Replacement of the methionine axial ligand in cytochrome c_{550} by a lysine: effects on the haem electronic structure

Ricardo O. Louro[a], Ellen C. de Waal[b], Marcellus Ubbink[b], David L. Turner[c,*]

[a] *Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Rua da Quinta Grande, 6, Apt. 127, 2780 Oeiras, Portugal*
[b] *Gorlaeus Laboratories, Leiden Institute of Chemistry, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands*
[c] *Department of Chemistry, University of Southampton, Southampton SO17 1BJ, UK*

Received 19 November 2001; revised 28 November 2001; accepted 28 November 2001

First published online 14 December 2001

Edited by Thomas L. James

Abstract The prosthetic group of low-spin haem proteins is an iron porphyrin with two axial ligands, typically histidine, methionine or lysine. Determining the geometry of the axial ligands is an important step in structural characterisation, particularly in the paramagnetic oxidised forms. This work extends earlier studies of the hyperfine nuclear magnetic resonance (NMR) shifts of haem substituents in bis-His and His–Met cytochromes to His–Lys co-ordination in the M100K mutant of *Paracoccus versutus* cytochrome c_{550}. The electronic structure of the His–Lys haem is shown to be similar to that produced by His–cyanide co-ordination, such that NMR can be used to determine the geometry of the His ligand.

Key words: Paramagnetic nuclear magnetic resonance; Cytochrome *c*; Solution structure; Site-specific mutagenesis; Axial ligand

1. Introduction

Several biologically important haem proteins have axial ligands connected to the iron via tetrahedral nitrogen. Some well known examples are the alkaline forms of cytochrome *c* where lysine 73 or 79 co-ordinates the iron [1,2], cytochrome *f* where the polypeptide N-terminus binds the iron [3], CooA, a transcription factor of the CAP family in which the iron is co-ordinated by the main chain nitrogen of a proline [4], and nitrite reductase, where the catalytic haem is coordinated in the fifth position by a lysine [5].

Electron paramagnetic resonance spectroscopy is an important tool for probing the crystal field of the haem iron [6], but it provides limited information about ligand geometry and may not even reveal the nature of the ligands [7]. Although nuclear magnetic resonance (NMR) spectroscopy is very much less sensitive, it can be used to determine the pattern of electron delocalisation in the haem directly [8] and, hence, provide information about ligand orientations with respect to the haem [9,10], as well as to characterise the ligands themselves. The nuclei in the haem substituents experience large hyperfine shifts that depend on the unpaired electron density on the carbon of the haem macrocycle to which they are attached. The relationship between these shifts, which are dominated by the Fermi contact interaction, the geometry of the axial ligands and the magnetic susceptibility tensor, has been clearly established for bis-His and His–CN^- haems *b* and *c*, and, less accurately, for His–Met ligation. Such information can be used to predict dipolar shifts, as an aid to signal assignment, and to improve the quality of solution structures of paramagnetic proteins [11–13].

The most comprehensive studies of Fermi contact shifts have been based on ^{13}C data, analysed in terms of the haem molecular orbitals, with the angle between the axial ligands related to the splitting of the frontier orbitals semi-empirically. More recently, it was shown that the ^1H shifts of haem methyl substituents could be fitted to empirical expressions to obtain the orientation of histidine ligands in bis-His and His–CN^- haems *c* [14,15]. Each of the methods has been used to predict the orientation of the axial His ligands in the trihaem cytochrome c_7 [15], and the recently reported crystal structure of the cytochrome [16] provides a test of their accuracy. The geometric parameters, θ and β, obtained from the semi-empirical analysis of ^{13}C-NMR data have an RMSD with respect to the crystal structure of 6°, whereas the empirical equation for ^1H-NMR data at 298 K gave an RMSD of 9°, and the improved and corrected version (equation 5 in [15]) gave 8°. Although the results obtained from ^1H-NMR agree slightly less well with the crystal structure, the differences are comparable to the combined experimental errors. The results from ^1H-NMR are also less precise than those from ^{13}C-NMR, and parameters are only available for data obtained at 298 K, but the relative ease of obtaining ^1H spectra makes the empirical method attractive.

In this work, we examine the possibilities for extending these methods to include ligands with tetrahedral nitrogen bound to the iron. We have chosen to study the effect of lysine in the His–Lys co-ordinated haem found in the Met100-Lys mutant [17] of cytochrome c_{550} from *Paracoccus versutus* (previously known as *Thiobacillus versutus* [18]). Since there are no three-dimensional structures available for this protein, either for the wild-type or for the mutant, the orientation of the His ligand is first established in the cyanide complex.

2. Materials and methods

The wild-type and Met100-Lys mutant of cytochrome c_{550} from *P. versutus* were obtained and purified as previously described [17]. The proteins were lyophilised and the NMR samples prepared by dissolving approximately 15 mg in 500 μl of ^2H_2O (99.9% atom ^2H). The

*Corresponding author. Fax: (44)-23-80593781.
E-mail address: dlt@soton.ac.uk (D.L. Turner).

0014-5793/02/$22.00
PII: S0014-5793(01)03272-0

cyanide derivative of the wild-type protein was prepared by adding solid KCN to the NMR tube and then readjusting the pH.

Experiments were performed in a Bruker DRX500 spectrometer using an inverse probe with z-gradients. NOESY spectra were collected with a mixing time of 30 ms and a sweep width of 24.5 kHz in both dimensions. The ^{1}H-^{13}C-HMQC experiments were acquired with a sweep width of 24.5 kHz in F_2 and 30 kHz in F_1. In both experiments a selective pulse of 750 ms was used for water pre-saturation. The spectra were calibrated in the ^{1}H dimension using the water signal as internal reference, and in the ^{13}C dimension using isotopically enriched lactate as external reference. The resonances of the haem substituents were assigned by combining the data from the NOESY and HMQC experiments performed at 298 K.

Molecular orbital calculations were performed using a FORTRAN program in which the ^{13}C hyperfine shifts were fitted to the model of the haem frontier molecular orbitals using the Marquardt method [9]. The diamagnetic reference for the haem methyls was taken as 12.1 ppm [19].

Values for β and ϕ were obtained from the empirical Eq. 1 (equation 5 in [15]):

$$\delta_i = \cos\beta\ [38.0\ \sin^2(\theta_i - \phi) - 4.1\ \cos^2(\theta_i + \phi) - 15.9] + 13.8 \pm 0.7 \quad (1)$$

where the sign for the final correction is negative for 2^1 and 7^1CH_3 and positive for 12^1 and 18^1CH_3. The angle θ_i is that between the vector connecting the ring methyl group i to the iron and the NC–NA vector. The sum of the squared differences between the observed ^{1}H chemical shifts of the four methyls and the calculated values was minimised using a commercial computer spreadsheet. In both cases, standard errors were estimated from regular statistical formulations [20].

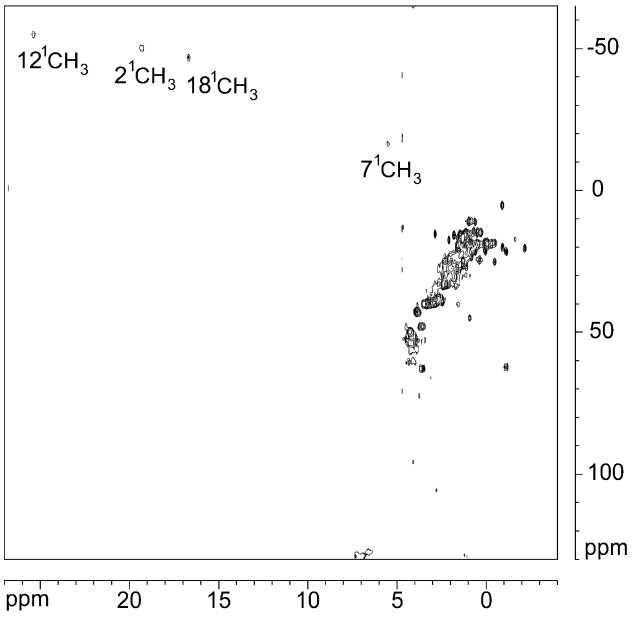

Fig. 1. ^{1}H-^{13}C-HMQC spectrum of oxidised M100K mutant of cytochrome c_{550} from *P. versutus* at 298 K. Labels indicate the resonances of the haem methyl groups.

3. Results and discussion

The chemical shifts of haem methyl groups in wild-type cytochrome c_{550} from *P. versutus*, together with those of its cyanide derivative, the M100K mutant, and, for comparison, cytochrome c_{550} from *Paracoccus denitrificans*, are reported in Table 1 and Fig. 1. These results confirm the ^{1}H assignment previously reported for the more downfield shifted methyl resonances of the M100K mutant [17], and extend the assignment to the 7^1CH_3 resonance, which is located close to the water signal. The ^{13}C shifts for the haem methyls are also reported.

This work was performed using samples with ^{13}C at natural abundance and it was not possible to observe the ^{13}C resonances associated with propionates 13 and 17 and methine protons 3 and 8 at this concentration. Thus, the ^{13}C and ^{1}H data sets to be compared are of similar completeness, although under more favourable conditions the data from the other four α-substituents of the haem can be incorporated into the ^{13}C molecular orbital calculations, increasing the precision of the results.

Table 2 reports the parameters obtained by fitting the chemical shifts of haem methyl groups from *P. versutus* cytochrome c_{550}, its cyanide derivative, and the M100K mutant. Only ^{1}H data observed at 298 K can be used for the empirical analysis, whereas all of the ^{13}C shifts derived from Table 1 were used. All values are reported using the convention of positive values for vectors lying in the sector defined by the nitrogens of pyrroles B and C or the nitrogens of pyrroles A and D. According to the parameterisation of the empirical equation for ^{1}H chemical shifts, the angle ϕ represents the average of the ligand plane projections in bis-His haems and the angle β is the dihedral angle between them. The analysis of ^{13}C shifts in terms of haem molecular orbitals yields the orientation of the rhombic perturbation of the molecular orbitals, θ, directly. This is determined by the average of the orientations of the histidine N_ε p-orbitals, perpendicular to the His plane, and hence is expected to be at right angles to ϕ. The angle θ is also the negative of the orientation of the y-axis of the g-tensor [10,21]. Finally, the splitting of the frontier orbitals, ΔE, is related empirically to the dihedral angle between His ligands through $\Delta E = 5\cos\beta$ [9].

The first objective is to establish the orientation of the His ligand in the mutant cytochrome. There is no crystal structure available for the mutant or the wild-type cytochrome c_{550} from *P. versutus*, but the structure of the highly homologous cytochrome c_{550} from *P. denitrificans* (84% amino acid se-

Table 1
Chemical shifts (ppm) of the ^{1}H and ^{13}C signals of haem methyls in the wild-type (wt) cytochromes c_{550} from *P. denitrificans*, Pd [23], and *P. versutus*, Pv, the cyanide derivative of Pv, and the M100K mutant of Pv

	wt Pd	wt Pv		PvCN				M100K			
	298 K	298 K		298 K		302 K		298 K		311 K	
	^{1}H	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
2^1	13.03	12.39	−32.7	20.26	−54.9	20.30	−51.3	19.84	−52.7	18.80	−48.0
7^1	29.00	29.04	−54.3	n.o.	n.o.	4.92	−15.5	5.20	−16.8	5.91	−16.1
12^1	16.75	16.59	−36.9	22.2	n.o.	21.37	n.o.	25.99	−57.4	24.72	−52.7
18^1	29.82	29.48	−62.5	18.97	−48.0	18.30	−46.6	16.80	−49.1	16.57	−45.4

The methyl groups are labelled according to IUPAC-UIB nomenclature. The label n.o. indicates signals not observed.

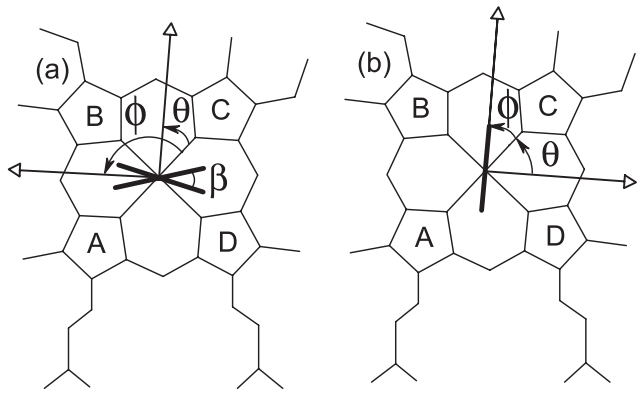

Fig. 2. Geometric parameters used to describe the haem ligands in the case of bis-histidine coordination (a), and histidine–cyanide coordination (b). In b, a single axial ligand determines the haem electronic structure such that $\beta \equiv 60°$; this diagram shows the actual orientation found in *P. versutus* cytochrome c_{550}.

quence identity) [22] has been determined (PDB accession code 1COT). Moreover, the chemical shifts of the haem methyl resonances in the oxidised form of the two wild-type proteins are closely similar, which indicates a similar haem environment [23,24]. There is no empirical equation developed for ^{1}H shifts in His–Met haems, but analysis of the ^{13}C chemical shifts of the *P. versutus* wild-type protein yields a rhombic perturbation at an angle, θ, of $-23°$, which is in reasonable agreement with the average of the ligand lone pair orientations ($-10°$) deduced from the crystal structure, given that only the four methyl shifts were used. However, the energy splitting of the frontier orbitals yields an approximate dihedral angle between the axial ligand planes (from $\Delta E = 5\cos\beta$) consistent with the angle of the His plane being at 33°, rather than 49° as in the crystal. Because such uncertainties are typical in the analysis of the geometry of His–Met ligation, we consider instead the complex with cyanide replacing the Met ligand, in which the relationship between the electronic properties of the haem and the ligand orientation is better defined.

Replacing the Met ligand with CN^- changes the spectrum dramatically. Analysis of the ^{13}C data from the His–CN^- complex yields an energy splitting of 2.57 kJ/mol, which is about half of the value expected for a bis-His haem with parallel planes and implies that the cyanide anion makes no contribution to the rhombic perturbation. Hence, θ should correspond to the normal to the His plane, which is found to be at an angle of 39° to the NC–NA vector. Although the empirical equation for fitting ^{1}H shifts was designed for bis-His haems, it has been shown to be applicable to His–CN^- complexes as well [15]. In this case, fitting yields a value for β close to 60°, such that $\cos\beta = 0.5$, as would be expected if only one ligand were contributing, and the value ϕ should correspond to the orientation of the single His plane (see Fig. 2). Thus, the two methods agree that the orientation of the His ligand is close to 40° in the cyanide complex, and this is not expected to vary by more than a few degrees as a result of substitution of the other axial ligand. This is also consistent with the angle of 49° found in the crystal structure 1COT, which has a resolution of 1.7 Å and carries its own uncertainty.

Table 2
Parameters obtained by two methods for analysing haem chemical shifts

		wt Pv	PvCN	M100K
^{1}H	ϕ (°)	–	40 (2)	34 (2)
	β (°)	–	56 (3)	56 (2)
^{13}C	θ (°)	−23 (1)	−51 (1)	−50 (1)
	ΔE (kJ/mol)	−1.81 (0.05)	−2.57 (0.08)	−2.63 (0.07)

The ^{1}H shifts measured at 298 K, reported in Table 1, were fitted to the empirical Eq. 1 [15]. The analysis of ^{13}C shifts in terms of haem molecular orbitals used a value of the hyperfine coupling constant Q_{cc} fixed at −36 MHz [11]. Values in parentheses indicate the standard error of the parameters, assuming 1 ppm uncertainty in the chemical shifts.

The chemical shifts observed for the M100K mutant of *P. versutus* cytochrome c_{550} are strikingly similar to those of the cyanide complex, which suggests immediately that the lysine makes little or no contribution to the rhombic perturbation. This is borne out by the analysis of ^{13}C chemical shifts, which is not affected significantly by the nature of the axial ligands on condition that the iron remains low-spin. The orientation of the rhombic perturbation is unchanged from that in the cyanide complex and the energy splitting implies that, if the two ligands had similar effects, there should be a dihedral angle between them of about 60°. That would require the mutation to cause the His ligand to rotate through 30° with respect to the wild-type protein and its cyanide complex, which is unlikely. A more plausible conclusion is that the orientation of the His ligand is unchanged and that lysine has similar effect to His, Met or CN^- on the axial field but a negligible effect on the rhombic anisotropy. In that case, the empirical analysis of ^{1}H shifts can be applied to His–Lys coordinated haems and the value of ϕ obtained can be interpreted as a direct measure of the His orientation.

In effect, the finding that lysine does not contribute significantly to the rhombic perturbation of the haem implies that the ligand has approximate threefold symmetry about the haem normal. That is reasonable in view of the similarity of the bonds in the tetrahedral CNH_2 group and the probable weakness of the interaction between the iron and the atoms bonded to the nitrogen, which is akin to hyperconjugation in methyl groups.

4. Conclusions

The results reported in this work demonstrate that the lysine axial ligand has little or no influence on the distribution of the unpaired electron in the haem frontier molecular orbitals, which defines the pattern of chemical shifts for the haem substituents. This situation is similar to that found for the cyanide ligand in cyanide derivatives [25]. In the absence of exogenous ligands, a radical change in the pattern of chemical shifts while the haem remains low-spin may be diagnostic of substitution of the sixth ligand by tetrahedral nitrogen. Equally, finding little change in the chemical shifts on complexation with cyanide may be a useful test for a tetrahedral nitrogen ligand in native haem proteins.

The good agreement between the results of the empirical and the molecular orbital methods shows that the parameterisation of the empirical equation [15] for haem methyl groups can also be used for the cases of oxidised haems with His–CN^- and His–Lys co-ordination, provided that data can be obtained at 298 K. In this way, it was possible to use the published ^{1}H NMR data available in the literature [1] to calculate the value

of the geometric parameter ϕ for both the Lys73 and Lys79 coordinated alkaline forms of the iso-1-cytochrome *c* from yeast (our unpublished results). The value obtained is within 10° of the orientation of the histidine reported in the structure obtained at low pH [26], showing that the perturbation of the axial His ligand by substitution of the sixth ligand is minimal. Thus, the straightforward application of the empirical method [15] should aid future efforts to determine the solution structures of haem proteins in which one of the axial ligands binds the iron through a tetrahedral nitrogen, such as the alkaline forms of the oxidised cytochrome *c* [1], in particular because it is not usually possible to measure NOE distance constraints for nuclei so close to the paramagnetic centre.

Acknowledgements: Funding was provided by EU Contract ERBFMRX-CT98-0218. R.O.L. is the recipient of a post-doctoral fellowship from FCT Praxis XXI programme (BPD/3585/2000).

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