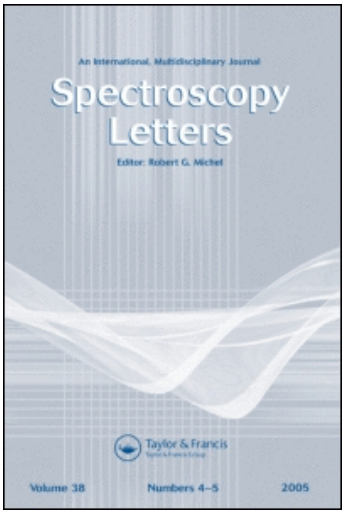

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NMR SPECTROSCOPIC STUDIES OF β-METHYL-PYRIDINE BINDING TO CYTOCHROME C

Key words: **β-methyl-pyridine, cytochrome c, 2D-EXSY, exchange rate constants, equlibrium constants**

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ABSTRACT

The binding of β-methyl-pyridine (β-MePy) to horse heart ferricytochorme c (cyt c) was studied by two-Dimensional exchange spectroscopy. Kinetic and equilibrium data for β-MePy binding to cyt c have been determined. Some hyperfine shifted resonances of β-MePy-cyt c have been assigned. Differences between β-MePy-cyt c and pyridine bound cyt c (py-cyt c) have been disscussed.

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INTRODUCTION

Ferricytochrome c (cyt c) can bind a wide range of ligands such as cyanide(CN^-), azide(N_3^-), imidazole(Im) and pyridine(Py) which displace Met80 and ligate to the heme iron(1-6). The previous work of our lab was involved in the affinity of pyridine and γ-picoline binding to cyt c and the resonances assignment of py-cyt c complex(7-9). However, the orientation and mobility of the bound ligand in the heme pocket and its interaction with the surrounding polypeptide was little covered. In this paper, we use an asymmetric, bulkier ligand, β-methyl-pyridine (β-MePy) to bind to cyt c. The presence of β-methyl will affect the orientation and mobility of β-MePy in heme crevice due to the stronger steric interaction with surrounding polypeptide. The differences in the kinetic data and in the hyperfine shifted pattern between β-MePy-cyt c and py-cyt c complex will reflect some structural information about the heme pocket. In order to elucidate the surroundings of the bound β-MePy and the origin of the asymmetric spin density redistribution among the four pyrroles induced by β-MePy binding to the iron, we have assigned some well resolved hyperfine shifted resonances of β-MePy complex of cytochrome c employing the 2D-EXSY experiment. The kinetic values and equilibrium constants for β-MePy binding to cyt c have been determined and the comparison between β-MePy-cyt c complex and py-cyt c complex also made.

EXPERIMENTAL

NMR experiments. Horse heart cytochrome c(VI) was obtained from Sigma Chemical Corp. and purified as previously described(10). Cyt c was dissolved in D_2O and incubated at 60°C for 3 hrs in order to exchange all the labile protons, and then lyophilized. β-methyl-pyridine was purified according to Heap et al.(11). The purity of these pyridine derivatives was checked by ^1H NMR. 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 the Silicon Graphics Indy workstation

using the X-WINNMR software of Bruker Corp. Chemical shifts were calibrated with respect to 1,4-dioxane at 3.743ppm. One-dimensional NMR spectra were obtained using a presaturation pulse for elimination of the residual water resonance. Two-dimensional exchange spectra(2D-EXSY) with the mixing time (τ_m) of 25ms were acquired using the phase sensitive NOESY pulse sequence(12) over a 35714.29Hz bandwidth. All two dimensional spectra were collected 2048(t_2)*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 $\sum I_{ij}=1$. 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 the system involving the chemical exchange between 2 sites, it has been shown that the peak amplitude in 2D-EXSY spectra was related to the exchange rate constant k', the relaxation rate and the mixing time τ_m by the expression(i)(14):

$$A=\exp(-R\,\tau_m), \qquad \text{(i)}$$

where A and R are given by

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

And

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

In A, the quantities I_{11}, I_{12} are two 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. R contains the kinetic parameters to be determined, namely, chemical exchange and longitudinal relaxation rates. R

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 the above equation is given by(15-17)

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

where $\ln D$ = diag ($\ln \lambda_i$). Thus R can be directly calculated from A.

In this paper, the binding of β-MePy to cyt c can be represented by reaction (1):

$$\text{Cyt c} + \beta\text{-MePy} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \beta\text{-MePy-cyt c} \qquad (1)$$

The magnetization exchange between the species is a first-order reaction:

$$M_{\text{cyt c}} \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} M_{\beta\text{-MePy-cyt c}} \qquad (2)$$

The relationships between the magnetization exchange rate constants k' and the reaction exchange rate constants k can be found in equation(3):

$k_1 = k'_1/[\beta\text{-MePy}]$, $k_{-1} = k'_{-1}$. (3)

And the apparent equilibrium constants K_{app} and the equilibrium constant K of the reaction are calculated from the following equations:

$$K = K_{app} \cdot \left[1 + \frac{[H^+]}{k_a}\right]$$
$$K_{app} = k_1 / k_{-1}. \qquad (4)$$

The thermodynamic values $\Delta H°$, $\Delta S°$, and the active energy of the reaction for cyt c with β-MePy were obtained according to the Van't Hoff and Arrhenius' equation:

$$\ln K = -\frac{\Delta H^o}{RT} + \frac{\Delta S^o}{R}$$

$$\ln k_1 = -\frac{E_a}{RT} + \ln A \qquad (5)$$

RESULTS AND DISCUSSION

1. Kinetics and affinity of cytochrome c for β-MePy

For β-MePy binding to cyt c at 303K, the chemical exchange is in two sites spin systems. According to the theory of kinetics by means of exchange spectroscopy(15-17) as shown above, the reaction amplitude matrix A is as follows:

$$A = \begin{vmatrix} 0.714 & 0.306 \\ 0.495 & 0.565 \end{vmatrix}$$

From the amplitude matrix A, the kinetic matrix R is calculated:

$$R = \begin{vmatrix} -22.1 & 22.4 \\ 36.2 & -33.0 \end{vmatrix}$$

Thus the magnetization exchange rate constants k'_1=36.2, and k'_{-1}=22.4. According to Eqn. 3, the reaction rate constants k_1=90.5 $s^{-1}\cdot mol^{-1}\cdot dm^3$, and k_{-1}=22.4 s^{-1}. Then the equilibrium constants K_{app} and K are calculated by Eqn.4 and 5 and listed in TABLE 1. Employing the same method, the rate constants for the forward and reverse reactions and the equilibrium constants at different temperatures at pH5.7 are obtained and given in TABLE 1.

From TABLE 1, it follows that the rate constants for the forward and reverse reactions increase with the increase of temperature, and so do the equilibrium constants. This is similar to the cases of pyridine binding to cyt c (8). The reason might be that increasing the temperature results in a more open heme crevice of cyt c, thereby enhancing the binding of exogenous ligands.

Comparing the equilibrium constants of β-MePy binding to cyt c with those of other ligands binding to cyt c at the same pH and temperature, the equilibrium constants of β-MePy binding to cyt c are greater than those of pyridine binding to cyt c(8). The reason of this phenomenon might be that the pK_a of β-MePy (5.68) is larger than pyridine(5.17).

TABLE 1. Rate and equilibrium constants of β-MePy binding to cyt c at different temperatures

T(K)	k_1	k_{-1}	K_{app}	K
298	36.0	15.8	2.28	4.45
300	50.5	17.9	2.82	5.52
303	90.5	22.4	4.04	7.90
306	147	27.4	5.37	10.5
308	225	34.8	6.47	12.7
313	494	46.1	10.7	20.9
318	987	61.1	16.0	31.3

FIG.1 presents the plots of lnK and lnk_1 versus 1/T. The thermodynamic values and the activation energy of the reaction can be obtained from TABLE 1 by least-square fitting and shown as follows:

$$\Delta H^\circ = 77.2 \text{ kJ·mol}^{-1}$$

$$\Delta S^\circ = 272 \text{ J·mol}^{-1}\text{·K}^{-1}$$

$$E_a = 132 \text{ kJ·mol}^{-1}$$

It is well known that the binding of ligand to cyt c needs a negative free energy change. In this case, the value ΔH° is quite positive, thus the reaction of cyt c with β-MePy is driven by a favorable entropy change in such a reaction condition. This is also the case of pyridine binding to cyt c(8), but different from the case of cyanide binding to myoglobin, where ΔS° is negative(18). The value of ΔS° in this reaction is much larger that of pyridine binding to cyt c(148 $\text{J·mol}^{-1}\text{·K}^{-1}$)(8).

Heme pocket provides a hydrophobic environment for the heme. The hydrophobic heme environment in the protein would be equivalent to a medium of low dielectric constant, which results in a negative ΔH° (19). On the other hand, conformational changes due to steric interaction of the bound ligand with the protein will contribute to a positive ΔH°. Being bulkier than pyridine, β-MePy would induce stronger steric hindrance in the heme pocket. Thus a more positive ΔH° is expected as shown in our results.

The activation energy for the forward reaction(E_a=132 KJ·mol^{-1}) also increases as compared

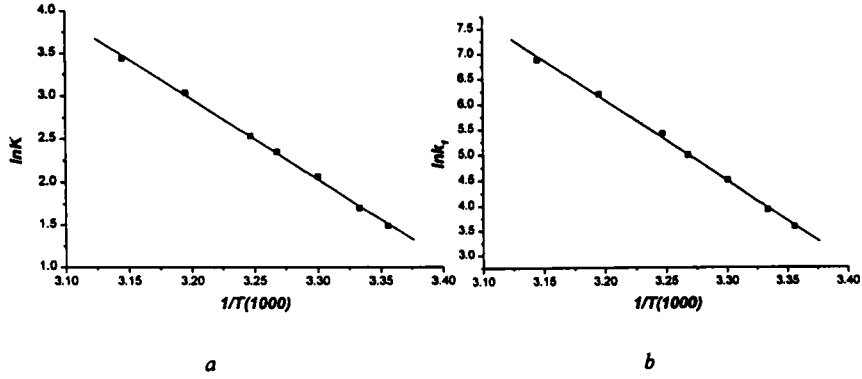

FIG.1 The plots of lnK (*a*) and lnk_f (*b*) versus 1/T in the reaction of β-MePy binding to cyt c.

to pyridine binding to cyt c (E_a=102 KJ·mol^{-1}). According to the solution structure of cyt c(20), the heme group is almost completely buried. Only about 15% of the potential accessible surface area of the heme is accessible to solvent. This indicates that cyt c has a close heme crevice, which represents a considerable barrier to the attacking ligand. It is reported that in the time range between 10^{-3} and 1 s large adjustments are possible in the ferricytochrome c so that the iron becomes open to substitution reactions by reagents such as cyanide and imidazole etc(21). It follows that the bulkier the ligand, the more difficult it could be for the ligand to ligate with heme iron. Thus under our circumstance, the binding of the β-MePy to cyt c needs more activation energy. Our result is close to the case of 1-methyl-imiazole binding to cyt c(22), where the activation energy is 142 KJ·mol^{-1}.

2. Proton Resonance Assignments of β-MePy-cyt c

The ^{1}H NMR spectra of cyt c, and β-MePy-cyt c are illustrated in FIG. 2. The exchange between the groups of native species and the substituted form can be visualized from the cross peaks in FIG.3, which represents portions of the 2D EXSY spectrum of a mixture of cyt c and

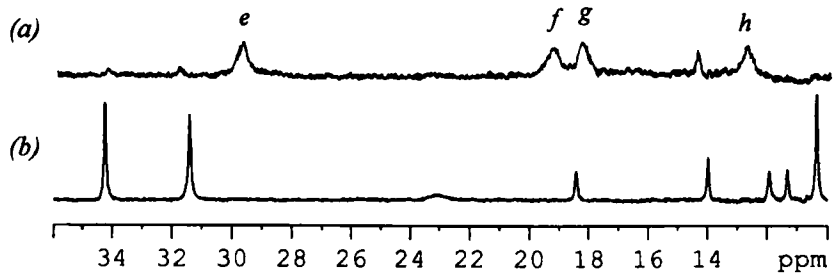

FIG.2 Downfield hyperfine shifted region of the ^1H NMR spectra in D_2O at 303K, pH5.7 of (*a*) cyt c with 0.8 mol dm^{-3} β-MePy-cyt c, (*b*) native cyt c only. In FIG.2, *e, f, g, h* denotes 8-CH_3, 3-CH_3, 5-CH_3 and 1-CH_3 of β-MePy-cyt c respectively.

β-MePy-cyt c at pH5.7, 303K. In FIG.3(a-b), four signals at 29.37ppm, 19.09ppm, 18.38ppm and 12.79ppm show correlation with signals at 33.87ppm, 31.53ppm, 10.34ppm and 7.21ppm, respectively. The chemical shifts of the latter four signals belong to the resonances of heme 8, 3, 5 and 1 methyl groups of cyt c(23-24). So the observation of the above across peaks unequivocally identifies signals at 29.27, 19.05, 18.38 and 12.84ppm as 8, 3, 5 and 1 methyl groups of β-MePy-cyt c. The propionic acid 7α(pro 7α) and 7α' protons of native cyt c are known at 18.11ppm and 11.45ppm. In FIG. 3 (c), the pro-7αH shows the exchange cross-peak at 5.85ppm which can be assigned to pro-7α of β-MePy-cyt c. Similarly, pro-7α'H of cyt c gives exchange cross-peak at 7.65ppm which can be identified to pro-7α' of β-MePy-cyt c.(FIG.3(b))

One of the Met80 βH of cyt c had been assigned to the peak at 11.74ppm(24), it show a exchange peak at 2.09ppm, so the signal at 2.09 can be assigned as Met80 βH of β-MePy-cyt c(FIG. 3b). The Met80 εCH_3 of cyt c shows correlation with the signal at 1.98 ppm (24) (FIG. 3d). Thus the latter resonance can be assigned to Met80 εCH_3 of β-MePy-cyt c . The above data about the exchange peaks of Met80 indicate that in β-MePy-cyt c, the coordination bond of Fe-S between Met80 and Heme is broken, and the side chain of Met80(εCH_3 and β-CH_2) has left their original positions in heme pocket and is situated in the region where the

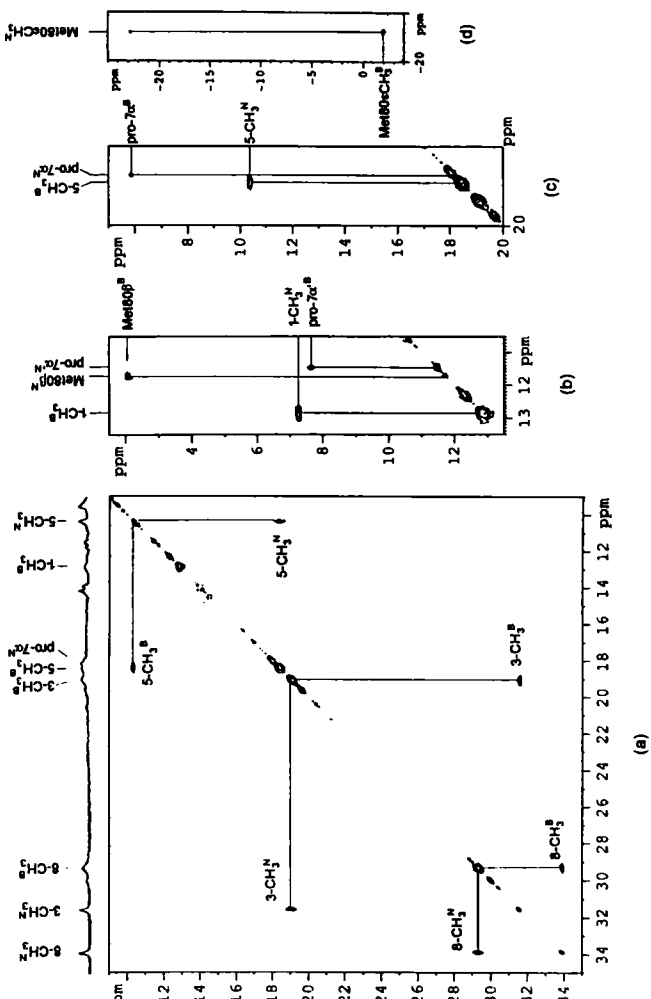

FIG.3 Portions of the two-dimensional EXSY spectrum of a mixture of β-MePy-cyt c and cyt c at pH5.7 and 303K with the mixing time of 25ms. One-dimensional spectrum is shown at the top. Resonances due to β-MePy-cyt c and cytc are labelled with B and N respectively. (*a* - *c*) portions of downfield region, (*d*) upfield region.

paramagnetic contribution to the resonances from heme iron is minor. This movement causes the chemical shifts of the side chain of Met80 to be close to those of the side chain of methionion in random peptide.

The assigned resonances of β-MePy-cyt c at pH5.7, 303K are shown in TABLE 2. The native cyt c has a pairwise pattern for the heme methyl shifts(25,28). The two most-shifted methyl resonances are 8-CH_3 and 3-CH_3. The shift values for the other two heme methyl signals are 10.34(5-CH_3) and 7.21(1-CH_3) (25). Upon β-MePy binding to cyt c, Met80 is detached from the iron and the heme methyl shift pattern changes significantly. The heme methyl resonances have a spread of only 16.43ppm, compared to a spread of 26.66ppm of cyt c. However, the most-shifted pair of the heme methyls in cyt c shifts upfield in β-MePy-cyt c while the other pair of heme methyls shifts downfield. This results in the disappearance of the pairwise pattern which indicates that β-MePy-cyt c has a higher symmetry for the electron spin distribution relative to cyt c.

In comparison with py-cyt c, the chemical shifts of heme methyls of β-MePy-cyt c are quite similar to those of py-cytc(8-9). The only difference in the heme methyl shift pattern is seen for 3-CH_3 and 5-CH_3. py-cyt c shows the hyperfine shifted pattern of 8> 5> 3> 1, while in β-MePy-cyt c, the pattern is 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 axes(26). It is also indicated that the histidine orientation has a considerable influence on the asymmetry of the heme electronic structure(27). In py-cyt c and β-MePy-cyt c, Met80 is detached from the heme iron and pyridine or β-MePy ligated to the iron. Thus for py-cyt c, it is the orientation of the axial histidine(His18) and the bound pyridine that determine the magnetic axes, while in the case of β-MePy-cyt c, the orientation of His18 and the bound β-MePy determine the magnetic axes. As β-Methyl has a stronger steric interaction with the surrounding polypeptide near the heme, the β-MePy might adopt a somewhat different orientation from that of pyridine in py-cyt c which affects the hyperfine shifted pattern.

TABLE 2. The Assigned Resonances of β-MePy-cyt c at 303K and pH5.7

Assignments	β-MePy-cyt c δ(ppm)	Cyt c δ(ppm)
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	7.24
pro-7α	5.85	18.11
pro-7α'	7.65	11.45
Met80 εCH_3	1.98	-23.05
Met80 βH	2.09	11.74

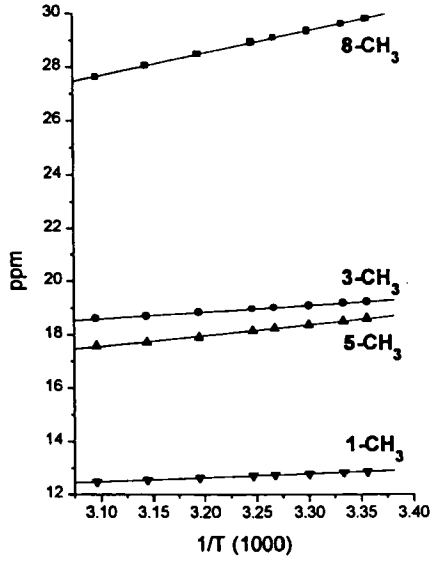

FIG. 4 The curie plot of β-MePy-cyt c at pH5.7.

The temperature dependence of the heme methyl resonances for β-MePy-cyt c is displayed in FIG.4 (the curie plot), from which it can be drawn that all the four heme methyls in β-MePy-cyt c exhibit normal Curie behavior. But for native cyt c, it has been reported that the shifts of heme methyls 1 and 5 have anti-Curie effect, which increases with the increase of temperature, while methyls 3 and 8 exhibit normal Curie behavior(28).The temperature dependence of the hyperfine shifts is related to the energy separation between the ground and the excited levels, which, in turn, is modulated by interactions betweeen 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 3kJ/mol(28).

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