DALTON
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Nuclear magnetic resonance spectroscopic studies of pyridine methyl derivatives binding to cytochrome c

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The binding of pyridine methyl derivatives (2-, 3- and 4-methylpyridine) to horse heart ferricytochrome c (cyt c) by displacing methionine-80 was studied by ^{1}H NMR spectroscopy to elucidate the effects of the different methyl substitution positions on the affinity and kinetics of binding to cytochrome c and the hyperfine-shifted NMR signals of the ligand–cytochrome c complex. Two-dimensional exchange spectroscopy (2D-EXSY) showed that except for 2-methylpyridine (2-mpy) these pyridine derivatives can form stable complexes with cytochrome c. The complexes 3-mpy–cyt c and 4-mpy–cyt c exhibit different hyperfine shift patterns compared to that of py–cyt c. The temperature dependence of the methyl resonances of 3-mpy–cyt c differs from those of 4-mpy– and py–cyt c. Kinetic and equilibrium data for the binding of 3- and 4-mpy to cyt c have been obtained by 2D-EXSY. Based on these data a comprehensive comparison between the binding properties of these pyridine derivatives and those of pyridine towards cyt c was made. The ^{1}H NMR resonances of 3-mpy–cyt c have also been assigned including the heme peripheral protons and some aliphatic and aromatic side chain protons.

Ferricytochrome c (cyt c) can bind a wide range of ligands such as cyanide, azide, imidazole (Him) and pyridine (py) which displace Met-80 and ligate to the heme iron.[1–7] Studies of these binding reactions will help obtain insight into the structural aspects of the heme surroundings and the source of the asymmetric electron spin density distribution of cytochrome c.[6,7] Binding of pyridine to cytochrome c has been studied by NMR methods.[6–9] In the present paper we have performed more comprehensive studies of the binding reactions of pyridine derivatives including 2-methyl- (2-mpy), 3-methyl- (3-mpy) and 4-methyl-pyridine (4-mpy) to cyt c employing 2D-EXSY (exchange spectroscopy). We have been investigating the effects of different positions of methyl substitution in pyridine on the affinity and kinetics of binding of pyridine derivatives to cyt c and on the hyperfine-shifted NMR signals for ligand–cytochrome c complexes by comparing the shift patterns and temperature dependences and through the comparisons of kinetic and equilibrium constants obtained by 2D-EXSY. The ^{1}H NMR resonances of 3-mpy–cyt c including those of the heme peripheral protons and some aliphatic and aromatic sidechain protons are further assigned. These protons are sensitive to the electronic state of the heme environment. The present studies thus help reveal the effect of the different methyl substitution positions in pyridine on the heme environment.

Experimental

NMR experiments

Horse heart cytochrome c(VI) was obtained from Sigma Chemical Corp. and purified as previously described.[10] It was dissolved in D_2O and incubated at 60 °C for 3 h in order to exchange all the labile protons, and then lyophilized. 3-Methylpyridine was purified according to Heap *et al.*,[11] 2-methyl- and 4-methyl-pyridine were reagent grade used without further purification. The purity of these pyridine derivatives was checked by ^{1}H NMR spectroscopy. The pH was adjusted by addition of small amounts of DCl or NaOD, with pH values not corrected for the isotope effect.

All NMR data were recorded on a Bruker Am500 spectrometer with an Aspect 3000 computer. All the data treatments were performed on a Silicon Graphics Indy workstation using the X-WINNMR software of Bruker Corp. 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 exchange spectra (2D-EXSY) with a mixing time (τ_m) of 25 or 50 ms were acquired using the phase-sensitive NOESY pulse sequence[12] over a 35 714.29 Hz bandwidth. All spectra were collected using $2048(t_2) \times 512(t_1)$ data points with 160 scans for each t_1 increment. After zero filling, which resulted in equal digital resolution in both dimensions, the time domain matrix was multiplied in both dimensions with the shifted sine bell function.

The integral values of the two-dimensional peaks were obtained by calculating from the spectra using a square frame and then normalized according to $\Sigma I_{ij} = 1$. The same frame was used for estimating 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 also normalized.[13]

Kinetics

For a system involving chemical exchange between two sites it has been shown that the peak amplitude in 2D-EXSY spectra is related to the exchange rate constant k', the relaxation rate and the mixing time τ_m by expression (1)[14] where A and R are given

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

by the matrices (2) and (3). In A the quantities I_{11}, I_{12} are two-

$$A = \begin{vmatrix} I_{11}/M_1 & I_{12}/M_2 \\ I_{21}/M_1 & I_{22}/M_2 \end{vmatrix} \quad (2)$$

$$R = \begin{vmatrix} -R_1 - k'_1 & k'_{-1} \\ k'_1 & -R_2 - k'_{-1} \end{vmatrix} \quad (3)$$

dimensional peak amplitudes measured in an experiment with mixing and normalized, and M_1, M_2 are the equilibrium magnetization values obtained from integration of the one-dimensional spectra and also normalized. The quantity R contains the kinetic parameters to be determined, namely chemical exchange and longitudinal relaxation rates. It can be obtained directly by first diagonalizing A and then calculating the eigenvector matrix X and its inverse X^{-1} so that $XDX^{-1} = A$ where D is the diagonal eigenvalue matrix. The solution to this equation is given[15–17] by (4) where $\ln D = \text{diag}\,(\ln \lambda_i)$. Thus R can be directly calculated from A.

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

In this paper, the binding of mpy to cyt c can be represented by reaction (5). The magnetization exchange between the

$$\text{cyt c} + \text{mpy} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{mpy–cyt c} \quad (5)$$

species is a first-order reaction (6). The relationships between

$$M_{\text{cyt c}} \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} M_{\text{mpy–cyt c}} \quad (6)$$

the magnetization exchange rate constants k' and the reaction exchange rate constants k can be found in (7a) and (7b), and the

$$k_1 = k'_1/[\text{mpy}] \quad (7a)$$

$$k_{-1} = k'_{-1} \quad (7b)$$

apparent equilibrium constants K_{app} and the equilibrium constant K of the reaction can be calculated from equations (8a) and (8b) where k_a is the dissociation constant of mpy. The

$$K_{app} = k_1/k_{-1} \quad (8a)$$

$$K = K_{app}\{1 + ([H^+]/k_a)\} \quad (8b)$$

thermodynamic values $\Delta H°$, $\Delta S°$, and the activation energy of the reaction of cyt c with mpy were obtained according to the Van't Hoff and Arrhenius equations (9) and (10).

$$\ln K = -\frac{\Delta H°}{RT} + \frac{\Delta S°}{R} \quad (9)$$

$$\ln k_1 = -\frac{E_a}{RT} + \ln A \quad (10)$$

Results and Discussion

Affinity of cytochrome c for pyridine methyl derivatives

Fig. 1 presents the hyperfine-shifted ^{1}H NMR spectra of cytochrome c at pH 5.7, 303 K in the presence of 2-, 3- and 4-mpy respectively. It is obvious that the presence of 3- and 4-mpy significantly alters the hyperfine-shifted resonances which indicates the formation of stable complexes with cytochrome c. However, there is little deviation in the spectrum of cyt c after the addition of 1 mol dm^{-3} 2-mpy. In fact, even when the amount of 2-mpy was increased to 2 mol dm^{-3} the spectrum still showed no critical alteration compared with the spectrum of native cyt c under the same conditions. This strongly suggests that 2-mpy cannot bind to cytochrome c which might be due to the 2-methyl group hindering the approach of 2-mpy to the heme plane.

For 3-mpy binding to cyt c at 303 K and pH 5.7 the chemical exchange is a two-site spin system. According to the theory of kinetics studied by means of exchange spectroscopy,[15–17] as shown above the reaction amplitude matrix A based on the integration of 8-CH_3 is as in equation (11). From this, the

$$A = \begin{vmatrix} 0.714 & 0.306 \\ 0.495 & 0.565 \end{vmatrix} \quad (11)$$

kinetic matrix R is calculated, equation (12). Thus the magnetization exchange rate constants $k'_1 = 36.2$ s^{-1} and $k'_{-1} = 22.4$ s^{-1}. According to equations (7a) and (7b) the reaction rate constants $k_1 = 90.5$ dm^3 mol^{-1} s^{-1} and $k_{-1} = 22.4$ s^{-1}. Then the equilibrium constants K_{app} and K are calculated by equations (8) and (9) and listed in Table 1. Employing the same method, the kinetic and equilibrium data for binding of 3- and 4-mpy to cyt c at different temperatures at pH 5.7 were obtained and given in Table 1.

$$R = \begin{vmatrix} -22.1 & 22.4 \\ 36.2 & -33.0 \end{vmatrix} \quad (12)$$

Table 1 Rate and equilibrium constants for binding of 3- and 4-mpy to cyt c at different temperatures

	3-mpy				4-mpy			
T/K	k_1	k_{-1}	K_{app}	K	k_1	k_{-1}	K_{app}	K
293					13.0	15.6	0.828	2.56
296.5					24.9	16.4	1.52	4.68
298	36.0	15.8	2.28	4.45				
300	50.5	17.9	2.82	5.52	54.7	17.4	3.14	9.70
303	90.5	22.4	4.04	7.90				
303.5					143	18.0	7.93	24.5
308	225	34.8	6.47	12.7				
313	494	46.1	10.7	20.9				

The errors of the rate constants are in the range 5–10%. Units are: k_1/dm^3 mol^{-1} s^{-1}, k_{-1}/s^{-1}, K/dm^3 mol^{-1}.

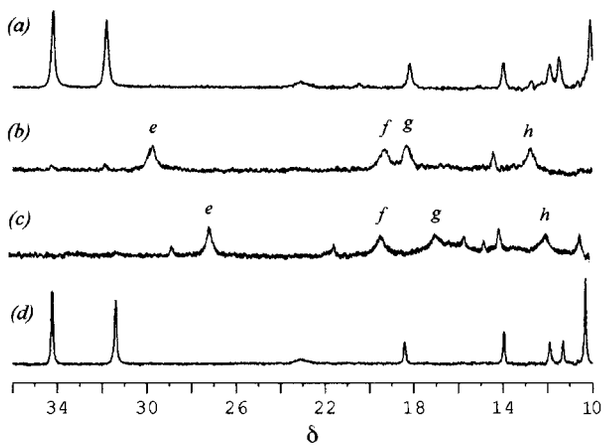

Fig. 1 Downfield hyperfine-shifted region of the ^{1}H NMR spectra in D_2O at 303 K, pH 5.7 of (*a*) cyt c with 1 mol dm^{-3} 2-mpy, (*b*) cyt c with 0.8 mol dm^{-3} 3-mpy–cyt c, (*c*) 0.6 mol dm^{-3} 4-mpy–cyt c and (*d*) native cyt c only; *e*, *f*, *g*, *h* denote 8-, 3-, 5- and 1-CH_3 of 3-mpy–cyt c and 4-mpy–cyt c respectively

From Table 1 it follows that the rate constants for 3- and 4-mpy for the forward and reverse reactions increase with increasing temperature, and so do the equilibrium constants. This is similar to the cases of pyridine binding to cyt c.[8] Comparing the equilibrium constants for binding of pyridine derivatives with that of pyridine to cyt c at the same pH and temperature (see Table 2) we get the following relationship: 4-mpy > 3-mpy > py. This is consistent with their pk_a values: 5.23 (py), 5.68 (3-mpy), 6.03 (4-mpy) respectively.[18]

The $\Delta H°$ and $\Delta S°$ for binding of 3- and 4-mpy to cyt c are

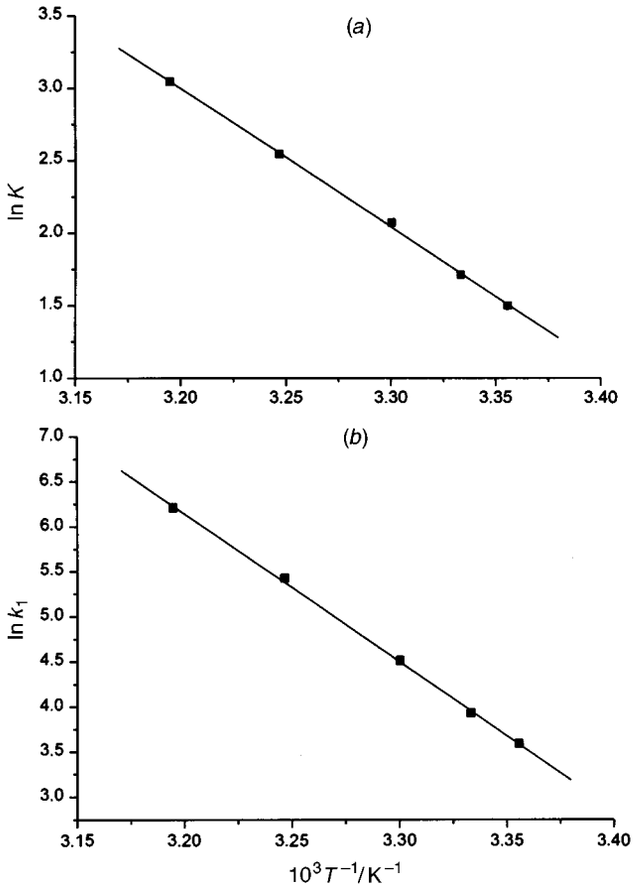

Fig. 2 Plots of ln K (*a*) and ln k_1 (*b*) *versus* $1/T$ in the reaction of 3-mpy with cyt c

obtained from Table 1 by least-squares fitting and listed in Table 2. Fig. 2 depicts plots of ln K and ln k_1 *versus* $1/T$ for the reaction of 3-mpy and cyt c obtained by least-squares fitting. As seen from Table 2, the $\Delta H°$ and $\Delta S°$ for binding of py, 3- and 4-mpy to cyt c are both positive. Although $\Delta H° > 0$ is unfavourable for the binding reactions, the positive $\Delta S°$ overweighs the negative effect of $\Delta H°$. This suggests that the reactions are driven by a favourable entropy change and the affinity of pyridine derivatives for cyt c arises from the positive $\Delta S°$. This is also the case for pyridine binding to cyt c.[8] However the values of $\Delta S°$ for the binding of 3- or 4-mpy to cyt c (272 and 548 J K^{-1} mol^{-1} respectively) are much larger than that for pyridine binding.[8,9] This might be related to the disruption of some secondary structure elements of cyt c after the binding of 3- or 4-mpy. Previous studies had shown that the binding of imidazole to cyt c not only induced the movements of some side-chains of cyt c, but also resulted in the disappearance of the 3_{10} helix (67–70), type II turn (75–78) and the distortion of the 50's helix.[19] The corresponding $\Delta S°$ value for this system was 184 J K^{-1} mol^{-1}. Being bulkier than imidazole, 4- or 3-mpy should result in larger $\Delta S°$ values due to greater steric interactions.

With the assistance of the molecular simulation software INSIGHT II[20] we constructed a model of imidazole, 4- and

Table 2 Thermodynamic parameters of the reactions of cyt c with py, 3- and 4-mpy respectively

Reaction	K/dm^3 mol^{-1}	$\Delta H°$/ kJ mol^{-1}	$\Delta S°$/ J K^{-1} mol^{-1}
py and cyt c[a]	1.40[c]	44	148
3-mpy and cyt c[b]	7.9[c]	77.2	272
4-mpy and cyt c[b]	24.5[d]	158	548

[a] From ref. 8. [b] This work. [c] Obtained at 303 K. [d] Obtained at 303.5 K.

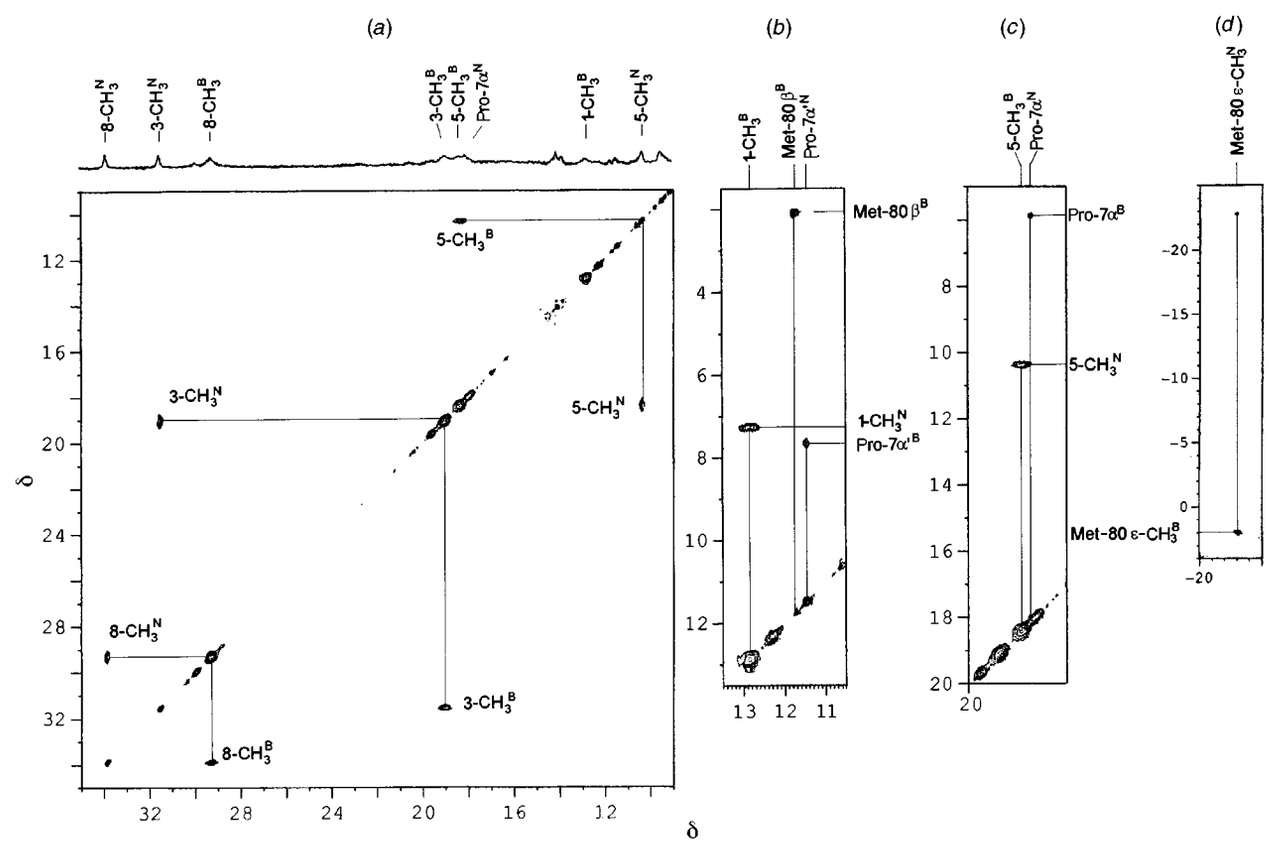

Fig. 3 Portions of the two-dimensional EXSY spectrum of a mixture of 3-mpy–cyt c and cyt c at pH 5.7 and 303 K, mixing time 25 ms. The one-dimensional spectrum is shown at the top. Resonances due to 3-mpy–cyt c and cyt c are labelled with B and N respectively. (*a*)–(*c*) Portions of downfield region, (*d*) upfield region

3-mpy binding to cyt c. It is found that imidazole can fit into the heme pocket more easily than 4- or 3-mpy. Furthermore, when 4-mpy binds to cyt c the 4-methyl group induces severe steric interactions with the peptide near Pro-71 because they are very close to each other. However, in the 3-mpy–cyt c system there is no such phenomenon. The 3-methyl group is located in the heme pocket and not so close to certain residues as that of 4-mpy. As a result, the binding of 4-mpy will induce more secondary structure changes or even gross conformational changes which result in the large ΔS° for this system. As 3-mpy will not induce so large structural changes as 4-mpy, the ΔS° of the 3-mpy–cyt c should be less than that for 4-mpy–cyt c. Further work on these calculations is in progress.

The heme pocket provides a hydrophobic environment for the heme.[21] This would be equivalent to a medium of low relative permittivity which results in a negative ΔH° for the binding reactions of pyridine derivatives to cyt c.[21] On the other hand, conformational changes due to steric interactions of the bound ligand (3- or 4-mpy) with the protein will contribute a positive ΔH°.[22] The contribution to ΔH° of the steric effect of binding of 3- or 4-mpy to cyt c overweighs that of the hydrophobic heme environment, so the total ΔH° is positive, reflecting the net effect of the protein. Being a much bulkier ligand, 3- or 4-mpy would induce greater steric hindrance in the heme pocket and more positive ΔH° than for pyridine are expected. The ΔH° for 4-mpy binding to cyt c is much larger than that for 3-mpy, indicating that substitution at the 4 position of pyridine will induce a greater steric effect than that at the 3 position.

Hyperfine shift pattern and temperature dependence of cytochrome c complexes with pyridine methyl derivatives

The ^{1}H NMR spectra of cyt c, 3-mpy–cyt c and 4-mpy–cyt c are illustrated in Fig. 1. Fig. 3 shows portions of the 2D-EXSY spectrum of a mixture of cyt c and 3-mpy–cyt c at pH 5.7, 303 K. The exchange between the groups of the native species and of the substituted form can be visualized from the cross-peaks. In Fig. 3(*a*) and 3(*b*) four signals at δ 29.37, 19.09, 18.38 and 12.79 show correlation with signals at δ 33.87, 31.53, 10.34 and 7.21, respectively. The chemical shifts of the latter four signals belong to heme 8-, 3-, 5- and 1-methyl groups of cyt c.[23,24] So the observation of the above cross-peaks unequivocally identifies the signals at δ 29.27, 19.05, 18.38 and 12.84 as due to the 8-, 3-, 5- and 1-methyl groups of 3-mpy–cyt c. Employing the same method, these heme methyls of 4-mpy–cyt c are assigned at δ 27.22, 19.89, 16.93 and 11.86 respectively at 303 K, pH 5.7 (figure not shown).

Native cyt c has a pairwise pattern for the heme methyl shifts.[25] The two most-shifted methyl resonances are 8- and 3-CH_3. The shift values for the other two heme methyl signals are δ 10.34 (5-CH_3) and 7.21 (1-CH_3).[23,25] Upon binding of 3- or 4-mpy to cyt c, Met-80 is detached from the iron and the heme methyl shift pattern changes significantly. The heme methyl resonances of 3-mpy–cyt c and 4-mpy–cyt c have a spread of only 16.43 and 15.36 ppm respectively, compared to a spread of 26.66 ppm for cyt c. The most-shifted pair of the heme methyls in cyt c shifts upfield for 3-mpy–cyt c and 4-mpy–cyt c while the other pair of heme methyls shifts downfield. This results in the disappearance of the pairwise pattern which indicates that these complexes have higher symmetry for the electron spin distribution relative to cyt c.

In comparison with py–cyt c, there is some difference between the chemical shift pattern of the heme methyls of 3- or 4-mpy–cyt c and that of py–cyt c.[8,9] The major difference is seen for 3- and 5-CH_3. The complex py–cyt c shows the hyperfine-shifted pattern 8 > 5 > 3 > 1, while for 3-mpy–cyt c and 4-mpy–cyt c the patterns are 8 > 3 > 5 >1. Such shift patterns primarily reflect a redistribution of the delocalized spin density among the four pyrroles. In cyt c the orientation of the axial Met is believed to determine the *x* and *y* magnetic

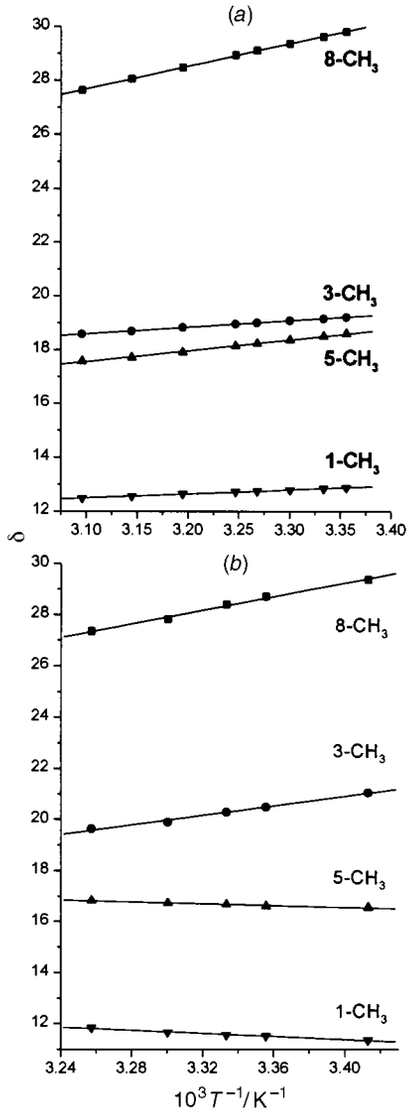

Fig. 4 Curie plots of 3-mpy–cyt c (*a*) and 4-mpy–cyt c (*b*) at pH 5.7

axes.[25,26] It is also indicated that the histidine orientation has a considerable influence on the asymmetry of the heme electronic structure.[27] In the cytochrome c complex with py, 3- and 4-mpy it is the orientation of the axial histidine (His-18) and the corresponding bound ligand that determines the magnetic axes. As a 3- or 4-methyl group has a stronger steric interaction with the surrounding polypeptide near the heme, 3- or 4-mpy might adopt a somewhat different orientation from that of pyridine in py–cyt c that affects the hyperfine shift pattern.

The hyperfine shift patterns of 3-mpy–cyt c and 4-mpy–cyt c are quite similar. Their difference also originates from the heme 3- and 5-CH_3. At 303 K and pH 5.7 the chemical shift difference between 3- and 5-CH_3 in 3-mpy–cyt c is 0.67 ppm while the value in 4-mpy–cyt c is enlarged to 2.96 ppm. This phenomenon reflects the different electronic states of heme induced by pyridine substituted with methyl at different positions.

The temperature dependences of the heme methyl resonances for 3-mpy–cyt c and 4-mpy–cyt c are illustrated in Fig. 4 (Curie plots), from which it can be concluded that all the four heme methyls in 3-mpy–cyt c exhibit no deviation from Curie's law while in the case of 4-mpy–cyt only 8- and 3-CH_3 obey Curie's law, the other methyls (5- and 1-CH_3) exhibiting anti-Curie behaviour. The behaviour of the heme methyls of 4-mpy resembles that of native cyt c. It has been reported that the shifts of heme methyls 1 and 5 of native cyt c have an anti-Curie effect, which increases with increasing temperature, while 3 and 8 exhibit normal Curie behaviour.[28] The temperature depend-

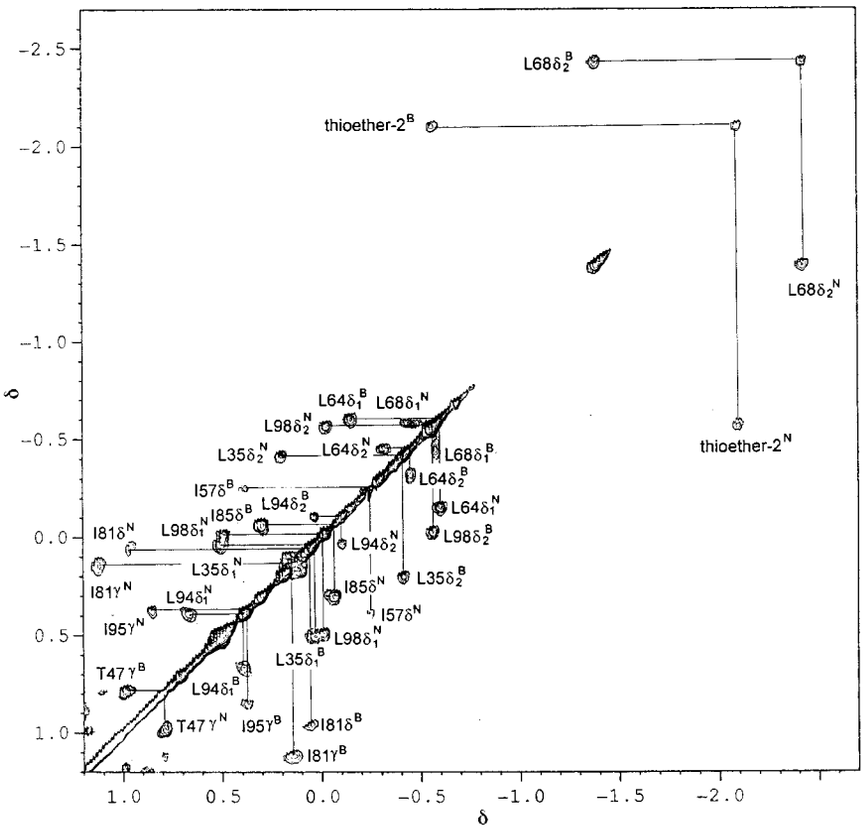

Fig. 5 Aliphatic region of the two-dimensional EXSY spectrum of a mixture of 3-mpy–cyt c and cyt c at pH 5.7 and 303 K, mixing time 50 ms. Resonances due to 3-mpy–cyt c and cyt c are labelled with B and N respectively

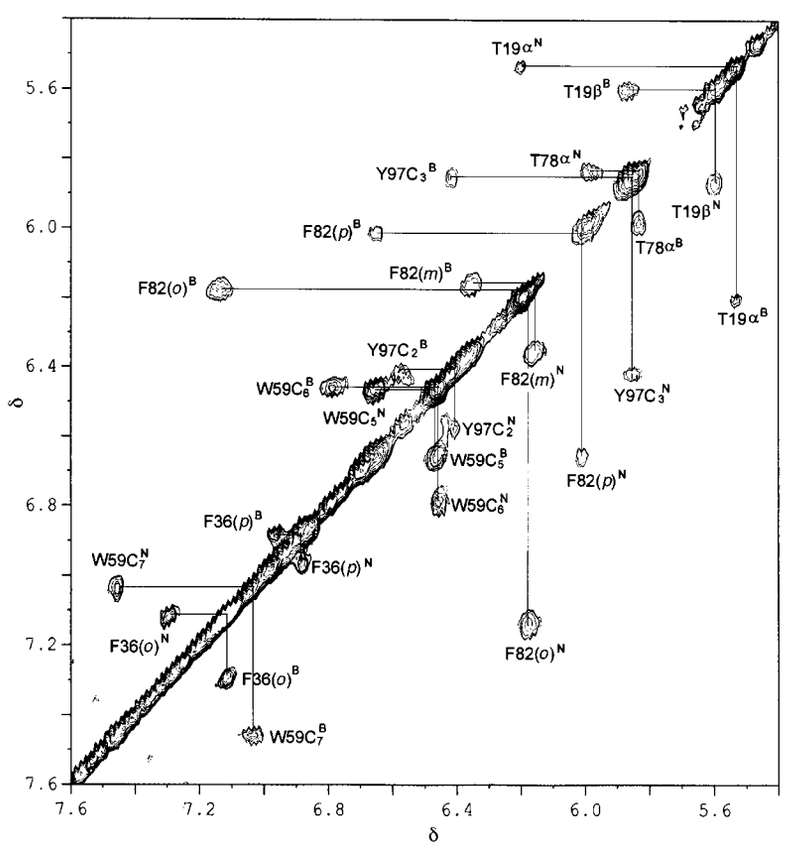

Fig. 6 Aromatic region of the two-dimensional EXSY spectrum of a mixture of 3-mpy–cyt c and cyt c at pH 5.7 and 303 K, mixing time 50 ms. Resonances due to 3-mpy–cyt c and cyt c are labelled with B and N respectively

ence of the hyperfine shifts is related to the energy separation between the ground and the excited levels, which, in turn, is modulated by interactions between the iron and the axial ligands.[25] The anti-Curie effect has been explained by a Boltzmann distribution between partially filled porphyrin 3e(π) molecular orbitals with an energy difference of 3 kJ mol^{-1}.[29]

Table 3 Assigned resonances of 3-mpy–cyt c at 303 K and pH 5.7

	δ	
Assignment	3-mpy–cyt c	cyt c
Heme 8-CH_3	29.27	33.88
3-CH_3	19.05	31.54
5-CH_3	18.38	10.34
1-CH_3	12.84	7.21
pro-7α	5.85	18.11
pro-7α′	7.65	11.45
Met-80 ε-CH_3	1.98	−23.05
Met-80 β-H	2.09	11.74
Leu-68 δ_2	−1.39	−2.43
Leu-68 δ_1	−0.58	−0.44
Thioether-2	−2.10	−0.56
Leu-64 δ_2	−0.32	−0.45
Leu-64 δ_1	−0.61	−0.14
Leu-98 δ_2	−0.55	−0.02
Leu-98 δ_1	−0.01	0.49
Leu-35 δ_2	−0.42	0.20
Leu-35 δ_1	0.03	0.51
Ile-57 δ	0.38	−0.25
Leu-94 δ_2	0.03	−0.11
Leu-94 δ_1	0.39	0.66
Ile-85 δ	0.30	−0.06
Ile-81 δ	0.05	0.97
Ile-81 γ	0.13	1.12
Ile-95 γ	0.36	0.85
Thr-47 γ	0.99	0.79
Thr-19 α	5.52	6.20
Thr-19 β	5.86	5.59
Thr-78 α	5.83	5.98
Phe-82 *p*	6.65	6.00
Phe-82 *m*	6.36	6.97
Phe-82 *o*	7.13	6.17
Tyr-97 C2	6.57	6.40
Tyr-97 C3	6.42	5.84
Trp-59 C5	6.46	6.65
Trp-59 C6	6.78	6.45
Trp-59 C7	7.03	7.45
Phe-36 *p*	6.96	6.77
Phe-36 *o*	7.11	7.28

The different temperature dependence of 4-mpy–cyt c from that of 3-mpy–cyt c also implies that the methyl substitution position in pyridine influences the behaviour of the mpy–cyt c complex.

Assignments of some resonances of 3-mpy–cyt c

Since the chemical shifts of many protons are expected to change in 3-mpy–cyt c and the native form of cyt c, chemical exchange correlation can be used to generate cross-peaks in a two-dimensional spectrum. As the full assignments of cyt c have been reported, exchange effects can be used to determine and establish the reliability of the proton assignments for 3-mpy–cyt c. Fig. 3 presents the hyperfine shift region of the 2D-EXSY spectrum recorded at pH 5.7, 303 K with a mixing time of 25 ms. The propionic acid 7α(pro-7α) and 7α′ protons of native cyt c are found at δ 18.11 and 11.45.[23,30] In Fig. 3(*c*) the pro 7α-H shows an exchange cross-peak at δ 5.85 which can be assigned to pro-7α of 3-mpy–cyt c. Similarly, pro-7α′H of cyt c gives an exchange cross-peak at δ 7.65 which can be identified as due to pro-7α′ of 3-mpy–cyt c [Fig. 3(*b*)].

One of the Met-80 β-H of cyt c had been assigned to the peak at δ 11.74,[31] it shows an exchange peak at δ 2.09, so the signal at δ 2.09 can be assigned as Met-80 β-H of 3-mpy–cyt c [Fig. 3(*b*)]. The Met-80 ε-CH_3 of cyt c is correlated with the signal at δ 1.98[31] [Fig. 3(*d*)]. Thus the latter resonance can be assigned to Met-80 ε-CH_3 of 3-mpy–cyt c. The above data on the exchange peaks of Met-80 indicate that in 3-mpy–cyt c the co-ordination bond Fe–S between Met-80 and heme is broken, and the side chain of Met-80 (ε-CH_3 and β-CH_2) has left its original position in the heme pocket and is situated in the region where the paramagnetic contribution to the resonances from heme iron is minor. This movement causes the chemical shifts of the side chain of Met-80 to be close to those of the side chain of methionine in the random coil.

In the diamagnetic region the 2D-EXSY experiment shows the major advantage of its greater selectivity over one-dimensional double resonance experiments. This is illustrated in Fig. 5 which shows the aliphatic region of the 2D-EXSY spectrum where the cross-peaks arising from chemical exchange between methyl peaks of Leu (L), Ile (I) and Thr (T) are indicated. With the known assignments for cyt c[9,23,24,31,32] the corresponding resonances of 3-mpy–cyt c can be unambiguously assigned from these connectivities. For example, Leu-68 δ_2-CH_3 of cyt c is known to resonate at δ −2.43. In Fig. 5 the exchange cross-peak (δ −1.39, −2.43) indicates the connectivity between the Leu-68 δ_2-CH_3 resonance of cyt c and the corresponding resonance of 3-mpy–cyt c at δ −1.39. Therefore the latter resonance can be assigned to the Leu-68 δ_2-CH_3 protons of 3-mpy–cyt c. This approach accounts for 19 methyl resonances which have been assigned to the specific groups of 3-mpy–cyt c.

The aromatic region of the two-dimensional EXSY spectrum of an equimolar mixture of 3-mpy–cyt c is shown in Fig. 6. The signal at δ 6.17 of cyt c corresponds to the resonance at δ 7.13 of 3-mpy–cyt c. This finding along with the previous assignment for cyt c[9,24,31,32] identifies the latter signal as due to Phe-82 *o*-H of 3-mpy–cyt c. Similarly, cross-peaks which are attributed from chemical exchange allow one to correlate resonances of the ligand-bound and the native species and to identify some aromatic ring protons of 3-mpy–cyt c. The assignments of heme-related protons are listed in Table 3. On the basis of the assignments of 3-mpy–cyt c, extensive resonance assignments can be made quickly from other two-dimensional spectra.

Comparing the chemical shifts of 3-mpy–cyt c with those of cyt c, some of the differences are near or larger than 1 ppm when passing from the native to the ligand-bound species such as Leu-68 δ_2, Phe-82 *o*, Ile-81 γ, *etc.* This can be attributed to perturbations in electronic and molecular structure. In comparison with the 2D-EXSY spectrum of py–cyt c, the two spectra are quite similar although there exist some differences at Leu-64, Phe-36, *etc.* which indicate that 3-mpy–cyt c has a similar ligand-dependent conformation to that of py–cyt c, although its methyl group induces some differences.

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