus N and a single $S = \frac{1}{2}$ ground state is given by

$$\delta_{fc} = (A_N/h)g_e\beta_e S(S+1)/3kT\gamma_N =$$
$$2\pi g_e\beta_e S(S+1)/3kT\gamma_N = K^{fc}Q_N\rho^\pi/\gamma_N T \quad (6)$$

where Q_N is a constant known as the McConnell constant[30] and is expressed in hertz. For methyl groups attached to sp^2 carbons with unpaired electron spin density ρ^π in the p_z orbital, the values $Q_H = 70-75$ MHz,[53,54] and $Q_C = -39$ MHz have been reported.[16,28,55,56] In a two-level system, in analogy with eqs 4 and 5, the Fermi contact shift for each nucleus is given by

$$\delta_{fc}(T) = K^{fc}(Q_N/\gamma_N T)(\rho_1{}^\pi f_1 + \rho_2{}^\pi f_2) \quad (7)$$

(c) Fit of the Hyperfine Shifts. The sum of eqs 5 and 6 can then be fit to the experimental hyperfine shifts using only $\rho_1{}^\pi$ and $\rho_2{}^\pi$ as adjustable parameters. The ρ values are then satisfactorily compared with the Hückel coefficients calculated from the simple Hückel program described previously.[54,57] The results are reported in Figure 3 and Tables 1 and 3. The good quality of the fits suggests that the main source of deviation from Curie law in these systems indeed arises from the temperature-dependent change in the population of the two levels, f_1 and f_2, which is reflected in both the metal-centered pseudocontact and contact shifts. The quality of the fit and the values of the ρ parameters are not much altered by changing ΔE_π within the two chosen limiting values (see Tables 1 and 3). The $\rho_1{}^\pi$ and $\rho_2{}^\pi$ values for each of the four methyl nuclei for both ^{1}H and ^{13}C (Table 3) consistently show alternation in

(51) Cruse, D. A.; Davies, J. E.; Gerloch, M.; Harding, J. H.; Macking, D. J.; McMeecking, R. F. CMMAG, a FORTRAN computing package, 1979.

(52) Stratemeier, H.; Hitchman, M. A.; Comba, P.; Bernhardt, P. V.; Riley, M. J. *Inorg. Chem.* **1991**, *30*, 4088−4093.

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(57) In these Hückel calculations carried out using the program MPOR-PH,[54] we have assumed that the orientation of the nodal plane of the highest-energy (half-filled) orbital is determined equally by the orientation of the nodal planes of the filled π-symmetry p orbital of Met-80 and that of the histidine imidazole, as concluded by Turner. This places the nodal plane of the π orbital of the heme at an angle of 14° clockwise from the nitrogens of pyrrole rings I and III and leads to the Hückel coefficients tabulated in Table 3.
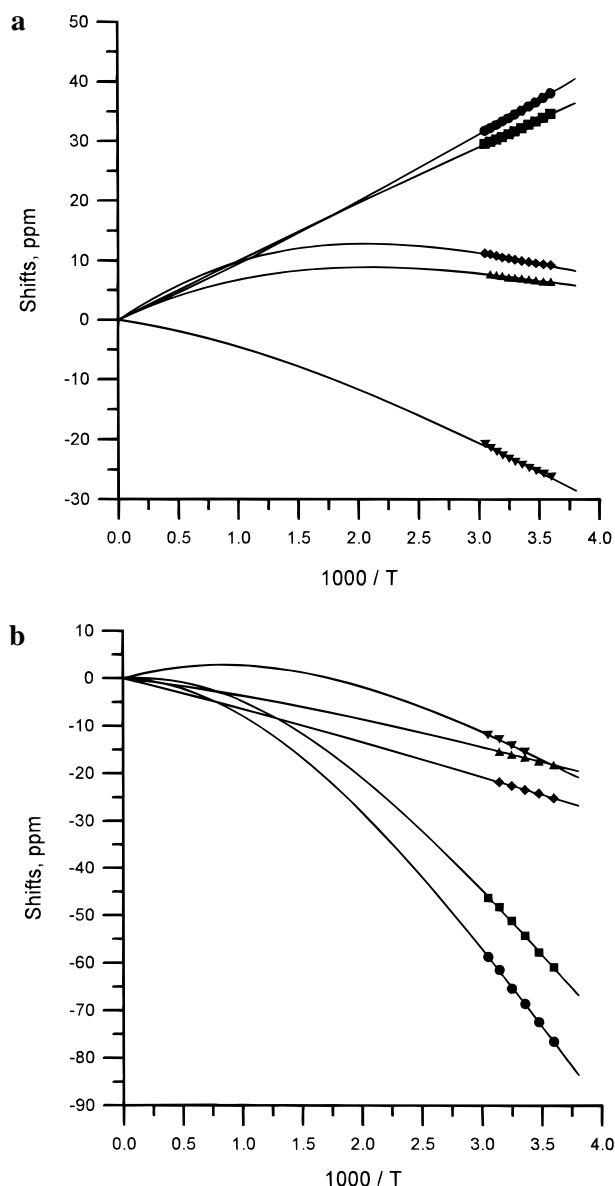

Figure 3. Curie plots for the isotropic shifts of (a) the protons and (b) the carbons of the methyl groups of ferricytochrome *c*: ▲, 1-Me; ■, 3-Me; ◆, 5-Me; ●, 8-Me; ▼, Met-Me. The lines represent the fit to the data with a $\Delta E_\pi = 355$ cm^{-1} (see text).

Table 3. Spin Density Coefficient Obtained from Hückel MO Calculation and from ^{1}H and ^{13}C Fermi Contact Shifts

posi-tion	ΔE, cm^{-1}	Hückel coefficients		^{1}H coefficient		^{13}C coefficient	
		ground state	excited state	ground state	excited state	ground state	excited state
1	355	0.00829	0.01177	0.001	0.021	0.004	0.021
	590			0.002	0.034	0.005	0.029
3	355	0.01029	0.00865	0.016	0.011	0.020	−0.003
	590			0.016	0.000	0.018	−0.017
5	355	0.00829	0.01177	0.000	0.035	0.005	0.025
	590			0.003	0.056	0.006	0.035
8	355	0.01750	0.00098	0.020	0.003	0.024	−0.005
	590			0.019	−0.008	0.022	−0.022
Met	355			−0.018	0.018	−0.002	0.030
Met	590			−0.015	0.040	0.001	0.040

magnitude, i.e., large for the ground state and small for the excited state or vice versa. This is a result that gives further support to the whole analysis. The ρ values for the axial methionine nuclei are also reported in Table 3 (see below).

(d) Ligand-Centered Pseudocontact Shifts. The appreciable spin density on the pyrrole carbons in one or the other of the two levels may also contribute a nonnegligible ligand-centered pseudocontact shift.[55,56] Goff[56] suggested, on the basis of analysis of the ^{13}C hyperfine shifts of model ferriheme complexes, that the ligand-centered dipolar term is of the same sign and is possibly comparable in magnitude to the contact term, at least for carbons that are themselves part of the π system, while Turner has assumed that this contribution should be negligible for methyl groups, for which the carbon is *not* involved in a π system.[16,28] The determination of the ligand-centered pseudocontact shift is a difficult task also for the case of a single level. A single level is characterized by a single set of g values but the ligand atoms contribute only slightly to g anisotropy because their λ is small. The contribution to $\Delta\chi$ from the anisotropy of the experimental value of $\langle S_z \rangle$ remains.[39] Therefore, if $\Delta\chi$ is taken to be the same and scaled for the spin densities all over the ligand, an overestimate or upper limit of the ligand-centered pseudocontact shift is obtained. Such an estimate can be obtained without increasing the number of parameters by recalling that (i) the χ tensor centered on any ith ligand nucleus is only scaled in magnitude but not in anisotropy, and thus, the ligand-centered pseudocontact shift maintains the same orientation as the metal-centered pseudocontact shift in the molecular frame; (ii) the geometric relationships between any heme substituent and the tensors centered on the adjacent pyrrole carbon are known; and (iii) the scaling factors for each electronic state are just proportional to the ρ^π values of that state.[56] The complete equation taking into account ligand-centered effects on all heme atoms can be written as

$$\delta_{hyp}(T) = [1 - \sum_i (\rho_{1i}{}^\pi f_1 + \rho_{2i}{}^\pi f_2)]\delta_{pc}(T) + \delta_{fc}(T) +$$
$$\sum_i K_i{}^{lc}[(\rho_{1i}{}^\pi f_1 + \rho_{2i}{}^\pi f_2)(298/T)([\Delta\chi_{1ax}{}^{298}G_{axi} +$$
$$({}^3/_2)\Delta\chi_{rh}{}^{298}G_{rhi}]f_1 + [\Delta\chi_{2ax}{}^{298}G_{axi} - ({}^3/_2)\Delta\chi_{rh}{}^{298}G_{rhi}]f_2)]$$
$$(8)$$

where $\delta_{pc}(T)$ is given by eq 5, $\delta_{fc}(T)$ is given by eq 7, and the sum i is over the ligands bearing spin density contributing to the ligand-centered pseudocontact shifts. For our purposes, the ligand-centered term can be limited to the adjacent pyrrole carbon. $K_i{}^{lc}$ is given by the reciprocal of the third power of the distance between methyl proton or carbon and the centers of electron density of the $2p_z$ orbital situated about 0.7 Å above and below the heme plane. The fit of the experimental data to eq 8 provides values for the ligand-centered contributions which are reported in Table 1. As is evident, the values, although small, are not negligible. However, the fit of the experimental data yields results of quality comparable to those obtained with the sum of eqs 5 and 7. The ligand-centered contributions are thus not negligible, but their influence on the goodness of the fit and the ρ values is modest. Therefore, ligand-centered pseudocontact shift contributions are consistent with the present data but are not required to account for the data within the approximations used.

(e) Overview of Spin Density Data. As is evident from the data of Table 3, the comparison between calculated Hückel coefficients and "experimental" spin densities obtained from the heme methyl shifts is quite acceptable (of the same order of magnitude) for the ^1H data, considering the crudeness of the simple Hückel calculations. The ground-state spin densities match the Hückel coefficients more closely than do the spin densities of the excited state, which should be approximately

the opposite order of sizes as compared to the ground state. Such behavior is to be expected for a fitting procedure such as the one used herein, and for the same reason, large spin densities match better than small ones. Small calculated spin densities may even turn out to be negative in some cases and may thus be assumed to be essentially zero. However, the "experimental" spin densities in the ground state are consistently larger for the carbon positions in the ground-state orbital when determined from ^{13}C data than from the ^1H data. This may indicate a larger Q_C/Q_H ratio than the value of 0.56. Larger Q_C/Q_H values have also been deduced from data on several other heme proteins.[38,58]

The ρ values obtained from the axial methionine nuclei deserve a comment. They have been calculated using the same McConnell Q values for the methyl protons and carbon as those for heme methyls, which is not necessarily correct. The fact that the ρ values on the sulfur atom calculated from the proton and carbon data on the methionine methyl group turn out to be so similar in size and positive in the excited state suggests that most of the unpaired spin density sensed by the two nuclei arises from a p_π orbital on the sulfur atom. This is consistent with the excited state being involved in π bonding. The sizable negative spin density for the methyl protons and near-zero spin density for the methyl carbon in the ground state may be indicative of a sizable contribution from spin polarization effects or from σ delocalization via the σ symmetry lone pair orbital of sulfur. The observed coefficients for the ground-state orbital must thus be the balance of these (negative) contributions *and* (positive) π contributions, because there must be a strong π bonding component in the ground state. Otherwise, the methionine ligand would not affect the orientation of the nodal plane of the π orbitals of the heme.

A closer look at the experimental and fitted data for the contact shift shows that there is a systematic variation in the calculated versus observed shifts for each of the heme methyls (Figure 3, Table S1). Although it is not much larger than the expected experimental error, there is a consistent trend that calculated heme methyl proton contact shifts are smaller at the highest and lowest temperatures, and larger at the middle temperatures, than the experimental contact shifts. The deviations are greatest for the protons that have the largest calculated contact shifts and smallest for those having the smallest contact shifts. The same trend is observed for the ^{13}C contact (or contact plus ligand-centered dipolar) shifts. The sizes of the deviations decrease as the value of ΔE_π decreases. Such deviations are not greater than ± 0.07 ppm for ^1H and not much larger for ^{13}C and are similar to the experimental error in each case. It is therefore difficult to determine whether these are meaningful trends or not. We could consider further reasons that may account for these minor deviations, such as other thermally accessible excited states, temperature-dependent chemical equilibria involving detachment of the methionine, inversion of the methionine chirality, temperature-dependent effects on the protein conformation that influence the energy gap between the two electronic states, ΔE_π, etc. All of these phenomena may introduce a temperature dependence in the energy separation between ϕ_1 and ϕ_2. Owing to the relatively simple two-state model used which, for example, neglects admixture of the d_{xy} orbital and second-order Zeeman effects,[43,44] as well as the very small sizes of the deviations observed, we have not pursued their analysis further.

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Conclusions

In this paper, we have addressed the so-called anomalous behavior of the hyperfine shifts of carbon nuclei and protons of methyls of the heme of cytochromes *c*. Indeed, the experimental evidence that two methyl protons, but not their carbons, have anti-Curie temperature dependence is at first glance surprising. However, it is the larger contribution of the metal-centered pseudocontact shifts for carbons and the opposite sign of the contact shift for the two nuclei that are the major contributors to the apparent difference in the Curie behavior of these two nuclei. The present analysis provides a complete explanation by (1) evaluating the metal-centered pseudocontact shifts of heme substituents at two temperatures from the pseudocontact shifts of nuclei not experiencing any contact shift, (2) evaluating the metal-centered pseudocontact shifts at all temperatures of interest, and (3) subtracting the metal-centered pseudocontact shifts from the hyperfine shifts to obtain contact plus ligand-centered pseudocontact shifts. Finally, the contact shifts for methyl protons and carbons are found to be consistent with theoretical expectations. Indeed, the spin densities of the pyrrole carbons estimated from the methyl proton contact shifts are in agreement to within 50% with those estimated from the methyl carbon contact shifts, even using unmodified literature values for the McConnell Q_C and ignoring the ligand-centered pseudocontact shift contribution to the carbon shifts. This agreement holds for both the ground and excited states.

In conclusion, the present analysis provides deep insight into the electronic distribution on the porphyrin ring in low-spin Fe(III) hemes and completes the previous detailed attempts of other workers. These results should serve as a useful reference point for future papers dealing with these systems.