Determination of the Principal Axes of the Magnetic Susceptibility Tensor for Horse Heart Oxidized Cytochrome c in Solution

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The magnetic axes in horse heart oxidized cytochrome c were calculated by magnetic anisotropy tensor determination utilizing the haem methyls (MATDUHM) and were compared with those calculated by the conventional least-squares search method (LSSM). For the backbone $C_\alpha H$ proton resonances, the calculated shifts using the LSSM axes correlate better with the observed shifts, *i.e.* the chemical shift difference between the resonances of the reduced and oxidized forms, than those using the MATDUHM axes. The correlation for the backbone NH proton resonances is almost comparable for the two sets of axes. Therefore the validity of MATDUHM was confirmed. Since the applicability of LSSM is limited to haemoproteins for which the crystal structures are known, MATDUHM is the only method so far available that can be applied to any iron(III) low-spin haemoproteins under various solution conditions to locate the magnetic axes.

In biological systems haemoproteins play central roles in energy metabolism utilizing molecular oxygen in addition to transporting dioxygen. Their active sites and various functions should involve specific interactions between the haem and the surrounding amino acid residues. Knowledge, at the atomic level, of the molecular structure of the active sites is indispensable for the understanding of their structure–function relationships. X-Ray structural analysis has provided a wealth of information on the structure of haemoproteins.[1] Their functional properties, however, are not fully understood on the basis of the crystal structure. For example, in the case of oxygen-binding haemoprotein, X-ray study does not reveal the path for approach of O_2 to the haem active site.[2–4] Hence, the analysis of the solution structure should provide useful information for interpreting functions in terms of the molecular structure.

The NMR technique is the most promising for providing detailed structural information on the active site of haemoprotein in solution.[5–7] Especially, the hyperfine-shifted resonances for paramagnetic haemoprotein have been used effectively to determine the electronic and molecular structure of its active site. The observed shift (δ_{obs}) of a hyperfine-shifted resonance is expressed as in equations (1) and (2) where δ_{dia} and δ_{hf} are the

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

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

diamagnetic and hyperfine shifts, respectively, and the shift of the iron(II) low-spin form or diamagnetic model complex is usually used as δ_{dia}; δ_c is the contact shift and $\delta^L{}_{pc}$ and $\delta^M{}_{pc}$ are the ligand- and metal-centred pseudo-contact shifts due to the magnetic dipolar field arising from the delocalized electron and the unpaired electron at the haem iron, respectively. Since δ_c and $\delta^L{}_{pc}$ are negligibly small for the resonances of non-co-ordinated amino acid protons, their δ_{hf} values should be equal to $\delta^M{}_{pc}$. The Hamiltonian (H_D) for the interaction of a nucleus (I) with unpaired electron(s) (S = total electron spin) is expressed as in equation (3) where D is the dipolar tensor for the

$$H_D = I \cdot D \cdot S \quad (3)$$

interaction between I and S.[8] Since D depends on the actual coordinates of the nucleus relative to the unpaired electron, H_D reflects the spatial relationship between the two spins. For a single unpaired electron, $\delta^M{}_{pc}$ is given in terms of the magnetic anisotropy for S as in equation (4) where χ_i are the principal

$$\delta^M{}_{pc} = [(3\cos^2\theta - 1)(2\chi_z - \chi_x - \chi_y) + 3\sin^2\theta\cos 2\Omega(\chi_x - \chi_y)]/4\pi 6r^3 \quad (4)$$

components of the molecular susceptibility tensor and r, θ, Ω is the polar coordinate system. Provided that the principal magnetic axes with respect to the haem are known, the spatial positions of non-co-ordinated amino acid residues relative to the unpaired electron can be quantitatively determined from the $\delta^M{}_{pc}$ values using equation (4) (see Fig. 1).

Two procedures have been proposed for determining the orientation of these axes for paramagnetic haemoproteins in solution. One is based on the components of the diagonal susceptibility tensor determined from measurement of the low-temperature g values of the lowest Kramer's doublets.[9–11] Even for ideal magnetic behaviour, questions remain about the application of those solid-state data to interpret solution NMR results.[12] It has been shown that the magnetic tensor orientation determined from low-temperature ESR measurements is inadequate for the analysis of solution NMR data.[13] The least-squares search method (LSSM) is the other approach and has been commonly and satisfactorily used for various haemoproteins, *e.g.* iron(III) low-spin forms of mitochondorial cytochrome c,[14] sperm whale myoglobin,[13] bovine cytochrome b_5,[15] horse heart cytochrome c[16,17] and yeast iso-1-cytochrome c.[18] This method requires not only the X-ray coordinates of the protein, but also the unambiguous assignments of many hyperfine-shifted NMR resonances.

The hyperfine-shifted haem methyl carbon NMR resonances for iron(III) low-spin haemoproteins are resolved outside of the ^{13}C NMR diamagnetic envelope where the carbon resonances of the apo-protein overlap severely and these resonances can be unambiguously assigned *via* ^1H–^{13}C correlation spectroscopy (COSY) connectivities.[19–23] We have shown that the combined analyses of the resonances of the haem methyl carbons and of the attached protons allows the determination of the principal

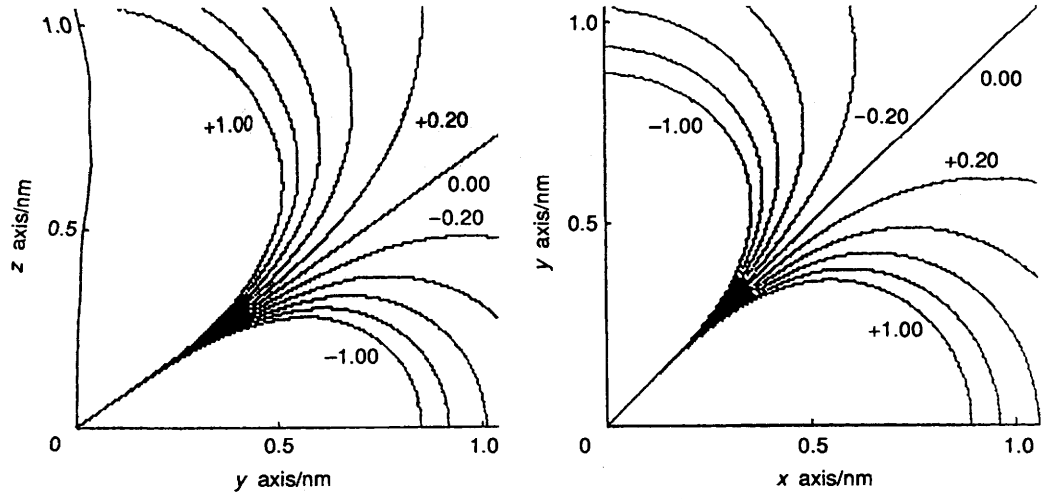

Fig. 1 The paramagnetic dipolar field in horse heart oxidized cytochrome c at 20 °C calculated by MATDUHM. The axial ($\delta^{M}_{pc\parallel}$) and rhombic ($\delta^{M}_{pc\perp}$) terms of equation (4) were plotted separately and δ^{M}_{pc} at any point in space is a sum of these terms. The numbers on the plots are shifts in ppm (positive sign indicates downfield shift). The $|\chi_{\parallel}/\chi_{\perp}|$ value of 1.70:1 was obtained from the calculation

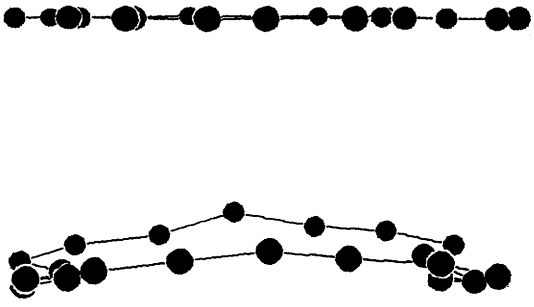

Fig. 2 The geometry of the haem porphyrin ring indicated in the cyt c single crystal[27] (lower) and used in MATDUHM (upper). The porphyrin ring is placed on the plane defined by the least-squares fit of the atomic coordinates of the four pyrrole nitrogens having D_{4h} symmetry in the upper structure. Only the ring carbons are indicated for simplicity

magnetic axes in haemin complex possessing magnetic anisotropy.[24–26] Since only the NMR results are used for the proposed method, MATDUHM (magnetic anisotropy tensor determination utilizing the haem methyls),[24–26] this method is applicable to any iron(III) low-spin haemoproteins under any solution conditions to locate their principal magnetic axes.

In this paper, MATDUHM is applied to horse heart oxidized cytochrome c, cyt c, to determine the principal magnetic axes. The availability of its crystal structure[27] and the exhaustive NMR signal assignments[18,28–32] allow a detailed comparison between the magnetic axes determined by MATDUHM and LSSM.[16,17]

Procedures of MATDUHM[25,26]

According to the McConnell's equation,[33,34] the δ_c values for the resonances of the haem methyl carbons and the attached protons are directly proportional to the unpaired electron density (ρ) in the p_z orbital of the pyrrole carbon to which the methyl group is covalently bonded. To the first approximation, δ^{L}_{pc} is also dependent on ρ. Therefore, plots of the quantity $\delta_c + \delta^{L}_{pc}$ for the haem methyl carbon resonance against that for the attached proton resonance should be linear and pass through the origin. Deviation of such a plot from a straight line is hence attributed to the difference in δ^{M}_{pc} between the haem methyl carbon and the attached proton. The main computational problem in MATDUHM arises from determining the best orientation of the Cartesian coordinates, such that the deviation of the $(\delta_c + \delta^{L}_{pc})$ plot from linearity is minimized. This requires calculation of δ^{M}_{pc} for the haem methyl carbons and protons with respect to all possible orientations (by every 1°) of a Cartesian coordinate system in space. Here, the origin of the coordinate system is set at the centre of mass for the four pyrrole nitrogens of the porphyrin (this origin does not always coincide with the position of the haem iron due to a distortion of the haem plane in the single crystal and, in the present protein, the origin is 4 pm away from the haem iron) (see below). Owing to the symmetric nature of equation (4), minimization converges to a few local minima such that the z axis lies close to the haem plane. The tensor orientation is selected on the premise that the z axis is generally along the normal to the haem plane.

An algorithm implemented in FORTRAN was run on a SUN 4 computer. The X-ray crystal coordinates of oxidized cyt c were kindly provided by Professor G. D. Brayer.[27] According to the reported crystal structure, the haem plane in the active site is slightly distorted (see Fig. 2). Such distortion of the porphyrin ring may result in functional consequences as recently suggested by Barkigia *et al.*[35] Since the spatial relationships between the haem iron and the haem methyl groups are crucial to our analysis, we tentatively consider that the porphyrin ring of the haem is in the plane defined by the least-squares fit of the atomic coordinates for the four pyrrole nitrogens with D_{4h} symmetry. The hydrogen-atom coordinates were generated from the heavy-atom coordinates using the program HGEN (FORTRAN source, MRC library files), with the assumption of standard amino acid geometries and a bond length of 0.1 nm. The centre of mass for the methyl protons was used to calculate the geometric factor for the haem methyl protons.

The procedures of MATDUHM for a given low-spin haemoprotein are summarized as follows.

(1) Assignments of both haem methyl ^1H and ^{13}C NMR resonances. The haem methyl ^1H signals can be assigned *via* nuclear Overhauser effect (NOE) connectivities[36] and then ^1H–^{13}C COSY[19–21,23] is effective for assigning the methyl carbon resonances.

(2) Estimation of $\delta_{hf}(=\delta_{obs} - \delta_{dia})$. For δ_{dia}, shifts of a diamagnetic form are desirable. However, the values of δ 3.73 and 13.85 for the haem ^1H and ^{13}C NMR resonances, respectively, obtained for the model complex (3,7,12,17-tetramethyl-8,13-divinylporphyrin-2,18-dipropanoato)zinc(II) in C_5D_5N[24] may also be used as δ_{dia} (see the following section).

(3) Definition of a reference coordinate system and calculation of the atomic coordinates for the haem methyl groups. The porphyrin ring of the haem is assumed to have D_{4h} symmetry and the centre of mass for the methyl protons is used for the coordinates for the ^1H.

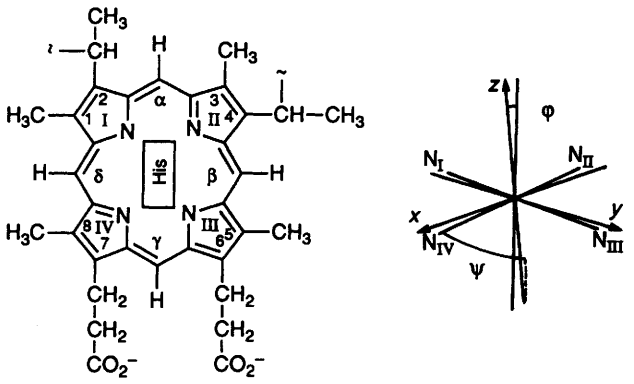

Fig. 3 The structure of the haem in cyt c (left) and the orientation of the principal magnetic axes with respect to the haem in cyt c, calculated by MATDUHM (right). The angle (φ) between the z axis and the normal to the haem plane is 9.5° and the angle (ψ) between the projection of the z axis on the haem plane and the N_{II}–N_{IV} axis is 57.9°

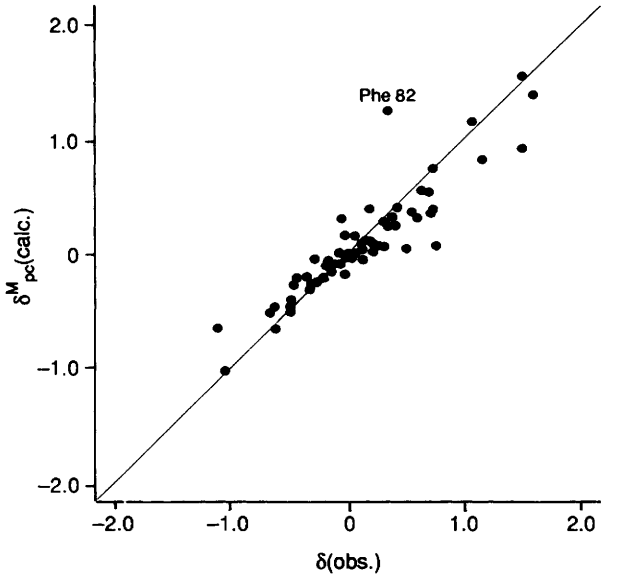

Fig. 4 Plot of δ^{M}_{pc}(calc.) *vs.* the chemical shift difference [δ(obs.)] of the backbone $C_\alpha H$ proton resonance between reduced[31] and oxidized cyt c.[32] The point for Phe 82 is indicated. Data for the Cys 14, Cys 17, His 18, Met 80 and 12 Gly residues are excluded (see text)

(4) Plot of δ_{hf} (^1H *vs.* ^{13}C) and least-squares fitting of the data points. The intercept of this line is fixed at the origin.

(5) Calculation of the sum of the distances (Σd) between the line selected in the preceding step and the individual data points.

(6) Rotation of a coordinate system, operated by Euler angles (α,β,γ), with respect to the reference frame, and calculation of δ^{M}_{pc} in equation (4). The number of computations can be reduced to 180^3 due to the symmetric nature of equation (4). For the calculation of δ^{M}_{pc} with a given (α,β,γ), $\chi_{\parallel}[=\chi_z - (\chi_x + \chi_y)/2]$ and $\chi_{\perp}(=\chi_x - \chi_y)$ are optimized for minimum Σd.

(7) Selection of $(\alpha,\beta,\gamma,\chi_{\parallel},\chi_{\perp})$ based on the Σd value. The z axis is assumed to be roughly along the normal to the haem plane.

Results and Discussion

Orientation of the Principal Magnetic Axes in cyt c determined by MATDUHM.—We used the assignments of the haem methyl ^1H and ^{13}C NMR resonances for both oxidation states of cyt c reported previously.[18,28–32] The orientation of the principal magnetic axes with respect to the haem, determined by MATDUHM, is schematically shown in Fig. 3 and the ratio of the magnetic anisotropy, $|\chi_{\parallel}/\chi_{\perp}|$, of 1.70:1 was obtained. The angle between the z axis and the haem normal is 9.5° and the z axis forms angles of 9.9 and 12.5° with the Fe–His 18 and Fe–Met 80 bonding vectors, respectively. Therefore the orientation of the z axis with respect to the haem appears to be different from that previously determined by LSSM.[16,17] This disagreement may be explained in terms of the nature of the two methodologies. In MATDUHM only the haem methyl groups are used in the calculation, while LSSM is based on a subset of the $C_\alpha H$ proton assignments and the X-ray structure. The $|\chi_{\parallel}/\chi_{\perp}|$ value of 1.70:1 is to be compared with the value of 1.73:1 calculated from the g values obtained from the single-crystal ESR measurements of cyt c at 4.2 K.[37]

Influence of the Uncertainty in δ_{hf} on the Result of MATDUHM.—Since MATDUHM relies on the δ_{hf} values for the haem methyl ^1H and ^{13}C NMR resonances, the uncertainty in δ_{hf} results in an alteration in the calculated orientation of the principal magnetic axes. Methodologies for assignment of all the haem methyl ^1H resonances in both diamagnetic and iron(III) low-spin haemoproteins have been established.[7,38] It has been shown that the assignment of the haem methyl ^{13}C NMR resonances for iron(III) low-spin haemoprotein can be made using the detection of the ^1H–^{13}C scalar connectivities.[19–23] At present assignments of the haem methyl ^{13}C NMR resonances of diamagnetic haemoproteins have been reported only for horse heart cyt c[30] and biosynthetically ^{13}C-enriched cyt c_{553}.[39] In the case where the δ_{dia} values are unavailable for the protein of interest, the value of 13.85 ppm, the average shift of the haem methyl ^{13}C NMR resonances of the diamagnetic model zinc complex in C_5D_5N,[40] may be used as δ_{dia} for the analysis. The calculation indicated that the ± 1.0 ppm change of δ_{hf} for the carbon resonances produces changes of ≈2° for the angle (φ) between the z axis and the normal to the haem plane and ≈5° for the angle (ψ) between the projection of the z axis on the haem plane and the N_{II}–N_{IV} axis, relative to the orientation shown in Fig. 3. Therefore a 1–3% change in δ_{hf} does not lead to a significant alteration in the orientation of the magnetic axes. It has been shown that the δ^{M}_{pc} values calculated from the magnetic axes determined using the δ_{dia} value of 13.85 for all the haem carbon resonances correlate well with the δ^{M}_{pc} values of the resonances of non-co-ordinated amino acid protons for the met-cyano form of sperm whale myoglobin.[25]

Evaluation of the Results calculated by MATDUHM.—Since the backbone structure of a protein in the solution state is expected to be similar to that in the crystal state, the amino acid $C_\alpha H$ proton shifts have been used as a basis data set for calculating the magnetic axes by LSSM.[16,17] Here, the results calculated by MATDUHM are evaluated on the basis of the backbone ^1H NMR shift data. The δ^{M}_{pc} values were calculated using the axes shown in Fig. 3 and the hydrogen-atom coordinates generated from heavy-atom coordinates reported by Bushnell *et al.*[27] The calculated δ^{M}_{pc} values [δ^{M}_{pc}(calc.)] are compared with the observed shift difference, δ(obs.), between reduced[31] and oxidized cyt c,[32] in Fig. 4. The $C_\alpha H$ protons of Cys 14, Cys 17, His 18 and Met 80 were excluded from the plot because their shifts are thought to be influenced by delocalized unpaired electrons. Additionally, the $C_\alpha H_2$ protons of 12 Gly residues were not considered due to the absence of stereospecific assignments. In spite of possible differences between the solution and crystal structures in both oxidation states and the structural fluctuations of the protein in solution, the agreement between the δ^{M}_{pc}(calc.) and δ(obs.) values is good. The δ^{M}_{pc}(calc.) and δ(obs.) values for the $C_\alpha H$ proton resonances, together with the distance from the haem iron, are plotted against the residue number in Fig. 5. A large discrepancy between the two values, arising from the contribution of δ_c, is apparent for the Cys 14, Cys 17, His 18 and Met 80 residues.

The difference (Δδ) between the δ(obs.) value of the $C_\alpha H$ proton resonance and the δ^{M}_{pc}(calc.) value calculated using the magnetic tensor determined by MATDUHM and LSSM[16,17] is plotted against the residue number in plots (*a*) and (*b*) of Fig. 6, respectively. The Gly residues and the residues experiencing

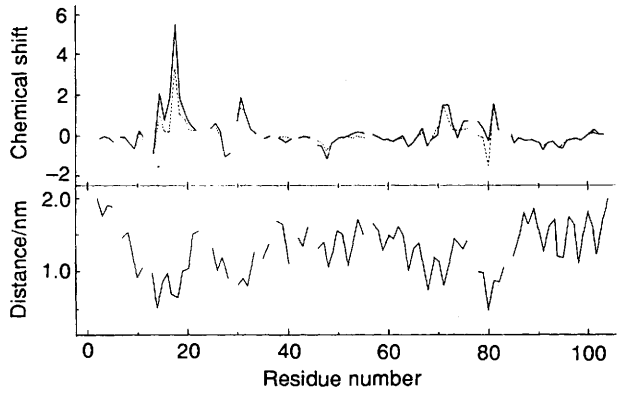

Fig. 5 Plot of δ^{M}_{pc}(calc.) (——) and δ(obs.) (– – –) for the backbone $C_\alpha H$ proton resonance *vs.* the amino acid sequence. The distances between the $C_\alpha H$ proton and the haem iron are also plotted

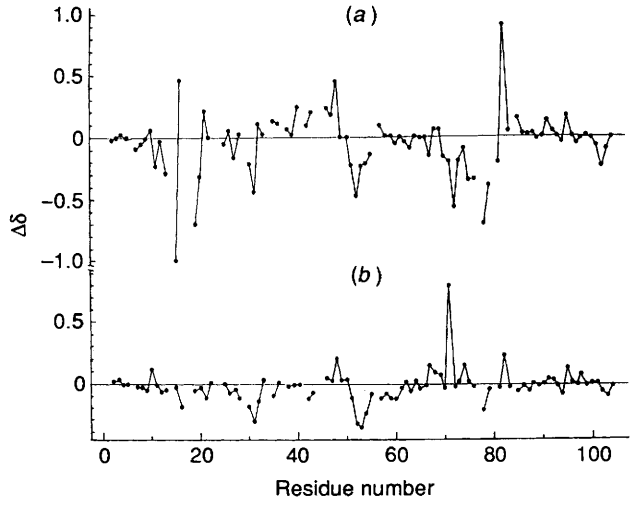

Fig. 6 Plots of the discrepancy $\Delta\delta[=\delta^{M}_{pc}(\text{calc.}) - \delta(\text{obs.})]$ for the backbone $C_\alpha H$ proton resonances *vs.* the amino acid sequence. The LSSM results (*b*) were obtained from ref. 16

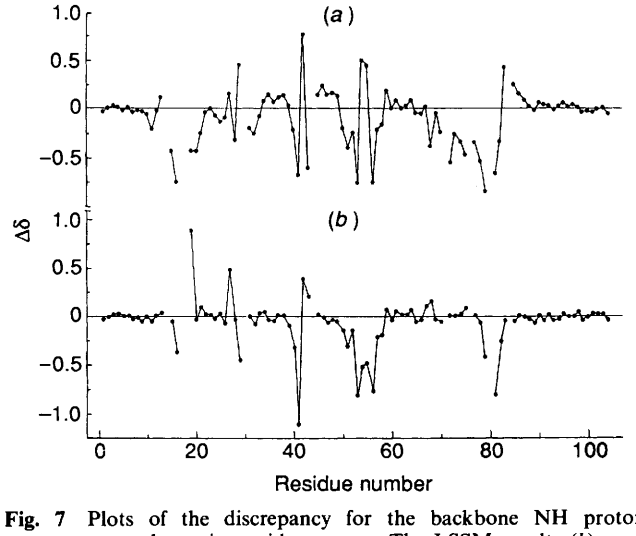

Fig. 7 Plots of the discrepancy for the backbone NH proton resonances *vs.* the amino acid sequence. The LSSM results (*b*) were obtained from ref. 16

sizable δ_c are excluded. The variance, $\Delta\sigma(=\Sigma\Delta\delta^2/n)^{\frac{1}{2}}$ (n = the number of assigned protons considered), is smaller for plot (*b*) than for (*a*), indicating that the magnetic axes reported by Feng and co-workers[16,17] predict the δ(obs.) values of the $C_\alpha H$ proton resonances better. The backbone NH proton resonances are similarly analysed in Fig. 7. The Δσ values are given in

Table 1 Difference between calculated and observed values

	Δσ(ppm)[a]		
Protein	$C_\alpha H$ protons[b]	NH protons[c]	Ref.
Horse heart cyt c	0.13 (86)	0.24 (98)	16, 17
	0.25 (86)	0.28 (98)	This work
Yeast iso-cyt c	0.21 (114)	0.24 (99)	18
Sperm whale myoglobin CN	0.44 (5)		13
Bovine cyt b_5	0.13 (65)	0.15[d] (72)	15

[a] $\Delta\sigma = \{(1/n)\Sigma[\delta^{M}_{pc}(\text{calc.}) - \delta(\text{obs.})]^2\}^{\frac{1}{2}}$; n the number of assigned resonances is indicated in parentheses. [b] The $C_\alpha H$ proton resonances of the Gly residues and the residues experiencing a sizable δ_c are excluded. [c] The NH proton resonances of the residues experiencing a sizable δ_c are excluded. [d] The Gly 42 residue (Δδ = 2.12) was excluded.

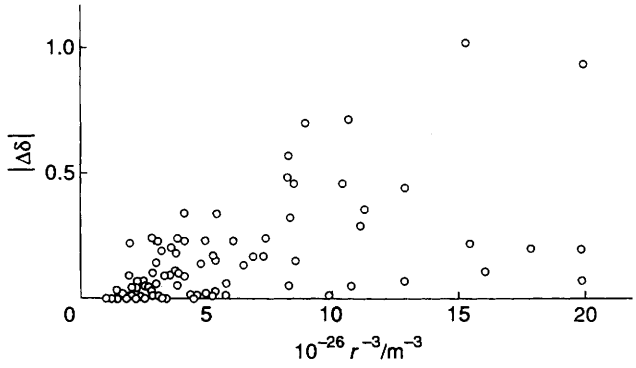

Fig. 8 The |Δδ| values for the backbone $C_\alpha H$ proton resonances plotted against r^{-3}

Table 1, together with the values calculated for other proteins for comparison. Since the chemical shift of the backbone protons is sensitive to hydrogen bonding as well as to the backbone conformation,[4,1] the δ(obs.) values should contain the effects of these differences between the two oxidation states of cyt c in addition to the δ^{M}_{pc} contribution. The Δσ values for the $C_\alpha H$ and NH protons are almost comparable for the results of MATDUHM and those of LSSM for the other proteins, because not only $C_\alpha H$ protons but also NH protons were considered in the calculation of LSSM. On the other hand, for the results of Feng and co-workers,[16,17] the Δσ value of the NH protons is much larger than that of the $C_\alpha H$ protons. This indicates that the orientation of the magnetic axes calculated by LSSM depends on the δ(obs.) data set used. Gao *et al.*[30] have pointed out that LSSM using only the $C_\alpha H$ protons as a data set tends to overestimate the effects of hydrogen bonding and conformational changes and that, on the other hand, LSSM using all the assigned protons, *i.e.* $C_\alpha H$, NH, and other side-chain protons, may underestimate these effects and suffers from the flexibility of the side-chain conformation. The MATDUHM approach is not subject to these problems.

The discrepancy, |Δδ|, for the results of MATDUHM is plotted against r^{-3} in Fig. 8, in order to analyse the relationship between |Δδ| and the field gradient of the paramagnetic dipolar field. If the principal magnetic axes calculated by MATDUHM are inadequate there should be a correlation between these two quantities, because the discrepancy should increase with r^{-3}. Such a correlation is not observed in the plot, indicating that MATDUHM is useful to calculate the orientation of the principal magnetic axes which can be used to estimate the δ(obs.) values of proton resonances in any portion of the protein. The large discrepancy for the $C_\alpha H$ proton of Phe 82 is discussed in terms of its conformational properties (see below). Although σ-type delocalization of unpaired electron density from the haem iron to the haem methyl nuclei, in practice, may not be completely ruled out and the distortion of the haem plane

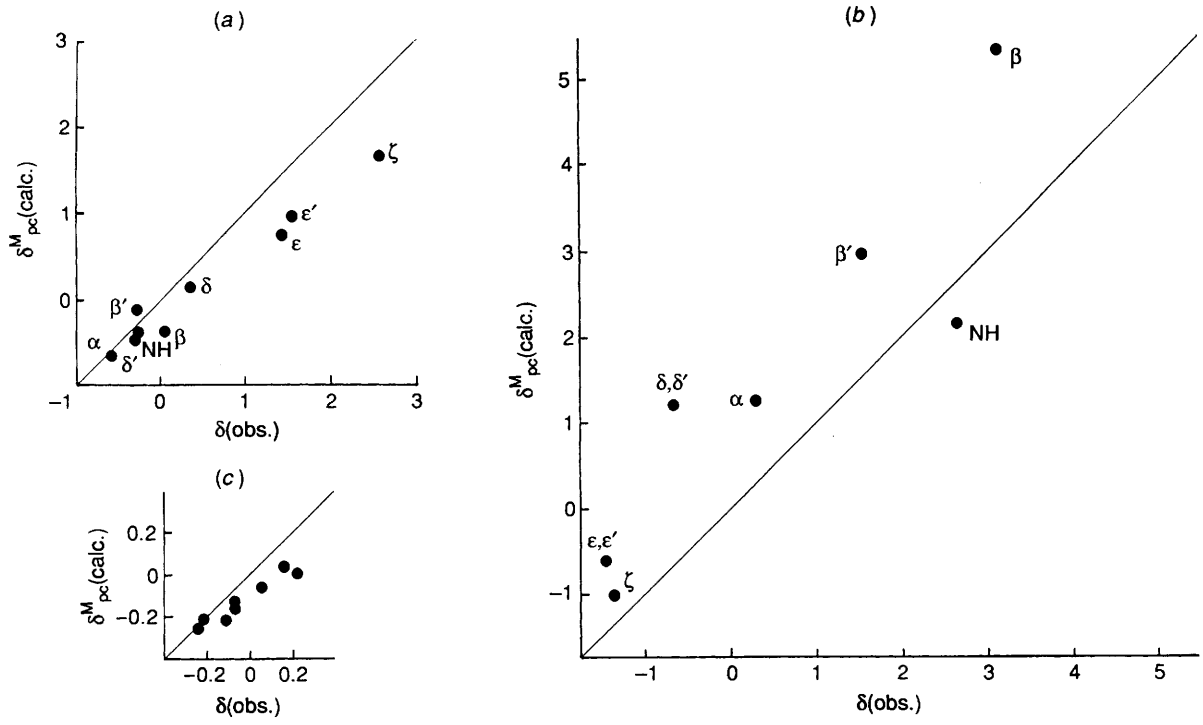

Fig. 9 Comparison of the δ^{M}_{pc}(calc.) values with the δ(obs.) values for Phe 10 (*a*), Phe 82 (*b*) and Tyr 97 (*c*). In (*b*) the mean of the δ^{M}_{pc}(calc.) values for $C_\delta H$, $C_\delta H'$ and $C_\varepsilon H$, $C_\varepsilon H'$ proton resonances are plotted

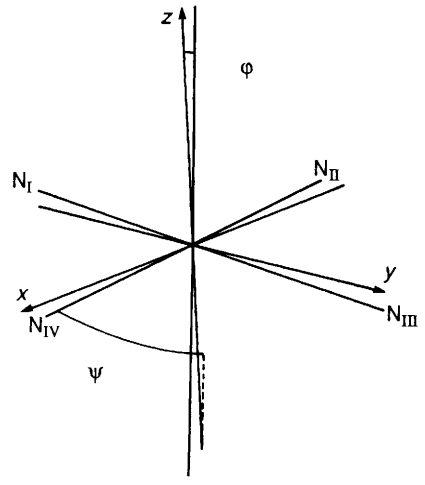

Fig. 10 The orientation of the principal magnetic axes in cyt c(CN) determined by MATDUHM. The φ and ψ angles are 5.1 and 62.4°, respectively

in the active site of haemoprotein in solution may actually be present, MATDUHM provides the principal magnetic axes of haemoprotein, which can be used to correlate the δ(obs.) value with the spatial relationship between protons and the unpaired electron in haemoprotein. Since the axes are determined solely by solution NMR data, MATDUHM is applicable to any haemoprotein under various solution conditions.

Analysis of the Side-chain Conformation.—The side-chain conformation of some aromatic amino acid residues was analysed using the magnetic axes calculated by MATDUHM. The δ^{M}_{pc}(calc.) values for Phe 10, Phe 82 and Tyr 97 proton resonances are given in Table 2 and they are compared with the δ(obs.) values in Fig. 9. The aromatic rings of Phe 10 and Tyr 97 are oriented in close proximity to each other and have been shown to be immobile.[31,32,41] The plot of δ^{M}_{pc}(calc.) *vs.* δ(obs.) for Phe 10 indicates that the δ^{M}_{pc}(calc.) value underestimates the δ(obs.) value for protons further away from the peptide backbone. Since Tyr 97 is oriented more than 1.3 nm from the haem iron, δ^{M}_{pc} is not so sensitive to its orientation relative to the haem. Although the shifts are small, plot (*c*) indicates that the δ^{M}_{pc}(calc.) value of the Tyr 97 proton resonances correlates well with the δ(obs.) value. Therefore the side-chain orientation of this residue relative to the haem in the solution state is likely to be similar to that in the crystal state. Hence, the results in plot (*a*) can be interpreted as that the phenyl plane of the Phe 10 residue in solution is moved slightly away from the haem relative to that indicated in the single crystal.

The Phe 82 residue has received considerable attention because it is proposed to play an important role in electron-transfer reactivity of the protein.[41–43] This residue is not in a helix region and its backbone NH proton is not involved in hydrogen bonding.[27] Furthermore, it has been shown to undergo a rapid 180° ring flip.[31,32] In plot (*b*) the δ^{M}_{pc}(calc.) values of not only the side-chain proton but also the backbone protons largely deviate from the δ(obs.) values. There is no aromatic amino acid residue close to Phe 82, the ring current of which possibly influences the shifts of its proton resonances. Therefore, the large deviation of plot (*b*) is attributed to the mobility of Phe 82 in solution and the presence of a substantial difference in its orientation relative to the haem between the structures in the solution and crystal states.

Orientation of the Principal Magnetic Axes in the Met-cyano Form of Oxidized cyt c.—The assignments for the haem methyl ^{13}C NMR resonances of the met-cyano form of oxidized cyt c [cyt-c(CN)] have been reported previously.[21] Using the δ_{dia} values reported for reduced cyt c,[30] the orientation of the magnetic axes in cyt-c(CN) was calculated by MATDUHM and the result is illustrated in Fig. 10. The orientation in cyt c(CN) is not significantly different from that in cyt c. Since the φ(ψ) angles are 4 (34°) and 6 (47°) for the met-cyano form of *Physter catodon*[25] and *Galeorhinus japonicus* myoglobins,[26] respectively, the small φ angle in these complexes may be reflected by the linear FeCN unit, although the tilt of CN with respect to the haem plane has been proposed.[13] It is impossible at present to compare, in a more quantitative manner, the orientations of the magnetic axes in the two cyt c complexes in terms of the difference in the symmetry of the ligand field around the haem iron.

Table 2 Chemical shift data for some amino acid residues[a]

Proton	δ_{obs}[b]	δ_{dia}[c]	δ^{M}_{pc}(obs.)[d]	$\delta^{M}_{pc\parallel}$[e]	$\delta^{M}_{pc\perp}$[e]	δ^{M}_{pc}(calc.)[f]
Phe 10 ($\Delta\sigma$ = 0.48)						
NH	8.43	8.66	−0.23	−0.206 8	−0.160 5	−0.367 2
$C_\alpha H$	3.44	4.06	−0.58	−0.418 6	−0.274 4	−0.693 0
$C_\beta H$	3.17	3.12	0.05	−0.129 0	−0.300 5	−0.429 5
$C_\beta H'$	2.75	3.00	−0.25	−0.013 39	−0.163 4	−0.176 8
$C_\delta H$	7.54	7.17	0.37	0.212 0	−0.048 63	0.163 3
$C_\delta H'$	6.92	7.18	−0.26	0.077 35	−0.556 5	−0.479 1
$C_\varepsilon H$	8.41	6.98	1.43	1.489	−0.541 8	0.947 0
$C_\varepsilon H'$	7.66	6.12	1.54	0.703 7	0.020 38	0.724 1
$C_\zeta H$	8.70	6.21	2.58	1.758	−0.084 56	1.673
Phe 82 ($\Delta\sigma$ = 1.29)						
NH	9.07	6.46	2.61	1.959	0.175 0	2.134
$C_\alpha H$	4.61	4.30	0.31	0.794 7	0.473 5	1.268
$C_\beta H$	3.71	0.61	3.10	4.621	0.686 6	5.308
$C_\beta H'$		2.18	1.53	1.534	1.454	2.988
$C_\delta H$	6.07	6.70	−0.63	−0.747 6	1.097	0.349 0
$C_\delta H'$				2.244	−0.197 4	2.047
$C_\varepsilon H$	6.04	7.49	−1.45	−1.197	0.130 8	−1.066
$C_\varepsilon H'$				0.418 3	−0.493 3	−0.075 04
$C_\zeta H$	5.89	7.23	−1.34	−0.564 0	−0.444 3	−1.008
Tyr 97 ($\Delta\sigma$ = 0.14)						
NH	7.94	8.13	−0.27	−0.122 4	−0.148 1	−0.270 6
$C_\alpha H$	4.17	4.26	−0.09	−0.036 64	−0.108 2	−0.172 1
$C_\beta H$	2.83	3.12	−0.29	−0.118 5	−0.197 6	−0.316 1
$C_\beta H'$	3.56	3.67	−0.09	−0.077 43	−0.139 6	−0.217 1
$C_\delta H$	6.41	6.54	−0.13	−0.023 28	−0.251 1	−0.274 4
$C_\delta H'$	7.16	7.08	0.08	0.007 424	−0.090 99	−0.086 56
$C_\varepsilon H$	5.73	5.46	0.27	0.077 44	−0.072 56	0.004 874
$C_\varepsilon H'$	6.86	6.67	0.19	0.226 9	−0.190 6	0.036 39

[a] Shifts in ppm at 20 °C and pH 5.7. [b] Shifts reported for oxidized cyt c.[32] [c] Shifts reported for reduced cyt c.[31] [d] $\delta_{obs} - \delta_{dia}$. [e] $\delta^{M}_{pc\parallel}$ and $\delta^{M}_{pc\perp}$ are the axial and rhombic δ^{M}_{pc} values corresponding to the first and second terms of equation (4). The values were calculated from the principal magnetic axes determined from MATDUHM and the coordinates of the corresponding hydrogen atoms, generated from the crystal structure of oxidized cyt c.[27] [f] $\delta^{M}_{pc\parallel} + \delta^{M}_{pc\perp}$.

Conclusion

It has been demonstrated that the orientation of the principal magnetic axes in paramagnetic haemoprotein, which is essential for quantitative interpretation of hyperfine-shifted NMR resonances in terms of the molecular/electronic structure of the haem active site, can be calculated solely from solution NMR data. This method is applicable to the iron(III) low-spin form of any haemoprotein under any solution condition to locate their principal magnetic axes and therefore is useful for the solution structural analysis of haemoprotein. Even though the X-ray crystal structure of the protein of interest has been reported, MATDUHM allows quantitative comparison of its solution structure with its crystal structure, as well as new NMR signal assignments to be made.

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