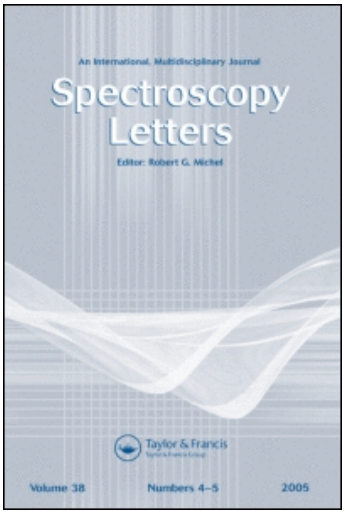

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^{1}H NMR Studies of Alkaline Isomerization of Cytochrome *C* in the Presence of Γ-Picoline

Dejian Ma[a]; Jun Lu[a]; Wenxia Tang[a]
[a] State Key Lab. of Coordination Chemistry, Coordination Chemistry Institute, Nanjing University, Nanjing, P.R., China

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^{1}H NMR STUDIES OF ALKALINE ISOMERIZATION OF CYTOCHROME *c* IN THE PRESENCE OF γ-PICOLINE

Key Words: Cytochrome *c*, γ-Picoline,
Alkaline isomerization, Kinetics, ^{1}H NMR

Dejian Ma, Jun Lu, Wenxia Tang*

State Key Lab. of Coordination Chemistry,
Coordination Chemistry Institute, Nanjing University,
Nanjing 210093, P.R. China

Abstract

The alkaline isomer of horse heart ferricytochrome *c* in the presence of γ-picoline (γ-MePy) is studied using ^{1}H NMR methods. We find that the lysine form of cyt *c* occurs at neutral pH environment in the presence of γ-MePy and increasing [γ-MePy] facilitates the transition from the native to the alkaline forms and stabilizes one of the two alkaline forms of cyt *c*. The conformational changes in cyt *c* induced by temperature are similar with that induced by [γ-MePy]. The kinetic behavior for the formation of the two alkaline isomers have changed in the presence of even small amounts of γ-MePy. It is indicated that the presence of γ-MePy stabilize Lys72 or 73 binding form and inhibit the formation of Lys79 binding alkaline isomer.

1. Introduction

The conformational isomerization of cytochrome *c* has been studied for many years using optical and NMR spectroscopy[1-4]. Recently, the alkaline conformation of ferricytochrome *c* formed on raising the pH to alkaline values (pK_a = 8.5 - 9 depending on species) has received particular experimental attention[5-12]. In the alkaline species, the heme iron retains the native imidazole ligand of His 18 while the sulfur atom of Met 80 is replaced by a strong-field ligand, which was suggested to be the ε-amino group of one of the nineteen lysine residues[7-8].

Point mutations and the alkaline conformational change in cyt *c* have been studied widely[10-12]. However, there is only a few studies on the influence of exogenous ligand to the alkaline isomerization of cyt *c*[4,13]. In this paper, the alkaline isomer of horse heart ferricytochrome *c* in the presence of γ-picoline(γ-MePy) is studied using ^{1}H NMR methods. The influence of variations in pH, temperature and concentration of γ-MePy to the native and alkaline forms of cyt *c* is reported. The kinetics of the formation of the two alkaline conformers is investigated using two-dimensional exchange spectroscopy (EXSY) method.

2. Materials and methods

Horse heart cyt *c* from Sigma Chemical Co. was purified as previously described[14]. NMR samples consisted of 6 mM cyt *c* in the presence of 6.7 mM γ-MePy (for 2D EXSY experiments), 5 mM cyt *c* in the presence of 0.4 M γ-MePy and so on (for other 1D NMR experiments). All the samples were prepared as previously described[15].

All the ^{1}H NMR experiments were performed on a Bruker AM 500 spectrometer equipped with an Aspect-3000 computer system. The detection of 2D EXSY spectra were achieved using the standard NOESY pulse sequence with mixing time of 50 ms[16]. The details of the methods are described else where[15].

3. Results and discussion

3.1. The pH dependence of ^{1}H NMR spectra of cyt *c* in the presence of γ-MePy

The pH dependent ^{1}H NMR spectra of cyt *c* were recorded at different pH values in the range 5.25 - 13.45 at 25 °C in the presence of 0.4 M γ-MePy. The down-field region of the spectra are given in Fig. 1. According to the resonance assignments of cyt *c* and γ-MePy cyt *c*[17-19], we find that a new set of signals appears when increasing pH to 6.80. The intensity of the new peaks increases as the pH increases (shown in Fig. 1). The chemical shifts of the new species are very

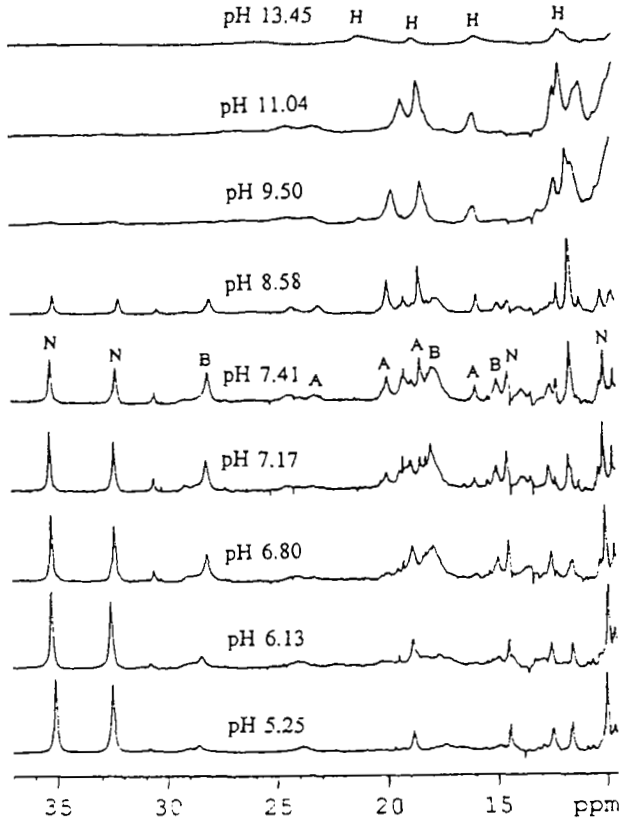

FIG. 1. Stack plot of the down-field region of the pH dependent ^{1}H NMR spectra of cyt *c* in the presence of 0.4 M γ-MePy in D_2O at 25 °C. $C_{cyt\,c}$ = 5 mM, N, native form; B, γ-MePy binding form; A, alkaline (lysine) form; H, hydroxide form.

similar to those observed for cyt f, which has been shown to possess a low-spin heme iron with His-Lys ligation[20]. From the spectral pattern, the new species is assigned to the alkaline (lysine) form of cyt *c*. The alkaline isomer of horse heart cyt *c* is formed on raising the pH to 9[11]. It is suggested that the presence of γ-MePy facilitates the alkaline transition.

Nall et al reported that the pK_{app} of alkaline transition for normal yeast iso-2-cyt *c* had a value of 8.45, while it decreased to 6.71 for Pro76Gly mutation[12]. The pK_a of yeast iso-1-cyt *c* was found to be 8.5 for the wild type, 7.7 for Ser82 and Gly82

mutations, 7.2 for Leu82 and Ile82 mutations[11]. In our previous work, the lysine form of cyt *c* occurred at neutral pH environment in the presence of pyridine(Py)[13]. Similar result is obtained in the presence of γ-MePy as mentioned above. This could be discussed in structural terms. The polypeptide chain around the sixth form of cyt *c* occurred at neutral pH environment in the presence of pyridine (Py)[13]. Similar result is obtained in the presence of γ-MePy as mentioned above. This could be discussed in structural terms. The polypeptide chain around the sixth ligand is flexible[21]. Appropriate point mutation or exogenous ligands could make major conformational change in this area which induce dramatic shift in pH dependent equilibrium between native form and alkaline protein[12]. The presence of γ-MePy disturb the conformation around Met80 which is required to stabilize the native form of cyt *c* at physiological pH[11]. The conformational change in the heme binding environment destabilize the native form relative to alkaline form of cyt *c*.

However in the interaction of cyt *c* with cyanide, imidazole and azide, no alkaline form of cyt *c* was observed in the proton NMR spectra at neutral pH[4,22-24]. The binding of cyanide to heme iron is very strong[25]. The cyano complex of cyt *c* is more stable than the lysine form of cyt *c*, which hinder the formation of alkaline isomer. Morishima et al found that cyano complex of cyt *c* showed no spectral change in pH range 7 - 10.5 and the alkaline form was observed only when pH increased to 11.5[4]. The binding ability of Im to heme iron is comparable to that of Py and γ-MePy[19,25]. However, different from Py and γ-MePy, Im cannot facilitate the alkaline transition[22]. This might be related to the molecular structure of Im. Im is less bulky than Py and γ-MePy. The conformational change caused by Im is not so obvious as that caused by Py and γ-MePy. Azide binds non-linearly and therefore forms an angle with the heme[26]. The steric interaction upon ligation appears to be small, which hardly effect the alkaline transition. When increasing pH value to 9, the deprotonated lysine will displace azide binding to heme iron and alkaline isomer will form as the pH dependent behavior in native cyt *c* since the binding of azide to iron is weaker than that of lysine[2].

The pK_a values are 5.25 and 6.02 for Py and γ-MePy respectively. At low pH values, the protonation of γ-MePy is more extensive than that of Py, which could influence the binding to heme iron. In the presence of Py, the alkaline isomer forms at pH 6.2 and both native cyt *c* and Py binding cyt *c* (pcyt *c*) lose their intensity with increasing pH above 6.2[13]. The pH value for the alkaline transition is pH 6.8

in the presence of γ-MePy. With increasing pH value from 6.80 to 7.41, the intensity of γ-MePy cyt *c* increases (Fig. 1). It seems that more deprotonated γ-MePy bind to heme iron with increasing pH. However, more importantly, the association constants for Py and γ-MePy to cyt *c* at 298 K are 2.93 M^{-1} and 10.9 M^{-1}, respectively[13,19]. The binding ability of γ-MePy to cyt *c* is stronger than that of Py. Even when the lysine form appears, γ-MePy can still compete with lysine to bind to heme iron while Py cannot. With increasing pH above 7.41, the lysine form becomes more stable than the γ-MePy cyt *c* complex and the intensity of the latter begins to decrease.

Under the experimental condition, the lysine form of cyt *c*, the native form and γ-MePy binding form can exist simultaneously while pH value spanning from 6.80 to 8.58(Fig. 1). When increasing pH to 9.5 and higher, no resonances from native cyt *c* and γ-MePy cyt *c* are observed in their original position. Only the lysine form is observed at pH values between 9.50 and 11.04. By further raising the pH to 13.45, those peaks of lysine form again disappear and the sample gives four broadened resonances in the down-field region as shown in Fig. 1. It is suggested that the internal and external ligands are replaced by the hydroxide anion at pH 13.45[4].

3.2. The effect of [γ-MePy] on the 1H NMR spectra of cyt *c*

The spectra of cyt *c* at 313 K and pH 9.5 with different concentration of γ-MePy were measured and are given in Fig. 2. The chemical shifts of heme 8-CH_3 resonance of native cyt *c* are unchanged with different [γ-MePy]. However, those of A1 and A2 move upfield with increasing [γ-MePy]. A1 moves faster than A2 as shown in Fig. 2. With increasing [γ-MePy], the total of the two lysine forms increases while the native form decreases. Actually, only one lysine form (A1) increases with increasing [γ-MePy]. The other lysine form (A2) loses its intensity simultaneously. The ratio of A1/A2 increases with increasing [γ-MePy].

NMR studies have been exhibited that there are two alkaline forms with very similar properties[5]. It has been suggested that Lys79 provides the sixth axial ligand in one of the two alkaline isomers and the most likely candidate for the other conformer is Lys72 or Lys73, though more extensive evidence is required[3,6-10]. Then, which lysine acts as the sixth ligand in A1 and which one in A2? In native cyt *c*, Lys72, 73 and 79 all locate close to heme iron. But, Lys72 and 73 exist in helix while Lys79 lies in random coil. When γ-MePy is present, the disturbation to

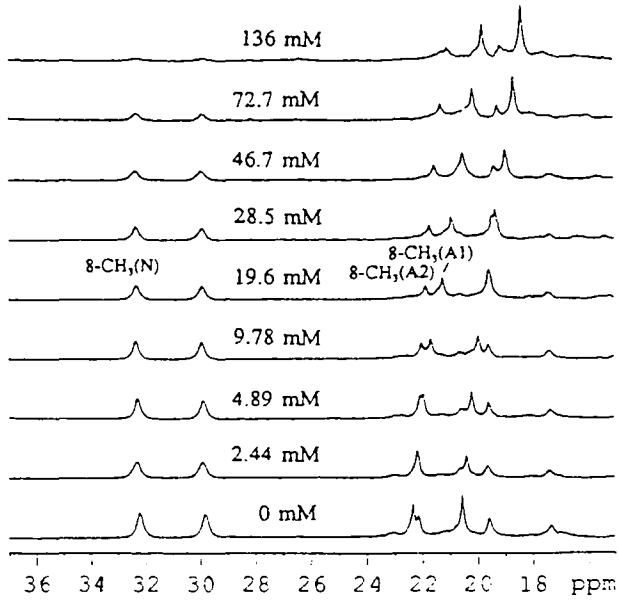

FIG. 2. Stack plot of the down-field region of the [γ-MePy] dependent ^{1}H NMR spectra of cyt *c* in D_2O at pH 9.5 and 40 °C. $C_{cyt\ c}$ = 5 mM, N, native form; A, alkaline form.

the conformation around the sixth ligand of cyt *c* is unavoidable. The polypeptide 79 - 85 including Met80 may swing away from heme and the binding of Lys72 or 73 may be more stable. So, it is suggested that Lys72 or 73 bind to heme iron in A1 and Lys79 bind to heme iron in A2. The ε-amino N of Lys79 displaces Met80 and binds to iron by rotating residue around its α-C atom[9]. The binding of Lys72 or 73 upsets the regular second structure. The alteration of the chemical shifts in Lys72 or 73 binding form is more noticeable than in the Lys79 binding form as mentioned above.

3.3. The effect of temperature on the alkaline isomers of cyt *c* in the presence of γ-MePy

The down-field region of the spectra of cyt *c* at pH 9.3 in the presence of 10 mM γ-MePy at different temperature are given in Fig. 3. The temperature dependent

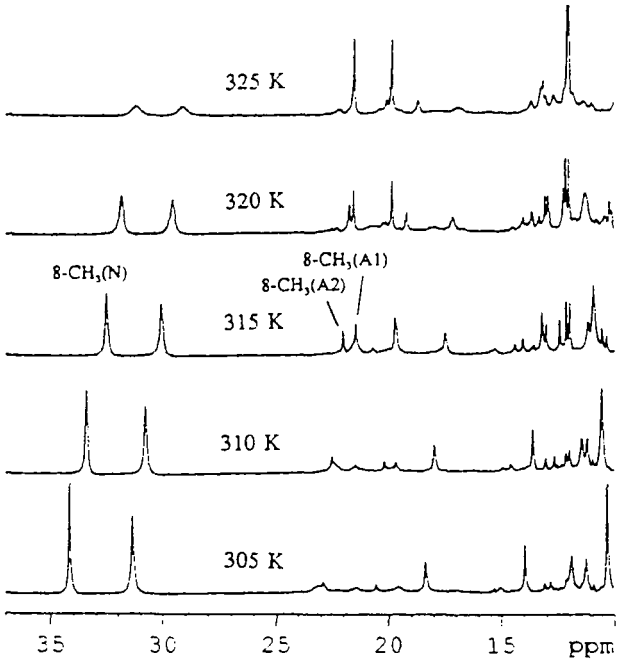

FIG. 3. Stack plot of the down-field region of the temperature dependent ^{1}H NMR spectra of cyt *c* in D_2O at pH 9.3 and in the presence of 10 mM [γ-MePy]. $C_{cyt\ c}$ = 5 mM, N, native form; A, alkaline form.

spectra are similar to those obtained with different concentration of γ-MePy. When temperature increases, the intensity of the total of the two lysine forms increases while that of the native form decreases. At temperatures below 313 K, the intensity of the heme 8-CH_3 signal of A1 is smaller than that of A2. When temperature is greater than 313 K, the intensity of A1 becomes larger than that of A2. It suggests that A2 is more stable than A1 at lower temperatures. When raising temperature, the protein becomes more flexible and A1 is more stable. This might be associated with the second structure of the protein. The random coil structure is considered to be more sensitive to the temperature than the regular second structure. This further implies that Lys79 binds to heme iron in A2. Davis et al reported that pH-induced and temperature-induced conformational changes in cyt *c* represented

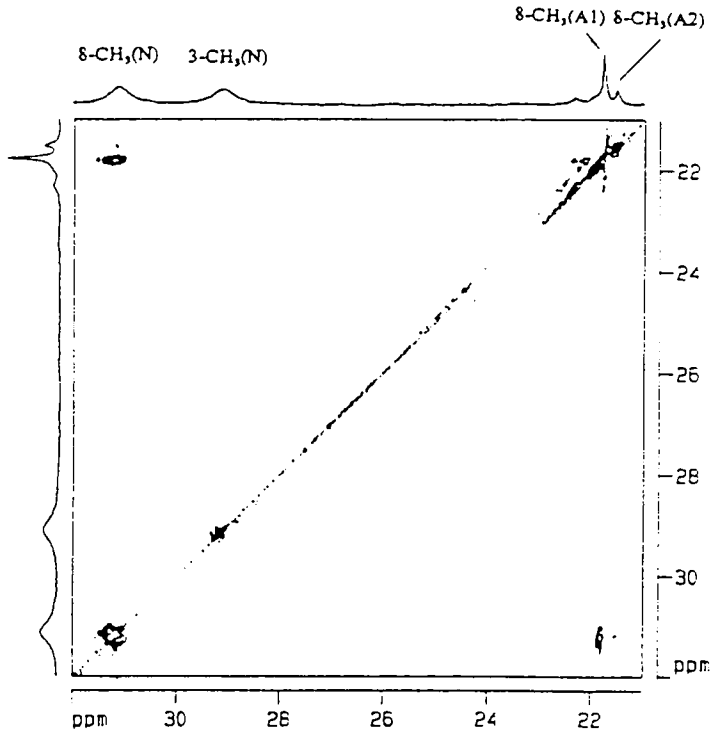

FIG. 4. The down-field region of 2D EXSY spectrum of cyt *c* at pH 7.2 and 50 °C in the presence of 6.7 mM γ-MePy with mixing time of 50 ms. $C_{cyt\ c}$ = 6 mM, N, native form; A, alkaline form.

identical rearrangement of protein chain[9]. It seems that the conformational changes in cyt *c* induced by [γ-MePy] are similar with that induced by temperature too.

3.4. Evaluation of the rate constant

In order to investigate the effect of γ-MePy on the kinetics of alkaline transition, the forward and reverse rate constants for the formation of the two alkaline isomers have been measured using 2D EXSY in the presence of 6.7 mM [γ-MePy] at 50 °C and pH 7.2. The exchange can be visualized from the cross-peaks between the heme methyl groups of the native species and those of the two alkaline forms (Fig. 4). There is no cross-peaks between the methyl groups of the two alkaline species.

Based on the heme 8-CH_3 group, forward rate constants of the transform from the native species to the two alkaline forms k_1 and k_2 are 3.89 s^{-1} and 0.94 s^{-1} respectively[27,28]. The corresponding reverse rate constants k_{-1} and k_{-2} are 5.59 s^{-1} and 4.63 s^{-1} respectively. So, the transition constants for the alkaline transition, K_1 and K_2, are 0.696 and 0.203 for A1 and A2 respectively. The ratio of K_1/K_2 is about 3.43. The intensity ratio of A1/A2 in the 1D spectrum is 3.42 (Fig. 4). It indicates that the two values are consistent with each other.

Dixon et al found that the kinetic behavior of the transitions between native and two alkaline forms is very similar when no external ligand is present. The forward rate constant (native to alkaline cyt *c*) has a value of 4.0±0.6 s^{-1} at 27 °C and is independent of pH. The reverse rate constant is pH-dependent[5]. However, the kinetic behavior for the formation of the two alkaline isomers have changed in the presence of very small amounts of γ-MePy. This further implies that the presence of γ-MePy stabilize only one form of alkaline isomers and inhibit the formation of the other which are Lys72 or 73 and Lys79 respectively.

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