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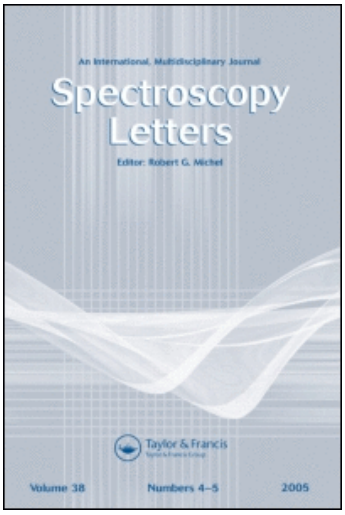

Spectroscopy Letters
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597299

NMR Spectroscopic Studies of the Effects of Nitrogenous Ligands on the Alkaline Isomerization of Cytochrome C

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To cite this Article Lu, Jun , Ma, Dejian , Hu, Jun , Tang, Wenxia and Zhu, Dexu(1999) 'NMR Spectroscopic Studies of the Effects of Nitrogenous Ligands on the Alkaline Isomerization of Cytochrome C', Spectroscopy Letters, 32: 4, 519 — 533
To link to this Article: DOI: 10.1080/00387019909350003
URL: http://dx.doi.org/10.1080/00387019909350003

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SPECTROSCOPY LETTERS, 32(4), 519–533 (1999)

NMR SPECTROSCOPIC STUDIES OF THE EFFECTS OF NITROGENOUS LIGANDS ON THE ALKALINE ISOMERIZATION OF CYTOCHROME C

Key words: nitrogenous ligands, cytochrome c, alkaline isomerization, NMR, exchange rate constants, 2D-EXSY

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ABSTRACT

The effects of pyridine derivatives and some other nitrogenous ligands(including 2-methyl-pyridine, 3-methyl-pyridine, 4-methyl-pyridine, 2,6-dimethyl-pyridine, imidazole and azide) on the alkaline isomerization of ferric cytochrome c were studied by ^{1}H-NMR. Bulky ligands like pyridine and its derivatives can facilitate the formation of alkaline form of cytochrome c and selectively stabilize one conformer of alkaline cytochrome c at enough

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concentration of such ligands. However, in the case of small ligands such as cyanide and azide, no such phenomena was observed. Being a ligand of medium size, imidazole has quite weak effect on alkaline isomerization of cytochrome c. It is interesting that 2-methyl-pyridine and 2,6-dimethyl-pyridine also have such effects on the alkaline isomerization of cytochrome c although they can not bind to the heme iron of cytochrome c due to the severe steric interactions between their methyl groups and the heme plane. This finding provides new evidence for the suggestion that the effects of pyridine and its derivatives on the alkaline isomerization of cytochrome c may not involve the binding of these exogenous ligands to the heme iron. The effect of 3-methyl-pyridine on the alkaline isomerization of cytochrome c was further quantitatively investigated by 2D-EXSY.

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INTRODUCTION

The alkaline isomerization of horse heart ferricytochrome c(cyt c) has attracted considerable attention and continues to be intensively studied [1-5]. Recently NMR studies of the hyperfine shift of cyt c and cyt c mutant (Lys79Ala) showed that two alkaline isomers coexisted in the solution of native cyt c when pH>9 and that in one of them Lys79 was the sixth ligand while in the other another lysine might serve as the sixth ligand to replace Met80 [3-5]. Factors affecting the alkaline transition pKa of cyt c, such as site specific mutation and high temperature, have been the subjects of many optical and NMR studies [6-9], but very few corresponding studies on exogenous ligands affecting alkaline isomerization have been reported. In our previous work[10,11], we showed that pyridine could induce alkaline transition at neutral pH and selectively enhanced the formation of one conformer of alkaline cyt c. However, many questions concerning this phenomenon remain to be answered. For example, in what manner pyridine participates the alkaline isomerization is still unknown. There are at least two possibilities for the role of pyridine during alkaline transition. First, pyridine binds to cyt c and forms an unstable complex which then transformed to alkaline cyt c. The other possible way is that the ligand just penetrates into the heme crevice and induces a structural modification in the heme binding domain of cyt c which facilitates the formation of the lysine form of cyt c.

To clarify how pyridine takes part in the alkaline isomerization of cyt c and to verify whether other nitrogenous ligands also have such effects, we made a further ^{1}H-NMR study on the alkaline isomerization by using several pyridine derivatives and other nitrogenous ligands. From the NMR spectra of cyt c at different pH or different ligand concentrations, it was found that pyridine derivatives could also induce alkaline transition at neutral pH and selectively facilitate the formation of one conformer of alkaline cyt c, while cyanide, azide did not seem to enhance the alkaline isomerization of cyt c and imidazole has quite weak effect on the alkaline transition. This result, together with the fact that 2-mpy and 2,6-mpy can not bind to cyt c, provides new evidence for the possible path by which pyridine influence the alkaline isomerization of cyt c.

To further verify the kinetic behaviors of alkaline transition in the presence of pyridine derivatives, the exchange rate constants between the native form and two individual basic forms with the presence of 3-methyl-pyridine(3-mpy hereafter) were further evaluated by 2D-EXSY. The result confirmed the effect of 3-mpy on the alkaline isomerization. It was also found that like pyridine, 3-mpy increased the forward rate constants of forming both of two conformers of alkaline cyt c, but the increase of the forward rate constants of forming one alkaline conformer(A1) was particularly great, thus leading to the final result that nearly one alkaline conformer (A1) exist at enough concentration of such exogenous ligands.

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EXPERIMENTAL

Horse heart cytochrome c (Type VI) from Sigma Chemical Co. was purified and lyophilized from D_2O before use[12]. NMR samples consisted of 5mmol/dm^3 cyt c(for one dimensional spectra), 8mmol/dm^3 cyt c pH9.28 with and without the presence of 10mmol/dm^3 3-mpy. 2-methy-pyridine(2-mpy), 4-methyl-pyridine(4-mpy), 2,6-dimethyl-pyridine(2,6-mpy), imidazole and azide were reagent grade and used without further purification. 3-mpy was purified according to Heap et al [13]. The purity of these reagents were checked by ^{1}H NMR. The pH was adjusted by addition of small amounts of DCl or NaOD, pH values were 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 the elimination of the residual water resonance. Two-dimensional exchange spectra(2D-EXSY) with the mixing time (τ_m) of 50ms were acquired using the

phase sensitive NOESY pulse sequence 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.

Kinetics. For the system involving the chemical exchange between N 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[14]:

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

where A and R are given by:

$$A=\begin{vmatrix} I_{11}/M_1 & I_{12}/M_2 & \cdots & a_{1N}/M_N \\ I_{21}/M_1 & I_{22}/M_2 & \cdots & I_{2N}/M_N \\ \cdots & \cdots & \cdots & \cdots \\ I_{N1}/M_1 & I_{N2}/M_2 & \cdots & I_{NN}/M_N \end{vmatrix}$$

$$R=\begin{vmatrix} -R_1-k_{12}-k_{13}-\ldots-k_{1N} & k_{21} & \cdots & k_{N1} \\ k_{12} & -R_2-k_{21}-k_{23}-\ldots-k_{2N} & \cdots & k_{N2} \\ \cdots & \cdots & \cdots & \cdots \\ k_{1N} & k_{2N} & \cdots & -R_N-k_{N1}-k_{N2}-\ldots-k_{N,(N-1)} \end{vmatrix}$$

In A, the quantities I_{11}, I_{12}, ... are two dimensional peak amplitudes measured in an experiment with certain mixing time and normalized. M_1, M_2, ... are the equilibrium magnetization values obtained from integration of the one dimensional spectra and were also normalized. R contains the kinetic parameters to be determined, namely, chemical exchange 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[14-16]:

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

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

RESULTS AND DISCUSSION

Effects of nitrogenous ligands on the alkaline isomerization

The pH dependent NMR spectra of cyt c were recorded at variant pH (from neutral to alkaline pH) in the presence of 3-mpy, 2-mpy or other nitrogenous ligands respectively. FIG.1a shows the downfield hyperfine shifted region of cyt c spectra as a function of pH in

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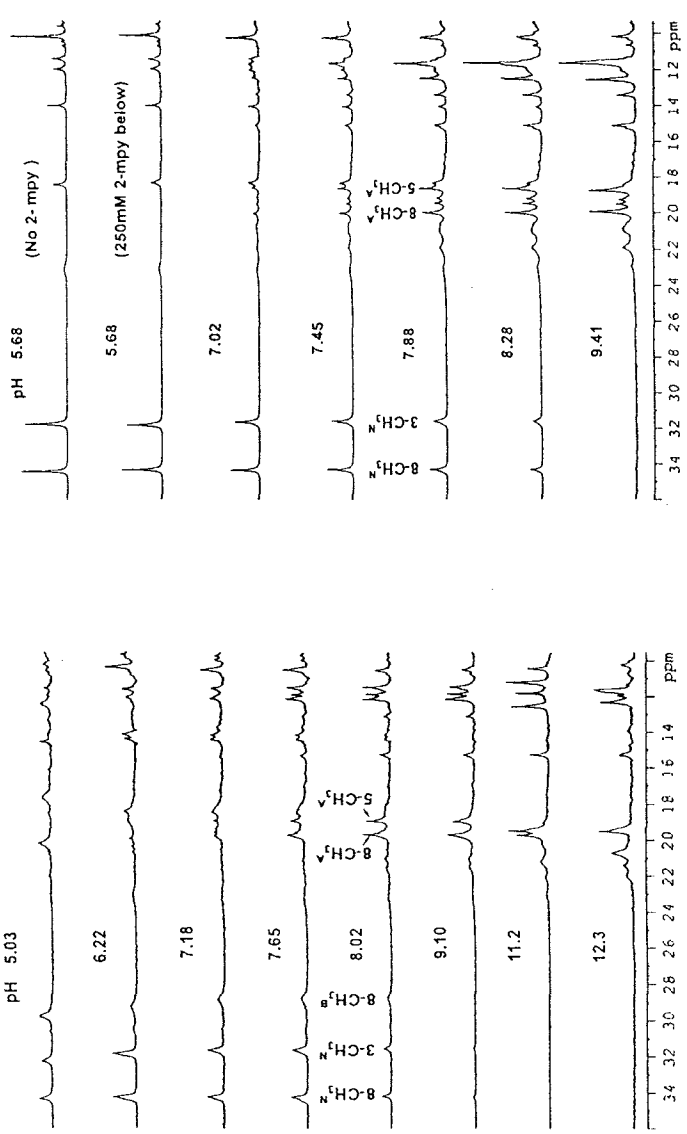

FIG. 1. The downfield hyperfine shifted region of cyt c spectra as a function of pH in the presence of 250mmol/dm^3 3-mpy (a) and 2-mpy(b) respectively at 303K. In this figure, N denotes native cyt c, A denotes alkaline cyt c, and B is ligand-bound cyt c.

the presence of 250mmol/dm^3 3-mpy at 303K. By comparison with the ^{1}H-NMR spectra of cyt c and 3-mpy-cyt c complex[17], it was found that two components existed at pH<7.65. One was the native form of cyt c and the other 3-mpy-cyt c complex. When the pH was increased above 7.65, a new set of signals appeared at the expense of those for native cyt c and 3-mpy-cyt c. Only the new species could be observed at pH9.1-12.3. The chemical shifts of the new species were similar to those observed for alkaline cyt c[3,4]. And the spectra pattern was close to that of pH dependent spectra in the presence of pyridine[10], in which these peaks were assigned to alkaline cyt c. Thus the new species was assigned to the alkaline form of cyt c[10,11]. So in the presence of 0.25M 3-mpy, the peaks of alkaline cyt c appeared above pH7.65, in contrast to the native lysine form which only appears at pH > 9 [10]. This result indicates that 3-mpy had an effect similar to that of pyridine to facilitate the alkaline isomerization of cyt c. Similar results were obtained for 4-mpy.

FIG.1b shows the downfield hyperfine shifted region of cyt c spectra as a function of pH in the presence of 250mmol/dm^3 2-mpy at 303K. Different from the spectra of cyt c with 3-mpy, there was only one component (native cyt c) existing at pH<7.02. This indicates that 2-mpy cannot bind to cyt c. In fact, the 2-mpy-cyt c complex could not be formed even at 1mol/dm^3 2-mpy due to severe steric interaction between 2-methyl and the heme plane[17]. When the pH rose above 7.02, the alkaline form of cyt c appeared and when it rose above pH9.5, only alkaline form of cyt c existed. This suggests that 2-mpy also had an effect of enhancing the alkaline isomerization although it could not bind to heme iron. Similar result were observed in the presence of 2,6-mpy.

However, in the case of azide, no such a phenomenon was observed. In the presence of 500mmol/dm^3 azide, the pH dependent spectra of cyt c kept nearly invariant in the pH ranges 5.8-10.0(Figure not shown). This experiment establishes that azides do not facilitate the alkaline isomerization of cyt c. The same is true of cyanide, which has no such an effect since the NMR spectrum of cyanoferriccytochrome c remains unchanged through the ranges from neutral to alkaline pH [1,18].

It has been reported that the alkaline form of cyt c was a mixture of two lysine-ligated conformers and that Lys79 was the sixth ligand in the one of two alkaline conformations[3,4]. Previous work[11] showed that pyridine not only facilitated alkaline transition but also selectively favored one isomer of alkaline cyt c over the other. To verify whether other nitrogenous ligands also had such selectivity, a series of NMR experiments of cyt c at pH9.2 and 318K with varying concentrations of ligands were carried out. From FIG.2 it is seen that at such a pH and temperature the NMR spectrum showed the presence of two alkaline conformers which were in equilibrium with the native form of the protein [4]. With the

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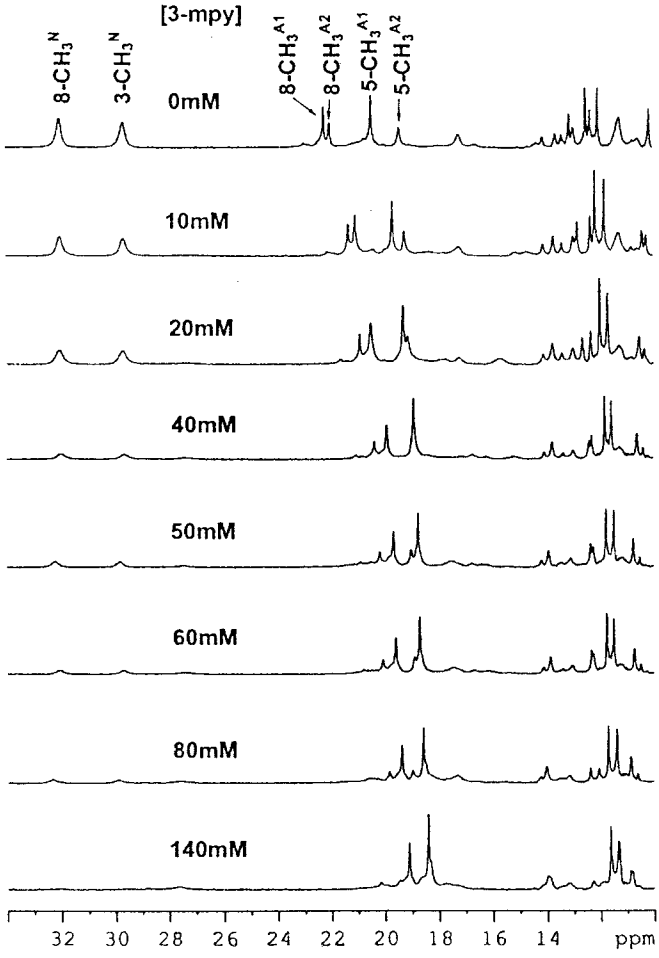

FIG.2 The downfield hyperfine shifted region of cyt c spectra as a function of 3-mpy concentration at 318K and pH9.2. N denotes native cyt c. A1 and A2 are two conformers of alkaline cyt c.

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increase of the concentration of 3-mpy, the magnitude of the resonance of native cyt c decreased and the magnitude of alkaline cyt c increased. Concurrently, the relative concentration of two alkaline cyt c conformers was altered. The intensity of one isomer (A1) increased with the addition of 3-mpy while the intensity of the other isomer(A2) decreased accordingly. At 80mmol/dm^3 3-mpy, there was mainly alkaline isomer A1 existing in cyt c solution.

Similar results were obtained when such experiments were performed using other ligands including 2-mpy, 2,6-mpy. FIG. 3 illustrates the downfield hyperfine shifted region of cyt c spectra as a function of 2.6-mpy concentration at pH9.2 and 318K. Increasing the concentration of 2,6-mpy not only led to the decrease in the intensities of the signals of native cyt c and one alkaline isomer but also enhanced the formation of the other isomer of alkaline cyt c (A1). At about 100mmol/dm^3 2,6-mpy, there was nearly one alkaline isomer (A1) of cyt c existing.

As neither 2,6-mpy nor 2-mpy could bind to heme iron, their effects on the alkaline isomerization strongly suggest that the binding to heme iron for these nitrogenous ligands might not be necessary for their effect of enhancing alkaline transition. That is to say, the effects of pyridine and its derivatives on alkaline isomerization were not through the path of forming ligand-cyt c complex but fulfilled in some other way. It would be possible that such a ligand penetrated into the heme crevice and induced conformational changes in the heme environment of cyt c, thus facilitating the formation of one alkaline isomer of cyt c. This is similar to the effect of pyridine on the heme environment of cytochrome b_{558} [19]. It has been suggested that the heme structure in cytochrome b_{558} is modulated by pyridine and becomes similar to that of cytochrome P450, suggesting that the 5th heme ligand(histidine) of the heme in pyridine-modified cytochrome b_{558} has been replaced with a nearby thiolate group without direct binding of pyridine to the heme[19].

FIG. 4 shows the downfield hyperfine shifted region of cyt c spectra as a function of imidazole concentration at pH9.2 and 318K. It seems that the addition of imidazole to the cyt c solution produced the imidazole-bound cyt c and resulted in minor changes in the chemical shifts of alkaline cyt c and relative concentration of the native cyt c and the two alkaline isomers. The intensity of imidazole-bound cyt c increased at the expense of native cyt c and alkaline cyt c. When there is no imidazole, the relative concentration between alkaline cyt c and native cyt c is about 1.3. When the concentration of imidazole increased to 100mmol/dm^3 this value changed to 2.2. This indicates that imidazole, being a medium size ligand, has minor effect on alkaline isomerization although this effect is very weak compared with those of pyridine derivatives.

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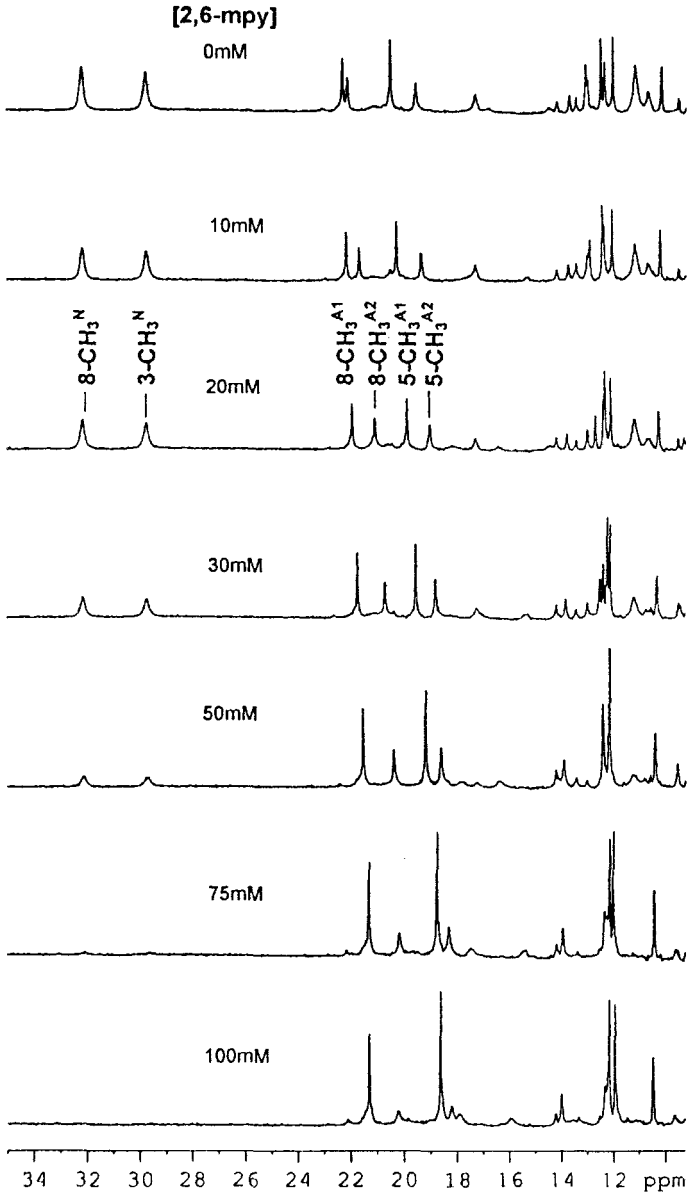

FIG.3. The downfield hyperfine shifted region of cyt c spectra as a function of 2,6-mpy concentration at 318K and pH9.2. N denotes native cyt c, A1 and A2 are two conformers of alkaline cyt c.

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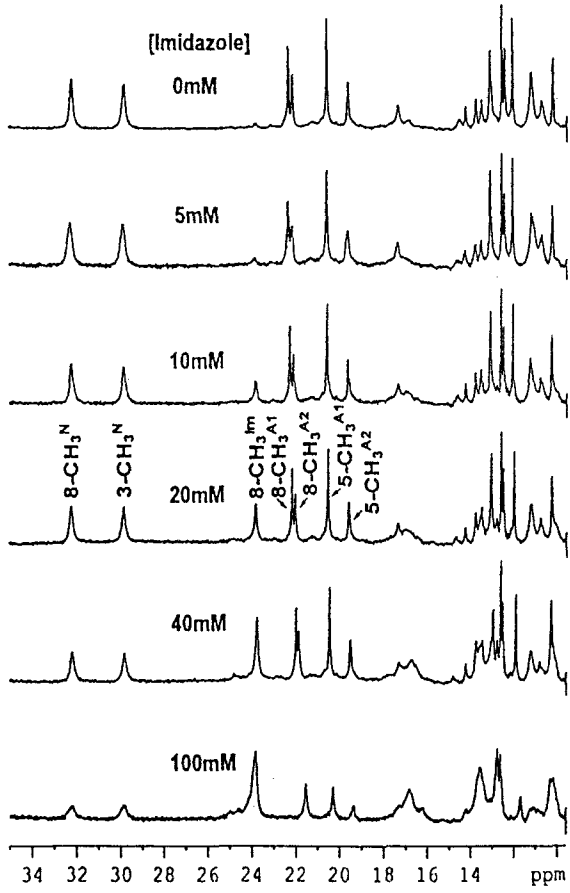

FIG. 4. The downfield hyperfine shifted region of cyt c spectra as a function of Imidazole concentration at 318K and pH9.2. N denotes native cyt c, A1 and A2 are two conformers of alkaline cyt c, Im denotes the imidazole-bound cyt c.

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As shown above, bulky ligands including pyridine derivatives (2-mpy, 3-mpy, 4-mpy, 2,6-mpy), like pyridine, can induce alkaline transition at neutral pH and selectively favor the formation of one conformer of alkaline cyt c, while small ligands like azide or cyanide cannot. A medium size ligand such as imidazole only has limited effect on alkaline isomerization. Therefore it is tempting to postulate that the size of such nitrogenous ligands is the controlling factor determining whether the ligand can affect the alkaline isomerization of cyt c. Only these bulky ligands can have a significant effect on the alkaline isomerization. The affinity constants of these ligands to cyt c might not be critical for their effects on alkaline isomerization. The binding constants of imidazole, pyridine,3-mpy and 4-mpy are comparable. However, pyridine, 3-mpy and 4-mpy all can significantly facilitate alkaline transition while imidazole has a very weak effect.

According to the ligand size, these ligands fall into three categories: (1) Ligands of small size such as cyanide and azide. Neither of the ligands can enhance the alkaline isomerization of cyt c. However, the case of cyanide is different from that of azide. The affinity constants for cyanide and azide are 3.16×10^3 $mol^{-1}dm^3$ and 4.3 $mol^{-1}dm^3$at 25°C respectively [20]. Possessing a very large affinity constant, cyanide binds so tightly to cyt c that it blocks the binding site and cannot be displaced by internal lysine of cyt c. So in the case of the cyanide complex, the NMR spectral changes were found to occur in alkaline pH only above pH11.5 while with native cyt c the alkaline form appeared around pH9 [1]. In the case of azide, which has a moderate affinity constant, it does not seem to affect alkaline isomerization of cyt c. This might be attributed to its small size so that it would not induce enough conformational changes to trigger alkaline isomerization at neutral pH. (2) Bulky ligands including pyridine and its derivatives. The affinity constants for pyridine, 3-mpy and 4-mpy are 1.4, 7.9 and 24.5 $mol^{-1}dm^3$ respectively [17]. 2-mpy and 2,6-mpy cannot bind to cyt c. It seems that these molecules only penetrated into the heme crevice and induced a structural modification in the heme environment which triggered the alkaline transition at neutral pH and selectively facilitated the formation of one alkaline isomer of cyt c. This result, together with the fact that 2-mpy and 2,6-mpy cannot bind to cyt c, strongly suggests that the effect of pyridine derivatives on the alkaline isomerization of cyt c might not involve the binding of these exogenous ligands to the heme iron. (3) Medium size ligand as imidazole. The behavior of imidazole resembles azide rather than pyridine. Its addition to cyt c solution at pH9.2 mainly produced imidazole-bound cyt c. However, it also has a weak effect to enhance alkaline isomerization of cyt c which might be due to that its size is not large enough to efficiently induce certain structural modification in the heme environment of cyt c.

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Kinetic analysis of alkaline isomerization in the presence of 3-mpy

Recently, two-dimensional (2D) EXSY has become popular for its power to deal with complex multi-sites exchange systems [11-14]. To further understand the kinetic and thermodynamic behaviors of the two alkaline isomers in their formation process and the effect of pyridine derivatives on them, 2D-EXSY experiments were employed to study the exchange rate constants between the alkaline isomers and native cyt c with and without the presence of 3-mpy. FIG.5 presents the partial downfield region of the 2D-EXSY spectrum of the mixture of native cyt c and two alkaline isomers(pH9.28, 50°C) with the presence of 10mmol/dm^3 3-mpy. The cross peak signal at 31.6ppm known as the heme 8-CH_3 group of cyt c connects the signals which are the heme 8-CH_3 of alkaline isomers A2 and A1 respectively. This indicates that there might exist the following equilibria in this system:

$$\begin{aligned} &\text{cyt c} \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} \text{alkaline cyt c isomer(A1)} + H^+ \\ &\text{cyt c} \underset{k_{31}}{\overset{k_{13}}{\rightleftharpoons}} \text{alkaline cyt c isomer(A2)} + H^+ \\ &\text{A1} \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} \text{A2}. \end{aligned} \qquad (1)$$

But from FIG.5 it is seen that there are no cross peaks between the two isomers, and this means that the exchange rate between them is too slow to be observed using this method. In this case, the magnetization exchanges between the species follow a first-order rate process and can be represented by:

$$\begin{aligned} &M_{\text{cyt c}} \underset{k'_{21}}{\overset{k'_{12}}{\rightleftharpoons}} M_{\text{cyt c(A1)}} \\ &M_{\text{cyt c}} \underset{k'_{31}}{\overset{k'_{13}}{\rightleftharpoons}} M_{\text{cyt c(A2)}} \end{aligned} \qquad (2)$$

According to the theory of kinetics by means of exchange spectroscopy [11-14], the reaction amplitude matrix A is as follows:

$$A = \begin{vmatrix} 0.15 & 0.028 & 0.033 \\ 0.037 & 1.2 & 0 \\ 0.073 & 0 & 1.1 \end{vmatrix}$$

As no cross peaks between A1 and A2 were observed, the corresponding items in amplitude matrix A ($a_{ij} = I_{ij} / M_i$) are supposed to be zero as shown above. From the amplitude matrix A, the kinetic matrix R was calculated, resulting in the rate constants k'_{12}=3.0s^{-1},

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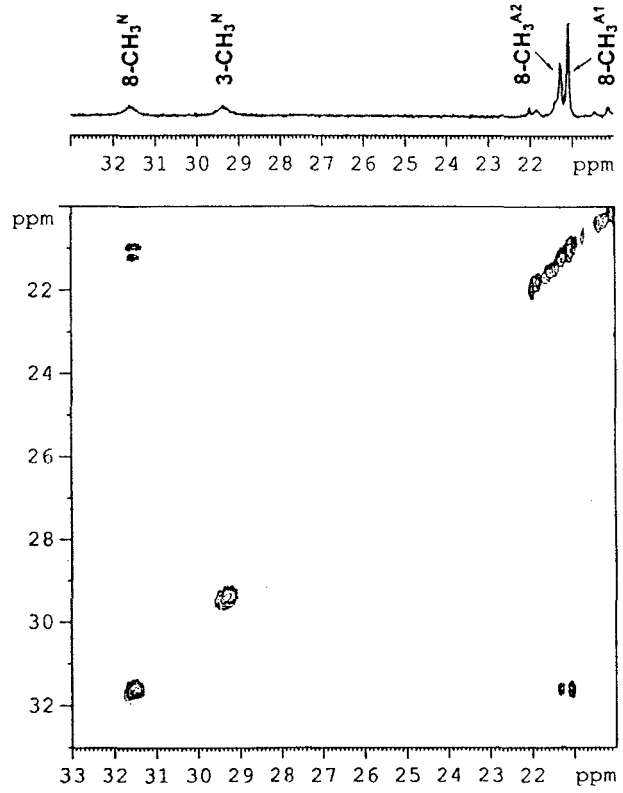

FIG. 5. Portions of the downfield region of the 2D-EXSY spectrum of the mixture of native cyt c and alkaline cyt c at 323K, pH9.28 in the presence of 10mmol/dm^3 3-mpy. N denotes native cyt c, A1 and A2 are two conformers of alkaline cyt c.

k'_{21}=1.3s^{-1}, k'_{13}=1.4s^{-1} and k'_{31}=1.1s^{-1}. However, the exchange pathway shown in Eqn.(2) does not reflect the reaction equilibria as shown in Eqn.(1). The relationships between the magnetization exchange rate constants and the reaction exchange rate constants can be found in Eqn.(3a):

$$k_{12}=k'_{12},\ k_{21}=k'_{21}/[H^+],\ k_{13}=k'_{13},\ k_{31}=k'_{31}/[H^+], \qquad (3a)$$

And the apparent equilibrium constants are:

$$K_{12}=k_{12}/k_{21} \quad \text{and} \quad K_{13}=k_{13}/k_{31} \quad . (3b)$$

Thus the reaction rate constants and equilibrium constants were determined and are listed in Table 1. The reaction rate constants and equilibrium constants of the system of pure cyt c and cyt c with pyridine reported before are also listed in Table 1.

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TABLE 1. The Exchange Rate Constants and Equilibrium Constants During the Alkaline Isomerization of Cytochrome c at pH9.28 and 323K

Reaction system	k_{12} (s^{-1})	K_{12} (mol dm^{-3})	k_{13} (s^{-1})	K_{13} (mol dm^{-3})	K_{12}/K_{13}
Cyt c with 3-mpy [a]	3.0	1.1×10^{-9}	1.4	6.4×10^{-10}	1.7
Pure cyt c [b]	0.62	3.4×10^{-10}	0.14	2.4×10^{-10}	1.4
Cyt c with pyridine [b]	1.0	7.7×10^{-10}	0.64	4.3×10^{-10}	1.8

Notes: [a] This work. [b] Ref. 11

In comparison with the pure cyt c system, both the exchange rate constants and the apparent equilibrium constants for the system containing 3-mpy have been significantly affected. The forward rate constants k_{12} and k_{13} are greater than those in the system of pure cyt c, indicating that the addition of 3-mpy has enhanced alkaline isomerization. This is in agreement with the above suggestion that 3-mpy interacts with cyt c and induces certain conformational changes in the heme environment thus facilitating alkaline isomerization.

Moreover, the difference between the equilibrium constants $\mathbf{K}_{12}$ and $\mathbf{K}_{13}$ is larger than that in pure cyt c system and comparable to that in the system containing pyridine [11]. This indicates that the concentration of A1 form has been selectively raised and the effect of 3-mpy is similar to that of pyridine. These results are in consistency with our above results that pyridine and its derivatives not only facilitate the transition of cyt c to alkaline cyt c, but also favor the specific alkaline isomer(A1).

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Date Received: October 23, 1998
Date Accepted: April 20, 1999

Downloaded At: 03:20 30 January 2011