# Notes

## $^1$H NMR Studies of Pyridine Binding to Cytochrome *c*

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### Introduction

Cytochrome *c*, which functions as an oxidation–reduction carrier in the respiratory chain, has been the most studied member of this important class of electron-transfer proteins.[1] One characteristic property of it is its coordination with iron-binding ligands such as cyanide ($CN^-$), azide ($N_3^-$), imidazole (Im), pyridine (Py), and various nitrogenous ligands.[2-7] Evidence suggests that these ligands bind by displacing the methionine-80 (Met80).[8,9] The substitution of cyt *c* by $CN^-$, $N_3^-$ and Im has been the subject of many NMR studies,[4,10-14] but NMR studies of Py binding to cyt *c* have scarcely been reported.[4,10]

In this paper, the nature of substitution of Met80 in horse ferricytochrome *c* by pyridine has been studied by $^1$H NMR methods. Saturation transfer experiments have been used to assign the heme methyl groups of Py–cyt *c*. The rate and equilibrium constants for Py binding to cyt *c* have been measured. The source of the asymmetric spin density distribution in heme groups of Py–cyt *c* and the reason for low affinity for the reaction have been discussed.

### Experimental Section

Horse cytochrome *c* (VI) was obtained from Sigma Chemical Co. and purified as previously described.[15] The NMR samples were prepared by dissolving weighed amounts of lyophilized cyt *c* in known volumes of $D_2O$ and known concentrations of neutral $D_2O$ solution of pyridine. All samples were adjusted to pD = 7.0.

Saturation transfer difference spectra were obtained on a Bruker AM-500 spectrometer, equipped with an Aspect-3000 computer system. Kinetic and equilibrium data for the binding of pyridine to cyt *c* were measured in the temperature range 303–319 K. Chemical shifts for all the spectra were referenced to dioxane at 3.74 ppm. The spin–lattice relaxation time $T_1$ for the heme methyl group (8-$CH_3$) of cyt *c* was measured by the routinely used inversion recovery method.

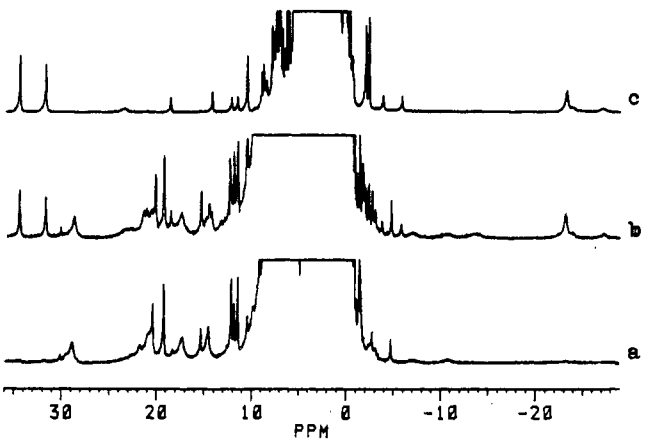


**Figure 1.** Downfield and upfield regions of $^1$H NMR spectra (pH = 7.0, 305 K): (a) [$^2H_5$]Py binding to cytochrome *c* ($C_{cyt\ c}$ = 5 mM, $C$[$^2H_5$]Py = 2.6 M); (b) a mixture of Py binding to cytochrome *c* and cytochrome *c* ($C_{cyt\ c}$ = 6 m M, $C_{Py}$ = 0.20 M); (c) cytochrome *c* ($C_{cyt\ c}$ = 5 mM).

The ligation of pyridine can be represented by reaction 1. The rate

$$\text{cyt } c + \text{Py} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \text{Py–cyt } c \qquad (1)$$

constants and equilibrium constants at different temperatures were measured by following the procedure described in ref 16. According to the Van't Hoff equations, the thermodynamic values of $\Delta H°$ and $\Delta S°$ were obtained.

### Results and Discussion

**Assignment of Some Hyperfine Shifted Resonances.** The saturation transfer method is used in conjunction with the recent hyperfine shifted resonance assignments for cyt *c*[17,18] to make unambiguous assignments for Py–cyt *c*. The $^1$H NMR spectra of cyt *c*, a mixture of cyt *c* and Py–cyt *c*, and Py–cyt *c* are illustrated in Figure 1. The resonances between cyt *c* and Py–cyt *c* can be differentiated in both the downfield and upfield regions of the spectrum. Difference spectra of the mixture of cyt *c* and Py–cyt *c* obtained upon saturation of cyt *c* 8-$CH_3$, 3-$CH_3$, and 1-$CH_3$ signals are seen in Figure 2a–c, showing saturation transfer to the corresponding peaks of Py–cyt *c* at 28.55, 17.18, and 14.68 ppm, respectively. The 5-$CH_3$ of cyt *c* is shown to be at 10.42 ppm, which overlaps with the resonance of Py–cyt *c* at 10.36 ppm. When the resonance at 10.42 ppm in the mixture of cyt *c* and Py–cyt *c* is irradiated, saturation is transferred to the resonances at 20.45 and 15.19 ppm (Figure 2d). The intensity of the resonance corresponding to 15.19 ppm compared with that for 1-$CH_3$ at 14.68 ppm in Py–cyt *c* indicates that it contains only one proton, which means it is not from the heme methyl group. Thus we can identify the peak at 20.45 ppm as the 5-$CH_3$ of Py–cyt *c*. Irradiation of the 5-$CH_3$ of Py–cyt *c* results in saturation transfer to the counterpart in cyt *c* at 10.42 ppm (Figure 2e), which verifies the above assignment.

In the difference spectra of the mixture of cyt *c* and Py–cyt *c* with saturation of the Met80 $\epsilon CH_3$, $\gamma$H, and $\beta$H of cyt *c*, which have been assigned previously as –23.32, –27.35, and 12.05 ppm,[18] the saturation is transferred to the corresponding resonances of Py–cyt *c* at 1.90, 2.49, and 2.13 ppm, respectively (Figure 3a–c).

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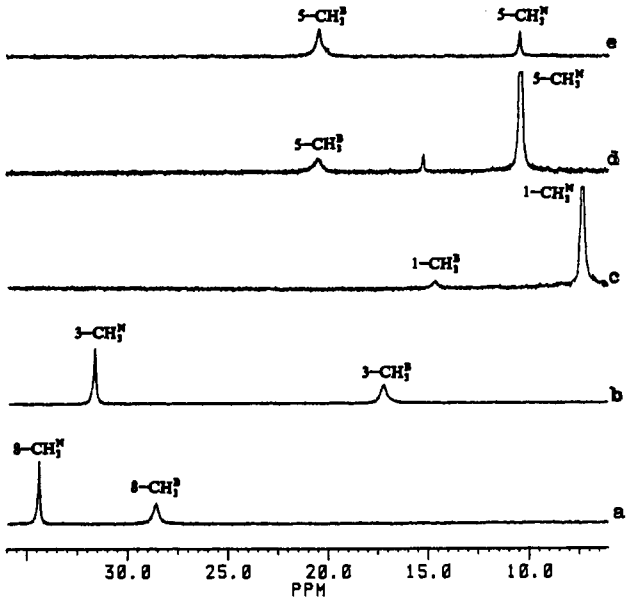


**Figure 2.** Saturation transfer difference spectra (pH = 7.0, 305 K) of a mixture of Py–cyt *c* and cyt *c* obtained upon saturation of cyt *c* signals of (a) 8-$CH_3$ at 34.35 ppm, (b) 3-$CH_3$ at 31.57 ppm, (c) 1-$CH_3$ at 7.29 ppm, and (d) 5-$CH_3$ at 10.42 ppm and of the Py–cyt *c* signal of (e) 5-$CH_3$ at 20.45 ppm. N = native cytochrome *c*; B = pyridine–bound cytochrome *c*.

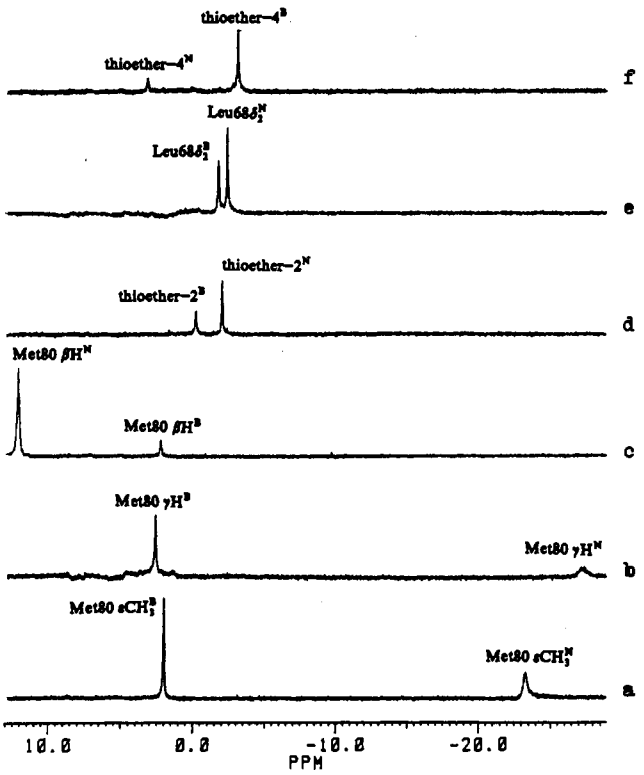


**Figure 3.** Saturation transfer difference spectra (pH = 7.0, 305 K) of a mixture of Py–cyt *c* and cyt *c* obtained upon saturation of cyt *c* signals of (a) Met80 $\epsilon CH_3$ at –23.32 ppm, (b) Met80 $\gamma$H at –27.35 ppm, (c) Met80 $\beta$H at 12.05 ppm, (d) thioether-2 at –211 ppm, and (e) Leu68 $\delta_2$ at –2.48 ppm and of the Py–cyt *c* signal of (f) thioether-4 at –3.21 ppm. N = native cytochrome *c*; B = pyridine–bound cytochrome *c*.

These results indicate that the Fe–S bond between the heme and Met80 is broken, and groups of Met80 (i.e. the $\epsilon CH_3$) move to the space where the paramagnetic contribution to the resonances from $Fe^{3+}$ is minor.

The resonances thioether-2 (Te-2) and Leu68$\delta_2$ of cyt *c* have been assigned in the literature.[17] From the saturation transfer difference spectra of the mixture of cyt *c* and Py–cyt *c* with irradiation at Te-2 and Leu68$\delta_2$ of cyt *c*, the corresponding resonances of Py–cyt *c* are obtained at –0.27 and –1.86 ppm (Figure 3d,e). After comparing the spectrum of cyt *c* with that of the mixture, we find that the resonance at –3.21 ppm must arise from Py–cyt *c*. Irradiation of the resonance results in the difference spectrum shown in Figure 3f, where a saturation transfer is observed at 3.06 ppm which has been assigned to Te-4 of cyt *c*.[17] So the peak at –3.21 ppm is from the Te-4 of Py–cyt *c*.

**Table I.** Heme Methyl Group Observed Isotropic, Dipolar, and Contact Shifts for Py–cyt *c* and cyt *c* at 205 K

| protein | heme methyl group | shift | | | |
|---|---|---|---|---|---|
| | | obsd | isotropic[a] | dipolar | contact |
| Py–cyt *c* | 8-$CH_3$ | 28.55 | 26.39 | –3.62[b] | 30.01 |
| | 5-$CH_3$ | 20.45 | 16.87 | –6.45[b] | 23.32 |
| | 3-$CH_3$ | 17.18 | 13.34 | –3.62[b] | 16.97 |
| | 1-$CH_3$ | 14.68 | 11.22 | –6.45[b] | 17.67 |
| cyt *c* | 8-$CH_3$ | 34.35 | 32.19 | –1.10 | 33.29 |
| | 3-$CH_3$ | 31.57 | 27.99 | –1.10 | 29.09 |
| | 5-$CH_3$ | 10.42 | 6.58 | –6.56 | 13.14 |
| | 1-$CH_3$ | 7.29 | 3.83 | –6.56 | 10.39 |

[a] Experimentally determined diamagnetic shift values of cytochrome *c* used to calculate the shifts.[17] [b] Reported EPR *g* values ($g_\perp$ = 3.29, $g_2$ = 1.99, $g_3$ = 1.48) used to calculate dipolar shifts.[10]

**Table II.** Rate and Equilibrium Constants of Pyridine Binding to Cytochrome *c* at Different Temperatures[a]

| $T$ (K) | $\tau$ (ms) | | $k_1$ | $k_{-1}$ | $K_{app}$ | $K$[b] |
|---|---|---|---|---|---|---|
| | 8-$CH_3^N$ | 8-$CH_3^B$ | | | | |
| 303 | 46.4 | 12.9 | 99.0 | 72.0 | 1.38 | 1.40 |
| 307 | 34.3 | 11.2 | 146 | 89.0 | 1.64 | 1.67 |
| 311 | 18.9 | 7.90 | 265 | 127 | 2.09 | 2.13 |
| 315 | 10.8 | 5.78 | 465 | 173 | 2.69 | 2.74 |
| 319 | 4.98 | 4.36 | 874 | 229 | 3.82 | 3.89 |

[a] N = native cytochrome *c*; B = pyridine-bound cytochrome *c*. [b] $K = K_{app}(1 + [H^+]/k_a)$, where a p$k_a$ value of 5.25 for pyridine is used.

The obtained chemical shifts of the heme methyl groups of Py–cyt *c* and cyt *c* and isotropic, dipolar, and contact shifts calculated according to the literature[10] are collected in Table I. It follows that the pattern of the heme methyl group contact shifts of Py–cyt *c* is similar to that of cyt *c*. But, in Py–cyt *c*, the 8,5-methyl groups are furthest downfield, as are the 8,3-methyl groups in cyt *c*. Such shift patterns reflect different distributions of spin density on pyrrole rings between the two proteins. In cyt *c*, the sulfur lone pair orbital of coordinated Met80 is pointed toward pyrrole ring IV as noted previously by Wüthrich.[19] The effect of lone pair interaction with the d$\pi$ orbitals is to direct the maximum spin density to the 8,3 groups. In Py–cyt *c*, because of the substitution of Met80 by pyridine, there exists no such effect. The Te-2,4 substituents in cyt *c* are more electron withdrawing than the propionic acid chains, which leads to the resonances of the 8,5-groups shifting furthest downfield.

**Affinity of Py Binding to cyt *c*.** The lifetime of both states, the rate constants for the forward and reverse reactions, and the equilibrium constants obtained at different temperatures are given in Table II.

The reaction rate constants for forward and reverse reactions increase with reaction temperature. The equilibrium constant obtained at 303 K ($K$ = 1.40 $M^{-1}$) is smaller than that obtained by Sutin and Yandell using a spectrophotometric method ($K$ = 2.4 $M^{-1}$). The difference may arise from small salt-induced perturbations of the protein structure ($I$ = 1.0 M).[20]

Figure 4 shows the plots of ln $K$ and ln $k_1$ versus $1/T$. The thermodynamic values of the reaction obtained by least-squares fitting are $\Delta H^\circ$ = 44.0 kJ·mol$^{-1}$ ($R$ = 0.993) and $\Delta S^\circ$ = 148

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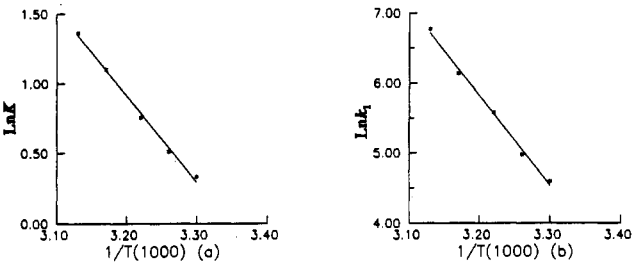


**Figure 4.** Variations of ln $K$ (a) and ln $k_1$ (b) of Py binding to cytochrome $c$ (pH = 7.0) with temperatures.

J·mol$^{-1}$·K$^{-1}$, which suggest that, at neutral pH, the reaction of cyt $c$ with Py is driven by a favorable entropy change. This can be rationalized in structural terms.

In general, heme proteins provide 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 $\Delta H°$ value,[21,22] while Py, a bulky ligand being bound to the heme, causes a steric effect on the protein which almost certainly results in movement of some side chains similar to those caused by cyanide binding.[21] The steric effects of the bound Py with protein will contribute to a positive $\Delta H°$ value. These factors interplay, and the apparent $\Delta H°$ measured reflects their net effect in protein. In Py–cyt $c$, steric interaction would be more significant, as evidenced by the positive $\Delta H°$ in our result.

The $\Delta S°$ for Py binding to cyt $c$ is positive. In cyt $c$, the binding of Py necessarily displaces Met80, so that the heat liberated by the formation of the Py–iron bond is compensated by the heat required to break the Fe–S bond and by the structural changes occurring overwhelmingly on the methionine-80 side of the heme, supported by NMR studies of iron–ligand substituted cytochrome $c$.[11,13] This also indicates that the environment of the sixth coordination site is sufficiently flexible to admit substantially bulky external ligands as compared with the case of metmyoglobin and methemoglobin in which Py hardly serves as an external ligand, due to denaturation.[4]

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