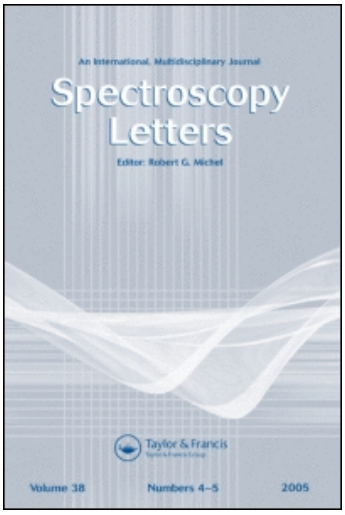

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2D EXSY STUDIES OF γ-PICOLINE BINDING TO CYTOCHROME *c*

Key Words: Cytochrome *c*, γ-Picoline, Thermodynamic parameter, NMR, 2D EXSY spectrum

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ABSTRACT

The binding of γ-picoline to horse heart ferricytochrome *c*(cyt *c*) by displacing methionine-80 has been studied using two dimensional exchange spectroscopy (EXSY) method. Some hyperfine shifted resonances arising from heme peripheral protons in the γ-picoline complex of cyt *c*(γ-MePy·cyt *c*) have been assigned. The rate and equilibrium constants of γ-picoline binding to cyt *c* have been measured and the thermodynamic values of ΔH°, ΔS° and the activation energy E_a for the forward reaction have been obtained. The patterns of the hyperfine shifts of γ-MePy·cyt *c* have been illustrated and the reason for the low affinity of γ-picoline to cyt *c* has been discussed.

INTRODUCTION

The biological function of cytochrome *c*(cyt *c*) is intimately linked with the heme electronic structure in the protein[1-2]. In the native protein molecule, the low-spin Fe(III) lies in the plane of

the porphyrin ring with the imidazole group of His18 and the sulfur atom of Met80 as two axial ligands. It has been shown by optical and NMR methods that Met80 dissociates more easily and can be displaced by intrinsic or extrinsic ligands when pH>9 or when extrinsic ligands are added[3-10]. The ligation of cyt *c* by cyanide(CN^-), imidazole(Im) and pyridine(Py) has been the subject of many NMR studies[3,6-10]. However, NMR studies about the ligation of cyt c by γ-picoline have scarcely been reported[3].

In this paper, two-dimensional exchange spectroscopy (2D EXSY) method has been used to study the kinetics and thermodynamics of γ-picoline binding to cyt *c* and to assign some hyperfine-shifted resonances in γ-picoline complex of cyt *c*(γ-MePy·cyt *c*). The patterns of the hyperfine shifts of γ-MePy·cyt *c* have been interpreted based on the heme electronic structural change. The thermodynamic values of $\Delta H°$, $\Delta S°$ and the activation energy E_a for the forward reaction have been obtained and elucidated in structural terms.

EXPERIMENTAL SECTION

Horse heart cytochrome *c*(VI) from Sigma Chemical Co. was purified as previously described[11]. After purification, it was dissolved in D_2O and incubated at 50°C for 5 hours in order to exchange all the labile protons, then lyophilized. γ-Picoline was chemically pure and was redistilled before used. Its purity was checked by ^1H NMR spectrum. The NMR sample consisted of 6mM cyt *c* and 0.42M γ-picoline(γ-MePy), which was prepared in D_2O and adjusted to pH5.7 by adding small amounts of DCl or NaOD. The pH readings were uncorrected for the isotope effect.

^1H NMR data were recorded on a Bruker AM 500 spectrometer equipped with an Aspect 3000 computer system and were processed using UXNMR software of Bruker spectrometer. One-dimensional spectra were obtained using 16K data points over a sweep width of 35.7kHz. Two-dimentional EXSY spectra were carried out using the phase-sensitive nuclear Overhauser effect spectroscopy(NOESY) pulse sequence[12] over a 35.7kHz bandwidth. Mixing time(τ_m) of 25ms was used. All two-dimensional spectra were collected with 2048(t_2)×512(t_1) data points and 160 scans for each t1 increment. After zero-filling, resulting in equal digital resolution in both

dimentions, the time-domain matrix was multiplied in both dimensions with a shifted sine bell function. The carrier was centered on the residual water peak which was suppressed by presaturation during the relaxation delay in both one- and two-dimentional experiments. Chemical shift values for all the resonances are referenced to internal 1,4-dioxane at 3.743 ppm.

Kinetic and equilibrium data for the binding of γ-picoline to cyt *c* were determined at a temperature range of 293-303.5K. The integral values of the two dimentional 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 dimentional integration and the I_{ij} data were corrected before normalization. The thermal equilibrium values of the magnetization were obtained from integration of the one-dimentional spectra and were also normalized[13].

RESULTS AND DISCUSSION

1. Some Hyperfine Shifted Resonance Assignments

2D EXSY method can be used to make unambiguous assignment for γ-MePy·cyt *c* in conjunction with the known assignments for native cyt *c*[14-16]. In the downfield and upfield regions of the EXSY spectrum, some resonances of cyt *c* and γ-MePy·cyt *c* are well resolved due to the large scalar interaction.

The EXSY spectrum features several peaks in the hyperfine-shifted region due to the effects of the paramagnetic ferric ion(Fig.1a). Four signals at 28.60ppm, 20.50ppm, 16.51ppm, 11.47ppm show connectivities with signals at 34.98ppm, 32.63ppm, 9.83ppm, 6.88ppm respectively. The chemical shifts of the latter four signals suggest that they belong to 8-CH_3,3-CH_3,5-CH_3,1-CH_3 of cyt *c*[14-15], so the observation of the above cross peaks unequivocally identifies signals at 28.60ppm, 20.50ppm, 16.51ppm, 11.47ppm as the heme methyl groups of γ-MePy·cyt *c*, 8-CH_3, 3-CH_3, 5-CH_3, 1-CH_3 respectively. In the upfield region of EXSY spectrum (Fig.1b),the signal at -24.07ppm was assigned to Met80ϵH_3 of cyt *c*[15-16]. It shows correlation with the resonance at 1.82ppm, which is identified as Met80ϵH_3 of γ-MePy·cyt *c*. In Fig.1a, the cross-peaks(14.31ppm, 14.90ppm) is due to the chemical exchange of His18βH between cyt *c* and γ-MePy·cyt *c*. As the

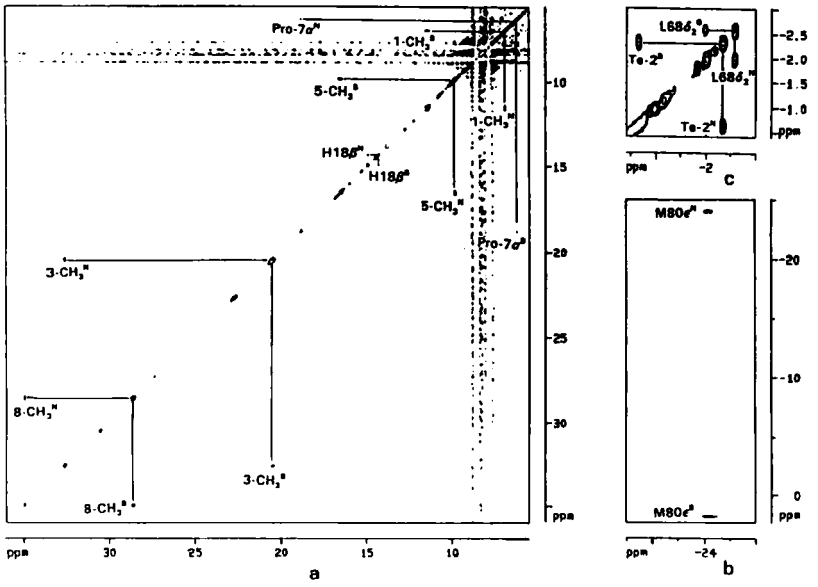

FIG.1. 2D EXSY spectrum of the mixture containing comparable amounts of cyt *c* and γ-MePy·cyt *c* ($C_{cyt\,c}$=6mM, $C_{\gamma\text{-picoline}}$=0.42M) at 298K and pH5.7 with mixing time 25ms. Chemical shifts in (a) and (c) are read in F_2 dimension while those in (b) are read in F_1 dimension. N indicates cyt *c*, B indicates γ-MePy·cyt *c*. (a)downfield region; (b)upfield region; (c)a region with Thioether-2 and Leu68δ_2H_3 indicated.

signal at 14.31ppm is assigned to His18βH of cyt c^{15-16}, the signal at 14.90 is assigned to His18βH of γ-MePy·cyt *c*. The resonance of propionic acid-7 αH (Pro-7αH) of cyt *c* occurs at 18.79ppm[15]. In Fig.1a, it shows a exchange cross-peak at 6.21ppm, which can be assigned to Pro-7αH of γ-MePy·cyt *c*. In Fig.1c, two signals at −0.66ppm and −2.01ppm correspond to the signals at −2.37ppm and −2.61ppm, respectively. The latter two signals belong to the resonances of Thioether-2 methyl protons(Te-2) and Leu68δ_2H_3 protons in cyt c^{14-16}, so the signals at −0.66ppm and −2.01ppm are assigned to the corresponding resonances in γ-MePy·cyt *c*. The results of the assignment are listed in Table 1.

TABLE 1

Hyperfine Shifted Resonance Assignments of γ-MePy·cyt *c* at 298K and pH5.7

	Chemical shifts[a]	
Assignment	γ-MePy·cyt *c*	cyt *c*
8-CH_3	28.60	34.98
3-CH_3	20.50	32.63
5-CH_3	16.51	9.83
1-CH_3	11.47	6.88
Pro-7α	6.21	18.79
Te-2	-0.66	-2.37
His18βH	14.90	14.31
Met80εH_3	1.82	-24.07
Leu68δ_2H_3	-2.01	-2.61

a Shifts of cyt *c* are also shown for comparison.

The data indicate that Met80εH_3 gives rise to a random coil ^1H chemical shift, which reflects that the Fe-S bond between heme iron and sulfur of Met80 is broken[17] and the Met80εH_3 group has moved into a position where the paramagnetic contribution to their resonances from heme iron is absent. It was thought that the electronic heme structure in ferricytochrome *c* was largely influenced by the mode of attachment of the axial methionine[18]. In ferricytochrome *c*, a lone-pair orbital of sulfur atom of Met-80 is directed towards pyrrole ring IV as previously noted[18]. The effect of the lone-pair interaction with the dπ orbital is that the large spin densities are localized on the porphyrin ring carbon atoms 3 and 8, whereas much smaller spin densities are on the ring carbons 1 and 5[14]. In γ-MePy·cyt *c*, there is no such effect and the spin density distribution changes a great deal due to the substitution of Met80 by γ-picoline, which is confirmed by the resonance assignments in Table 1. In γ-MePy·cyt *c*, the shift sequence for the resonances of the four heme methyl groups does not change compared with in cyt *c*. However, the resonances of heme 8-CH_3, 3-CH_3 move towards upfield while 5-CH_3, 1-CH_3 move towards downfield compared with those of cyt

c. The shift spread of the four heme methyl groups in γ-MePy·cyt *c* is 17.13ppm, which is much narrowed compared with that of 28.10ppm in cyt *c*. These changes imply that the electron density distribution in the heme plane has been altered after methionine was displaced by γ-picoline as the axial ligand coordinated to the heme iron and reflect that the asymmetric distribution of unpaired electron spin density in γ-MePy·cyt *c* is not so high as in cyt *c*.

2. Affinity of γ-Picoline to Cyt *c*

The ligation of γ-picoline to cyt *c* can be represented by the chemical equation as follows:

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

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

$$M_{cyt\ c} \underset{k_{BA}}{\overset{k_{AB}}{\rightleftharpoons}} M_{\gamma\text{-}MePy\ cyt\ c} \qquad (2)$$

For a system involving chemical exchange between N sites, it has been shown that peak amplitude in two-dimentional EXSY spectrum is related to the exchange rate constants, the relaxation rate and the mixing time τ_m by the expression(3)[19-20]:

$$A = \exp(-R\tau_m) \qquad (3)$$

where A is the amplitude matrix whose elements are defined by equation(4):

$$A_{ij} = \frac{I_{ij}}{M_j^0} \qquad (4)$$

and R is the dynamic matrix which contains the parameters to be determined.

$$R = \begin{bmatrix} \rho_A & -k_{BA} \\ -k_{AB} & \rho_B \end{bmatrix} \qquad (5)$$

From the solution of Eq.(5), we can calculate the magnetization exchange rate constants[20]. The substitution reaction rate constants k_1, k_{-1} and the equilibrium constant K are calculated from the following equations:

$$k_1 = \frac{k_{AB}}{[\gamma\text{-}MePy]} \tag{6}$$

$$k_{-1} = k_{BA} \tag{7}$$

$$K_{app} = \frac{k_1}{k_{-1}} \tag{8}$$

$$K = K_{app}(1+\frac{[H^+]}{k_a}) \tag{9}$$

where K_{app} is the apparent equilibrium constant of the reaction, and ka is the dissociation constant of γ-picoline.

In our experiments, based on the heme 8-CH_3 groups, we obtained the following amplitude data from the 2D EXSY spectrum recorded at 300K:

$$A = \begin{bmatrix} 0.675 & 0.276 \\ 0.363 & 0.695 \end{bmatrix}$$

from which the kinetics matrix is calculated to be as follows:

$$R = \begin{bmatrix} 20.6 & -17.4 \\ -23.0 & 19.3 \end{bmatrix}$$

the substitution reaction rate constants and the equilibrium constant are calculated to be

$$k_1 = 54.7s^{-1}M^{-1}, \quad k_{-1} = 17.4s^{-1}$$
$$K_{app} = k_1/k_{-1} = 3.14M^{-1}, \; K = 9.70M^{-1}$$

The rate constants for the forward and reverse reactions and the equilibrium constants at different temperatures are obtained in a similar way and are given in Table 2.

TABLE 2

Rate and Equilibrium Constants of γ-Picoline Binding to Cyt *c* at Different Temperatures

T(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
300	54.7	17.4	3.14	9.70
303.5	143	18.0	7.93	24.5

The thermodynamic values $\Delta H°$, $\Delta S°$ and the energy of activation for γ-picoline binding to cyt *c* were obtained according to the Van't Hoff and Arrhenius' equations by least-square fitting as shown in Fig.2.

$$\ln K = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \qquad (10)$$

$$\ln k_1 = -\frac{E_a}{RT} + \ln A \qquad (11)$$

$\Delta H°$ = 158 kJ·mol^{-1} R = −0.994

$\Delta S°$ = 548 J·mol^{-1}·K^{-1}

E_a = 169 kJ·mol^{-1} R = −0.995

The thermodynamic values suggest that the reaction of γ-picoline binding to cyt *c* at pH5.7 is driven by a favorable entropy change[21]. The affinity of γ-picoline to cyt *c* is not strong. This can be rationalized in structural terms[5,21]. Cyt *c* was thought to provide a hydrophobic environment for the heme. Hydrophobic environment is equivalent to a medium of lower dielectric constant, which results in a negative $\Delta H°$ value. On the other hand, γ-picoline is a bulky ligand and is sterically hindered from entering the heme pocket. The steric effect upon ligation which almost certainly includes side-chain movement of some residues will contribute to a positive $\Delta H°$. These factors interplay, and the $\Delta H°$ measured reflects their net effect in protein. In γ-MePy·cyt *c*, the steric interaction is

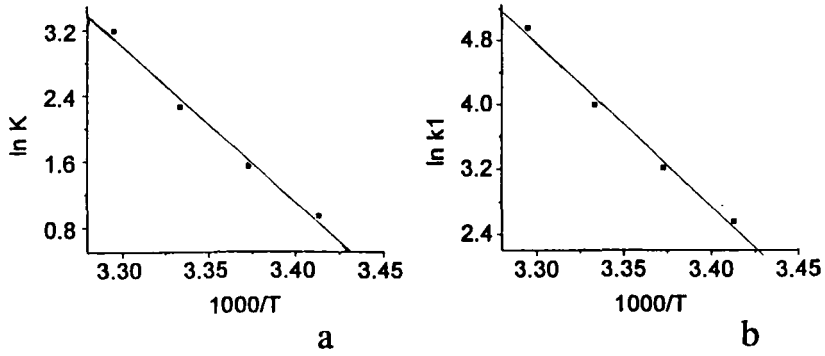

FIG.2. Variations of ln K (a) and ln k_1 (b) of γ-picoline binding to cyt *c* with temperatures(pH=5.7).

more significant, which is evidenced by the positive ΔH° in our result.

The positive ΔS° for γ-picoline binding to cyt *c* may also be examined in structural terms[5,8,21]. Structural changes due to the ligation of γ-picoline for cyt *c* is inevitable, which involves the displacement of Met80 by γ-picoline binding to heme iron. The heat liberated by the formation of Fe-N(γ-picoline) bond is compensated by the heat required to break Fe-S(Met80) bond and by the structural changes occurring overwhelmingly on the methionine-80 side of the heme. It is suggested that the less ordered structure due to tertiary structural changes accompanying ligation may contribute to the positive ΔS°. The activation energy E_a reflects the energy barrier for the binding process[21]. A large E_a for the forward reaction is expected, since γ-picoline is a bulky ligand and it is not easy for γ-picoline binding to heme iron of cyt *c* by breaking the Fe-S bond and displacing Met80.

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