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吡啶、2-甲基吡啶存在下细胞色素 c 碱式异构化和配体-细胞色素 c 配合物的电化学研究

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用半胱氨酸修饰的金电极研究了吡啶、2-甲基吡啶存在下细胞色素 c 碱式异构化和配体结合细胞色素 c 的电化学。在此电极上, 细胞色素 c 可发生准可逆的电极反应而吡啶结合细胞色素 c 和 2-甲基吡啶结合细胞色素 c 在循环伏安图上只给出还原峰。高浓度($1.27 mol \cdot L^{-1}$)的吡啶和 2-甲基吡啶可诱导碱式细胞色素 c 在中性条件下生成。进一步的研究表明, 这种诱导作用与配体和细胞色素 c 的键合无关。

关键词:　循环伏安　细胞色素 c　碱式异构化
分类号:　O614.81　O626　O646

Electrochemical Study on the Alkaline Isomerization of Cytochrome c and Ligand-Binding Cytochrome c in the Presence of Pyridine and 2-methyl-pyridine

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The alkaline isomerization of horse heart cytochrome c and ligand-binding cyt c in the presence of pyridine and 2-methyl-pyridine has been studied using cyclic voltammetry at a gold disc electrode modified with cysteine over a range of solution pH. At this modified electrode, cyt c shows a quasi-reversible electrode process while the cyclic voltammograms of alkaline cyt c and py•cyt c only give cathodic peaks. At neutral pH, high concentrations ($1.27 mol \cdot L^{-1}$) of py and 2-mpy caused the formation of alkaline cyt c which appeared only when pH was above 9 in the absence of such ligands. These results confirm the conclusion of previous NMR studies that py and its derivatives induce the formationof the alkaline cyt c at neutral pH. Further investigation indicates that the inducement function is not involved in the ligand's coordination to the heme iron.

Keywords:　**cyclic voltammetry**　**cytchrome c**　**alkaline isomerization**

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0 Introduction

Cyt c is one of the most extensively studied electron-transfer proteins. As pH rises, the protein undergoes a series of conformational changes and gives rise to the so-called alkaline form of cyt c with a pKa about 8.5 ~ 9 depending on species[1~4]. The alkaline isomerization of cyt c has attracted considerable attention in recent years. NMR studies of the hyperfine shift of horse heart cyt c and horse heart cyt c mutants 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[2,3].

As well known, cyt c undergoes a substitution reaction with exogenous ligands[5-8]. Recent studies have discovered that ligands, such as py and its derivatives, can facilitate the formation of the alkaline form of cyt c and selectively stabilize one conformer of alkaline cyt c at enough concentration of suchligands[7,8]. In other words, in the presence of these ligands, the alkaline form of cyt c appears at neutral pH. In addition, site-specific variants of cyt c have been described that are surprisingly altered in their conformational stability[9]. Amino acid substitution at certain positions yields proteins in whichthe alkaline form is the most thermodynamically stable form of cyt c at neutralpH. For example, the pKa of wild-type yeast cyt c is 8.5 while the pKa of Ser82 and Ile82 mutated yeast cyt c is 7.7 and 7.2 respectively[16]. These results suggest that both exogenous ligands and amino acid substitution at specific positions have effects on the alkaline isomerization of cyt c.

Cyclic voltammetry, as an efficient method to investigate metalloproteins, is suitable to the identification and electrochemical characterization of interconverting species in complex protein solutions. The electrochemistry of cyt c has been widely studied in recent two decades[10~15,17]. Important considerations include electrode materials and their pre-treatment, protein concentration, pH and ionic strength as well as the exact nature of the other species present in solution. It appears that the nature of the electrode/solution interfaces is a critical factor in the electron-transfer process. For most proteins, to obtain an identified and good-shaped voltammogram, promoters or chemically modified electrodes should be employed[18-19]. However, most previous electrochemicalstudies only focused on the thermodynamic properties and electrochemical parameters of native cyt c. The electrochemistry of the alkaline form of cyt c and ligand-binding cyt c in the presence of exogenous ligands has been rarely reported. In this paper, we report a cyclic voltammetry study on the alkaline isomerization of cyt c and ligand-binding cyt c in the presence of py and 2-mpy usinga cysteine modified gold disc electrode.

1 Experimental

1.1 Materials

Horse heart cyt c (type Ⅵ) was purchased from Sigma Chemical Co. It was used without further purification. Py and 2-mpy were of chemical purity and wereredistilled before used. Lcysteine was bought as a biochemical reagent in the form of hydrochloride salt. All other reagents were of analytical grade. All solutions were prepared with deionized water.

1.2 Measurements

Electrochemical experiments were performed using a PAR M273 potentiostat (PAR Princeton, NJ) which was connected to a COMPAQ computer and controlled by PAR M270 software. The electrochemical cell was of all-glass construction, approximately 2 mL in volume, equipped with a conventional three-electrode system. A modified gold disc electrode was used as the working electrode, with a platinum wire as counter and a saturated calomel electrode as reference electrodes. Priorto use, the gold electrode was polished using fine alumina suspension and then washed with distilled water. The modification of the electrode was completed by dipping the gold electrode in 10 mmol·L^{-1} cysteine solution for 2 hours and then rinsing with distilled water. Oxygen was removed by bubbling anaerobic argon into the solution for 15min. The cell system was calibrated by 0.1 mol·L^{-1}KCl including 1.0 mmol·L^{-1} $K_3Fe(CN)_6$. A nice cyclic voltammogram was observed with an $E^{0'}$ of 240 mV vs SCE and the peak separation of 70 mV. All experiments reported were carried out in a mixed buffer system consisting sodium phosphate(0.02 mol·L^{-1}), $NaClO_4$(0.1 mol·L^{-1}), cyt c (0.5 mmol·L^{-1}) and py/2-mpy (1.27 mol·L^{-1}). The pH was adjusted with small amount of NaOH and $HClO_4$. All the potential reported in this paper are referenced to SHE unless otherwise stated.

The ^1H NMR experiments were performed on a Bruker AM 500 spectrometer equipped with an Aspect 3000 computer system. Assignments were made using UXNMR software on a Silicon Graphics Indy workstation. All the samples were prepared in D_2O and the pH was adjusted by adding small aliquots of DCl. The pH readings were uncorrected for the isotope effect.

2 Results and discussion

2.1 Cyclic voltammetry of cyt c at different pH

Table 1 Results of Cyclic Voltammetric Studies of cyt c, py-cyt c and 2-mpy-cyt c Systems (0.5 mmol · L^{-1} cyt c, 1.27mol · L^{-1} py/2-mpy, 0.02mol · L^{-1} sodium phosphate buffer, 0.1mol · L^{-1} $NaClO_4$)

system	pH	native cyt c $E^{0'}$/mV(ΔE_p/mV) vs SHE	py · cyt c $E_{p,c}$/mV vs SHE	alkaline cyt c $E_{p,c}$/mV vs SHE
cyt c	7.1	245(110)	—	—
	9.6	—	—	-170
py + cyt c	5.6	282(116)	16	—
	7.1	278(112)	—	-140
2-mpy + cyt c	5.6	308(172)	—	—
	6.9	266(180)	—	—
	7.2	—*	—	-250

— no peak

—* weak response not to be properly recognized

Fig. 1 shows the voltammograms of cyt c at a gold disc electrode modified with cysteine at neutral and alkaline pH conditions. At neutral pH (7.1), the voltammogram of native cyt c shows a couple of quasi-reversible redox peaks with an $E^{0'}$ of 245 mV and ΔE_p of 110 mV (Table 1). The midpoint potential $E^{0'}$ compares favorably with the value described in previous literature[10, 15].

While at alkaline pH (9.6), the formation of alkaline cyt c from native cyt c gives a cathodic

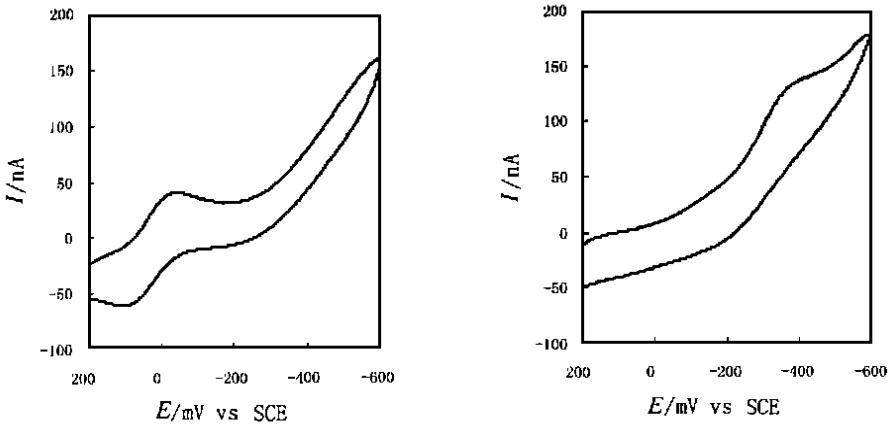

Fig. 1　Cyclic voltammograms of 0.5 mmol · L^{-1} cyt c at a cysteine modified Au, 0.1 mol · L^{-1} $NaClO_4$, 0.02 mol · L^{-1} sodium phosphate buffer, scan rate: 50 mV · s^{-1} (a) pH 7.1 (b) pH 9.6

reduction peak without a corresponding oxidation peak. The cathodic peak potential $E_{p,c}$ is − 170 mV, which is close to the results obtained before[15]. This phenomenon can be interpreted as follows: as pH rises(> 9), Met80 is substitutedby a surface lysine and cyt c transforms into the alkaline form. When ferric alkaline cyt c is reduced, its product ferrous alkaline cyt c, is unstable and converts to ferrous native cyt c immediately, so the oxidation peak could not observed unless the scan rate is high enough[16]. However, we did not observe the oxidation peak of ferrous alkaline cyt c although we tried to increase the scan rate to 2 V•s^{-1}.

2.2　Cyclic voltammetry of cyt c in the presence of py at different pH

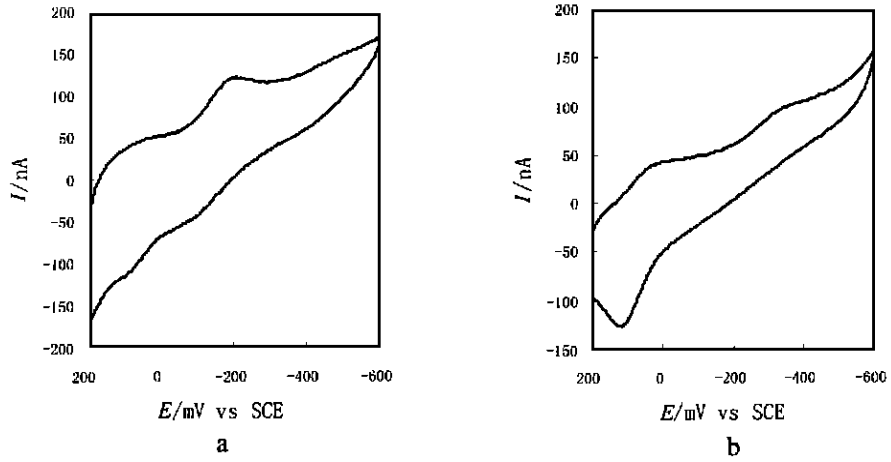

Fig. 2　Cyclic voltammograms of 0.5 mmol · L^{-1} cyt c at a cysteine modified Au electrode, 1.27 mol · L^{-1} py, 0.1mol · L^{-1} $NaClO_4$, 0.02 mol · L^{-1} soduim phosphate buffer, scan rate: 50 mV · s^{-1} (a) pH 5.6 (b) pH 7.1

Fig. 2 is the cyclic voltammograms of cyt c in the presence of py at different pH. At pH 5.6, comparing with Fig. 1a, cyclic voltammogram (Fig. 2a) shows a new peak except the peaks of cyt c. Previous NMR studies showed that py could displace Met80 of cyt c and produce py•cyt c

when pH is less than 6.4 .[7]According to those results, the peak can be assigned tothe reduction peak of py•cyt c. It is interesting that the oxidation peak corresponding to reduced py•cyt c is missed. This is because reduced py•cyt c is unstable and disassociates to ferrous native cyt c and py rapidly. At neutral pH, quite different from cyt c in the absence of py, the peak of alkaline cyt c appears in the cyclic voltammogram (Fig. 2b). These results show that in the presence of 1.27 mol•L^{-1} py, native cyt c and alkaline cyt c can coexist at neutral pH while previous studies indicated that alkaline cyt c appeared only when pH is raised to above about 9 in the absence of exogenous ligands.

NMR studies on py binding to cyt c have been thoroughly investigated and it was suggested that py can facilitate the formation of alkaline cyt c at neutral pH[7]. Our work in this paper supported this conclusion. However, whether this function is related to the binding of py to cyt c is still unclear. Trying to answer this question we carried out the following experiments.

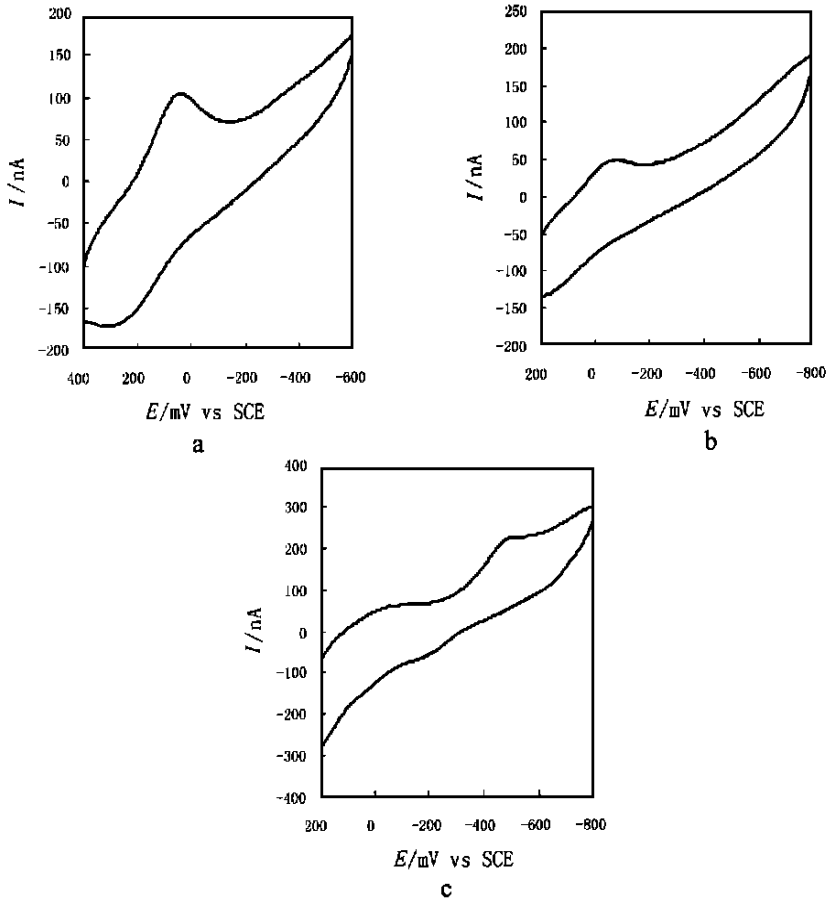

Fig. 3　Cyclic voltammograms of 0.5 mmol · L^{-1} cyt c at a cysteine modified Au electrode, 1.27 mol · L^{-1} 2-mpy, 0.1mol · L^{-1} NaClO4, 0.02 mol · L^{-1} sodium phosphate buffer, scan rate: 50 mV · s^{-1}
(a) pH 5.2 (b) pH 6.9 (c) pH 7.2

2.3　Cyclic voltammetry of cyt c in the presence of 2-mpy at different pH

Fig. 3 gives the cyclic voltammograms of cyt c in the presence of 2-mpy. At pH 5.6, the

cyclic voltammogram shows only one couple of redox peaks of native cyt c without anynew peak (Fig. 3a), indicating that 2-mpy can not bind to cyt c due to the severe spatial interaction between the molecule and the heme. Similar to py-cyt c, 2-mpy can also induce the formation of alkaline cyt c at neutral pH (Fig. 3c). At the same pH and in the same buffer system, we find that the potentials inthe presence of py or 2-mpy are different from those in the absence of these molecules(Table 1). Moreover, the presence of py or 2-mpy causes a positive shift of the midpoint potential of cyt c and the midpoint potential in the presence of 2-mpy is higher than that in the presence of py. Comparing to the potential in the absence of such molecules, there are also some noticeable changes in the peak potential of the alkaline cyt c in the presence of py or 2-mpy. These variations in the redox potentials may be attributed to the following reasons: in the presence of py or 2-mpy, the accessibility of these molecules to the active site of cyt c causes the exposure of the heme to the solvent and the hydrophobicity of the interior of the protein changed[20,21].

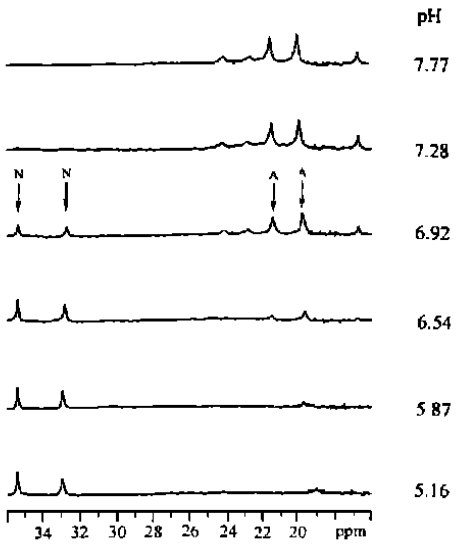

Fig. 4 Downfield hyperfine-shifted region of the ^{1}H NMR spectra in D_2O at 298 K at different pH, 5 mmol · L^{-1} cyt c, 1.27 mol · L^{-1} 2-mpy (Resonances due to native cyt c and alkaline cyt c are labeled with N and A respectively.)

To verify the effects of 2-mpy on the alkaline isomerization of cyt c, we made the 1H NMR experiment (Fig. 4). The NMR spectra of cyt c in the presence of2-mpy show that even when pH decreases to about 5, there is no new peak except the peaks of native cyt c. This shows that different from py, 2-mpy can not bind to cyt c. At neutral pH (about 7.0), the resonance peaks of alkaline cyt c appear at the expense of the decrease of peaks of native cyt c. When pH is above7.28, no other peaks are observed except the peaks of the alkaline form of cyt c. All these indicate that 2-mpy also can facilitate the formation of the alkaline form of cyt c at neutral pH although it can not directly bind to the heme iron.

The details that how py and 2-mpy can make alkaline cyt c form at neutral pH is still not very clear. However, our experiments gave some useful clues to that procedure. 2-mpy can not bind to cyt c because of the severe interaction between its methyl group and the heme, but this molecule also has the ability to facilitate the formation of alkaline cyt c at neutral pH as py does. This phenomenon suggests that the effects of py and 2-mpy on the alkaline isomerization of cyt c may not be involved in the direct binding of these molecules to the hemeiron of cyt c. These molecules only penetrate into the heme pocket and modulatethe environment of the heme vicinity and then cause the alkaline form more stable than the nature form.

Other examples of conformational changes induced by not directly altering the axial coordina-

tion in metalloproteins have also been reported. ESR studies suggested that py induced a structural modification in the heme environment of cyt b 558 by shifting the fifth heme ligand to a nearby thiolate group without direct binding of py to the heme and this procedure caused a cyt P450-like structure of cyt b_{558}[22]. In cyt c, replacement of Phe82 with histidine generates a mutant cyt c in which His18 and Met80 are coordinated in the iron Ⅱ complex, whereas His18 and His82 are coordinated in the iron Ⅲ state[23].

3 Conclusion

The suggestions that py and its derivatives can induce the formation of the alkaline form of cyt c[7, 8] are confirmed by using cyclic voltammetry. The affinity constant for py binding to cyt c is 1.4 $L \cdot mol^{-1}$[8], while 2-mpy cannot bind to cyt c. It seems that these molecules only penetrate into the heme pocket and induce a structural modification in the heme surrounding environment which trigger the alkaline transition at neutral pH. Results of this work and NMR studies strongly suggest that the affect of py and its derivatives on the alkaline isomerization of cyt c might not be involved in the binding of these exogenous ligands to the heme iron.

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