Hyperfine Shifts in Low-Spin Iron(III) Hemes: A Ligand Field Analysis

Ivano Bertini,*[a,b] Claudio Luchinat,[a,c] and Giacomo Parigi[a,c]

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The hyperfine shifts of heme nuclei in low-spin iron(III) porphyrins of known geometry are predicted from: (i) a ligand field analysis, and (ii) the Kurland and McGarvey theory (R. J. Kurland, B. R. McGarvey, *J. Magn. Reson.* **1970**, *2*, 286–301). The ligand field parameters are optimized to reproduce the experimentally available *g* values and magnetic susceptibility anisotropy, the latter being obtained from the analysis of pseudo-contact shifts. Simple known geometric relationships (I. Bertini, C. Luchinat, G. Parigi, F. A. Walker, *J. Biol. Inorg. Chem.* **1999**, *4*, 515–519; N. V. Shokhirev, F.A. Walker, *J. Biol. Inorg. Chem.* **1998**, *3*, 581–594), are used to relate the contact shifts to the various heme positions. The systems investigated are: metMyoglobin cyanide, cytochrome b_5 and cytochrome *c*. It is shown that the contact hyperfine coupling constants recalculated from this approach are different to those obtained through the simple and generally used McConnell equation, and that the deviation can be more or less severe, depending on the position on the heme and on the orientation of the axial ligands. The contact shift tensor is highly anisotropic and therefore the shift is dependent on the orientation of the molecule in a magnetic field. This theoretical analysis is necessary to interpret ENDOR spectra and NMR spectra in partially oriented systems.

Introduction

Contact shifts are generally and incorrectly assumed to be isotropic because the hyperfine coupling constant is isotropic.[1−4] However, contact shifts depend on the induced electron magnetic moment at the nucleus, and such magnetic moments are orientation-dependent because spin-orbit coupling causes anisotropy in the expected value of S_z, $<S_z>$. This effect is well-known[5] but does not show up in solution where the anisotropy is averaged out.[1−4]

Dipolar shifts due to the electron-nucleus coupling are known to be anisotropic.[2−6] They have an average value of zero in the absence of spin-orbit coupling and if all the orientations in solution are equally probable. In the presence of spin-orbit coupling, *g* and the magnetic susceptibility χ become anisotropic and the average dipolar coupling is different from zero. This effect gives rise to the well-known pseudo-contact shift.[2−6]

The interest here is in low-spin iron(III) heme systems. The *g* values of the ground state are available from EPR spectroscopy.[7−10] The magnetic susceptibility anisotropy, $\Delta\chi$, is available from the analysis of the ^{1}H pseudo-contact shifts, which are easily obtained for heme proteins [11−24]. Indeed, protons separated from the metal ion by several chemical bonds experience only pseudo-contact shifts, which then provide both magnetic susceptibility anisotropy and the principal directions of the magnetic susceptibility tensor. By fitting the *g* and $\Delta\chi$ values and directions with any ligand-field approach, the proper wave functions of the three lowest orbital levels are obtained, which are the basis set for the Kurland and McGarvey approach to determine: (i) the contact shifts for each orientation of the molecule in the external magnetic field, and (ii) the average value of isotropic rotation. The calculated contact shifts are then compared to the contact shifts of the heme methyl protons, which are estimated from the experimental hyperfine shifts by subtracting the calculated pseudo-contact shifts from the known magnetic susceptibility anisotropy tensor. Here, a simple analytical approach[9,25−29] is used. The contact shifts along the heme positions are predicted by assuming a $\sin^2\theta$ dependence for the ground state and a $\cos^2\theta$ dependence for the first excited state, where θ is the angle between the iron-proton vector and the direction of the (average) p-orbital nodal plane(s) of the π-bonding axial ligand(s)[21,30]. The analysis of the experimental contact shifts on the basis of this theoretical approach is quite instructive. The systems analyzed are: cyanometmyoglobin, rat microsomal cytochrome b_5 and horse heart cytochrome *c*.

Finally, since the technology of partial orientation is rapidly developing,[31−39] we want to address the problem of the effect of partial, but extensive, orientation on the contact (and pseudo-contact) shifts. This prediction is extremely important for the analysis of ENDOR spectroscopy and is not yet available in the literature, whereas extensive orientation in solution is not yet obtainable without severe broadening of the NMR lines.

Theory

Contact Shift

The Fermi contact contribution to the paramagnetic NMR shift can be calculated by using the Kurland and

[a] CERM, Magnetic Resonance Center, University of Florence, Via L. Sacconi, 6, 50019 Sesto Fiorentino, Italy
Fax: (internat.) +39-055/420-9271
E-mail: bertini@cerm.unifi.it
[b] Department of Chemistry, University of Florence
E-mail: luchinat@cerm.unifi.it
parigi@cerm.unifi.it
[c] Department of Soil Science and Plant Nutrition, University of Florence

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McGarvey equation,[5] as it depends on the thermally populated electronic states:

$$\delta_i^{con} = \frac{1}{kT\sum_{\Gamma n} e^{-\varepsilon_\Gamma/kT}}\left(\sum_{\Gamma n,\Gamma m} e^{-\varepsilon_\Gamma/kT} < \Gamma n \mid \mu_i \mid \Gamma m >< \Gamma m \mid A_{Fi} \mid \Gamma n > + \right. \quad (1)$$

$$\left. - kT \sum_{\Gamma n,\Gamma m\,(\Gamma \neq \Gamma')} \frac{e^{-\varepsilon_\Gamma/kT} - e^{-\varepsilon_{\Gamma'}/kT}}{\varepsilon_\Gamma - \varepsilon_{\Gamma'}} < \Gamma n \mid \mu_i \mid \Gamma' m >< \Gamma' m \mid A_{Fi} \mid \Gamma n > \right)$$

Here i represents any of the principal directions of the molecular axes, $|\Gamma n>$ are the eigenstates of the electronic multiplets, ε_Γ is the energy of each multiplet state $|\Gamma>$, k is the Boltzmann constant and T is the temperature. Furthermore,

$$\mu_i = -\mu_B(L_i + 2S_i)$$

$$A_F = \frac{8\pi g_e \mu_B}{3}\delta(r)S$$

where δ indicates the Dirac δ operator, μ_B is the electron Bohr magneton, g_e is the electron g factor and L and S are the electron-orbital and spin operators. The eigenstates $|\Gamma n>$ can be provided by a ligand-field analysis.[9,25−29]

A pictorial scheme of the electronic levels of low-spin iron(III) heme proteins[26] is shown in Figure 1. On the left-hand side, the splitting of the d_{xy}, d_{yz}, d_{xz} orbitals (with energies E_0, E_1, E_2) is provided by the low symmetry, whereas on the right-hand side the effect of spin-orbit coupling is shown (and the orbital energy levels are labeled E'_0, E'_1, E'_2). The figure also shows the energy of the excited states, ε_1 and ε_2.

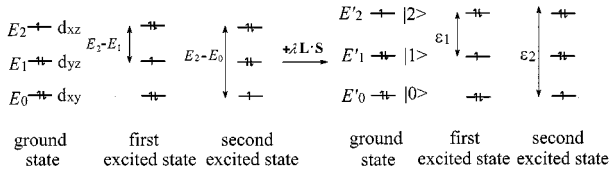

Figure 1. The low-symmetry splitting of the orbitally degenerate $^2T_2$ ground state and first and second excited states of low-spin iron(III)

This simple three-state analysis provides the g values for the ground state and the molecular χ values as follows [9,25−29]:

$$g_x = 2[2\xi_0\zeta_0 - \eta_0^2 - \sqrt{2}\eta_0(\xi_0 - \zeta_0)]$$

$$g_y = 2[-2\xi_0\zeta_0 - \eta_0^2 - \sqrt{2}\eta_0(\xi_0 + \zeta_0)] \quad (2)$$

$$g_z = 2[-2\xi_0^2 + \eta_0^2]$$

$$\chi_{ii} = -\frac{\mu_0\mu_B^2}{4\sum_{\Gamma n} e^{-\varepsilon_\Gamma/kT}}\sum_{\Gamma n}\sum_{\Gamma' m}(g_{\Gamma\Gamma'})_i^2 \frac{e^{-\varepsilon_\Gamma/kT} - e^{-\varepsilon_{\Gamma'}/kT}}{\varepsilon_\Gamma - \varepsilon_{\Gamma'}} \quad (3)$$

where μ_0 is the vacuum permeability, $(g_{\Gamma\Gamma'})_i$ are the electron g values in the ith direction[29] and ξ, η, and ζ are the coefficients of the ground and the two excited-state Kramer doublets, given by the following linear combination of the $|m_l, m_s>$ states:

$$|\Gamma,+> = \xi_\Gamma|1,-1/2> + \eta_\Gamma|0,1/2> + \zeta_\Gamma|-1,-1/2>$$

$$|\Gamma,-> = \xi_\Gamma|-1,1/2> + \eta_\Gamma|0,-1/2> + \zeta_\Gamma|1,1/2> \quad (4)$$

for $\Gamma = 0$, 1 and 2.

The fitting of the experimental data requires E_1 and E_2 of Figure 1 and the spin-orbit coupling constant. The orbital reduction parameter is taken to be 1.

By performing the calculations of Equation (1) with $|\Gamma n>$ provided by the three Kramer doublets, for external magnetic field directions along the principal directions of the g (or χ) tensor, the following components of the contact shift tensor are obtained:

$$\delta_x^{con} = a_x A_0/h + b_x A_1/h + c_x A_2/h$$

$$\delta_y^{con} = a_y A_0/h + b_y A_1/h + c_y A_2/h \quad (5)$$

$$\delta_z^{con} = a_z A_0/h + b_z A_1/h + c_z A_2/h$$

The a, b and c coefficients derive from the specific $|\Gamma n>$ functions weighted by the Boltzmann population, and $A_{0,1,2}/h$ are the hyperfine coupling constants (expressed in MHz; $\frac{A_i}{h} = \frac{\gamma_N}{2\pi}\frac{8\pi g_e\mu_B}{3}K_i$, where γ_N is the proton magnetogyric ratio and K_i represents the proportionality constant between the orbital-spin density and the electron-spin density at the nucleus[5]) for the ground and excited levels, respectively. In fact, since the hyperfine contact constants A are different for the different electronic states, three such terms are needed. The functional form of these terms has been extensively studied in previous works for the particular case of low-spin iron(III) heme systems,[17,21,30,41−49] and will be summarized below.

The average contact shift in solution is obtained by averaging over all orientations,

$$\delta_{av}^{con} = \frac{1}{3}\left[\delta_x^{con} + \delta_y^{con} + \delta_z^{con}\right] \quad (6)$$

For completeness, the equation for the average dipolar shift, which is the pseudo-contact shift, is also reported:[5,40]

$$\delta^{pc} = \frac{1}{24\pi}\frac{1}{r^3}\left\{\left[2\chi_{zz} - (\chi_{xx} + \chi_{yy})\right](3n^2 - 1) + 3(\chi_{xx} - \chi_{yy})(l^2 - m^2)\right\} \quad (7)$$

where χ_{ii} is the magnetic susceptibility of the molecule in the i direction, r is the distance of the observed nucleus from the atom bearing the unpaired electron spin, and l, m and n are the direction cosines of the metal-nucleus vector with respect to the principal directions of the χ-tensor.

Functional Form of A_0, A_1 and A_2

In low-spin heme complexes, A_0 and A_1 for heme methyl substituents are a function of the angle between the metal-pyrrole II direction and the direction of the (average) p-orbital nodal plane(s) of the π-bonding axial ligand(s)(Figure 2).[17,21,41−43] A_0 depends on the spin density according to[30]

$$A_0 = a\sin^2(\gamma_i - \varphi) \quad (8)$$

where a is a proportionality constant which depends on the amount of orbital-spin density and γ_i is the angle between the metal-ith-proton direction and the metal-pyrrole II axis (equal to 112.5, 22.5, 292.5 and 157.5 for methyls 1, 3, 5 and 8, respectively).

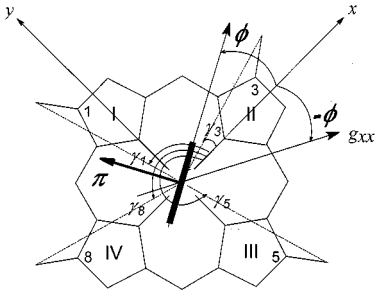

Figure 2. Schematic representation of the heme moiety; the γ_i angles for the four methyl groups are defined as the angles between the metal−methyl ith vector and the metal−pyrrole II direction; the Φ angle defines the direction of the average p-orbital nodal plane of the π-bonding axial ligand(s), and the -Φ angle the resulting direction of the g_{xx} and χ_{xx} axis

The second A value, A_1, is related to the heme spin-density pattern originating from the excited π-MO. It has been assumed that A_1 is equal to A_0, but shifted by 90°, and thus[30]

$$A_1 = a\cos^2(\gamma_i - \varphi) \quad (9)$$

The third A value, A_2, is related to the spin density on a MO to which iron contributes mostly with the d_{xy} orbital. Its value is given by a constant owing to the symmetry of the d_{xy} orbital. Furthermore, its contribution is small as found in those systems with a d_{xy} ground state.[44−49]

Results and Discussion

The simultaneous reproduction of the observed g values and the anisotropy of the magnetic susceptibility values, $\Delta\chi$, was obtained with the parameters reported in Table 1 for cyanometmyoglobin, cytochromes b_5 and c. The values of E_1, E_2, and λ are somewhat different to those calculated by fitting the g values only,[9,26] but the overall picture is the same. The values of E_1 and E_2 vary by about 10% when calculated through an angular overlap approach, which takes into consideration all the excited levels (program CAMMAG [50,51]). This provides a difference in the calculated contact shifts of about 5%, which is considered negligible. Therefore, the three-orbital approximation is sufficient.

In cyanometmyoglobin, the electron energy levels are 0, 692, and 915 cm^{-1} for the lowest three orbitals, d_{xy}, d_{yz}, and d_{xz}, respectively, so that the difference in energy between the ground and the two excited states is 223 and 915 cm^{-1}. These states are perturbed by spin-orbit coupling (with spin-orbit coupling constant $\lambda = 240$ cm^{-1})[52] to 0, 348, and 1023 cm^{-1} (Figure 1). These energy values provide $g_x = 0.93$, $g_y = 1.86$, $g_z = 3.40$ (see Table 1), in good agreement with the experimental values,[8,53] and $\chi_x = 3.12\cdot10^{-32}$, $\chi_y = 4.15\cdot10^{-32}$, $\chi_z = 7.35\cdot10^{-32}$ m^3, at 298 K, which provide $\Delta\chi_{ax} = 3.71\cdot10^{-32}$ m^3 and $\Delta\chi_{rh} = -1.03\cdot10^{-32}$ m^3, again in good agreement with the experimental values (see Table 1). The values of $\delta^{con}_{x,y,z}$, for external magnetic field directions along the principal directions of the g (or χ) tensor, have thus been calculated (in ppm):

$$\delta_x^{con} = 14.7A_0/h + 9.9A_1/h + 0.5A_2/h$$

$$\delta_y^{con} = 19.9A_0/h + 7.7A_1/h + 0.6A_2/h$$

$$\delta_z^{con} = 36.3A_0/h + 0.7A_1/h + 0.9A_2/h$$

at 298 K, where $A_{0,1,2}/h$ are expressed in MHz. Since A_2 is reasonably much smaller than A_0 and A_1,[44−49] and since the coefficients for the second excited state are usually smaller than the others, we will neglect the second excited state contribution hereafter.

The average solution values of the calculated contact and pseudo-contact shifts for the methyl protons, found by setting a fixed value for the a parameter in Equations (8) and (9), are reported in Table 2. It can be seen that the shift pattern is well reproduced for both the contact and the pseudo-contact contributions. For cyanometmyoglobin, for instance, the calculated contact shifts for the 1, 3, 5, and 8 methyl protons are 21.5, 8.2, 26.3, and 12.0 ppm, respectively, in comparison with the experimental values 18.4, 6.7, 27.8, and 15.8 ppm, and the calculated pseudo-contact shifts for the 1, 3, 5, and 8 methyl protons are −3.0, −5.8, −3.0, and −5.6 ppm, respectively, in comparison with the experimental values −3.4, −5.7, −3.3, and −6.5 ppm.[12] It should be noted that the angular dependence of the contact constants is of the same type as that of the pseudo-contact shift. Therefore, the sum of contact and pseudo-contact shifts is provided by an equation which contains the linear combination of $\sin^2$ and $\cos^2$ terms. This justifies the use of a recently proposed heuristic equation.[30]

Similarly, satisfactory results were obtained for cytochrome b_5 and cytochrome c (Table 2). In cytochromes b_5 and c the φ angle in Equations (8) and (9)[43] is determined by the presence of two histidines or a histidine and a methionine as axial ligands.[54] The a values needed to best reproduce the experimental shifts are within ±25% from those found for MetMbCN.

A comparison between the contact hyperfine constants calculated with the Kurland and McGarvey equation and the McConnell equation is reported in Table 3. It is interesting to compare the analysis of the contact shifts through

Table 1. Energy values of the excited orbitals, E_1 and E_2, obtained by fitting the experimental g and χ values of MetMbCN, cytochrome b_5 and cytochrome c. ε_1 and ε_2 are the energies of the excited states; the energy values are expressed in cm^{-1}, the values of the magnetic susceptibility are expressed in m^3; the a, b, and c coefficients of Equation (5) are provided for the three proteins;[a] the experimental data are from: MetMbCN, for g see ref.[8,53] and for $\Delta\chi$ see ref.[18]; cytochrome b_5: for g see ref.[9] and for $\Delta\chi$ see ref.[20]; cytochrome c:for g see ref.[26] and for $\Delta\chi$ see ref.[19,21]

MetMbCN		Experimental	Calculated	Experimental	Calculated			
$E_1 = 692$	$\varepsilon_1 = 348$	$g_x = 0.93$	$g_x = 0.93$	$\Delta\chi_{ax} = 3.78\cdot10^{-32}$	$\Delta\chi_{ax} = 3.71\cdot10^{-32}$	$a_x = 14.7$	$b_x = 9.9$	$c_x = 0.5$
$E_2 = 915$	$\varepsilon_2 = 1023$	$g_y = 1.89$	$g_y = 1.86$	$\Delta\chi_{rh} = -1.01\cdot10^{-32}$	$\Delta\chi_{rh} = -1.03\cdot10^{-32}$	$a_y = 19.9$	$b_y = 7.7$	$c_y = 0.6$
		$g_z = 3.45$	$g_z = 3.40$			$a_z = 36.3$	$b_z = 0.7$	$c_z = 0.9$
Cytochrome b_5		Experimental	Calculated	Experimental	Calculated			
$E_1 = 554$	$\varepsilon_1 = 483$	$g_x = 1.43$	$g_x = 1.43$	$\Delta\chi_{ax} = 2.83\cdot10^{-32}$	$\Delta\chi_{ax} = 2.83\cdot10^{-32}$	$a_x = 17.2$	$b_x = 6.1$	$c_x = 0.4$
$E_2 = 963$	$\varepsilon_2 = 1050$	$g_y = 2.23$	$g_y = 2.23$	$\Delta\chi_{rh} = -1.06\cdot10^{-32}$	$\Delta\chi_{rh} = -1.52\cdot10^{-32}$	$a_y = 23.7$	$b_y = 3.8$	$c_y = 0.6$
		$g_z = 3.03$	$g_z = 2.99$			$a_z = 32.7$	$b_z = 0.7$	$c_z = 0.9$
Cytochrome c		Experimental	Calculated	Experimental	Calculated			
$E_1 = 437$	$\varepsilon_1 = 441$	$g_x = 1.25$	$g_x = 1.25$	$\Delta\chi_{ax} = 2.68\cdot10^{-32}$	$\Delta\chi_{ax} = 2.99\cdot10^{-32}$	$a_x = 16.9$	$b_x = 7.6$	$c_x = 0.6$
$E_2 = 791$	$\varepsilon_2 = 895$	$g_y = 2.25$	$g_y = 2.25$	$\Delta\chi_{rh} = -1.25\cdot10^{-32}$	$\Delta\chi_{rh} = -1.73\cdot10^{-32}$	$a_y = 25.0$	$b_y = 4.4$	$c_y = 1.0$
		$g_z = 3.06$	$g_z = 3.06$			$a_z = 35.0$	$b_z = 0.5$	$c_z = 1.4$

[a] In the present work, the theoretical treatment always results in labeling g_x as the smallest of the three g-tensor components (in absolute value). The same holds for the χ-tensor components. Therefore, $\Delta\chi_{rh}$ values are always negative.

Table 2. Calculated and experimental contact and pseudo-contact shifts for MetMbCN [12], cytochrome b_5 [20] and cytochrome c;[19] the shifts (in ppm) are reported for methyl protons 1, 3, 5, and 8; the methyl proton shifts were corrected for an average higher shift value of the protons of methyls 5 and 8 and an average lower shift value of the protons of methyls 1 and 3 (±1 and ±2.4 ppm for cytochrome c and for cytochrome b_5 and MetMbCN, respectively);[30,43] the empirical a values in Equations (8) and (9) are 1.2, 0.9 and 1.4 for MetMbCN, cytochrome b_5 and cytochrome c, respectively

	Contact shift calculated	experimental	Pseudo-contact shift calculated	experimental
MetMbCN	21.5/8.2/26.3/12.0	18.4/6.7/27.8/15.8	−3.0/−5.8/−3.0/−5.6	−3.4/−5.7/−3.3/−6.5
Cytochrome b_5	6.3/13.5/11.2/6.2	8.6/16.0/17.7/2.0	−0.9/−5.9/−0.9/−2.3	−1.5/−4.7/−1.5/−2.8
Cytochrome c	9.9/29.6/11.9/33.0	7.7/30.1/11.0/35.4	−5.9/−1.2/−5.9/−1.6	−4.4/−1.1/−4.9/−1.9

the Kurland and McGarvey approach and the McConnell approach. In the latter case, the contact shift is given by[1]

$$\delta^{con} = \frac{g_e \mu_B S(S+1)}{3kT\gamma_N}\frac{A'}{\hbar} \qquad (10)$$

where A' is the McConnell hyperfine coupling constant. The data reported in Table 3 show that the value of A'/h is not directly correlated to the values of A_0/h and A_1/h, and the deviation is different for the different methyl protons. Data reported in Table 3 provide an indication of the error inherent in use of the simple McConnell equation. If the difference in energy between the ground and excited states increases, the spin-orbit coupling does not efficiently mix the different d orbitals, the g anisotropy decreases and A' approaches A_0. It should be noted that A' differs from A_0 even in the limits of zero and infinite temperatures (Table 3). In the first case, this is because $<S_z>$ contains an orbital contribution which makes it different from the Curie value used in the McConnell equation. In the second case, the contact coupling constants of each level differ from one another. Only in a case where $A_0 = A_1 = A_2$, which is unrealistic for methyl protons in heme porphyrins, would A' coincide with A_0.

Figure 3 shows how anisotropic the contact shift of methyl protons in cyanometmyoglobin is predicted to be. The contact shift is reported for different orientations of the external magnetic field in either the xy, xz, or yz planes. NMR experimentalists of low-spin hemeproteins have always ignored this anisotropy, because they are dealing with an averaged shift in solution and only the average contact contribution to the hyperfine interaction is obtained. It is obvious, however, that in the case of partially oriented systems, the average should be calculated by providing a different weighting in the different directions, and therefore anisotropy must be considered.

In Table 4 the contributions to the contact shift along the three main directions have been reported for the methyl protons. Data show that the anisotropy can be very large, so that such an effect must be taken into account in the presence of partial orientation. Appropriate equations are reported in the Appendix.

It should be noted that, since contact shifts are usually larger than pseudo-contact shifts, the effects originating from the anisotropy in the contact term are similarly expected to be larger than those related to the anisotropies in the dipolar term. Therefore, such dependencies should be carefully considered in ENDOR spectroscopy, where any

Table 3. Contact shifts of the methyl protons, δ^{con}, of MetMbCN, cytochrome b_5 and cytochrome c, obtained with the Kurland and McGarvey equation with the values of A_0/h and A_1/h calculated from Equations (8, 9) and the resulting coupling constant A'/h back-calculated from the values of δ^{con} and from the McConnell equation, for three different temperatures; A/h values are expressed in MHz, and the shifts in ppm; note that in the table the heuristic correction made in Table 2, and suggested in ref.[43] was not made

MetMbCN								
	δ^{con} 298 K	A'/h (McConnell)	A_0/h (McGarvey)	A_1/h (McGarvey)	δ^{con} 1 K	A'/h (McConnell)	δ^{con} 3130 K	A'/h (McConnell)
1-CH_3	23.9	0.902	0.960	0.199	6306	0.799	1.440	0.571
3-CH_3	10.6	0.400	0.199	0.960	1316	0.167	0.941	0.373
5-CH_3	23.9	0.902	0.960	0.199	6306	0.799	1.440	0.571
8-CH_3	9.6	0.362	0.142	1.017	941	0.119	0.904	0.358
Cytochrome b_5								
	δ^{con} 313 K	A'/h (McConnell)	A_0/h (McGarvey)	A_1/h (McGarvey)	δ^{con} 1 K	A'/h (McConnell)	δ^{con} 3130 K	A'/h (McConnell)
1-CH_3	8.7	0.345	0.267	0.610	2066	0.262	0.805	0.319
3-CH_3	15.9	0.630	0.610	0.267	4714	0.597	0.998	0.396
5-CH_3	8.7	0.345	0.267	0.610	2066	0.262	0.805	0.319
8-CH_3	3.8	0.151	0.035	0.842	271	0.034	0.674	0.133
Cytochrome c								
	δ^{con} 293 K	A'/h (McConnell)	A_0/h (McGarvey)	A_1/h (McGarvey)	δ^{con} 1 K	A'/h (McConnell)	δ^{con} 3130 K	A'/h (McConnell)
1-CH_3	10.9	0.404	0.239	1.152	1764	0.223	1.154	0.457
3-CH_3	30.6	1.135	1.152	0.239	8475	1.073	1.681	0.666
5-CH_3	10.9	0.404	0.239	1.152	1764	0.223	1.154	0.457
8-CH_3	32.0	1.187	1.221	0.171	8980	1.137	1.720	0.682

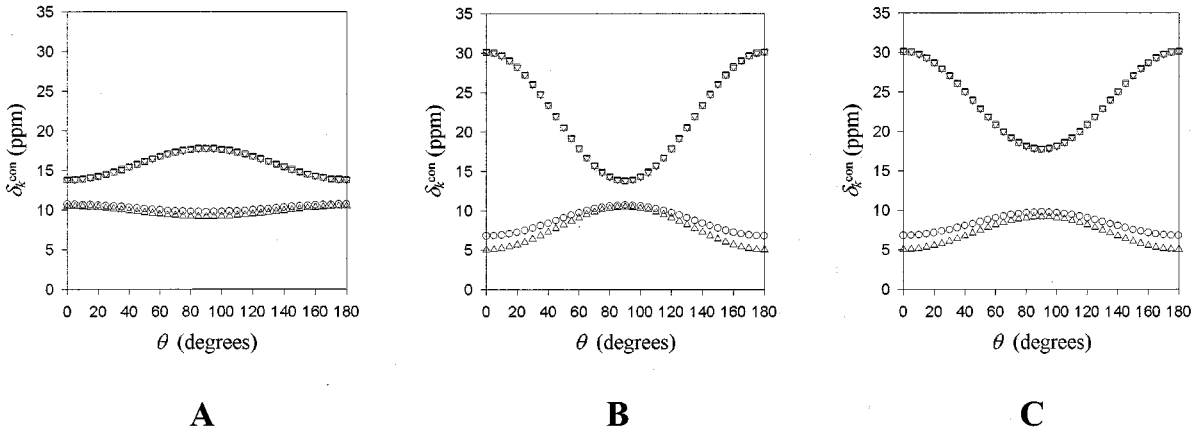

Figure 3. Contact shift calculated for different k orientations of the molecule in a magnetic field for the four methyl protons (□ = 1-CH_3, ○ = 3-CH_3, ▽ = 5-CH_3, Δ = 8-CH_3); panel A shows the shifts for the magnetic field B_0 in the xy plane, where θ is the angle between B_0 and the metal-pyrrole(II) direction; in panels B and C, θ is the angle between B_0 and the z axis, with B_0 in the xz (B) or yz (C) planes; φ is set to −2°, as found experimentally for MetMbCN[60,61]

angular dependence is usually ascribed to the dipolar interaction, and assuming an isotropic contact contribution would be erroneous.

Concluding Remarks and Perspectives

The McConnell equation (Equation 10) is generally used to analyze contact shift patterns. As outlined on several occasions,[5,55] such an equation only holds for one unpaired electron in a single orbital with g near to 2. Low-spin Ru^{III} and Fe^{III} have g values very different from 2, and also have low-lying excited levels.[28,29,53] In these cases a much more complex analysis is needed. In the case of heme proteins, the hyperfine shifts are provided by both contact and pseudocontact contributions. The latter are absolutely dominant for protons several bonds away from the metal ion, and allow for the determination of magnetic susceptibility anisotropies (according to Equation 7) together with the

Table 4. Anisotropic contributions to the contact shifts (in ppm), calculated for MetMbCN, cytochrome b_5, and cytochrome *c*

Methyl		MetMbCN[12,59]	Cytochrome b_5[20]	Cytochrome *c*[19,21]
1-CH_3	*x*	12.3	8.2	9.0
	y	15.9	8.5	7.8
	z	27.0	9.1	6.3
	average	18.4	8.6	7.7
3-CH_3	*x*	7.9	12.2	21.0
	y	7.2	15.6	29.4
	z	5.0	20.2	39.9
	average	6.7	16.0	30.1
5-CH_3	*x*	18.6	16.8	12.9
	y	24.0	17.6	11.1
	z	40.7	18.7	9.0
	average	27.8	17.7	11.0
8-CH_3	*x*	20.1	3.0	24.3
	y	17.6	2.1	34.6
	z	9.8	0.9	47.3
	average	15.8	2.0	35.4

principal direction of the χ tensor and a refinement of the solution structure.[19,20,40,56] From the knowledge of the *g* and Δχ values, the contact shift of the heme protons can be estimated. A ligand-field analysis, together with the Kurland and McGarvey approach, allows for a detailed and instructive analysis of the contributions of the various electronic levels. Such an analysis is absolutely necessary when the hyperfine coupling is analyzed by ENDOR spectroscopy and when proton hyperfine shifts are analyzed in partially oriented systems.

Finally, magnetically anisotropic molecules in a high magnetic field experience partial orientation, which should result in a variation in the hyperfine shifts due to residual dipolar coupling between the unpaired electron(s) and the resonating nucleus. When the contact shift is anisotropic, there is also a variation in the contact contribution. This is the first time that such an effect has been accurately calculated for heme proteins, although dipolar coupling in those systems is well known in ENDOR spectroscopy.[57] When contact shift occurs, its anisotropy also contributes to the change in hyperfine shifts in partially oriented systems. This has always been neglected in ENDOR and NMR spectroscopy, despite the warnings of McGarvey,[5,55] which have largely been ignored.

Residual electron-nucleus dipolar couplings are a new class of structural constraints and may become significant when systems with $S > 1/2$ and/or more efficient orienting solvents are used. When the contact interaction is operative, further details of the electronic structure of the systems under investigation become available.

Appendix

In the presence of partial orientation, an orientational *D* tensor should be defined, with principal components D_x, D_y, and D_z. The residual contact shift, i.e. the difference between the averaged contact shift in the absence and in the presence of partial orientation, $\Delta\delta^{con}$, is given by

$$\Delta\delta^{con} = \tilde{\delta}_x^{con} D_x + \tilde{\delta}_y^{con} D_y + \tilde{\delta}_z^{con} D_z \qquad (A.2)$$

In general, the *D* and *g* tensors are not diagonal in the same frame. Therefore, the shifts must be calculated by evaluating the weighted average between the shifts along the main directions of the orientational *D* tensor, so that in Equation A.1

$$\tilde{\delta}_i^{con} = \delta_x^{con} \sin^2\alpha\cos^2\beta + \delta_y^{con} \sin^2\alpha\sin^2\beta + \delta_z^{con} \cos^2\alpha \qquad (A.2)$$

where $\tilde{\delta}_i^{con}$ are provided by Equation (5), with α and β equal to the polar angles that define the position of the $i = x, y, z$ axes of the *D* tensor in the frame of the *g* tensor.

Analogously, the residual dipolar shift contribution is provided by the following equation

$$\Delta\delta^{dip} = \tilde{\delta}_x^{dip} D_x + \tilde{\delta}_y^{dip} D_y + \tilde{\delta}_z^{dip} D_z \qquad (A.3)$$

with[4]

$$\tilde{\delta}_i^{dip} = \frac{1}{4\pi r^3}\left[3\left(\chi_{zz}\cos\alpha\cos\vartheta + \chi_{xx}\sin\alpha\cos\beta\sin\vartheta\cos\varphi + \chi_{yy}\sin\alpha\sin\beta\sin\vartheta\sin\varphi\right)\right.$$
$$\left(\cos\alpha\cos\vartheta + \sin\alpha\cos\beta\sin\vartheta\cos\varphi + \sin\alpha\sin\beta\sin\vartheta\sin\varphi\right) - \qquad (A.4)$$
$$\left.\left(\chi_{zz}\cos^2\alpha + \chi_{xx}\sin^2\alpha\cos^2\beta + \chi_{yy}\sin^2\alpha\sin^2\beta\right)\right]$$

where α and β are the polar angles that define the position of the main axis *i* of the *D* tensor in the frame where χ is diagonal, and are the polar angles that define the position of the electron−proton direction in the same frame (see Figure 4). Integration of Equation (A.4) over all space gives Equation (7). Equations (A.1−A.2) and (A.3−A.4) may prove useful whenever extensive orientation in solution

without too severe broadening in the NMR lines is obtained.

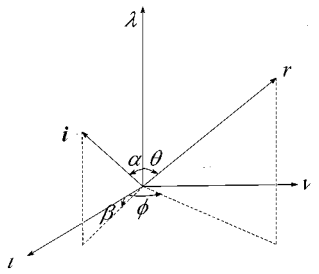

Figure 4. Schematic representation in the χ frame of the α and β polar angles defining the position of the *i* direction and of the θ and φ polar angles defining the proton-electron *r* direction

For completeness, we mention the limit values of Equations (A.1−A.2) and (A.3−A.4) when the *D*, *g* and χ tensors are diagonal in the same frame. This may be the case for molecules experiencing partial orientation in a field as a result of the anisotropy of the χ tensor itself.[58] For the residual contact shift, $\tilde{\delta}_i^{\mathrm{con}}$ in Equation (A.1) are provided by Equation (5). For the residual dipolar shifts, $\tilde{\delta}_i^{\mathrm{dip}}$ in Equation (A.3) reduce to:

$$\tilde{\delta}_x^{\mathrm{dip}} = \frac{\chi_{xx}\left(3l^2-1\right)}{4\pi r^3} \qquad \tilde{\delta}_y^{\mathrm{dip}} = \frac{\chi_{yy}\left(3m^2-1\right)}{4\pi r^3} \qquad \tilde{\delta}_z^{\mathrm{dip}} = \frac{\chi_{zz}\left(3n^2-1\right)}{4\pi r^3}$$

In the axial case and in the hypothesis that the components of the *D* tensor are proportional to those of the χ tensor, the following relationships can be derived:

$$D_{\parallel} = \frac{1+\frac{B_0^2}{2\mu_0 kT}\chi_{\parallel}}{3+\frac{B_0^2}{2\mu_0 kT}\chi_{\parallel}+2\frac{B_0^2}{2\mu_0 kT}\chi_{\perp}} \qquad D_{\perp} = \frac{1+\frac{B_0^2}{2\mu_0 kT}\chi_{\perp}}{3+\frac{B_0^2}{2\mu_0 kT}\chi_{\parallel}+2\frac{B_0^2}{2\mu_0 kT}\chi_{\perp}}$$

so that $D_{\parallel} + 2D_{\perp} = 1$. Substituting into Equation (A.3) provides the pseudo-contact shift

$$\delta^{\mathrm{pc}} = \frac{1}{3+\frac{B_0^2}{2\mu_0 kT}\chi_{\parallel}+2\frac{B_0^2}{2\mu_0 kT}\chi_{\perp}} \cdot \frac{1}{4\pi r^3}\left[\left(3\chi_{\parallel}\cos^2\theta-\chi_{\parallel}\right)\left(1+\frac{B_0^2}{2\mu_0 kT}\chi_{\parallel}\right)+2\left(\frac{3}{2}\chi_{\perp}\sin^2\theta-\chi_{\perp}\right)\left(1+\frac{B_0^2}{2\mu_0 kT}\chi_{\perp}\right)\right]$$

where θ is the angle between *r* and $\chi_{\parallel}$. To first order, the above equation can be approximated as

$$\delta^{\mathrm{pc}} = \frac{(3\cos^2\theta-1)(\chi_{\parallel}-\chi_{\perp})}{12\pi r^3}\left(1+\frac{B_0^2}{6\mu_0 kT}(2\chi_{\parallel}+\chi_{\perp})\right) \quad (A.5)$$

A similar equation has already been reported.[58] A remarkable feature of this equation is that the correction to the pseudo-contact shifts owing to orientation $\left(\frac{B_0^2}{6\mu_0 kT}(2\chi_{\parallel}+\chi_{\perp})\right)$ depends on the magnitude of the magnetic susceptibility but is independent of its anisotropy.

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