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ARTICLE

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# Ligand orientation and haem electronic structure in ferricytochrome *c″* from *Methylophilus methylotrophus* studied by $^{13}$C NMR

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**Abstract** Cytochrome *c″* from *Methylophilus methylotrophus* contains a single haem with bis-histidine coordination which couples electron and proton transfer by reversible detachment of one of the axial ligands on reduction. $^{13}$C NMR of the haem substituents is now used to determine the orientations of the two histidine ligands of the ferricytochrome. The relative orientation of the ligands is found to be nearly perpendicular (an angle of 85±2° between the histidine planes was obtained), which is consistent with the near-axial g-tensor determined by EPR. Although the absolute orientation of the axial ligands is not well defined in the presence of near-axial symmetry, $^{13}$C NMR is found to be a useful complement to EPR for obtaining quantitative information for systems of this type.

**Key words** Cytochrome *c″* · Ligand orientation · *Methylophilus methylotrophus* · $^{13}$C NMR · Heme electronic structure · Fermi contact shift

## Introduction

Cytochrome *c″* is an unusual monohaem protein (15 kDa) present in the obligate methylotroph *Methylophilus methylotrophus.* About half of the sequence, 61 residues in the N-terminus of the protein, is known, including the haem attachment site which shows the –CXXCH– sequence typical of haem *c* proteins. NMR studies (Santos and Turner 1988) have shown that the haem iron is low spin and hence six-coordinate in the oxidised protein, but that it is high spin when reduced. NMR also showed that the two axial ligands in the oxidised form are histidines and that there is a single histidine ligand in the reduced form. EPR studies (Berry et al. 1990) on the ferricytochrome revealed spectroscopic features similar to those found in model compounds in which the axial ligand planes are forced into a perpendicular orientation by steric constraints (Walker et al. 1986; Safo et al. 1991). Such highly axial g-tensors have been proposed to be diagnostic of approximately perpendicular ligand orientations in bis-histidine cytochromes as well as in model compounds (Walker et al. 1986).

The midpoint redox potential of this protein shows a strong pH dependence (redox-Bohr effect) in the physiological pH range. This redox-Bohr effect is principally determined by the ionization of a group with a pK of 8.4 which is found only in the reduced form and is assigned to the detached His ligand (Costa et al. 1992). The protein is therefore able to couple electron and proton transfer by a novel mechanism which involves a drastic change in the haem ligands. Complete assignment of the haem proton NMR resonances in the oxidised form and analysis of nuclear Overhauser enhancements (NOEs) between these protons and neighbouring amino acid sidechains showed that propionate 13 is largely exposed to the solvent, providing a possible route for proton transfer (Costa et al. 1993). That work also suggested that His 52, which immediately follows the haem binding site in the amino acid sequence, with cysteines at positions 48 and 51, is the ligand which remains attached upon reduction. Further elucidation of the structure of the haem cleft may reveal the mechanism for destabilising the other His ligand in the reduced protein and would represent an important step towards understanding the dramatic structural change.

Attempts to crystallise the protein for study by X-ray diffraction have been unsuccessful and therefore NMR remains as the most promising technique for obtaining structural information. However, since only half of the sequence is known, it is not yet possible to apply standard methods of assignment and hence to determine the structure in so-

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lution from measured NOEs. Recent studies (Turner et al. 1995; Turner 1995; Banci et al. 1995; Pierattelli and Turner 1996) of the paramagnetic shifts of $^{13}C$ nuclei attached to the haems of low spin oxidised cytochromes have demonstrated a clear relationship with the orientations of the axial ligands. Here, this relationship is ued in order to define the haem coordination geometry of cytochrome *c″* more precisely.

## Materials and methods

Cytochrome *c″* was purified from *M. methylotrophus* (NCIB 10 515) grown on methanol under nitrogen-limited conditions and generously supplied by I.C.I. Bioproducts Ltd, as described previously (Costa et al. 1992).

Heteronuclear $^{1}H\{^{13}C\}$ multiple quantum correlation (HMQC) experiments (Bendall et al. 1982) were run on samples of ca. 6 mM cytochrome *c″*, in $^{2}H_2O$ at pH* 5.5 using a Bruker AMX 500 spectrometer. Chemical shift values are referenced to internal, 1,4-dioxane, designated 3.77 ppm for $^{1}H$ and 66.6 ppm for $^{13}C$ relative to 3-(trimethylsilyl)-propanesulfonic acid (sodium salt).

## Results and discussion

The $^{13}C$ chemical shifts of the haem substituents and axial ligands in ferricytochrome *c″* were straightforwardly assigned from the known shifts of the attached protons (Costa et al. 1993) and are listed in Table 1. The paramagnetic shifts are obtained by subtracting the diamagnetic contribution from the observed shifts but, since there is no diamagnetic form of this protein from which reference shifts can be measured, they are approximated by values taken from model compounds (Turner 1995). The paramagnetic contributions to the $^{13}C$ shifts of nuclei positioned $\alpha$ to the haem provide a more reliable measure of the unpaired electron distribution than $^{1}H$ shifts, both because of the elimination of geometric factors and the uncertainty over the appropriate coupling constant for protons. This is demonstrated by the fact that the haem methyl resonances are proportionately more closely spaced in the $^{13}C$ dimension of the HMQC spectrum, illustrated in Fig. 1, than in the $^{1}H$ dimension, as expected for near axial symmetry. Also, the close similarity between the shifts of the $\alpha$CH and the $\beta CH_2$ carbons of the two His ligands confirms that there is no significant difference in their binding in the oxidised protein.

The dipolar contribution to the paramagnetic shifts of the carbons $\alpha$ to the haem may be neglected as a first approximation (referred to as Model I), and the shifts treated as pure Fermi contact interactions which can be fitted to a model of $\pi$ molecular orbitals with perturbed $D_{4h}$ symmetry (Shulman et al. 1971; Turner et al. 1995). According to

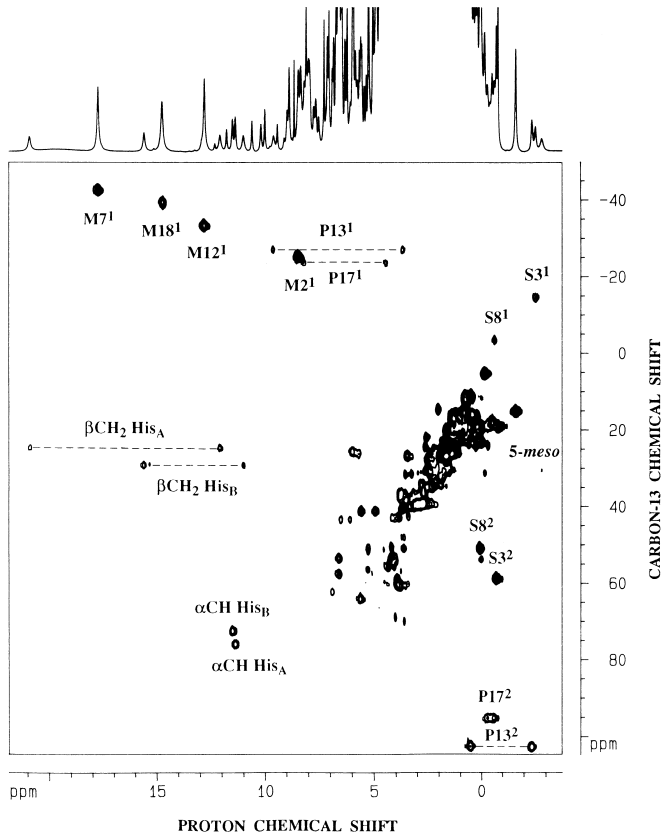


**Fig. 1** HMQC spectrum of 6 mM ferricytochrome *c″* in $^{2}H_2O$ at 295 K, pH * 5.5, correlating the $^{1}H$ and $^{13}C$ chemical shift of directly bonded nuclei. The resonances assigned to the haem methyl groups (*M*), thioether bridges (*S*), haem propionate groups (*P*) and the axial ligands (*His*) are labelled

**Table 1** $^{13}C$ chemical shifts of haem substituents and axial ligands in ferricytochrome *c″* from *Methylophilus methylotrophus* at pH* 5.5, referred to dioxane at 66.6 ppm

| Assignment | Chemical shift (ppm) | | |
|---|---|---|---|
| | 278.5 K | 295.0 K | Diamagnetic references |
| Haem | | | |
| $2^1$ | –29.0 | –26.8 | 12.1 |
| $7^1$ | –47.5 | –44.2 | 12.1 |
| $12^1$ | –37.8 | –34.6 | 12.1 |
| $18^1$ | –43.3 | –40.6 | 12.1 |
| $3^1$ | –19.4 | –15.9 | 35.3 |
| $3^2$ | 59.3 | 57.2 | |
| $8^1$ | –7.3 | –5.1 | 35.3 |
| $8^2$ | 50.0 | 49.5 | |
| $13^1$ | –33.0 | –28.4 | 22.8 |
| $13^2$ | 106.1 | 101.3 | |
| $17^1$ | –28.1 | –25.1 | 22.8 |
| $17^2$ | 97.0 | 93.9 | |
| 5CH | nd | 29.0 | 96.2 |
| $His_A$ | | | |
| $\alpha$-CH | 76.1 | 74.5 | |
| $\beta$-$CH_2$ | 23.4 | 23.1 | |
| $His_B$ | | | |
| $\alpha$-CH | 72.3 | 71.1 | |
| $\beta$-$CH_2$ | 28.0 | 27.7 | |

**Table 2** Results of fitting the observed paramagnetic shifts of $^{13}C$ nuclei $\alpha$ to the haem in ferricytochrome *c″* to a model of $\pi$ molecular orbitals with perturbed $D_{4h}$ symmetry. The paramagnetic shifts are treated as pure Fermi contact shifts ($\delta_{FC}$) in the first approximation (Model I). The results of this approximation may be compared with the separation of the paramagnetic shifts into metal centred dipolar shifts ($\delta_{pc}$) and Fermi contact shift ($\delta_{FC}$) in Model II (see text). All shifts are given in ppm and the parameters used in the fits are listed in Table 3

| | Observed | Model I $\delta_{Fc}$ | Model II | |
|---|---|---|---|---|
| | | | $\delta_{FC}$ | $\delta_{pc}$ |
| 278.5 K | | | | |
| $2^1$ | –41.05 | –46.11 | –38.09 | –8.31 |
| $3^1$ | –54.72 | –54.04 | –44.51 | –9.77 |
| $7^1$ | –59.62 | –56.17 | –48.61 | –8.02 |
| $8^1$ | –42.57 | –48.24 | –42.19 | –6.19 |
| $12^1$ | –49.89 | –46.11 | –38.09 | –8.07 |
| $13^1$ | –55.82 | –54.04 | –44.51 | –9.52 |
| $17^1$ | –50.90 | –56.17 | –48.61 | –7.36 |
| $18^1$ | –55.44 | –48.24 | –42.19 | –6.04 |
| 295.0 K | | | | |
| $2^1$ | –38.90 | –43.79 | –36.23 | –7.57 |
| $3^1$ | –51.22 | –50.87 | –41.96 | –8.89 |
| $7^1$ | –56.27 | –52.77 | –45.62 | –7.31 |
| $8^1$ | –40.36 | –45.69 | –39.89 | –5.64 |
| $12^1$ | –46.72 | –43.79 | –36.23 | –7.35 |
| $13^1$ | –51.20 | –50.87 | –41.96 | –8.67 |
| $17^1$ | –47.90 | –52.77 | –45.62 | –6.71 |
| $18^1$ | –52.73 | –45.69 | –39.89 | –5.51 |

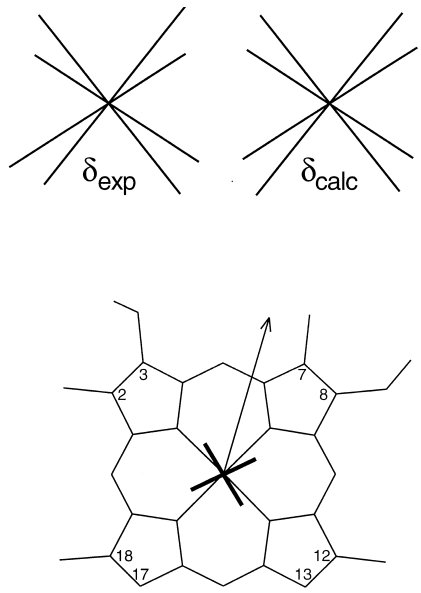


**Fig. 2** Schematic representation of the paramagnetic shifts of $^{13}C$ nuclei $\alpha$ to the haem in ferricytochrome *c″* and the orientations deduced for the His ligands. The shifts are represented by vectors directed towards the substituent nuclei with lengths proportional to the total paramagnetic shifts observed at 278.5 K ($\delta_{exp}$) or calculated according to Model II ($\delta_{calc}$), which are listed in Table 2. The orientations of the His ligands are shown superimposed on the haem structure (from which the propionate groups have been deleted for clarity and with IUPAC numbering for the pyrrole $\beta$ carbons). The *arrow* represents the orbital mixing parameter, $\theta$ in Table 3, which corresponds to the bisector of the acute angle between the normals to the His planes

the model, the $^{13}C$ Fermi contact shifts are proportional to the unpaired spin density on the pyrrole $\beta$ carbons, which in turn depends on two unperturbed molecular orbital coefficients and the strength and orientation of a rhombic perturbation. We assume that the molecular orbital coefficients, which we label $c_1$ and $c_5$, are constant for low spin haems and employ the values which were recently optimised for horse cytochrome *c* (Turner 1995). The strength of the rhombic perturbation is reflected by the splitting of the $\pi$ molecular orbitals, which are originally degenerate, and the unpaired electron density is simply approximated by a Boltzmann distribution between them. The paramagnetic shifts measured at 278.5 K and 295.0 K can therefore be fitted simultaneously using only three parameters: a mixing parameter for the unperturbed partially occupied molecular orbitals, the energy separation of the orbitals after mixing, and a scaling parameter represented by the factor for the hyperfine coupling constant.

The result of fitting the $^{13}C$ shifts is given in Table 2. The parameters relevant to the haem geometry are the orbital mixing parameter $\theta$, and the splitting of the energy, levels, $\Delta E$. The scaling factor, Q, represents the polarisation of the 1s electrons of the substituent $^{13}C$ nucleus by $\pi$ electron density on the pyrrole $\beta$ carbon to which it is attached, and it is in good agreement with the value of $Q^C_{C'C}$ determined theoretically by Karplus and Fraenkel (1961). Attempts to fit the data with the two molecular orbital coefficients, $c_1$ and $c_5$, allowed to vary in place of the scaling factor resulted in unacceptably large errors for all parameters. However, the fitted values of $\theta$ and $\Delta E$ vary by only ±10% with the ratio $c_1/c_5$ in the range 1.8 – 2.4, which has been found in various simple Hückel calculations (Shulman et al. 1971; Turner 1995), and so the parameters obtained using the coefficients from horse cytochrome *c* ($c_1/c_5 = 2.06$) appear to be representative. The mixing parameter, $\theta$, has been shown to follow the orientation of the bisector of the acute angle, $\beta$, between the normals to the His planes in several bis-His cytochromes, and the energy level separation shows a monotonic dependence on $\beta$ (Turner et al. 1995; Banci et al. 1995; Pierattelli and Turner 1996). The form of the dependence is not yet well characterised, but it may be approximated as $\Delta E \approx 5\cos\beta$ kJ/mol (Turner et al. 1995), yielding $\beta \approx 85°$ for cytochrome *c″*. The observed and calculated values of the paramagnetic shifts are illustrated in Fig. 2, together with the orientations of the axial ligands which may be deduced.

The analysis could be improved by using calculated values for the dipolar shifts in order to separate the Fermi contact contributions from the total paramagnetic shifts. Accurate values for the dipolar shifts require the magnetic susceptibility tensor to be measured, which may be achieved by fitting the paramagnetic shifts of several protons in the polypeptide (Keller and Wüthrich 1972). However, since neither spatial coordinates nor diamagnetic shifts are available, this is not yet possible for cyto-

chrome *c″*. A less satisfactory alternative is to extrapolate the principal values of the magnetic susceptibility tensor from the low temperature g values measured from EPR spectra (Horrocks and Greenberg 1973). It is then essential that all three g values should be measured and it is also necessary to assume a value for the spin-orbit coupling constant. Even then, the orientation of the tensor is unknown, though it is reasonable to make the approximation that the largest component is normal to the haem plane and that the second largest component is rotated through the negative of the angular mixing parameter obtained from the $^{13}C$ Fermi contact shifts (Oosterhuis and Lang 1969; Byrn et al. 1983). Unfortunately, only $g_{max}$ can be determined for cytochrome *c″* at neutral pH and so the energies of the Kramers doublets cannot be fitted directly. We therefore make the additional assumption that the axial distortion parameter in cytochrome *c″* is typical of bis-histidine haems *c* with a value of 2.8 $\lambda$ (Thomson and Gadsby 1990), where $\lambda$ is the spin-orbit coupling constant. The observed value of $g_{max}$ is then fitted with a rhombic distortion of 0.42 $\lambda$, giving $g$ values 3.65, 1.38, and 0.15; the low values calculated for $g_{med}$ and $g_{min}$ being in accordance with the failure to observe signals in the EPR spectrum. The principal values of the magnetic susceptibility tensor may then be calculated at any temperature by assigning a value to the spin orbit coupling constant, which we take to be 340 $cm^{-1}$. It is then possible to fit the observed shifts to the sum of the metal centred dipolar shift and the Fermi contact contribution calculated on the assumption that the magnetic susceptibility tensor rotates in the opposite sense to the molecular orbitals and that the direction of $g_{max}$ is normal to the haem plane. The averaged coordinates of haems from several crystal structures were used for this purpose. The results of applying this approximation (referred to as Model II), are also given in Table 2.

The factor for the hyperfine coupling constant, which scales the Fermi contact shifts, naturally changes between the two models in order to compensate for the dipolar contribution, but the change in the energy separation is relatively small. Subtracting the dipolar contribution also has the effect of rotating the pattern of Fermi contact shifts slightly, which is indicated by the change in the orbital mixing parameter. The cross peak observed in the HMQC spectrum for the 5CH group at 295 K provides a further test of the analysis. Taking the diamagnetic reference for the $^{13}C$ shift to be the average of the *meso* carbon shifts in horse ferrocytochrome *c*, 96.2 ppm (Santos and Turner 1992), and subtracting the calculated dipolar shift of –38.3 ppm, we find a Fermi contact contribution of –28.9 ppm. The Fermi contact shift may be calculated using the unperturbed molecular orbital coefficients obtained for horse cytochrome *c* (Turner 1995), together with the constants tabulated by Karplus and Fraenkel (1961) and the parameters from Table 2, yielding a value of –33.7 ppm at 295 K. In view of the approximations made, including neglect of any ligand centred dipolar interaction (Kurland and McGarvey 1970) for the 5CH $\pi$ orbital (for which $\rho = 0.0019$), this level of agreement indicates that the parameters obtained from the analysis are of the correct order.

The structural information which may be deduced from these parameters is illustrated in Fig. 2, and its quality may be evaluated by comparing the results obtained with the different sets of assumptions represented by Models I and II. Firstly, the model necessarily predicts that the pattern of Fermi contact shifts should be centrosymmetric whereas the measured paramagnetic shifts of diametrically opposed substituents differ by up to 13 ppm, and differences of up to 20 ppm have been observed in several other haem proteins (Timkovich 1991; Turner et al. 1995; Turner 1995; Banci et al. 1995; Pierattelli and Turner 1996). A small part of the differences may be attributed to variations in the diamagnetic shifts, but the pattern may also be distorted by neighbouring residues, by the asymmetry of the haem substituents, and distortion of the haem geometry. This reduction in symmetry is usually not serious since the Fermi contact shifts may span a range of 50 ppm in systems with moderately large rhombic perturbations and the each pair of shifts is effectively averaged in the fitting procedure. For example, in the present case we find that the rmsd for fitting individual paramagnetic shifts is 4.5 ppm, but this is reduced 1.8 ppm for the pairwise averages. Such deviations may provide an additional source of information once data from a large number of haem proteins is available for comparison but now we shall only consider the implications for the significance of the parameters $\Delta E$ and $\theta$.

The orbital mixing parameter should be well defined according to the model if the axial ligands have nearly parallel orientations since the in-plane anisotropy then approaches a maximum, but the apparent energy level splitting will become increasingly uncertain since the populations are represented by a Boltzmann distribution and $\Delta E$ may be several times kT. This is a limiting factor in determining a precise relationship betwen $\Delta E$ and the dihedral angle between axial ligands, $\beta$, on the basis of the data presently available. Conversely, as the limit of axial symmetry is approached, $\theta$ tends towards infinite uncertainty but $\Delta E$ approaches a zero crossing point and is very well defined. Of course, this does not imply that the Kramers doublets dominated by the iron $d_{xz}$ and $d_{yz}$ orbitals become degenerate: these doublets are split by the spin orbit coupling even in the absence of a rhombic perturbation, but the coefficients of the orbitals then become equal.

In the case of cytochrome *c″*, the observed paramagnetic shifts span a range of only 19 ppm and, if allowance is made for distortion of ±5 ppm from inversion symmetry of the paramagnetic shifts and the parameters of Models I and II are compared (see Table 3), the orientation of the rhombic perturbation may be quoted as 30±15°. This error limit will also dominate the uncertainty in the absolute orientation deduced for each of the axial ligands according to the model. However, since $\Delta E$ is found to be 500±150 J/mol, the relative orientation, $\beta$, deduced for the two ligands from the relation $\Delta E \approx 5\cos\beta$ kJ/mol (Turner et al. 1995) is 85° with an uncertainty of only ±2°, and this value is only weakly dependent on the maximum value ascribed to $\Delta E$ in the presence of parallel ligands.

In contrast, EPR studies of haem proteins with near-axial symmetry such as cytochrome *c″* yield a single datum,

**Table 3** Parameters obtained by fitting the paramagnetic shifts of $^{13}C$ nuclei $\alpha$ to the haem in ferricytochrome *c″*. The orbital mixing parameter, $\theta$, is equivalent to the orientation of the rhombic distortion and the energy level splitting, $\Delta E$, may be compared with the rhombic crystal field parameter, V. The scaling factor, Q, takes the same form as the hyperfine parameter $Q^{C}_{C\cdot C}$ (Karplus and Fraenkel 1961). The standard errors given in parenthesis assume an experimental error of ±1.0 ppm for the chemical shifts

| | $\theta$ degrees | $\Delta E$ J/mol | Q MHz |
|---|---|---|---|
| Model I | 35.58 (2.25) | –484.47 (31.78) | –39.66 (0.20) |
| Model II | 25.44 (2.91) | –565.07 (49.78) | –33.62 (0.20) |

$g_{max}$, which should approach a maximum value as the axial ligands approach $S_4$ symmetry (Walker et al. 1986; Berry et al. 1990; Safo et al. 1991) and is therefore rather insensitive to the precise angle. The value of $g_{max}$ at this maximum also depends on the axial distortion parameter, which can only be approximated, and so EPR allows only qualitative conclusions to be drawn about the relative orientation of the axial ligands. The absolute orientation of the rhombic perturbation can only be obtained from EPR studies of single crystals and of course this becomes infinitely uncertain as axial symmetry is approached, as in NMR.

## Conclusion

This work provides additional data for understanding the relationship between the geometric and electronic structures of low spin haems as well as refining the model of the haem cleft in ferricytochrome *c″*. There is a growing number of studies which demonstrate a clear relationship between the pattern of Fermi contact shifts of $^{13}C$ nuclei positioned $\alpha$ to haems and the orientation of the axial ligands. This relationship is now used to deduce the ligand geometry in ferricytochrome *c″* directly from $^{13}C$ data, allowing the relative orientation of the axial ligands, which was deduced from EPR to be roughly perpendicular, to be made quantitative. Despite the existence of secondary effects on the Fermi contact shifts which spoil the inversion symmetry predicted by the model, the effective splitting of the $\pi$ molecular orbitals is obtained with reasonable accuracy and provides an extremely sensitive measure of the rhombic perturbation in near-axial haems. Since it is usual to measure only one of the three *g* values in EPR studies of near-axial systems and it is relatively insensitive to the magnitude of the rhombic perturbation, $^{13}C$ NMR should become a valuable complement to EPR in systems of this type.

The absolute orientation of the two ligands may be deduced from their relative orientation together with the orientation of the rhombic perturbation. NMR has some advantages over EPR in this respect since it avoids the need for single crystal studies, but values obtained from NMR or EPR necessarily become imprecise in near-axial systems. The result obtained for ferricytochrome *c″* is roughly midway between alignment with the *meso* carbons, as found in (tetraphenylporphinato)iron(III) complexes and many cytochromes, and alignment with iron nitrogen bonds, but there is no obvious reason for the instability of one His ligand in the reduced form. In fact the reduced cytochrome would be expected to be stabilised by the low rhombic splitting generated by near perpendicular ligands (Walker et al. 1986).

There is an interesting parallel between the detachment of the His ligand in ferrocytochrome *c″* and the model proposed for the reduction of nitrite by cytochrome $cd_1$ nitrite reductase from *Thiosphaera pantotropha* (Fülöp et al. 1995) in which the substrate binds to a five-coordinate Fe(III), the reduced product is displaced by a sixth ligand, and the six-coordinate ferric protein is returned to the active state by the transfer of one proton and one electron. The redox-linked spin state transition observed in cytochrome *c″* may therefore have some relevance as a model system and is clearly worthy of further investigation. The rearrangement could be triggered by axial movement of the fixed His ligand stabilising a movement of the high spin Fe(II) out of the porphyrin plane or by an active reordering or hydrogen bonds to the detachable ligand. Future studies will be directed towards identifying the hydrogen bonding of the His $N_1H$ protons in order to clarify the mechanism further.

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