DALTON

Nuclear magnetic resonance studies of pyridine binding to cytochrome c†

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The binding of pyridine by displacement of the methionine-80 residue from heart ferricytochrome c (cyt c) has been studied by ^{1}H NMR spectroscopy. Owing to the low stability of the pyridine-bound form of cytochrome c, the so-called alkaline isomer (lysine form) of cyctochrome c appears at pH > 6 in the presence of 0.45 mol dm^{-3} pyridine, in contrast to the native lysine form which only appears at pH > 9. A mixture of native cytochrome c and pyridine without the lysine form can only be obtained at pH < 6. The bound pyridine is replaced by a lysine as the pH value increases. Native cytochrome c, pyridine-bound cytochrome c and the lysine form can exist simultaneously at neutral pH. A pure lysine form can be obtained at pH > 8 in the presence of 1.27 mol dm^{-3} pyridine. Using the known resonance assignments for native cytochrome c, some hyperfine-shifted resonances arising from haem peripheral protons and two axial ligands and some side-chain resonances of the aliphatic and aromatic protons of pyridine-ligated cyt c (pcyt c) have been assigned. The experimental dipolar shifts for protons belonging to the non-co-ordinated residues have allowed the identification of the plausible orientation and magnitude of the *g* tensor. The sources of the asymmetric spin density distribution in the haem group of pcyt c are discussed. The substitution reaction rate constants and the equilibrium constant for pyridine binding to cyt c have been evaluated.

The structure, function and folding of cytochrome c (cyt c) are intimately linked with the presence of a covalently attached iron-(II) or -(III) haem group with axial ligands provided by the side chains of His-18 and Met-80.[1] The second axial ligand, Met-80, is essential to the structure and function of cytochrome c. It has been shown by optical and NMR spectroscopy that Met-80 dissociates more readily and can be displaced by intrinsic or extrinsic ligands when pH > 9 or when extrinsic ligands are added.[2–5] A NMR study of the hyperfine shift distinguished six different forms of iron(III) cytochrome c depending on the pH of the protein solution.[6] The so-called alkaline conformation of cytochrome c, formed on raising the pH to alkaline values (pK_a = 8.5–9 depending on the species), has received particular experimental attention.[7–10] The NMR studies indicated that there are two isomers in the alkaline conformation and Lys-79 provides the sixth axial ligand in one of these forms.[3,10] The time course of cytochrome c folding under native conditions can be divided into four stages, a burst phase, a fast phase, an intermediate phase and a slow phase.[1] The ligation of Met-80 occurs during the intermediate phase. The presence of an exogenous ligand serves as a kinetic trap which leads to the accumulation of a long-lived intermediate and makes it experimentally observable. In this sense the involvement of exogenous ligands is a useful approach for investigating the role of haem ligation in cyt c folding and the pH dependence.

The substitution of cyt c by CN^-, N_3^- and imidazole (Him) has been the subject of many NMR studies, but corresponding studies on pyridine (py) binding have scarcely been reported.[4,11] In this paper the nature of the substitution of Met-80 in horse heart ferricytochrome c by pyridine is investigated by ^{1}H NMR methods. A new finding that the alkaline form (lysine form) of cytochrome c appears at neutral pH in the presence of pyridine is first reported. This fact had been neglected by previous NMR studies on pyridine binding to cyt c which were carried out at neutral pH.[4,11] This work also extends previous NMR studies of the pyridine-bound form (pcyt c), two-dimensional exchange spectroscopy (EXSY) having been used to obtain the kinetics of ligand exchange and assign the haem peripheral protons and some resonances of the side chains. The orientation and magnitude of the *g* tensor for pcyt c has been estimated from the pseudo-contact shifts of protons involving residues not directly bound to the haem iron.

† *Supplementary data available* (No. SUP 57206, 2 pp.): pseudo-contact shifts of side-chain protons. See Instructions for Authors, *J. Chem. Soc., Dalton Trans.*, 1997, Issue 1.

Experimental

Horse heart cyt c (Type VI) from Sigma Chemical Co. was purified and lyophilized once from D_2O before use.[12] The NMR samples consisted of 5 mmol dm^{-3} cyt c (for one-dimensional spectra), 8 mmol dm^{-3} cyt c and 0.45 mol dm^{-3} C_5D_5N (for one- and two-dimensional EXSY experiments), or 5 mmol dm^{-3} cyt c containing 2.0 mol dm^{-3} C_5D_5N (for one-dimensional experiment) in D_2O. The pH was adjusted by the addition of small amounts of DCl or NaOD; pH values were not corrected for the isotope effect. No electrolyte was added and the ionic strength effects were neglected.

All NMR data were recorded on a Bruker AM500 spectrometer with an Aspect-3000 computer system. Chemical shifts were calibrated with respect to 1,4-dioxane at δ 3.743. One-dimensional NMR spectra were obtained using a presaturation pulse for elimination of the residual water resonance. Two-dimensional EXSY was carried out using the phase-sensitive nuclear Overhauser effect spectroscopy (NOESY) pulse sequence[13] over a 35 714.29 or 8064.52 Hz bandwidth. Mixing times (τ_m) of 25 or 50 ms were used. All two-dimensional spectra were collected with 2048 (t_2) × 512 (t_1) data points and 160 scans for each t_1 increment. After zero-filling, resulting in equal digital resolution in both dimensions, the time-domain matrix was multiplied in both dimensions with a shifted sine bell function.

The integral values of the two-dimensional peaks were obtained by direct reading from the spectra using a square frame, and then normalized according to $\Sigma I_{ij} = 1$. The same frame was used to estimate the average noise integral value in order to remove the noise effects from the quantitative two-dimensional integration, and the I_{ij} data were corrected before

normalization. The equilibrium magnetization values were obtained by integration of the one-dimensional spectra and were also normalized.[14]

Kinetics

For a spin system involving chemical exchange between N sites within a molecule or between molecules, the peak amplitude in two-dimensional EXSY spectra is related to the exchange rate constant k, the relaxation rate and the mixing time τ_m by expression (1),[15] where A is the amplitude matrix the elements

$$A = \exp(-R\tau_m) \quad (1)$$

of which are defined by equation (2) and R is the dynamic

$$A_{ij} = I_{ij}/M_j^0 \quad (2)$$

matrix which contains the parameters to be determined. The solution of equation (3) is given by (4),[16] where X is the square

$$\begin{vmatrix} \rho_A & -k_{BA} \\ -k_{AB} & \rho_B \end{vmatrix} \quad (3)$$

$$R = \frac{-\ln A}{\tau_m} = \frac{-X(\ln \Lambda)X^{-1}}{\tau_m} \quad (4)$$

matrix of eigenvectors of A, such that $X^{-1}AX = \Lambda = \mathrm{diag}(\lambda_i)$ and $\ln \Lambda = \mathrm{diag}(\ln \lambda_i)$, with λ_i the ith eigenvalue of A. The equilibrium magnetization, M_j^0, which is necessary for the computation, can be evaluated by simply replacing each M_j^0 by ρ_j, the relative population of each site, so the off-diagonal elements of R may in fact be calculated from a single two-dimensional spectrum obtained with $\tau_m = 0$ provided the stoichiometry of the spin system is known (from integration of one-dimensional spectrum, for example).[16] Thus a single two-dimensional spectrum with mixing suffices to calculate the exchange rate constants.

g Tensor

In the absence of the second-order Zeeman interaction, for an iron(III) low-spin haem protein, the theoretical pseudo-contact shift $\delta(\mathrm{pc})_{calc}$ is determined by the parameters of the electronic g tensor as in equations (5a) and (5b),[17–19] where μ_0 is the

$$\delta(\mathrm{pc})_{calc} = \frac{\mu_0}{4\pi} \cdot \frac{\mu_B S(S+1)}{9\pi k T r^3} [g_{ax}(3\cos^2\theta - 1) + 1.5 g_{eq} \sin^2\theta \cos 2\Omega] \quad (5a)$$

$$g_{ax} = g_z^2 - \tfrac{1}{2}(g_x^2 + g_y^2);\ g_{eq} = (g_x^2 - g_y^2) \quad (5b)$$

permeability of a vacuum, μ_B the Bohr magneton, T the absolute temperature and k is Boltzmann's constant. The position of each proton is defined by its polar coordinates (r, θ, Ω) in the reference system of the electron-spin g tensor [Fig. 1(*a*)]. The axial and equatorial anisotropies of the g tensor, g_{ax} and g_{eq}, are defined [equations 5(b)] in terms of the principal components g_i ($i = x$, y or z). Equation (5a) assumes that the unpaired electron spin is effectively localized at the haem iron, which then acts as a point dipole.

To calculate the orientation and magnitude of the g tensor for oxidized pyridine cytochrome c, we have written a VB (version basic) program based on a program from Ivano Bertini. All the possible orientations of the g tensor were sampled, and for each calculated orientation the best agreement between the calculated and experimental shifts $\delta(\mathrm{pc})_{calc}$ and $\delta(\mathrm{pc})_{obs}$ was sought varying the g-tensor component. Once the first estimate of the best orientation had been found the calculation was repeated with finer angle variation around the first estimate.

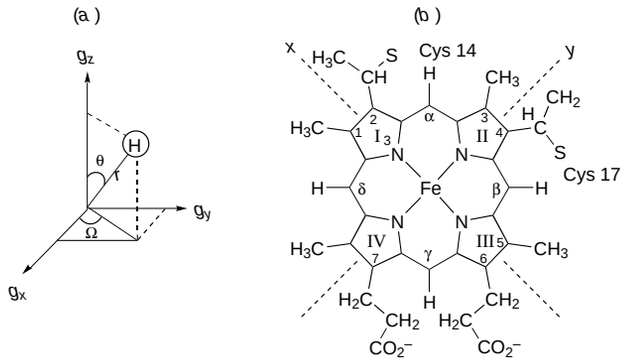

Fig. 1 Nomenclature for positioning each proton in the g-tensor system (*a*) and for identifying haem-related groups (*b*). The X and Y axes of the haem-based coordinate system are indicated; Z is normal to the haem plane. The pyrrole rings, viewed from Met-80, are numbered I–IV in a clockwise direction

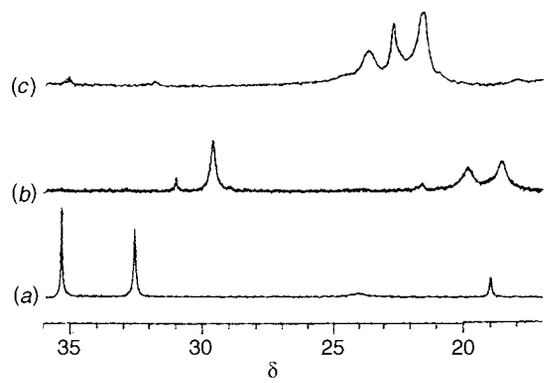

Fig. 2 Downfield hyperfine-shifted region of the 500 MHz ^{1}H NMR spectra in D_2O at 298 K of (*a*) 5 mmol dm^{-3} cyt c at pH 5.7, (*b*) d^5 5 mmol dm^{-3} cyt c and 2.0 mol dm^{-3} C_5D_5N at pH 5.7 and (*c*) cyt c 4 mmol dm^{-3} at pH 10.1

The observed pseudo-contact shifts $\delta(\mathrm{pc})_{obs}$ were estimated from the observed shift values for oxidized pyridine-bound cytochrome c obtained in this work by subtracting the diamagnetic contribution. Since the Fe–S bond in ferrocytochrome c is more stable than that in ferricytochrome c, Met-80 cannot be replaced by pyridine in the reduced form. Thus, the shift values of reduced cytochrome c were used to approximate those of reduced pyridine cytochrome c.[20] Proton coordinates were generated from heavy-atom coordinates of horse heart cytochrome c[21] by using the BIOSYM molecular graphics program with the usual assumption of standard amino acid geometries. Crystal structure coordinates, defined with respect to the unit cell, were transferred to a haem-based reference system with the X axis along the porphyrin N^1–N^3 direction, the Y axis along the porphyrin N^2–N^4 direction, and the Z axis normal to these two vectors [Fig. 1(*b*)].[22,23]

Results and Discussion

pH Dependence of NMR spectra of cyt c in the presence of pyridine

The downfield hyperfine-shifted portions of the ^{1}H NMR spectra of cyt c, pcyt c and the alkaline form of cytochrome c are shown in Fig. 2. Downfield and upfield hyperfine-shifted portions of the pH-dependent 500 MHz ^{1}H NMR spectra of cyt c with 0.45 mol dm^{-3} pyridine at 298 K are given in Fig. 3. By comparison with the ^{1}H NMR spectra of cyt c and pcyt c, it is found that two components exist at pH < 5.7. One is native cytochrome c and the other is pcty c. The intensities of the signals of cyt c increase as the pH decreases. With increasing pH above 6.20 a new set of signals appears at the expense of

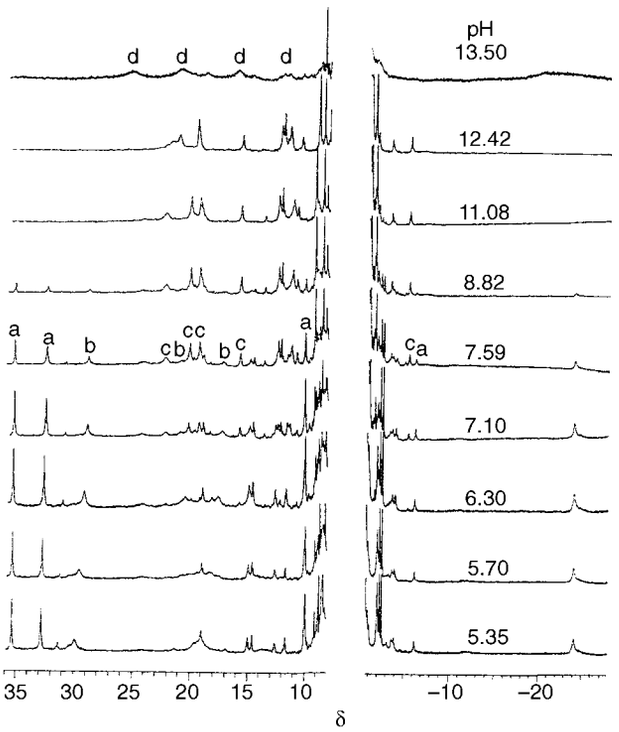

Fig. 3 Downfield and highfield hyperfine-shifted portions of the pH-dependent 500 MHz ^{1}H NMR spectra of cyt c in the presence of 0.45 mol dm^{-3} C_5D_5N in D_2O at 298 K. Four species, a–d, are observed in the pH range studied, assigned to native cyt c, pcyt c, the lysine form of cyt c and the hydroxide form of cyt c, respectively

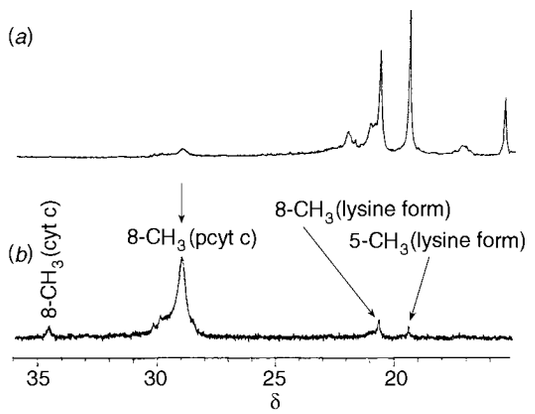

Fig. 4 (*a*) Downfield hyperfine-shifted portions of the 500 MHz ^{1}H NMR spectrum of cyt c in the presence of 1.27 mol dm^{-3} C_5D_5N in D_2O at 298 K and pH 7.4. (*b*) Saturation-transfer difference spectrum for the saturation of the 8-CH_3 resonance of pcyt c in the presence of 1.27 mol dm^{-3} C_5D_5N in D_2O at 298 K and pH 7.4. Saturation transfer is observed for the signal at δ 20.62 for the lysine form of cyt c

those for native cyt c and pcyt c. Only the new species can be observed at pH 9–12. It has been shown by optical and NMR spectroscopy that His-18 remains bound to oxidized haem iron and cannot be displaced by intrinsic or extrinsic ligands at pH > 9 or by the addition of extrinsic ligands.[1,24] The chemical shifts of this new species are very similar to those observed for cyt f, which has been shown to possess a low-spin haem iron with His-Lys ligation.[25] Fig. 2(*c*) shows the downfield hyperfine-shifted portion of the alkaline form of cyt c. From the spectral pattern, the new species is assigned to this (lysine) form. The pH range over which cyt c, pcyt c and the lysine form can exist simultaneously depends on the pyridine concentration. Native cyt c still exists at pH 8.82 in the presence of 0.45 mol dm^{-3} py. However, at 1.27 mol dm^{-3} py, no resonance of native cyt c is observed at pH > 7.4 and only the lysine form is observed at pH > 7.9. This indicates that increasing pyridine concentration stabilizes the lysine form and facilitates the transition from the native to the lysine form.

The alkaline isomer of horse heart cytochrome c is formed on raising the pH to 9.[6,10] However, the pH-dependent NMR spectra of pcyt c show that the change from native cyt c to the lysine form occurs at pH 6.0, which is much lower than that for the native form.

The NMR studies indicate that there are two isomers in the alkaline conformation and that Lys-79 provides the sixth axial ligand in one of these forms; the identity of the axial ligand to the haem iron in the second conformer of wild-type ferricytochrome c remains unknown though presumably it is located in the region between residues 66 and 104.[3,10] Elöve *et al.*[1] found that the presence of 200 mmol dm^{-3} imidazole prior to and during refolding had a slight effect on the folding of the N- and C-terminal helices of cyt c. Our NMR studies gave similar results.[24] This indicates that Lys-86, -87 or -88 which are located in the C-terminal helices cannot bind to the haem iron in alkaline ferricytochrome c. Therefore, the most likely candidates for this role at present are Lys-72 and -73, although extensive conformational changes will be required to accommodate these residues.

Since the binding of pyridine to haem is weak, Lys-79 (or -72, -73) will compete with pyridine to bind to Fe. That the lysine form appeared in the presence of pyridine shows that it is more stable than pcyt c under highly basic conditions. It does not appear with cyanide-bound cytochrome c[2] indicating that the stabilization of the lysine form is weaker than that of the cyanide form. Bearing in mind the pK_a values of 5.2 and 9.0 for pyridine and lysine respectively,[26] protonation of the lysine is more extensive than that of pyridine with decreasing pH. A decrease in pH will lead to the formation of pcyt c instead of the lysine form. A decrease in the concentration of neutral pyridine and Lys-79 (or -72, -73) with decreasing pH due to protonation also leads to the formation of native cyt c. When the pH is decreased below 5.7 only the pyridine and native forms can be observed.

Separate observation of three different species in the spectrum of pyridine-bound cyt c at pH 7.4 allows the corresponding resonances of the forms of the protein to be related through the saturation transfer. We have reported the saturation transfer between native cyt c and pcyt c in a previous article.[12] The rate constant for conversion of native cyt c into the lysine form had been reported at 4.0 ± 0.6 s^{-1} at 300 K,[3] which is far less than that into the pyridine form. As illustrated in Fig. 4, the saturation of the haem 8-CH_3 proton signal of pcyt c exhibits a weak connectivity to the signal at δ 20.66 for the lysine form of cyt c, which allows one to assign this signal to the haem 8-CH_3 of the lysine form. A weaker connectivity to the signal at δ 19.38 is due to the transfer NOE to the haem 5-CH_3. These results also confirmed that the new species is the lysine form of cyt c. The details will be reported elsewhere.

Proton resonance assignments for pcyt c

Since the lysine form appears at pH > 6.2 all the NMR experiments were carried out at pH 5.7. The one-dimensional ^{1}H spectra of a mixture of cyt c and pcyt c are illustrated at the top of Fig. 5. Compared with those of native cyt c and pcyt c shown in Fig. 2, it is found that some resonances of cyt c and pcyt c both downfield and upfield are well resolved due to the large scalar interaction. The exchange between groups of the native species and of the substituted form can be visualized from the cross-peaks in a two-dimensional spectrum.[3,27] From the previous assignment of native cyt c,[28–30] such a spectrum can be used to assign the groups of pcyt c.

In Fig. 5(*a*), the two-dimensional EXSY spectrum of a mixture of cyt c and pcyt c, four signals at δ 29.61, 18.43, 19.70, 13.96 show correlation with those at δ 35.26, 32.67, 9.97 and 8.00, respectively. The chemical shifts of the last four signals

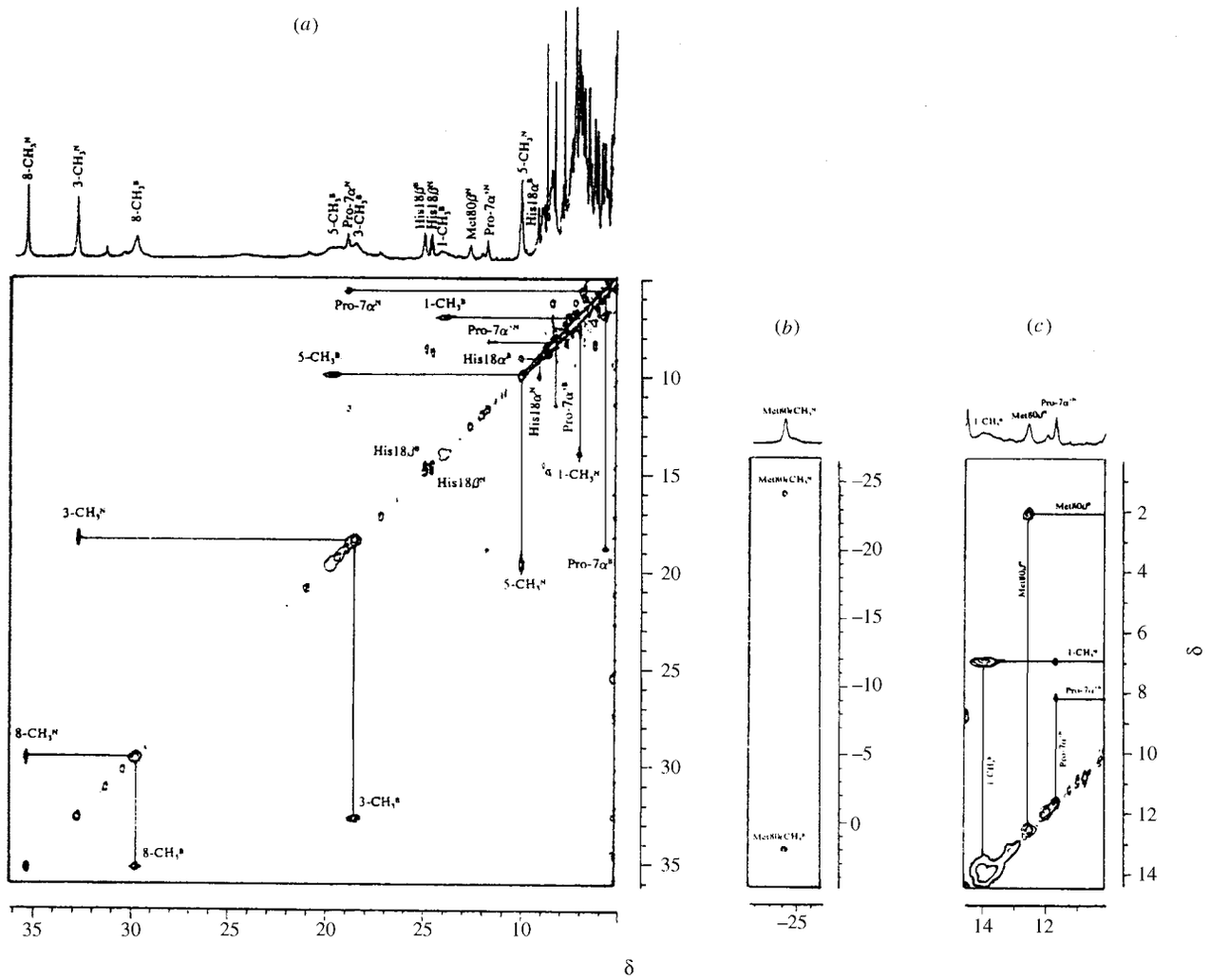

Fig. 5 Portions of the two-dimensional EXSY spectrum of a mixture of pcyt c and cyt c at pH 5.7 and 298 K with a mixing time of 25 ms. One-dimensional spectra are shown at the top. Chemical shifts of the resonance for pcyt c (B) and cyt c (N) are read in the F_2 dimension. (*a*) Downfield region, (*b*) upfield region and (*c*) portion of downfield region with Met-80 β-H indicated

suggest that they belong to the haem 8-, 3-, 5- and 1-methyl groups of cyt c,[28,30] so the observation of the above cross-peaks unequivocally identifies its first four signals as the corresponding methyl group resonances of pcyt c. The propionic acid 7α(pro 7α) and 7α′ protons of native cyt c have been assigned at δ 18.83 and 11.64.[20,28] In Fig. 5(*a*) these two signals show exchange cross-peaks at δ 5.68 and 8.20 respectively besides the NOE cross-peak between them. They can thus be assigned as pro 7α-H and 7α′-H of pcyt c. With the previous assignment of His-18 of cyt c,[30] the cross-peak between δ 9.03 and 9.94 confirmed that the signal at 9.94 can be assigned to His-18 α-H of pcyt c, and that the cross-peak between δ 14.49 and 14.85 shows that the resonance at δ 14.85 can be assigned to His-18 β-H of pcyt c. The Met-80 ε-CH_3 of cyt c at δ −24.23 shows an exchange cross-peak at δ 1.92 which can be assigned as Met-80 ε-CH_3 of pcyt c [Fig. 5(*b*)]. One of the Met-80 β-H of cyt c had been assigned to the peak at δ 12.53;[30] it shows an exchange peak at δ 2.06 [Fig. 5(*c*)], which is therefore assigned as due to Met-80 β-H of pcyt c. These data indicate that the S–Fe bond between Met-80 and haem is broken and this group gives rise to a random-coil ^{1}H chemical shift. The 8-, 5-methyl groups are furthest downfield for pcyt c, as are the 8-, 3-methyl groups of cyt c. Such shifted patterns reflect the different distributions of spin density on the pyrrole rings of cyt c and pcyt c, which will be discussed below.

In the poorly resolved diamagnetic region the two-dimensional EXSY experiments shows the major advantage of the greater selectivity available over one-dimensional double-resonance experiments. This is illustrated in Fig. 6 which displays the aliphatic region of the two-dimensional EXSY spectrum where the cross-peaks arising from chemical exchange between the methyl peaks of Leu, Ile and Thr occur. With the known assignments for cyt c,[29–31] the corresponding resonances for pcyt c can be unambiguously assigned from these connectivities. For example, Leu-68 δ_2 of cytochrome c is known to resonate at δ −2.61; in Fig. 6 the exchange cross-peak (δ −2.06, −2.61) indicates the connectivity between the Leu-68 δ_2H_3 resonance of cyt c and the corresponding resonance of pcyt c at δ −2.06. This approach accounts for 24 methyl resonances which have been assigned to the specific groups of pcyt c.

The aromatic region of the two-dimensional EXSY spectrum of an equimolar mixture of pcyt c and cyt c is shown in Fig. 7. The signal at δ 7.80 of cyt c corresponds to that at δ 7.36 of pcyt c. This finding along with the previous assignment for cyt c[29,31] identifies the latter signal as due to Tyr-67 (*o*)-H of pcyt c. Similarly, cross-peaks arising from chemical exchange allow one to correlate resonances of the ligand-bound and native species and to identify some aromatic ring protons of pcyt c. The assignments of the haem-related protons are listed in Table 2 and other assigned resonances of pcyt c are given in SUP 57206.

Dipolar shift calculations

Using two-dimensional EXSY spectra the assignments made for cyt c have subsequently been transferred to the corresponding pcyt c species. Some of the chemical shift differences are

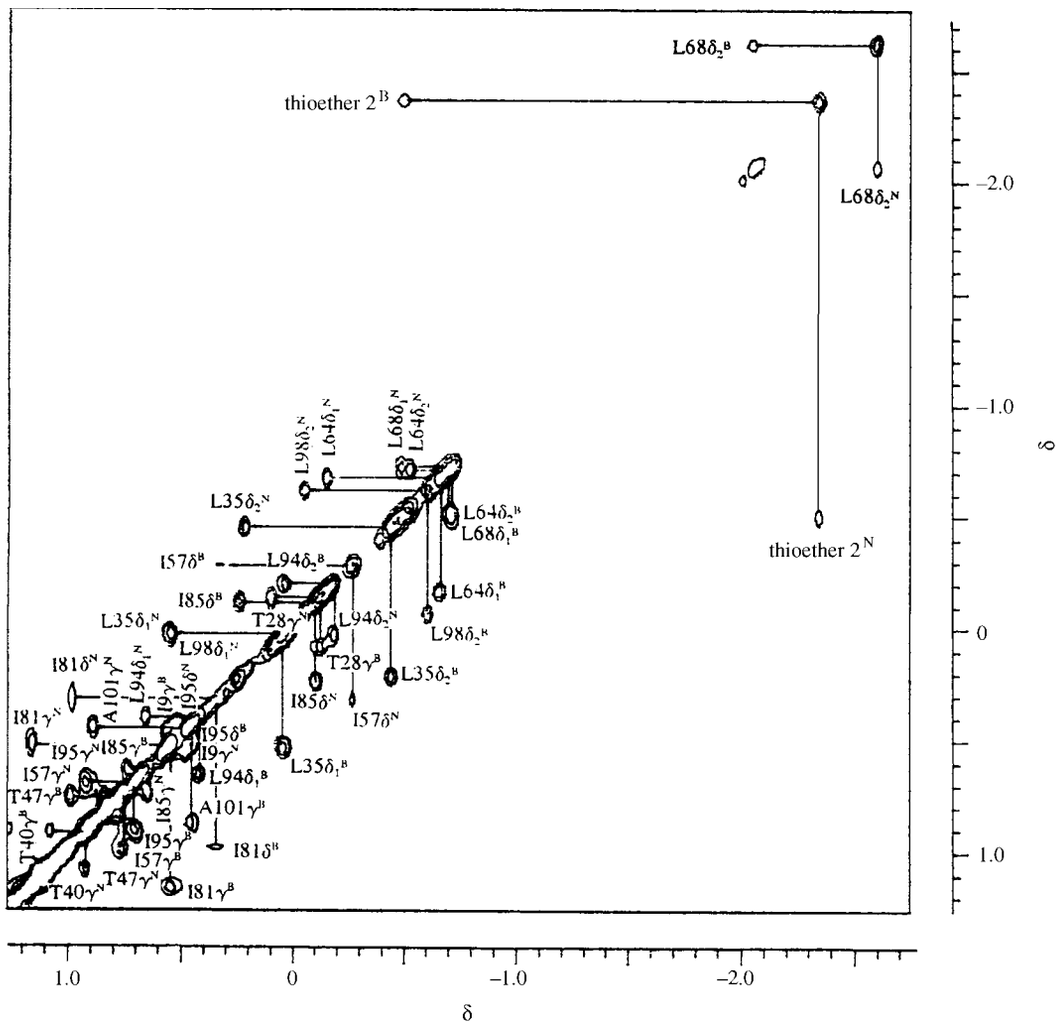

Fig. 6 Aliphatic region of the two-dimensional EXSY spectrum of a mixture of pcyt c and cyt c at pH 5.7 and 298 K with a mixing time of 50 ms. Chemical shifts of the resonance for pcyt c (B) and cyt c (N) are read in the F_2 dimension

larger than 1 ppm when passing from the native to the ligated species. This can be attributed to perturbations in electronic and molecular structure.

To determine the magnitude and the orientation of the principal axes of the *g* tensor in pcyt c, dipolar shift calculations have been performed for the proton resonance of non-co-ordinated residues assigned in this research. As shown in Table 1, the five *g* parameters (α, β, γ, g_{ax} and g_{eq}) are calculated to be 316, 5 and 16°, 5.04 and −2.54 respectively. Some differences are found on comparing the five parameters of pcyt c and those of native cyt c. For cyt c the direction of the *Z* axis is significantly tilted away from the haem normal and falls accurately along the Fe–S bond.[23] For pcyt c the direction of the *Z* axis is tilted by 5° from the perpendicular to the average plane passing through the four haem pyrrole nitrogens, consistent with expectation that the axial ligands are essentially perpendicular to the haem plane. When β is small, $\alpha + \gamma$ approximates the rotation of the orthogonal to the g_{ax} and g_{eq} axes relative to the haem *X* and *Y* axes [Fig. 1(*b*)]. In native cyt c $\alpha + \gamma$ is approximately 360°, which means that the g_x and g_y axes of the *g* tensor in solution match the natural haem reference system.[23] However, $\alpha + \gamma$ is close to 330° in pcyt c, and the direction of the *Y* axis falls along the direction of the haem 8-CH_3 towards Fe. This indicates that the g_x and g_y axes in solution make an angle of −30° with the natural haem reference system in pcyt c. The electronic structure of the haem is affected by the co-ordination of the sixth axial ligand.

The 8-, 5-methyl groups are furthest downfield for pcyt c as are the 8-, 3-methyl groups of cyt c, which reflects different distributions of spin density on the pyrrole rings between cyt c and pcyt c (Table 2). In the oxidized haem the unpaired electron density causes a number of haem-related protons to exhibit large hyperfine shifts, reflecting scalar Fermi-contact interactions due to proton–electron spin-density overlap, in addition to the dipole–dipole pseudo-contact shift. Analysis of the pseudo-contact shift contribution allows the contact shifts of the haem-related protons to be evaluated (Table 2).

The pseudo-contact shifts of haem peripheral protons between native cyt c and pcyt c are somewhat different, moreover their contact shifts (δ_c) are significantly different. The contact shifts around the haem of native cyt c reveal a highly asymmetric distribution of unpaired electron spin, as has been noted before.[23] In cyt c, substitutions on one diagonal (pyrroles II and IV) exhibit much larger downfield (positive) shifts than on the other (pyrroles I and II). Thus large downfield shifts occur at 3-CH_3, 8-CH_3 and the α-H of propionate 7, and lesser, although still large, shifts at 1-, 5-CH_3. For pcyt c the contact shifts of 3-, 8-CH_3, pro 7*α*, α-H and Cys-17 α, β-H decreased, but those of 1-, 5-CH_3 and thiother 2 increased. Thus the contact shifts of substituents on pyrroles II and IV decreased while those of substituents on pyrroles I and III increased. The asymmetric distribution of unpaired electron spin is not as high as in cyt c. It should be pointed out that 8- and 5-CH_3 (pyrroles III and IV) exhibit larger downfield contact shifts than do 1-, 3-CH_3, which leads to the 8-, 5-methyl groups being furthers downfield for pcyt c. In cyt c the sulfur lone-pair orbital of co-ordinated Met-80 points toward pyrrole ring IV as noted previously by Wüthrich. The effect of the lone-pair inter-

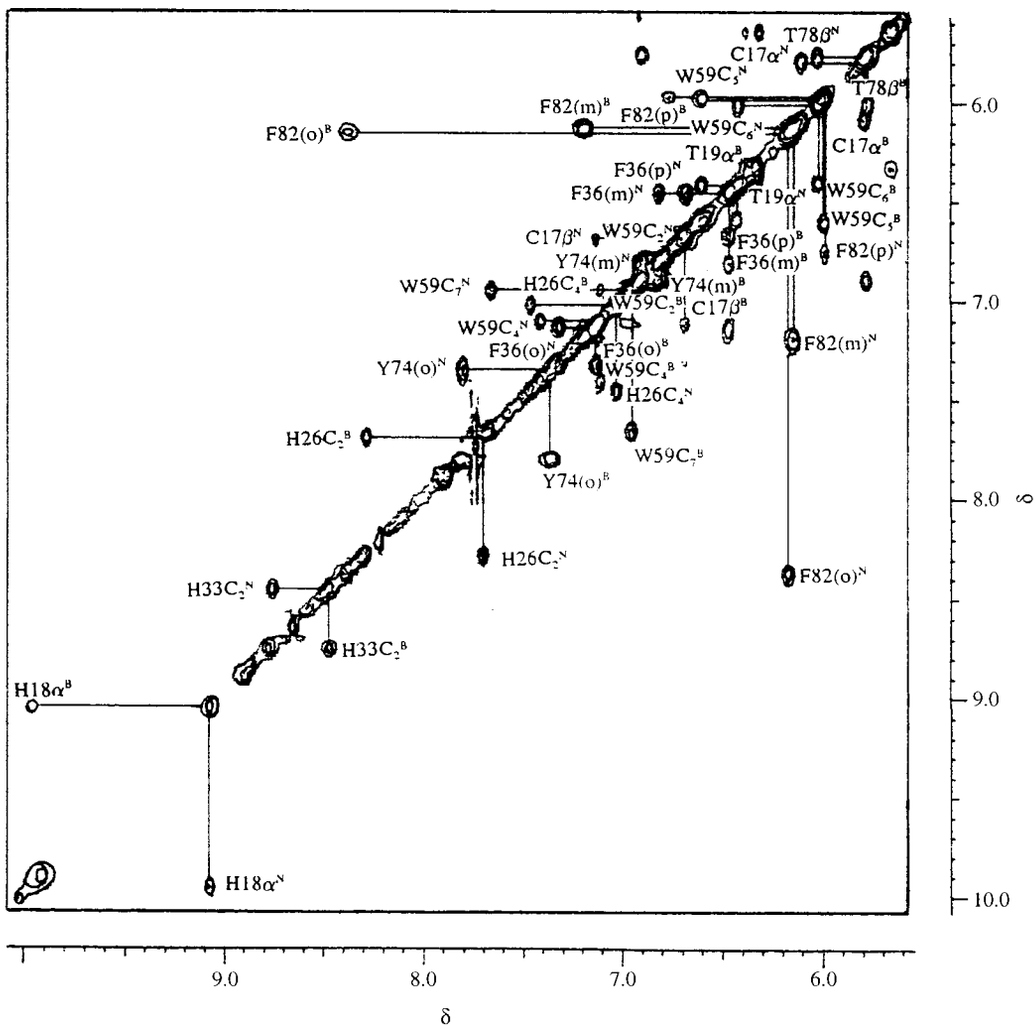

Fig. 7 Aromatic region of the two-dimensional EXSY spectrum of a mixture of pcyt c and cyt c at pH 5.7 and 298 K with a mixing time of 50 ms. Details as in Fig. 6

action with the d_π orbital is to direct the maximum spin density to the 8,3 groups. As we illustrated before,[12] there is no such effect when Met-80 is substituted by pyridine. The thioether 2,4 substituents in cyt c are more electron withdrawing than are the propionic acid chains, which leads to the resonances of the 8,5 groups being shifted furthest downfield.

The divergence $\Sigma [\delta(\mathrm{pc})_{\mathbf{obs}} - \delta(\mathrm{pc})_{\mathbf{cal}}]^2/N$ of pcyt c obtained here is larger than that of native cyt c,[23] significant differences between observed and calculated pseudo-contact shifts being observed for some protons. As mentioned above, during the calculation, the chemical shifts of reduced cyt c were used for those of reduced pcyt c, and the proton coordinates of oxidized cyt c were used for those of oxidized pcyt c. Conformational differences between the reduced and oxidized cyt c produce diamagnetically based chemical shift differences δ(d), so do conformational differences between oxidized cyt c and oxidized pcyt c, δ(py), and between oxidized cyt c in solution and the crystal, δ(x). In addition, protons in oxidized pcyt c in solution are subject to pseudo-contact shifts, δ(pc, s), which vary with their position in space relative to the paramagnetic centre.[23] Thus the observed chemical shift differences can be expressed as in equations (6a)–(6c).

$$\delta(\mathrm{pc})_{\mathbf{obs}} = \delta(\mathrm{d}) + \delta(\mathrm{py}) + \delta(\mathrm{pc, s}) \quad (6a)$$

$$\delta(\mathrm{pc, s}) = \delta(\mathrm{pc})_{\mathbf{calc}} + \delta(\mathrm{py}) + \delta(\mathrm{x}) \quad (6b)$$

$$\delta(\mathrm{obs}) - \delta(\mathrm{pc})_{\mathbf{calc}} = 2\delta(\mathrm{py}) + \delta(\mathrm{d}) + \delta(\mathrm{x}) \quad (6c)$$

The chemical shift difference resulting from pyridine substitution is relatively larger than δ(d) and δ(x) which arise from structural differences among the different forms of native cyt c. Therefore, the significant differences between observed and calculated pseudo-contact shifts can be related to the structure differences between cyt c and pcyt c. Besides, for Met-80, significant differences are found to occur for the side-chain of residues Leu-64, Ile-57, -81, Thr-40, -78, Trp-59, Tyr-74 and Phe-82. This indicates that these side-chains may rearrange after Met-80 is replaced, in agreement with our previous results based on NOE connectivities of imidazole-bound cytochrome c.[24]

Evaluation of the rate constant

Since two-dimensional NMR exchange spectroscopy (EXSY) was proposed by Jeener *et al.*,[32] the study of slow molecular dynamics which is dominated by a number of rate processes such as chemical exchange and molecular relaxation has become well established. The ligation of py can be represented by reaction (7). The magnetization exchange between the

$$\mathrm{py} + \mathrm{cyt\ c} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{py{-}cyt\ c} \quad (7)$$

species is a first-order reaction (8). In our experiments the

$$M_{\mathbf{cyt\ c}} \underset{k_{\mathrm{BA}}}{\overset{k_{\mathrm{AB}}}{\rightleftharpoons}} M_{\mathbf{pcyt\ c}} \quad (8)$$

chemical exchange proceeds in two-site spin systems, and the amplitude data matrix *A* [based on 8-CH_3, Fig. 5(*a*)] was

Table 1 The haem electronic *g*-tensor parameters for oxidized pcyt c with those of cyt c for comparison

	Principal axes			Anisotropy factors		Orientation			
Source	g_x	g_y	g_z	g_{ax}	g_{eq}	α	β	γ	Divergence [d]
cyt c, EPR [b]	1.25	2.25	3.06			≈0	≈0	≈0	
cyt c [c]	2.25	2.59	3.32	5.15	−1.65	106	13	251	0.004
pcyt c, EPR [d]	1.48	3.29	1.99						
pcyt c				5.04	−2.54	316	5	16	0.047

[a] $\Sigma\,[\delta(pc)_{obs} - \delta(pc)_{calc}]^2/N$, where N is the number of groups used in the search for the g tensor. [b] Measured by Mailer and Taylor [22] in single crystals of horse heart cytochrome c at 4.2 K, with β to within ±3°, α and γ to ±5°. [c] From Feng *et al.*[23] for horse heart cytochrome c at 293 K, pH 5.7. [d] From ref. 4.

Table 2 Assignments, calculated pseudo-contact and contact shifts of haem-related protons of pcyt c at pH 5.7 and 298 K. The corresponding shifts for cyt c are shown for comparison

	δ_{obs}		δ_{pc}			δ_c	
Proton	pcyt c	cyt c	pcyt c_{obs} [b]	cyt c [a]	pcyt c_{calc} [b]	cyt c [a]	calc. [b]
8-CH_3	29.61	35.26	−2.7		−4.38	36.4	32.4
3-CH_3	18.43	32.67	−2.4		−1.25	31.6	16.21
5-CH_3	19.70	9.97	−5.2		−6.54	11.3	22.37
1-CH_3	13.96	8.00	−4.3		−6.29	7.6	15.55
pro 7α-H	5.68	18.83	−1.6		−1.47	17.3	4.02
7α′H	8.20	11.64	−1.5		−1.14	8.9	5.10
thioether 2	−0.50	−2.36	−3.7		−2.29	−0.4	0.05
Met-80 ε-CH_3	1.92	−24.23	11.7		−6.2/4.20	−33.3	
β-H	2.06	12.53	16.8/15.9		17.42	0.6/0.5	
His-18 α-H	9.97	9.06	5.1		6.18	0.30	0.14
β-H	14.85	14.49	7.4/8.1		5.65/4.3	5.6/6.3	8.12/9.47
Cys-17 α-H	5.80	6.09	0.9		1.90	1.0	−0.33
β-H	6.67	7.12	0.6/−1.5		2.55	5.0/7.1	2.59

[a] At 293 K and pH 5.7.[23] [b] This work.

obtained [expression (9)]. From equation (4) the dynamic

$$\begin{vmatrix} 0.799 & 0.260 \\ 0.252 & 0.688 \end{vmatrix} \quad (9)$$

matrix was obtained through a simple matrix calculation [expression (10)] from which rate constants $k_{AB} = 14.2\ s^{-1}$ and

$$\begin{vmatrix} 11.4 & -14.6 \\ -14.2 & 17.6 \end{vmatrix} \quad (10)$$

$k_{BA} = -33.5\ s^{-1}$ were calculated. However, the exchange pathway shown in equation (8) does not reflect the reaction equilibrium as shown in (7). The relationships between the magnetization exchange rate constants and the substitution reaction rate constants can be found in equations (11). Thus the reaction

$$k_1 = k_{AB}/[py],\ k_{-1} = k_{BA} \quad (11)$$

rate constants and equilibrium constant are determined as $k_1 = 31.5\ dm^3\ mol^{-1}\ s^{-1}$, $k_{-1} = -14.6\ s^{-1}$ and $K_{app} = 2.16\ dm^3\ mol^{-1}$.

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