Mapping Paramagnetic Metal-centred Dipolar Field in Haemoprotein Using Haem Methyl Carbon and the Attached Proton Resonances

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A novel approach to mapping the paramagnetic metal-centred dipolar field in ferric low-spin haemoprotein is presented and the proposed method provides a new unique tool to determine the active site structure of haemoprotein in solution.

The NMR spectroscopy of paramagnetic haemoproteins has provided a wealth of information about the chemical environments of the prosthetic haem group.[1] In particular, the paramagnetic metal-centred dipolar field due to the unpaired electron in the ferric low-spin form of haemoprotein has been used as unique probe in determining the structure of the active site, because the metal-centred pseudo-contact shift (δ^{M}_{pc}) can be interpreted in terms of the actual coordinates of the nucleus relative to the unpaired electron by use of eqn. (1).

$$\delta^{M}_{pc} = \frac{1}{4\pi}\cdot\frac{1}{2r^3}[(3\cos^2\theta - 1)(\tfrac{3}{2}\chi_{zz} - \tfrac{1}{2}\chi_{xx} - \tfrac{1}{2}\chi_{yy}) + \sin^2\theta\cos 2\Omega\cdot(\chi_{xx} - \chi_{yy})] \quad 1$$

Where χ_{ii} are the principal components of the molecular susceptibility tensor, r is the distance between the electron and nucleus spins, and θ and Ω are the angles between r and the z axis and between the projection of r in the xy plane and the x axis, respectively.[2] Therefore, provided that the magnetic anisotropy tensor is determined, the spatial relationship between the haem and the nearby non-coordinated amino acid residues can be determined from the analyses of the hyperfine shifts of the amino acid resonances because their hyperfine shifts are purely δ^{M}_{pc} in origin. The least-squares fitting of the observed hyperfine shifts for a large number of assigned proton resonances arising from non-coordinated amino acid residues to eqn. 1 is a common approach for locating the orientation of the magnetic susceptibility tensor in haemoprotein.[3,4] However, even for ideal magnetic behaviour, question remains about the application of the solid data to the interpretation of solution NMR results,[1b] the g-tensor components measured for a single crystal or frozen sample are used in the present method. In addition, the precise molecular coordinates of the protein are indispensable for this method and such data for most ferric low-spin haemoproteins are not available.

We have shown that the magnetic susceptibility tensor in haemin complex with axially symmetric anisotropy can be analytically determined from the combined analyses of the observed hyperfine shifts for the haem methyl carbon and the attached proton resonances.[5] We demonstrate in this report that our method can be further extended to the system of ferric low-spin haemoprotein in which the assumption of axial symmetry in the magnetic anisotropy is no longer valid. The proposed method, MATDUHM (Magnetic Anisotropy Tensor Determination Utilizing Haem Methyls), is evaluated with regard to the experimental results obtained for the ferric low-spin cyanide complex of sperm whale myoglobin-(metMbCN).

The upfield hyperfine shifted portion of the 67.8 MHz ^{13}C NMR spectrum of metMbCN in ^2H_2O, p^{2}H 7, at 35 °C is illustrated in Fig. 1. Four haem methyl carbon resonances of metMbCN are clearly observed[6] and the plot of their hyperfine shifts *vs.* those of the attached proton resonances are shown in (*a*) of Fig. 2. The hyperfine shift (δ_{hf}) of these resonances is expressed as the sum of the contact shift (δ_c), the ligand-centred pseudo-contact shift (δ^{L}_{pc}), and δ^{M}_{pc}, *i.e.* $\delta_{hf} = \delta_c + \delta^{L}_{pc} + \delta^{M}_{pc}$. Because both δ_c and δ^{L}_{pc} of the haem methyl carbon and the attached proton resonances are directly proportional to the unpaired electron density (ρ) at the pyrrole carbon to which the methyl group is covalently bonded,[7] they vanish simultaneously as $\rho \rightarrow 0$. Hence, the plot of $\delta_c + \delta^{L}_{pc}$ for the haem methyl carbon resonance *vs.* that of the attached proton resonance should fall on a straight line that passes through the origin. The deviation of the plot for each haem methyl data from this straight line, as indicated by broken lines in the figure, therefore, can be attributed to the contribution of δ^{M}_{pc} to their δ_{hf} values. We have calculated their δ^{M}_{pc} values for all possible orientations of a magnetic tensor, *i.e.* a Cartesian coordinate system with its origin at the haem iron was rotated in 1° gradations throughout a space. Then $\delta_{hf} - \delta^{M}_{pc}$ ($= \delta_c + \delta^{L}_{pc}$) of the haem methyl carbon resonance was plotted *vs.* the attached proton resonance. Here, the computational problem is to find the tensor orientation such that the deviation of the plots for $\delta_c + \delta^{L}_{pc}$ from a straight line is minimized subject to eqn. 1. This minimization process was based on the summation of the distances between the plots and the straight line. For computation, an algorithm implemented in FORTRAN was run on a SUN 4 computer. Although the direction of the z-axis can be analytically obtained from the present method, the symmetric nature of eqn. 1 does not allow the determination of its sense. Therefore, we assumed that the z-axis is oriented toward the proximal residue. In addition, as expected from the spatial

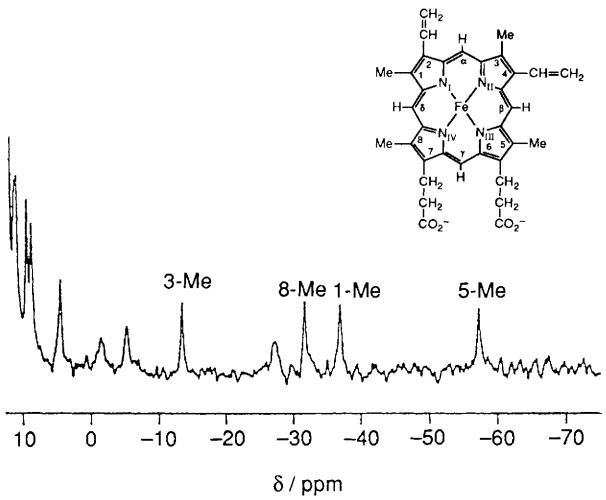

Fig. 1 Upfield hyperfine shifted region of the 67.8 MHz ^{13}C NMR spectrum of metMbCN in ^2H_2O at p^{2}H 7.0 and 35 °C. The haem methyl carbon resonances are observed at −55.5, −35.2, −29.9, and −11.5 ppm.[6] The structure and the numbering system of the haemin is shown in the inset.

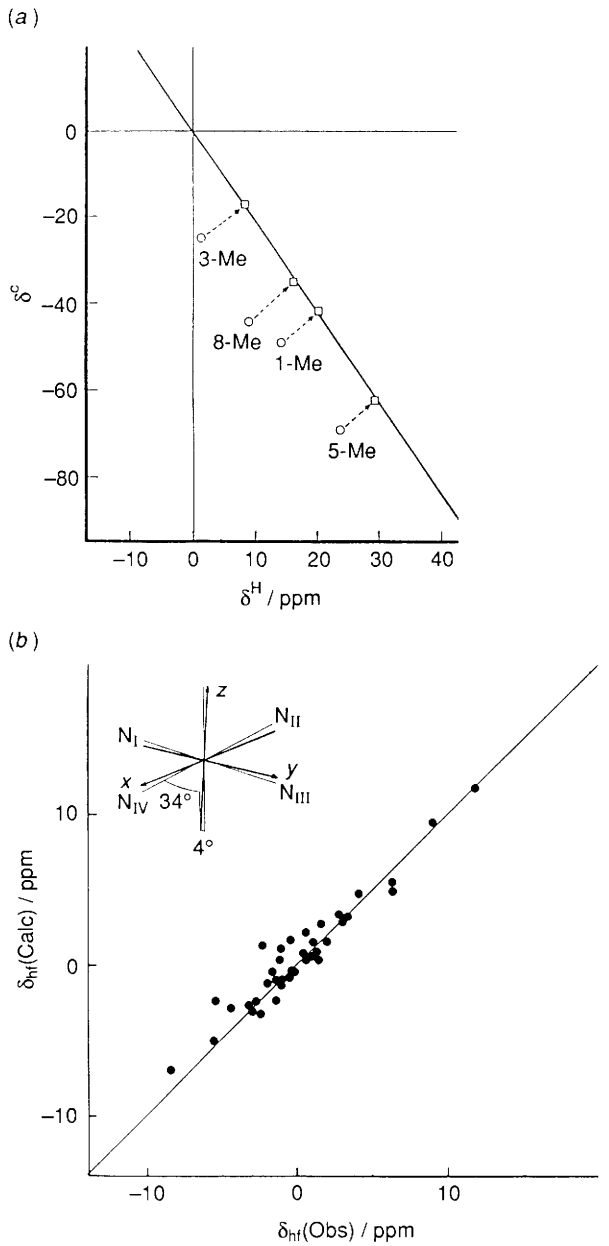

Fig. 2 (*a*) Plots of δ_{hf} of the haem methyl carbon resonance *vs.* that of the attached proton resonance for all the haem methyl groups in metMbCN(O). The shifts of the haem methyl carbon resonances in zinc protoporphyrin IX in [$^2H_5$] pyridine[5] and the haem methyl proton resonances in carbonmonoxy Mb[10] were used as the diamagnetic reference shifts for the corresponding resonances of metMbCN. According to the crystal structure,[8] the plane of the haem is not usually flat, but is distorted to some extent in the active site of haemoproteins and such distortion of the haem plane is quite random among the reported crystal structures. Since the spatial position of the haem methyl group with respect to the haem iron critically affects the results of our calculation, fourfold symmetry of the porphyrin ring was assumed in the analysis. The centre of mass among the haem methyl proton nuclei was used to calculate the geometric factors of the haem methyl protons. Four data points are mapped onto a straight line passing through the origin in the process of MATDUHM and the results from the computation are indicated by □.

(*b*) Plot of δ_{hf}(Calc) against δ_{hf}(Obs) for the assigned proton signals of non-coordinated amino acid residues. δ_{hf}(Obs)s were obtained from ref. 4. The determined tensor orientation with respect to the haem is illustrated in the inset. The angle between the *z*-axis and the normal to the haem plane is 4° and the projection of the *z*-axis onto the haem plane and the N_{II}–Fe–N_{IV} axis is 34°.

relationship among the haem methyl groups and the haem iron, the tensor orientations in which their *z*-axes lie close to the haem plane are obtained as local minima in our calculation. Such orientations were discarded.

The orientation of the magnetic susceptibility tensor determined by MATDUHM is shown in the inset of Fig. 2(*b*). The *z*-axis of the obtained principal magnetic axes does not coincide with the normal to the haem plane and the angle between these axes was found to be 4°. The obtained magnetic tensor orientation is different from the previously reported results,[4,8] and the reason for this is not clear. The hyperfine shifts of proton resonances for some non-coordinated amino acid resonances are calculated from their coordinates in the carbonmonoxy form of sperm whale myoglobin and the magnetic tensor orientation determined by MATDUHM and the calculated shifts [δ_{hf}(Calc)] are compared with the observed shifts [δ_{hf}(Obs)] in Fig. 2(*b*). Although the correlation between δ_{hf}(Obs) and δ_{hf}(Calc) is better for the results of Emerson and La Mar,[4] it appears that MATDUHM satisfactorily predicts the δ_{hf} values for non-coordinated amino acid proton resonances.

Advantages of MATDUHM over the conventional least-squares fitting approach are (i) no crystal structure for the protein is needed, (ii) the results from the ESR measurement of a single crystal or frozen sample are not essential, and (iii) tensor orientation at any given temperature can be easily estimated. (i) and (ii) make MATDUHM applicable to any haemoprotein for mapping their paramagnetic metal-centred dipolar field. The structural determination of the active sites of various haemoproteins using MATDUHM is in progress in our laboratory.

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