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THE EFFECTS OF TEMPERATURE AND ELECTROLYTE ON THE REDOX POTENTIAL OF CYTOCHROME *C* AT A CHEMICALLY MODIFIED MICROBAND GOLD ELECTRODE

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Abstract—The dependence of formal potential ($E^{0\prime}$) on temperature for cytochrome *c* in phosphate buffer solution at various pH was investigated by the microband gold electrode modified with a new promoter 4,6-dimethyl-2-mercaptopyrimidine (DMMP). The temperature dependence of the $E^{0\prime}$ showed a biphasic character in slightly alkaline solution with a turning point at *ca.* 45°C. However, the $E^{0\prime}$ values decreased monotonically with the increasing temperature in acidic and neutral media. The thermodynamic parameters of the electron transfer reaction for cytochrome *c* at the chemically modified microband electrode were estimated. The $E^{0\prime}$ and the changes of standard Gibb's free energy (ΔG^0), entropy (ΔS^0) and enthalpy (ΔH^0) for the reduction of ferricytochrome *c* at 25°C and pH 7.0 were 0.272 V (vs. NHE), $-26.2\,kJ\,mol^{-1}$, $-123.7\,J\,mol^{-1}\,K^{-1}$ and $-63.1\,kJ\,mol^{-1}$, respectively. The effect of buffer component on the $E^{0\prime}$ was also studied in binding (phosphate) and nonbinding (Tris/cacodylic acid) neutral buffer media. The results showed that the $E^{0\prime}$ in Tris/cacodylic acid buffer solution shifted positively by about 10 mV in comparison with that in phosphate buffer solution.

Key words: microband gold electrode, promoter, thermodynamics, cytochrome *c*, 4,6-dimethyl-2-mercaptopyrimidine.

INTRODUCTION

The electrochemistry of metalloproteins is a subject of great interest. Mammalian cytochrome *c* is a water—soluble heme protein that exists in the cytosol between the inner and outer membranes of mitochondria. It plays an important role in the biological respiratory chain, its function is to receive electrons from cytochrome *c* reductase and deliver them to cytochrome *c* oxidase. More recently, the electrochemistry of cytochrome *c* has been studied extensively at various electrode surfaces[1–3] due to the fact that some analogies exist between the electrode reactions of the metalloproteins and their interactions in biological redox system, the information obtained from studying the interactions of the metalloproteins with electrodes will help in improving understanding of the electron transfer mechanism of the metalloproteins *in vivo*. However, electrochemical studies of cytochrome *c* at bare metal electrodes have shown significant irreversible characteristics. The modification of electrode surface with appropriate compounds, called promoters, paved a way to increase the electron transfer rate between cytochrome *c* and electrode[4]. Recently, we have reported that the 4,6-dimethyl-2-mercaptopyrimidine (DMMP) can be used as a new and effective promoter for the redox reaction of cytochrome *c* when it is adsorbed on the microband gold electrode surface[5]. In the present work, the effects of temperature, pH and electrolyte on the redox potential of cytochrome *c* are studied at the microband gold electrode modified with a new promoter DMMP.

Microelectrodes have received considerable attention in some applications due to their many advantages in comparison with conventionally sized electrodes, it has attracted great interest for the determination of biomolecules in special environment, for example, it have been used to monitor the level of neurotransmitter and its release in brain tissue of a live animal or a single cell[6]. Although many studies of the electrochemistry of cytochrome *c* have been made by various electrodes[1–5, 7–9], to our knowledge, there is no report of electrochemistry of cytochrome *c* at a gold microelectrode, and there are few reports of studies of the redox thermodynamics of cytochrome *c* [7–9]. In order to obtain more redox information of cytochrome *c*, it is necessary to study the redox thermodynamics of cytochrome *c* and acquire its thermodynamic parameters at various electrode surfaces. The goal of this work is to obtain more knowledge about how and how much the environmental conditions, such as temperature and pH etc., affect the redox behaviour of cytochrome *c*. Such information would facilitate to understand better the electron transfer reaction of cytochrome *c* both *in vitro* and *in vivo*. Simultaneously, this work will present a method for the studying electrode reaction thermodynamics by a microband gold electrode.

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EXPERIMENTAL PROCEDURES

Chemicals

Horse heart cytochrome *c* (Type VI, Sigma Chemical Co.) and 4,6-dimethyl-3-mercaptopyrimidine (DMMP) (Aldrich Chemical Co.) were used as received without further purification. All other chemicals were of analytical grade. The phosphate and Tris/cacodylic acid buffer solutions were made up from Na_2HPO_4 and KH_2PO_4, tris(hydroymethyl)aminomethane and cacodylic acid (hydroxydimethylarsine oxide), respectively. The ionic strength of all buffer solutions is 0.2 M. Water, twice distilled from quartz apparatus, was used in all experiments.

Instrumentation

A PAR M270 Electrochemical System (EG&G, USA) was used for electrochemical experiments. A nonisothermal electrochemical cell was employed in which a platinum wire was used as the counter electrode, a saturated calomel electrode (*sce*), which was isolated from the thermostated region and remained at room temperature, as a reference electrode and a chemically modified microband gold electrode (*ca.* 0.1 μm × 1.0 cm) as the working electrode.

Procedures

The fabrication and method of polishing of the microband gold electrode were the same as previously reported[5]. The electrode was modified by immersing the electrode into 5×10^{-3} M DMMP solution (pH 7.0) for some time in order to apply a film, and then transferring it to cytochrome *c* solution. Voltammetric experiments were performed in the potential range of -0.15–$+0.3$ V (vs. *sce*) at different temperatures which was controlled by a thermostat to ± 0.1°C. After deaeration with pure N_2 for 30 minutes, the electrochemical experiments were carried out under a N_2 atmosphere. All electrochemical measurements were performed inside a Faraday cage. Formal potentials ($E^{0\prime}$) were estimated from the median of the anodic and cathodic peak potentials obtained at low scan rate. The *sce* was assumed to be 244 mV relative to NHE.

In order to test the nonisothermal behaviour of the cell used in this work, a model system of $K_3Fe(CN)_6$ was chosen. The formal potential of the redox couple at a gold band microelectrode decrease linearly with increasing temperature. In our nonisothermal cell, only the temperature of the redox half-cell of interest was varied. The temperature of the *sce* half-cell was maintained at constant room temperature. As described previously[10], the temperature coefficient of thermal junction potential was only a few microvolts per degree, they can be neglected in comparison with the overall temperature coefficient of the nonisothermal cell. A reaction entropy change of 144.8 J mol^{-1} K^{-1} is obtained from dE^0/dT, and the formal potential for reduction of $K_3Fe(CN)_6$ at 25°C is 0.42 V (vs. NHE), these values are in good agreement with those previously reported[10] at a conventionally sized electrode. These results prove that the cell behaved nonisothermally.

RESULTS AND DISCUSSION

Voltammetric behavior of cytochrome c at DMMP/Au microband electrode

Figure 1 shows the cyclic voltammograms of cytochrome *c* at various scan rates (20–100 mV s^{-1}). The ratio of anodic to cathodic peak currents at various scan rates, i_{pa}/i_{pc}, is almost unity. Both cathodic and anodic peak currents increase linearly as a function of the square root of the scan rate. The separation of anodic and cathodic peak potentials at low scan rates is about 68 mV. A further increase in the scan rate resulted in a large separation of peaks. For example, a separation of *ca.* 94 mV occurs at 100 mV s^{-1}. These results indicate that the electrode reaction is quasi—reversible process. Using Nicholson's method, the heterogeneous electron transfer rate constant of cytochrome *c* at DMMP/Au electrode was evaluated to be *ca.* 6.6×10^{-3} cm s^{-1}. Similar voltammograms are also observed at various temperatures and pH. The $E^{0\prime}$ value obtained at pH 7.0 phosphate buffer solution is 28 mV. However, at a bare Au microband electrode, the electrochemical response of cytochrome *c* is not observed, therefore the DMMP adsorbed on the Au electrode surface can acts as an effective promoter for promoting direct redox reaction of cytochrome *c*.

The dependence of $E^{0\prime}$ on temperature

The cyclic voltammograms of cytochrome *c* at the DMMP/Au microband electrode at different temperatures (pH 7.0 with an ionic strength of 0.2 M) are similar to that shown in Fig. 1. But with increasing temperature, the cathodic and anodic peak poten-

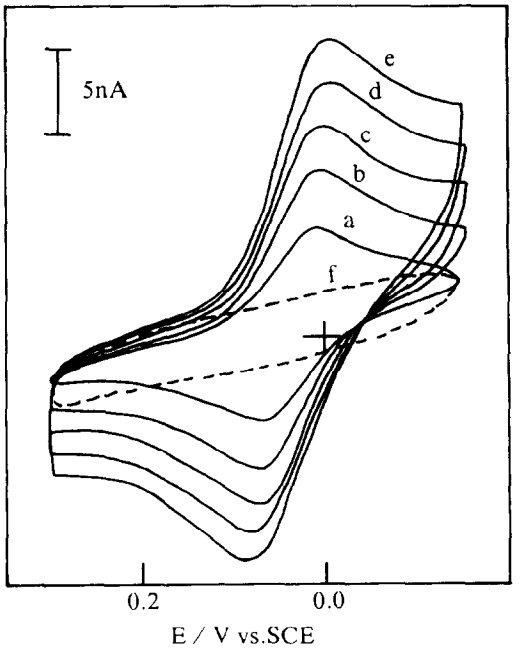

Fig. 1. Cyclic voltammograms of 3.4×10^{-4} M cytochrome *c* at DMMP/Au microband electrode in phosphate buffer (pH 7.0) at (a) 20 mV.s^{-1}; (b) 40 mV s^{-1}; (c) 60 mV s^{-1}; (d) 80 mV s^{-1}; (e) 100 mV s^{-1}, and (f) without cytochrome *c* at 20 mV s^{-1}.

tials shift in the negative direction, which is similar to the behaviour of the $K_3Fe(CN)_6$ redox system. However, the negative shift of the cathodic peak is larger than that of the anodic peak. These results are similar to those obtained at a glassy carbon electrode[7]. The difference is probably due to the fact that the conformation of the ferro- and ferricytochrome *c* is affected differently by temperatures[11, 12]. The effect of temperature on the conformation of ferri- and ferrocytochrome *c* has been widely investigated[11–14]. The heme group in ferrocytochrome *c* is neutral, thus ferrocytochrome *c* is very stable and can retain its native conformation in the temperature range 4–97°C at pH 7.0 and from pH 4 to 12 at 25°C[11] except for minor conformational changes. Ferricytochrome *c* has a net positive charge +1 on the heme group, and hence electrostatic repulsion between the positively charged heme group and the positively charged lysine residues reduces its stability. However, with the increasing temperature, the Fe-S (the sulfur of the Met-80 residue) bond in ferricytochrome *c* gradually weakens[12] resulting from the increasing bond length due to the increasing of vibration energy. The residues adjacent to the Met-80 group are also flexible[9], the crevice around the solvent—exposed heme edge allows greater exposure of the heme to solvent, which stabilizes the positively charged heme group of ferricytochrome *c*. Therefore, the reduction of ferricytochrome *c* requires more negative potential. As a result, a more negative shift of the cathodic peak with increasing temperature is observed. The values of $E^{0\prime}$ as a function of temperature in phosphate buffer (pH 70) are shown in Figure 2b. Clearly, the $E^{0\prime}$ decreases linearly with increasing temperature.

The temperature dependence of the formal potential of a redox couple can be used to evaluate the reaction center entropy change, ΔS^0_{rc}. This parameter is $-58.5\,J\,mol^{-1}\,K^{-1}$ in pH 7.0 phosphate buffer. The changes of standard Gibb's free energy (ΔG^0), entropy (ΔS^0) and enthalpy (ΔH^0) for the cell reaction:

ferricytochrome c + $1/2H_2$ → ferrocytochrome c + H^+

can be calculated at 25°C from these equations, $\Delta G^0 = -FE^{0\prime}$, $\Delta S^0 = \Delta S^0_{rc} - 65.2\,J\,mol^{-1}\,K^{-1}$[15], and $\Delta H^0 = \Delta G^0 - T\Delta S^0$. The values obtained using the DMMP/Au microband electrode at pH 7.0 phosphate buffer are $-26.2\,kJ\,mol^{-1}$, $-63.1\,kJ\,mol^{-1}$, and $-123.7\,J\,mol^{-1}\,K^{-1}$ for ΔG^0, ΔH^0 and ΔS^0 respectively. These values are in good agreement with those previously reported using the glassy carbon electrode[7] and PySSPy—modified gold electrode[8].

The effect of pH on $E^{0\prime}$

Figure 2 shows the temperature dependences of $E^{0\prime}$ for cytochrome *c* at various pH. In slightly acidic and neutral solutions, the $E^{0\prime}$ shifts monotonically in the negative direction with increasing temperature. In pH 5.9 phosphate buffer, a ΔS^0 of $-114.5\,J\,mol^{-1}\,K^{-1}$ is obtained. In pH 8.2 slightly alkaline solution, the biphasic behaviour is observed

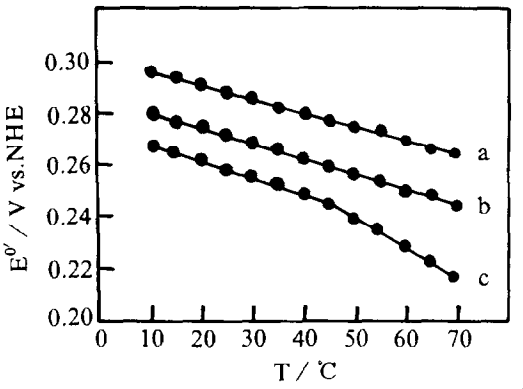

Fig. 2. Effects of pH on the temperature dependence of $E^{0\prime}$ for the reduction of ferricytochrome *c* in phosphate buffer solutions: (a) pH 5.9, (b) pH 7.0, (c) pH 8.2. Results shown are the averages of three separate experiments.

with turning point at *ca.* 45°C, a ΔS^0 of $-127.4\,J.mol^{-1}\,K^{-1}$ and $-175.9\,J\,mol^{-1}\,K^{-1}$ are obtained below and above 45°C. This biphasic dependence of $E^{0\prime}$ is reversible with respect to the temperature. This resembles well the temperature dependence of the absorbance of cytochrome *c* at 695 nm, the decrease of the absorbance with increasing temperature was explicitly biphasic in alkaline solutions but not in acid and neutral solution[16]. Further studies[17–19] showed that the 695 nm band was eliminated by two causes: conformational changes in the protein moiety of cytochrome *c*, and the disruption of the linkage between the heme iron (in ferri-state) and the sulfur of Met-80. If the disruption of Fe(III)—S(Met-80) linkage occurs, it will result in a large negative shift of the formal potential of cytochrome *c*, because the disruption of Fe(III)—S(Met-80) linkage with replacement by other ligand-results in a larger shift of potential toward a more negative direction than native cytochrome c[8]. Thus, the parallel of the temperature dependence in the biphasic behaviour between the $E^{0\prime}$ and the adsorbance at 695 nm of cytochrome *c* suggests that the conformation of the protein moiety in cytochrome *c* molecules changes biphasically with temperature with an intersection point at *ca.* 45°C in an alkaline solution.

The effects of the chloride ion on $E^{0\prime}$

Using the optically transparent thin-layer electrode (OTTLE) technique, Kreishman *et al.*[20, 21] reported that chloride ion caused the discontinuous change of bulk water structure at 42°C due to hydration of chloride ion, $Cl^- + 3H_2O \rightarrow Cl(H_2O)_3^-$, which resulted in a drastic biphasic relationship between $E^{0\prime}$ of cytochrome *c* and temperature in a neutral NaCl solution. Taniguchi *et al.*[8] using a PySSPy—modified gold electrode and Dong *et al.*[7] using a bare glassy carbon electrode studied the effect of chloride ion on the temperature dependence of $E^{0\prime}$ of cytochrome *c*. Their experimental results showed that chloride ion does not contribute to the temperature dependence of $E^{0\prime}$ of cytochrome *c*. In the present experiments, the effect of chloride ion on the temperature dependence of $E^{0\prime}$ is examined again using a DMMP/Au microband electrode.

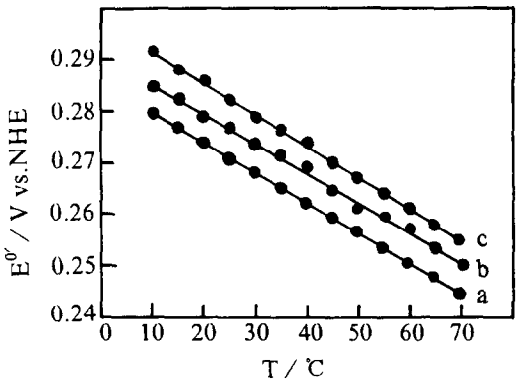

Fig. 3. Temperature dependence of the formal potential for cytochrome *c* in pH 7.0 phosphate (a), phosphate + NaCl (b) and Tris/cacodylic acid (b) buffer solutions. Results shown are the averages of three separate experiments.

The values of $E^{0'}$ as a function of temperature in the neutral NaCl solution are shown in Figure 3b. Clearly, the $E^{0'}$ value also decreases linearly with increasing the temperature without biphasic character, which is similar to that in the absence of chloride ion.

The effect of the buffer component on $E^{0'}$

Two different buffer systems, phosphate and Tris/cacodylic acid buffer solution, which provide extremes for binding and nonbinding media, are selected to probe the effect of buffer component on the formal potential as a function of temperature, which arise from anion binding to cationic lysine residues of cytochrome *c*. Figure 3(a) and 3(c) show the temperature dependence of $E^{0'}$ in phosphate (a) and Tris/cacodylic acid (c) buffers, respectively. It can be seen that the formal potential in phosphate buffer shifts negatively by about 10 mV in comparison with that in Tris/cacodylic acid at same temperature. This difference is attributed to anion binding in the case of phosphate buffer, but not in Tris/cacodylic acid. As mentioned previously, electrostatic repulsion exists between the positively charged heme group and lysine residues. The binding of phosphate anion to the positively charged lysine residues results in an opening of the heme crevice of ferricytochrome *c*, increasing the solvent exposure of the heme group. Thus the charge repulsion between the positively charged heme group of ferricytochrome *c* and the positively charged lysine residues is reduced. An increased stability of the positively charged oxidized form relative to the structure present in the Tris/cacodylic acid nonbinding system would occur. Thus the reduction potential for ferricytochrome *c* in phosphate buffer is more negative than that in Tris/cacodylic acid buffer.

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