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ARTICLE

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Determination of solution structures of paramagnetic proteins by NMR

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Abstract Standard procedures for using nuclear Overhauser enhancements (NOE) between protons to generate structures for diamagnetic proteins in solution from NMR data may be supplemented by using dipolar shifts if the protein is paramagnetic. This is advantageous since the electron-nuclear dipolar coupling provides relatively long-range geometric information with respect to the paramagnetic centre which complements the short-range distance constraints from NOEs. Several different strategies have been developed to date, but none of these attempts to combine data from NOEs and dipolar shifts in the initial stages of structure calculation or to determine three dimensional protein structures together with their magnetic properties. This work shows that the magnetic and atomic structures are highly correlated and that it is important to have additional constraints both to provide starting parameters for the magnetic properties and to improve the definition of the best fit. Useful parameters can be obtained for haem proteins from Fermi contact shifts; this approach is compared with a new method based on the analysis of dipolar shifts in haem methyl groups with respect to data from horse and tuna ferricytochromes *c*. The methods developed for using data from NOEs and dipolar shifts have been incorporated in a new computer program, PARADYANA, which is demonstrated in application to a model data set for the sequence of the haem octapeptide known as microperoxidase-8.

Key words Paramagnetic proteins · Cytochromes · Solution structure · NMR · Dipolar interactions · Magnetic properties

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Introduction

One of the main bottlenecks in the area of structural biology is the development of techniques for obtaining high resolution structures in solution, so that structural modifications can be followed as physical and chemical conditions are changed (e.g. temperature, redox potential, and added substrates or inhibitors). In many cases, X-ray crystallography gives excellent results, but conditions within the crystal are not easy to control. The solution structures of a variety of diamagnetic proteins have been obtained from NMR data, predominantly from measurements of proton-proton dipolar cross relaxation in the form of nuclear Overhauser enhancements (NOE) (Wüthrich 1989; Güntert et al. 1991). The NOE intensities are related approximately to interproton distances, and, hence, may be converted into distance constraints through empirical calibration. Such NMR studies are best suited to diamagnetic globular proteins since the assignment of the spectra of paramagnetic proteins and the interpretation of NOEs are complicated by paramagnetic contributions to the chemical shifts, which may alter the usual patterns drastically, and the relaxation rates, which are dominated by electron-proton interactions in the vicinity of prosthetic groups. The competing relaxation pathways reduce NOE intensities, increasing the apparent interproton distances and making lower-limit distance constraints unreliable and upper limits excessively loose. In the presence of large paramagnetic effects, the number of constraints obtained around the metal (active) centre may be small. In less extreme cases, the additional spread of resonances induced by paramagnetic shifts generally results in an increase of spectral resolution and is also a valuable aid to assignment in larger proteins. Furthermore, the paramagnetic effects are governed by geometric functions, thus containing valuable structural information (Xavier et al. 1993). Some compensation for the reduction in NOE intensities may also be achieved by using the initial structures as input to relaxation matrix calculations with terms for electron-nuclear dipolar relaxation which are proportional to the inverse sixth

power of the distance between each proton and the paramagnetic centre (Bertini et al. 1996).

In addition to reducing NOE intensities, the dipolar interaction between nuclei and rapidly relaxing electrons induces hyperfine shifts which are approximated quite well by the so-called metal centred pseudocontact shift (Bertini and Luchinat 1986):

$$\delta_i^{pc}(\text{ppm}) = \frac{10^6}{12\pi r_i^3} \cdot \left[(3\cos^3\theta - 1)\Delta\chi_{ax} + \frac{3}{2}\sin^2\theta\cos 2\phi\,\Delta\chi_{eq} \right] \quad (1)$$

in which the metal is at the origin of the principal axes of the magnetic susceptibility tensor, with axial and equatorial anisotropies $\Delta\chi_{ax}$ and $\Delta\chi_{eq}$, and the nucleus has spherical polar coordinates r, θ, ϕ. Proton dipolar shifts are not sufficient to define atomic coordinates since each particular value corresponds to a complete surface. However, structures may be determined from dipolar shifts together with some other property which has a different dependence on geometry, such as paramagnetic relaxation (Barry et al. 1971) or nuclear Overhauser enhancements (Barry et al. 1974). Alternatively, the dipolar shifts of protons in a predetermined structure may be used to characterise the magnetic susceptibility tensor of the compound. This is straightforward in low-spin haem proteins because the position of the metal is rigidly defined and the reduced protein can be used to provide diamagnetic reference shifts (Keller et al. 1972). Then, by measuring the shift difference of protons in the oxidised and reduced protein, it is possible to test the accuracy of a structure or to detect redox-state-related changes in structure (Williams et al. 1985).

Such procedures have been applied with increasing refinement to horse cytochrome *c*, which has also been the subject of several X-ray studies. A simple analysis of the absolute difference between dipolar shifts observed in the ferricytochrome and shifts calculated using anisotropies extrapolated from EPR data with an optimised orientation for the magnetic susceptibility tensor showed that there is little difference between the structure of the proteins in solution and in the crystal, or between the oxidised and reduced forms (Williams et al. 1985). Subsequently, the procedure was improved by fitting all five parameters of the magnetic susceptibility tensor and evaluating deviations between observed and calculated shifts with respect to the local gradient of the dipolar field (Feng et al. 1990). In later work, the tensor was fitted after excluding anomalous shifts on a statistical basis (Turner and Williams 1993). This work also used information from ^{13}C shifts, which are much more sensitive to alterations to the diamagnetic shifts which may result from changes in structure, to show that there are indeed slight redox-state-related movements in horse cytochrome *c* in the region of Trp 59.

Most recently, Qi et al. attempted to determine redox-state-related structural changes in horse cytochrome *c* directly in solution and they concluded that the effects are remarkably large (Qi et al. 1996). The dipolar shifts of the

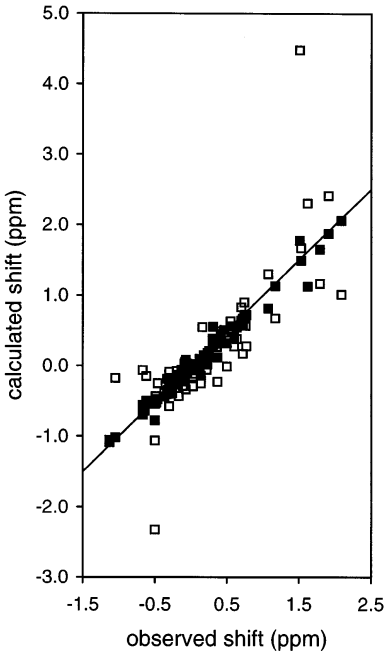

Fig. 1 Comparison of observed and calculated alpha CH proton dipolar shifts in horse cytochrome *c* fitted to the X-ray structure 1crc (Sanishvili et al. 1995), *filled symbols*, or the solution structure 1ocd (Qi et al. 1997), *empty symbols*

alpha CH protons were calculated in order to validate the structure obtained for the ferricytochrome but these results were not compared with calculations based on X-ray structures. The coordinates of the solution structure, 1ocd, were not made available until July of this year but they now provide an excellent example of the value of dipolar shift calculations in testing structures. The atomic coordinates used in this work were obtained from the Protein Data Bank at Brookhaven and the four-character PDB identification code is given in each case. We fitted the 87 unambiguous alpha CH dipolar shifts according to the method described by Turner et al. (1993) with an assumed error of ±0.05 ppm in each shift and varied the positional uncertainty of the protons, instead of rejecting data, until the RMSD was reduced to unity. This showed that the structural model obtained by NMR would be compatible with the shifts of these backbone protons only if the coordinates had an uncertainty of ±2.01 Å. Furthermore, the iron to Met80 sulphur bond is tilted by 40° from the perpendicular to the haem. For comparison, the X-ray structures 1hrc (Bushnell et al. 1990) and 1crc (Sanishvili et al. 1995) yielded positional uncertainties of ±0.29 Å and ±0.28 Å, respectively, with the same input data. Observed and calculated shifts are shown in Fig. 1. It is clear, therefore, that the X-ray structure of the ferricytochrome is a far better approximation to the structure in solution than the NMR structure. The solution structure, 2frc, of the ferrocytochrome (Qi et al. 1996) gives slightly better agreement, with a positional uncertainty of ±1.92 Å, but reveals a change in chirality of thioether 3^1CH and a near-planar Met80 sulphur. However, the characteristic distortion of the haem plane found in the crystal structures is reproduced with extraordinary preci-

sion in each case, having an RMSD for the macrocycle of just 0.1 Å with respect to 1crc. This deviation is smaller than that found among X-ray structures of similar cytochromes and may be compared with an RMSD of 0.2 Å for a range of crystal structures with respect to a flat haem. Thus, it appears that the oxidised and reduced solution structures are both of very low resolution and that the distorted haem must originate from X-ray data, modified only slightly by the force field. The simple test of the quality of the solution structure of horse ferricytochrome *c* provided by this metal centred dipolar shift calculation demonstrates clearly that it would be unwise to use it as the basis of any discussion of redox-state-related structure changes.

Dipolar shifts have also been used to provide additional constraints in structure refinement (Barry et al. 1971; Gochin and Roder 1995; Banci et al. 1996). As Banci et al. (1996) have argued, it is unsatisfactory to refine a structure in the absence of the constraints, such as NOE data, which were used to generate the initial structure. Nevertheless, Gochin and Roder (1995) have demonstrated a refinement procedure based on dipolar shifts and a force field, using a crystal structure of horse ferricytochrome *c* as a starting point. This work reveals a number of difficulties. First, the inclusion of dipolar shifts was found to have relatively little effect on the structure compared with minimisation using only the force field. In part, this may result from neglecting the rapid rotation of methyl groups since a significant reduction in the deviation between observed and calculated shifts may be achieved by a spurious reorientation of the methyl protons, which changes the calculated dipolar shifts by up to 15% in this particular structure. More importantly, the minimisation was performed with respect to a fixed and predetermined magnetic susceptibility tensor and the claimed precision, based on the gradient of the dipolar field, of up to 0.1 Å in the coordinates of the refined structure took no account of possible errors in the parameters of the tensor itself. Since the global minimum in fitting magnetic parameters is typically broad and the parameters are quite strongly coupled, small changes in the atomic coordinates or in the set of dipolar shifts can produce significant changes in the values.

The procedure introduced by Banci et al. (1996) and demonstrated in application to the cyanide complex of the Met80Ala mutant of *Saccharomyces cerevisiae* iso-1-ferricytochrome *c* was improved by the simultaneous use of constraints from NOE data and dipolar shifts. Each structure was minimised with respect to a predetermined magnetic susceptibility tensor, as in the earlier example, and inevitably reduced the RMSD across a family of starting structures derived from NOE data because each structure is required to conform to the same dipolar field. Since families of computed structures are analysed for the purpose of evaluating uncertainties in the structural parameters, it would be more satisfactory to compute the magnetic properties of each structure independently.

In this article, we demonstrate how structural and magnetic properties may be obtained simultaneously by a straightforward extension of structure calculations in torsion angle space. Such calculations commonly take random structures as starting points and it is impractical to fit a magnetic susceptibility tensor to the set of dipolar shifts until the approximate folding of the protein has been established by the application of short-range distance constraints derived from NOE data. Thus, it is convenient to adopt the variable target function approach (Braun et al. 1985) and introduce the magnetic parameters and constraints based on dipolar shifts at a late stage in the calculation of each structure. Alternatively, starting values for the parameters of the magnetic susceptibility tensor in haem proteins may be obtained from independent ^{13}C NMR and EPR measurements, with the relatively long-range dipolar shift constraints being given very little weight initially in order to avoid being trapped in local minima. However, if independent experimental data for the tensor orientation is available, then it can be included as an additional constraint throughout the calculation, which should improve the eventual resolution of the structure.

While this article was in preparation, Banci et al. (1997) published another NMR structure of horse ferricytochrome *c* based on NOE data and dipolar shifts, improving the methodology which they had demonstrated earlier (Banci et al. 1996). The resulting structure is significantly better than that of Qi et al. (1996), with an uncertainty of ±1.06 Å for the alpha CH coordinates, calculated as described above. The similarity of the haem macrocycle to that in the X-ray structure 1crc is still more remarkable, with an RMSD of just 0.08 Å, and so it would appear that the method used is still not capable of resolving haem distortions without input from X-ray data. The major advance in methodology lay in including the axes of the magnetic susceptibility tensor as parameters for each individual structure calculation. However, the anisotropies were predetermined and fixed, as in their earlier work.

The present work demonstrates that the anisotropy of the magnetic susceptibility tensor is highly correlated both with its orientation and with the calculated three dimensional structure but that it is straightforward to avoid bias by calculating all of the magnetic parameters together with each structure. It is also apparent that there is still room for considerable improvement in the methodology used for determining the structures of paramagnetic proteins in solution from NMR data. This is essential if solution structures are to become sufficiently accurate to provide a more useful basis for understanding the behaviour of proteins in solution than structures obtained from crystals.

Computational methodology

Since force field parameters are not well optimised for haems and the use of force fields is inappropriate unless all of the structural water molecules have been located, our computational procedure, PARADYANA, is based on the DYANA program of Güntert et al. (1997) which uses only repulsive van der Waals interactions. Protein structures are defined by a set of torsion angles, with standardised bond lengths and bond angles for the amino acids, and the tor-

sion angles are adjusted to fit the NMR data either by direct minimisation or by simulated annealing.

Mathematical expressions for the derivatives with respect to torsion angles of a target function based on dipolar shifts have been presented by Banci et al. (1996) and these equations must be supplemented by derivatives with respect to torsion angles which define the orientation of the magnetic susceptibility tensor and with respect to its anisotropies. The contribution to the target function made by the dipolar shifts may be written:

$$\begin{aligned} T_d &= w_d \sum_i w_i \left[\Theta(|\Delta\delta_i| - t)\right]^2 ; \\ \Delta\delta_i &= \delta_i^{\text{obs}} - \delta_i^{\text{calc}} ; \\ \delta_i^{\text{calc}} &= \frac{10^6}{12\pi r_i^5}\left\{\Delta\chi_{ax}\left[3(\boldsymbol{r}_i\cdot\boldsymbol{r}_z)^2 - r_i^2\right]\right. \\ &\qquad \left. + \frac{3}{2}\Delta\chi_{eq}\left[(\boldsymbol{r}_i\cdot\boldsymbol{r}_x)^2 - (\boldsymbol{r}_i\cdot\boldsymbol{r}_y)^2\right]\right\} \end{aligned} \tag{2}$$

where the function Θ is as defined by Güntert et al. (1991) and here gives the maximum of the difference between the calculated and observed shifts, in ppm, and a cutoff, t, set to 0.05 ppm as used by Banci et al. (1996). The overall weighting of the dipolar contribution, w_d, may be used to control the influence of dipolar shifts in a variable target function and the weighting of each individual value, w_i, may be used to reflect different experimental errors and to average over the shifts of methyl protons. The vector $\boldsymbol{r}_i$ gives the position of proton i, while $\boldsymbol{r}_x$, $\boldsymbol{r}_y$, $\boldsymbol{r}_z$, are unit vectors representing the principal axes of the magnetic susceptibility tensor and the $\Delta\chi$ terms represent its axial and equatorial anisotropy. These vectors are all defined with the same origin as the tensor, which is normally the coordinate of a metal atom. The dipolar shifts contribute to the partial differentials of the target function with respect to all torsion angles which affect the position of a proton relative to the origin of the tensor:

$$\begin{aligned} \frac{\partial T_d}{\partial\phi_j} &= -2w_d \sum_i w_i \Theta(|\Delta\delta_i| - t)\,(\Delta\delta_i / |\Delta\delta_i|)\,\frac{\partial\delta_i^{\text{calc}}}{\partial\phi_j} ; \\ \frac{\partial\delta_i^{\text{calc}}}{\partial\phi_j} &= \boldsymbol{e}_j'\cdot(\boldsymbol{r}_i'\wedge\boldsymbol{Q}_i) - (\boldsymbol{e}_j'\wedge\boldsymbol{r}_j')\cdot\boldsymbol{Q}_i ; \\ \boldsymbol{Q}_i &= \frac{10^6}{4\pi r_i^5}\left\{\Delta\chi_{eq}\left[(\boldsymbol{r}_i\cdot\boldsymbol{r}_x)\boldsymbol{r}_x - (\boldsymbol{r}_i\cdot\boldsymbol{r}_y)\boldsymbol{r}_y\right]\right. \\ &\qquad \left. + 2\Delta\chi_{ax}\left[(\boldsymbol{r}_i\cdot\boldsymbol{r}_z)\boldsymbol{r}_z - \frac{1}{3}\boldsymbol{r}_i\right]\right\} - \frac{5\delta_i^{\text{calc}}}{r_i^2}\,\boldsymbol{r}_i \end{aligned} \tag{3}$$

which is equivalent to the expression given by Banci et al. (1996) but expressed in a form which is more readily computed. The composite vector $\boldsymbol{Q}_i$, together with the vector $\boldsymbol{r}_i'$, which gives the position of proton i with respect to whatever origin is being used for the structure calculation, contain all of the necessary information about the proton. The information about each torsion angle, ϕ_j, is given by the direction of a unit vector along the bond, $\boldsymbol{e}_j'$, and the coordinate of the atom at the immobile end of the torsion, as explained by Güntert et al. (1991). The expression for torsion angles which move the metal centre with respect to a stationary proton simply takes the opposite sign.

The simultaneous calculation of the three dimensional atomic structure and the magnetic properties requires partial differentials for the target function also with respect to the anisotropies of the magnetic susceptibility tensor. These have a similar form to those for torsion angles, with simple expressions for the partial differentials of the calculated dipolar shifts:

$$\begin{aligned} \frac{\partial\delta_i^{\text{calc}}}{\partial\Delta\chi_{ax}} &= \frac{10^6}{12\pi r_i^5}\left[3(\boldsymbol{r}_i\cdot\boldsymbol{r}_z)^2 - r_i^2\right]; \\ \frac{\partial\delta_i^{\text{calc}}}{\partial\Delta\chi eq} &= \frac{10^6}{8\pi r_i^5}\left[(\boldsymbol{r}_i\cdot\boldsymbol{r}_x)^2 - (\boldsymbol{r}_i\cdot\boldsymbol{r}_y)^2\right]. \end{aligned} \tag{4}$$

Finally, a series or torsion angles, ϕ_k, allows the orientation of the principal axes of the magnetic susceptibility tensor to be optimised with respect to the molecular axis system; these torsions do not affect the distance, r_i, between the protons and the metal centre. The method of computation is similar to that adopted for the other torsion angles, as set out in Eq. (3), but it can also be formulated as:

$$\begin{aligned} \frac{\partial\delta_i^{\text{calc}}}{\partial\phi_k} &= -\mathbf{e}_k'\cdot(\mathbf{r}_i'\wedge\mathbf{R}_i) - (\mathbf{e}_k'\wedge\mathbf{r}_k')\cdot\mathbf{S}_i ; \\ \mathbf{S}_i &= \frac{10^6}{4\pi r_i^5}\left\{\Delta\chi_{eq}\left[(\mathbf{r}_i\cdot\mathbf{r}_x) - (\mathbf{r}_i\cdot\mathbf{r}_y)\right] + 2\Delta\chi_{ax}(\mathbf{r}_i\cdot\mathbf{r}_z)\right\}\mathbf{r}_i ; \\ \mathbf{R}_i &= \frac{10^6}{4\pi r_i^5}\left\{\Delta\chi_{eq}\left[(\mathbf{r}_i\cdot\mathbf{r}_x)\mathbf{r}_x' - (\mathbf{r}_i\cdot\mathbf{r}_y)\mathbf{r}_y'\right] + 2\Delta\chi_{ax}(\mathbf{r}_i\cdot\mathbf{r}_z)\mathbf{r}_z'\right. \end{aligned} \tag{5}$$

where the primed vectors, $\boldsymbol{r}_x'$, $\boldsymbol{r}_y'$, $\boldsymbol{r}_z'$, are the sum of unit vectors which represent the principal axes of the magnetic susceptibility tensor and the coordinate of the metal centre in the molecular axis system.

In application to haem proteins, since porphyrins show considerable flexibility, the distance between the pyrrole nitrogens and the iron are variable and so it is not convenient to include the magnetic axis system in the haem fragment. Instead, we attach the axes to one of the axial ligands such that only the ligand-iron bond length is implicitly predetermined. The construction of suitable fragments for the DYANA library is illustrated in Fig. 2.

Although empirical determination of the magnetic susceptibility tensor normally requires that a large number of dipolar shifts should be available for nuclei with known coordinates, coordinates for atoms in the semi-rigid porphyrin can always be approximated for haem proteins of unknown structure. It is also relatively simple to assign the resonances of oxidised and reduced haems, but the paramagnetic shifts include large contributions from Fermi contact interactions with the delocalised electron. This problem can be circumvented by using ^{13}C shifts, which are dominated by the Fermi contact interaction, and neglecting the dipolar shift in order to characterise the partially filled haem molecular orbitals. Since the ligand field of the iron is approximately cubic with the z axis close to the perpendicular to the haem plane, the in-plane orienta-

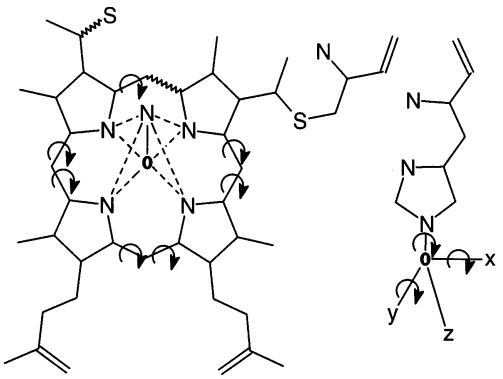

Fig. 2 Flexible haem fragment used for structure determination with the programs DYANA and PARADYANA. The haem plane is allowed some flexibility by rotation of the rigid pyrrole rings about bonds to the meso carbons. The additional variable torsion angles are indicated by arrows and constrained links by zig zags. The His fragment carries a set of pseudoatoms which represent the principal axes of the magnetic susceptibility tensor. Ligation to the haem is ensured by upper-limit distance constraints between the pyrrole nitrogens and the tensor origin and to the imidazole nitrogen, which are indicated by dashed lines with respect to the N-o portion of the His fragment. These constraints are sufficiently loose to accommodate the flexibility of the haem as well as a tilt of the His ligand or movement of the tensor origin out of the haem plane

tion of the rhombic perturbation may be used to obtain the approximate *x* and *y* axes of the tensor (Oosterhuis and Lang 1969). The anisotropies may then be extrapolated from EPR measurements (Horrocks and Greenberg 1973) to complete the description of the tensor.

An alternative method has been proposed which relies on the assumption that the Fermi contact shifts of the haem methyl protons are directly proportional to those of the methyl carbons (Yamamoto et al. 1989; 1990). This is a poor approximation since ratios for $\delta_C^{Fc}/\delta_H^{Fc}$ between –2.0 and –3.1 have been reported (Banci et al. 1994), but, in principle, it allows the Fermi contact contributions to be cancelled so that the dipolar shifts of the methyl groups can be extracted for fitting. This procedure is applicable only to the methyl substituents because the propionate and thioether or vinyl groups have proton shifts which depend strongly on their geometry, which is generally unknown. Also, the 2^1CH_3 and 12^1CH_3 groups have inversion symmetry and, hence, the method yields only three independent values. The original description of the method stated that the magnetic susceptibility tensor could be obtained without reference to a crystal structure or to EPR, which may have been an illusion created by finding a local minimum in the region of published values for the five parameters of the tensor by simple grid searching since it is plainly not possible to extract five parameters from three experimental values. Furthermore, the dipolar shifts relate to points in a plane which contains the origin and so they cannot be used to define the anisotropies or the orientation of the *z* axis. Instead, we propose to adapt the procedure used with haem molecular orbitals and approximate the *z* axis with the haem perpendicular and calculate the anisotropy from EPR data. Only two parameters then remain to be fitted: the ratio of the ^{13}C and ^1H Fermi contact shifts, *k*, and the in-plane rotation of the magnetic axes, θ. Thus, for each methyl group, we have

$$\left[\Delta\delta_{Ci}^{para} - \delta_{Ci}^{dip}(\theta)\right] - k\left[\Delta\delta_{Hi}^{para} - \delta_{Hi}^{dip}(\theta)\right] = 0 \qquad (6)$$

where the observed paramagnetic shifts, $\Delta\delta_i^{para}$, are simply the difference between shifts in the oxidised and diamagnetic reduced forms and the dipolar shifts, δ_i^{dip}, are calculated using the anisotropies fixed by EPR data and the variable, θ. The accuracy of the calculation of the ^1H dipolar shifts is maximised as usual by using twelve coordinates to allow for three rotation of the methyl group.

Results

Empirical tensor determination

The available data on mitochondrial cytochromes *c* provide some convenient examples for testing the relationship between atomic structure and magnetic properties. First, we shall consider empirical fitting of the magnetic susceptibility tensors in the proteins from horse and tuna. As noted in the introduction, dipolar shifts for 87 unambiguous alpha CH protons are available for horse cytochrome *c* (Feng et al. 1989; Wand et al. 1989), but only 60 shifts are available for tuna ferricytochrome *c* (Gao et al. 1989) and the corresponding diamagnetic shifts for horse ferrocytochrome *c* were used. Results of fitting these data, with RMSD values and standard errors based on an assumed uncertainty of ±0.05 ppm for each chemical shift and ±0.5 Å for each coordinate, are listed in Table 1.

We note that the quality of the fit for the data from the tuna cytochrome is significantly worse than that from horse and that this is entirely attributable to the quality of the NMR data, both the smaller number of shifts and the approximate diamagnetic references, since fitting the shifts of the first to the coordinates of the second or vice versa gives virtually identical χ^2 values. Even so, the orientations found for the tensors in each case are quite similar, though the anisotropies differ by more than would be expected on the grounds of the different temperatures at which the spectra were acquired (293 K for horse and 313 K for tuna ferricytochrome *c*). The most surprising feature of these data is an apparently significant difference in the tilt of the magnetic *z* axis with respect to a molecular axis system based on the pyrrole nitrogens (Williams et al. 1985), as is commonly used, despite the fact that the distortions of the haem planes in crystal structures for horse and tuna cytochromes *c* are closely similar, with RMSD's between the macrocycles of ca. 0.1 Å. This difference is eliminated by fixing the molecular *z* axis perpendicular to the best plane through the porphyrin (see Table 1) and, since haems displays a wide range of distortions, we consider that this stricter definition of the molecular axis system is essential for a detailed analysis of the factors

Table 1 Parameters obtained for magnetic susceptibility tensors fitted empirically to alpha CH paramagnetic shifts. Results are given for NMR data from horse cytochrome *c* with coordinates from two different crystal structures, and for tuna cytochrome *c* using the coordinates of the 'outer' 3cyt molecule (Takano and Dickerson 1981). In each case, standard errors for the Euler angles and anisotropies are given in parenthesis for an assumed ±0.05 ppm error in the shifts and ±0.5 Å in the atomic coordinates, and the first line employs a molecular axis system based on the pyrrole nitrogens whereas the second employs the best plane and centre of gravity of the haem

Data set	RMSD	α (deg)	β (deg)	γ (deg)	$\alpha+\gamma$ (deg)	$\Delta\chi_{ax}\times10^{32}$ (m^3)	$\Delta\chi_{eq}\times10^{32}$ (m^3)
Horse/1crc	0.85	–110.2 (5.8)	–11.5 (0.9)	–241.9 (5.9)	7.9 (2.4)	3.30 (0.11)	–1.43 (0.11)
	0.85	–114.2 (4.5)	–14.3 (0.9)	–237.8 (4.8)	7.9 (2.4)	3.30 (0.11)	–1.43 (0.11)
Horse/1hrc	0.87	–106.4 (5.2)	–12.9 (0.8)	–245.2 (5.4)	8.4 (2.3)	3.35 (0.11)	–1.48 (0.11)
	0.87	–112.4 (4.5)	–14.5 (0.9)	–239.0 (4.8)	8.6 (2.3)	3.35 (0.11)	–1.47 (0.11)
Tuna/3cyt	2.01	–110.0 (5.6)	–15.7 (1.2)	–245.0 (6.6)	5.1 (4.5)	2.69 (0.13)	–0.92 (0.12)
	2.00	–114.6 (5.6)	–15.5 (1.2)	–240.3 (6.6)	5.2 (4.5)	2.68 (0.13)	–0.91 (0.12)

Table 2 The in-plane rotation of the rhombic perturbation (θ) and energy splittings (ΔE) of the haem π molecular orbitals obtained by fitting paramagnetic shifts of ^{13}C or ^{1}H nuclei in haem substituents in horse and tuna ferricytochrome *c*. Dipolar shifts calculated from anisotropies extrapolated from EPR data are included in the first set of results (A) and neglected in the second (B). Nuclei positioned α to the haem in all eight substituents are included except where it is noted that the data set is restricted to the four methyl groups. The results obtained by the method of cancelling Fermi contact shifts are shown for comparison (C). The standard errors in parenthesis are based on an assumed experimental error of ±0.5 ppm for ^{13}C shifts and ±0.2 ppm for the ^{1}H shifts

Temperature (K)	Horse		Tuna	
$\Delta\chi_{ax}\times10^{32}$ (m^3)	303.00		298.00	
$\Delta\chi_{eq}\times10^{32}$ (m^3)	3.33		3.44	
	–1.92		–1.90	
	θ (deg)	ΔE (kJ/mol)	θ (deg)	ΔE (kJ/mol)
A. including dipolar shifts:				
^{13}C	–5.11 (0.16)	–6.56 (0.14)	–5.12 (0.15)	–6.04 (0.12)
^{13}C methyls only	–4.50 (0.25)	–8.16 (0.52)	–4.66 (0.25)	–7.56 (0.41)
^{1}H methyls only	–4.81 (0.30)	–7.07 (0.32)	–4.67 (0.29)	–7.26 (0.35)
B. neglecting dipolar shifts:				
^{13}C	–6.60 (0.20)	–4.03 (0.05)	–6.56 (0.19)	–3.85 (0.05)
^{13}C methyls only	–5.59 (0.30)	–4.60 (0.14)	–5.73 (0.29)	–4.45 (0.13)
C. cancellation				
^{1}H and ^{13}C methyls only	+2.12 (1.60)		+5.15 (1.81)	

which control the orientation of the magnetic *z* axis (see e.g. Brennan and Turner 1997).

The in-plane rotation of the magnetic axes, approximated by the sum of the Euler angles α and γ when the tilt of the *z* axis is small, is far less sensitive to the definition of the molecular axes; this is what we seek to determine by using chemical shifts solely from the haem. A full set of ^{1}H and ^{13}C assignments for the haem substituents in horse cytochrome *c* has been available for some time (Santos and Turner 1987, 1992; Wand et al. 1989; Feng et al. 1989; Gao et al. 1989) and, recently, similar data has been obtained for the protein from tuna (Sukits and Satterlee 1997). Thus, we may compare the results of various methods for analysing the data in each case. First, we take the full calculation of molecular orbitals based on eight ^{13}C shifts, with and without the inclusion of dipolar shifts calculated from EPR data (Mailer and Taylor 1972; Hori and Morimoto 1970), then restrict the input to the four methyl groups and, finally, compare the results obtained from the method of cancelling the Fermi contact shift contributions to the ^{1}H and ^{13}C shifts of the methyl substituents which was described in the previous section. The results are listed in Table 2 with standard errors based on an assumed experimental error of ±0.5 ppm for ^{13}C shifts and ±0.2 ppm for the ^{1}H shifts.

Comparison with the result of empirical fitting (Table 1) shows that the magnitude of the equatorial anisotropy is overestimated by extrapolation from EPR data and, hence, the orientation of the rhombic perturbation obtained from ^{13}C shifts with neglect of the dipolar contribution may be no less accurate than that obtained with the dipolar shift included.

The method of cancelling the Fermi contact contribution necessarily involves calculation of the dipolar shifts and yields the orientation of the magnetic axis directly, which is of opposite sign to that of the rhombic perturbation, as expected (Oosterhuis and Lang 1969). However, the precision and the accuracy both appear to be reduced and, since experiments required to obtain the input data also provide the information necessary for analysing the haem molecular orbitals, this method might be useful simply to cross check results from ^{13}C Fermi contact shifts.

The calculations based on haem molecular orbitals also have the advantage of providing some information about the *relative* orientation of the axial ligands, which appears to be the most important factor in determining the orbital energy splitting parameter, though the value clearly depends on the model used (Turner 1993, 1995).

The most surprising result is that reasonable values are obtained from haem molecular orbitals fitted to the paramagnetic shifts of the methyl protons. Dipolar shifts cannot be neglected for the ^{1}H paramagnetic shifts of the haem methyl groups because they are comparable with the Fermi contact contributions (Turner 1993). The success of fitting the ^{1}H shifts in this case may be fortuitous: the rotation of the rhombic perturbation is small in these cytochromes and so there is little difference between the magnetic and perturbation axes. The results of analysing ^{13}C shifts have been tested in a wide variety of haem proteins and found to be a good predictor of the magnetic axes but it would be more convenient to use ^{1}H shifts of the haem methyl groups since they are more readily available. Although the variability of the ^{1}H hyperfine coupling constants (Bolton et al. 1962; Eaton and Phillips 1965; LaMar et al. 1978) may distort the result in some situations, which will be tested in future, the method may prove useful at least for obtaining rough estimates of the orientation of the magnetic axes.

The paramagnetic shifts of ^{13}C nuclei positioned α to the haem do not depend on the geometry of the substituent and the analysis is, therefore, not restricted to methyl groups. This has a further advantage insofar as the shifts of nuclei in the β positions may be predicted and used as a test of the consistency of assignments (Pierattelli et al. 1996; Salgueiro et al. 1997). Thus, for example, the table of ^{13}C shifts of the haem and adjacent amino acids in tuna ferricytochrome *c* presented by Sukits and Satterlee (1996) is strikingly similar to that presented earlier for the protein from horse (Turner 1995), though there are significant differences in the ^{13}C chemical shifts of 10CH (β-*meso*) and also of $17^{2}CH_2$ (propionate $7\beta CH_2$). This suggests that there are errors in the assignments: the 10CH shift in horse ferricytochrome *c* has already been shown to agree with the molecular orbitals deduced from the ^{13}C shifts of the haem substituents (Turner 1995) and the strong correlation found between the ^{13}C shifts of propionate α and βCH_2 groups (Salgueiro et al. 1997) indicates that the assignment made for tuna ferricytochrome *c* should be revised in this case also.

Structure calculations

Having examined the utility of dipolar shift calculations for testing the quality of structures and resonance assignments, we shall now demonstrate the simultaneous calculation of the three dimensional structure and the magnetic properties of a haem complex using the program PARADYANA. These calculations are based on a model dataset derived from the structure of horse cytochrome *c* (Bushnell et al. 1990) for a sequence of eight residues which includes the haem binding site. For each case, the six structures with the smallest target functions out of 400 random starting structures are illustrated in Fig. 3. The first family of structures is based on 23 upper-limit distance constraints of less than 4.0 Å between backbone protons only. This restricted set of constraints leaves enough flexibility to reveal the effect of the dipolar shifts clearly, but it is sufficient to establish the face of the haem to which the ligand is attached. The haem macrocycle is constrained to be flat for clarity of illustration since the input data chosen for this model calculation are not sensitive to any distortion. Secondly, the dipolar shifts of 29 protons obtained from the empirical fit, excluding the Cys and His βCH_2 and the imidazole protons, are fitted simultaneously with the distance constraints, with five additional structural parameters to describe the magnetic susceptibility tensor. In the final example, these data are fitted with the in-plane rotation of the magnetic axes constrained to 5±5°, determined as described above.

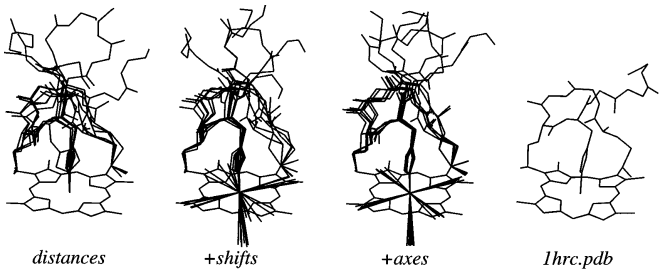

Fig. 3 Models of a haem octapeptide structure calculated using the programs DYANA and PARADYANA. The dataset is described in the text: all calculations include upper distance limits for the backbone protons, dipolar shifts are then included for the amino acid protons and the magnetic axes are shown, then the in-plane orientation of the axes is constrained as well on the basis of the analysis of haem molecular orbitals. The six structures with the lowest target functions are shown in each case with the haems superimposed and the original fragment from the structure 1hrc (Bushnell et al. 1990) is shown for comparison

Each additional constraint naturally reduces the spread of the family of structures (backbone RMSD 1.15, 1.01, and 0.85 Å for the three families, respectively) but the target functions are not altered significantly by constraining the magnetic axes (the range for the six best structures is 0.72–0.98 without axis constraint and 0.68–0.97 with). The variations in the anisotropy of the magnetic susceptibility clearly reflect the variability of the structures; $\Delta\chi_{ax}$ and $\Delta\chi_{eq}$ have average values (with the standard deviation over six structures given in parenthesis) of 3.35 (0.24) and $-1.71\ (0.40)\times10^{-32}$ m^3 without constraints on the axes but $3.49\ (0.16)$ and $-1.59\ (0.29)\times10^{-32}$ m^3 when the constraint is introduced. The C-terminal residues, which have small dipolar shifts and relatively few distance constraints, are poorly defined in each case. However, the residues of the haem binding sequence have their definition improved significantly by the inclusion of dipolar shifts and this behaviour should be more representative of residues in the interior of a full-sized protein. Finally, although this is a model data set, the sequence used corresponds to the haem octapeptide known as microperoxidase-8. It has already been

shown that the orientation of the His ligand in the real haem octapeptide-cyanide complex is similar to that found in the native protein (Brennan and Turner 1997) and so this calculation is indicative of the resolution which may be achieved once a comprehensive set of experimental NOEs and paramagnetic shifts becomes available for the complex.

Conclusion

The approximate theories which describe dipolar shifts in paramagnetic proteins and Fermi contact shifts in low-spin oxidised haems provide a valuable framework for testing the quality of NMR data as well as that of three dimensional protein structures. We have shown that the ^{1}H data available for tuna ferricytochrome *c* is compatible with the crystal structure, though it is more limited than that available for horse ferricytochrome *c*, but the ^{13}C data published recently (Sukits and Satterlee 1996) requires revision. It is also clear that the structures obtained for horse cytochrome *c* in solution (Qi et al. 1996; Banci et al. 1997) have too little resolution to be used to identify differences between the oxidised and reduced forms.

Measured dipolar shifts may also be used to provide additional constraints for structure determination. Since atomic coordinates are a prerequisite for optimising the parameters of the magnetic susceptibility tensor and these parameters are sensitive to small changes in the coordinates, it is necessary to calculate the three dimensional structure in conjunction with the magnetic properties. This can be achieved by the straightforward extension of the distance geometry algorithm of Güntert et al. (1991, 1997), as demonstrated here using the program PARADYANA. The imposition of a single susceptibility tensor obtained from the average of several structures calculated from distance constraints alone (Banci et al. 1996) may well bias the outcome. Nor is it sufficient to optimise the tensor orientation for individual structures while using predetermined anisotropies (Banci et al. 1997) since the magnetic parameters are highly correlated.

However, it is possible to use Fermi contact shifts to provide an independent measure of the orientation of the principal axes of the tensor in haem proteins and this can be used as an additional constraint. The method described by Yamamoto et al. (1989, 1990) for separating and using the dipolar shifts of haem substituents appears to be unworkable: the calculations which we have presented here required extensive modifications to the principle in order to obtain any results and those results are less precise than those from molecular orbital calculations based on the same data.

The analysis of ^{1}H data in terms of haem molecular orbitals, which is derived ultimately from the work of Shulman et al. (1971), gives surprisingly good results when applied to the paramagnetic shifts of haem methyl protons. Although it is reasonable to neglect dipolar shifts in the analysis of ^{13}C resonances from the haem, their accurate calculation is crucial in the analysis of ^{1}H shifts. Including the effects of thermal population and orbital mixing in the magnetic field (Horrocks and Greenberg 1973) allows a more realistic anisotropy to be obtained from EPR *g*-values, and it is also essential to take account of the opposite sense of rotation of the magnetic axes and of the rhombic perturbation to the ligand field (Oosterhuis and Lang 1969). Nonetheless, the small angle of rotation observed in horse and tuna ferricytochrome *c* makes these examples particularly favourable and it remains to be seen whether reliable parameters may be obtained from ^{1}H data or if analysis of ^{13}C shifts will be necessary in general.

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