Investigation of the electrode reaction of cytochrome *c* and pyrroliquinoline quinone at self-assembled monolayers of amino acid

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Abstract

Self-Assembled monolayers of carboxyl-terminated alkanethiols, which is negatively charged in pH 7.0, were usually used to facilitate the electron transfer between the positively charged protein and the electrode. In case of L-cysteine, as it has both positive and negative group, it can be a candidate for a new modifier to facilitate positively charged protein or negatively charged protein. Our investigation of L-cysteine shows that the electron transfer occurs successfully to both cytochrome *c* (cyt. *c*) and pyrroloquinoline quinone (PQQ). By using 1-ethyl-3-(3-dimethlyaminopropyl) carbodiimide (EDC), we made a covalent bond between cyt. *c* and monolayer. Then PQQ was electrostatically adsorbed to the same monolayer. Cyclic voltammograms show that both molecules do not interfere each other and electron transfer is appreciable.

Key words : Self-Assembled Monolayer, Cytochrome *c*, Pyrroliquinoline quinone, L-Cysteine, Cyclic voltammetry

1. Introduction

The formation of self-assembled monolayers (SAMs) on electrode surfaces has become an important research topic in electrochemistry. The modification of electrodes with these highly organized molecular assemblies, which enables us to study the electron-transfer kinetics in specific and well-controlled microenvironments, has been under extensive investigations during the last few years.[1-3]

SAMs of alkanethiols with specific terminal groups on gold electrodes have been applied successfully for protein electrochemistry recently.[4-7] For example, carboxylic acid-terminated alkanethiol monolayers were used to provide a favorable surface for reversible electrochemistry of cytochrome *c* which is a positively charged protein. An acid-terminated alkanethiol monolayer covered with a polycation, poly-L-lysine, was found to give reversible electron transfer for cytochrome b_5 which is a negatively charged protein.[8]

On the other example, the redox properties of quinoproteins, *i.e.* enzymes containing the quinonoid cofactor (pyrroloquinoline quinone (PQQ)), have been studied by electrochemical methods in immobilized states.[9,10] The PQQ enzymes were used to prepare amperometric biosensors. The artificial electron transfer mediators such as 2,6-dichlorophenolindophenol, ferrocene, and Os complex were used to provide electrical communication between the electrode and enzymes. Without the apoenzyme (*i.e.* an inactive enzyme that must associate with a specific cofactor molecule or ion in order to fuction), PQQ is chemically very active and capable of catalyzing many different oxidation reactions, *e.g.* oxidation

NADH, amines, amino acids, alcohol, glucose *etc.* Therefore it is not surprising that the chemistry of PQQ has been studied extensively. However, only a few reports of the electrochemical properties of PQQ have been published, including potentiometric titration and cyclic voltammetry studies. A new approach that is monolayer immobilization of quinones on a gold electrode surface via chemisorption of sulfur-containing reagents (*e.g.* cystamine) has recently been developed.

In previous work, thiol reagents were described as dual functionality or X-Y promoters because of the high affinity of the X group for the metal electrode surface and the stable interaction of the Y group with the protein surface.[11] So the investigation have been focused upon monolayers which have one kind of Y can bind only same proteins. Hill found that L-cysteine, which has both the amine and carboxylate groups, can enhance the electron transfer on cytochrome *c* through electrostatic bonding.[12]

In this paper, using L-cysteine we investigated the electrochemical behavior of positively charged protein (cyt. *c*) and negatively charged one (PQQ) on same monolayer.

2. Experimental

Horse heart cytochrome *c* (type VI), 1-ethyl-3-(3-(dimethylamino) propyl) carbodimide hydrochloride (EDC), L-cysteine and PQQ (methoxatin, 4,5-dioxo-1H-pyrolo-[2,3-f]-quinoline-2,7,9-tricarboxylic acid) were purchased from Sigma Chemical Co. and Fluka and used as received. All the other reagents were of analytical reagent grade quality and were used without further purification. Ultrapure water from a Millipore Milli-Q was used throughout this work.

A standard three-electrode cell was used with a Ag/AgCl

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reference electrode and a Pt wire counter electrode. A gold disk electrode (geometrical area ca. 0.02 cm^2) was treated with piranha solution (1 : 3, 30% hydrogen peroxide : concentrated sulfuric acid) for 10 min at 80°C, followed by rinsing with ethanol excessively. A cyclic voltammogram recorded in 0.5 M H$_2$SO$_4$ was used to determine the purity of the electrode surface just before modification.

A gold electrode was soaked in a solution of 1.0 mM L-cysteine in water for 5 h. The electrode was then rinsed thoroughly with water to remove the physically adsorbed L-cysteine. A cytochrome c in buffer solution was introduced to the cell cavity and allowed to equilibrate for 10~15 min. An aliquot of concentrated EDC in buffer solution was then added to this solution, and the immobilization reaction was allowed to proceed at room temperature for 2 h. The final solution condition were ~250 µM cytochrome c and ~10 mM carbodiimide in pH 7.5, HEPES (N-[2-Hydroxyethyl] piperazine-N-[2-ethanesulfonic acid]) buffer. The cytochrome c/EDC solution was then removed from the sample cavity, the cell was thoroughly rinsed several times and refilled with 20 mM, pH 7.0 phosphate buffer, and cyclic voltammograms of the covalently immobilized cytochrome c were acquired. Argon bubbling was used to remove oxygen from the solution in the electrochemical cell.

The cyt. c and cysteine-modified electrode was used for the cyclic voltammetry study of PQQ which was electrostatically adsorbed. This electrode was incubated for ca. 30 min in a 3.0 mM solution of PQQ in 20 mM, pH 7.0 phosphate buffer. The electrode was rinsed thoroughly with water to remove the non adsorbed PQQ.

3. Results and Discussion

Cytochrome c interacts strongly with COOH-terminated monolayer as its lysine residues makes the interfacial carboxylate/ammonium ion paring. The PQQ molecule has three carboxylic groups and the charge interaction with the positively charged amino groups on the electrode surface results in strong attraction. The promoting effect for cytochrome c and PQQ electrochemical reaction is usually provided by an organic monolayer which lowers down a barrier to electron transfer. The effect strongly depends on the charge state of the surface (Fig. 1).

As it was mentioned before, L-cysteine has both carboxylate and amine groups and it is zwitterion form at pH 7.0. L-cysteine binds to the gold electrode via the thiol group and provide a functional groups that can interact with both the lysines of cytochrome c and the carboxylic group of PQQ (Fig. 2).

At first cytochrome c was immobilized by using EDC (Fig. 3). EDC, a zero-length cross-linking reagent, has been routinely to form stable and covalent protein/protein complexes through the formation amide bonds between complementary amino and carboxyl groups. Fig. 4 shows cyclic voltammograms of cytochorme c on 20 mM phosphate buffer solution. A redox response of cytochorome c was observed. An anodic peak and a cathodic peak were observed at +133 mV and 81
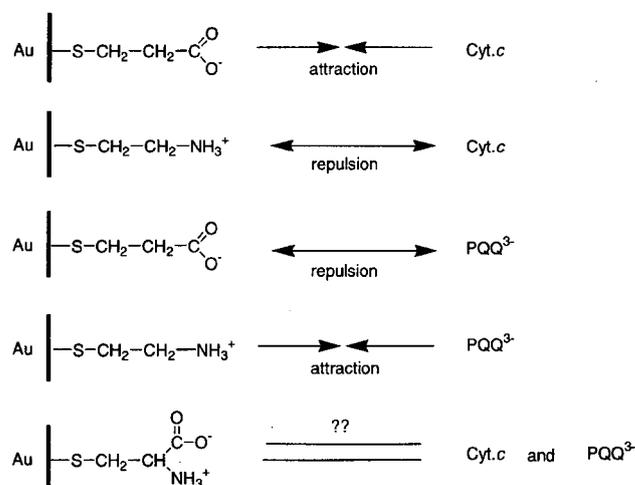

Fig. 1. Charge effect of different monolayers on the electrochemistry of Cyt. c and PQQ.
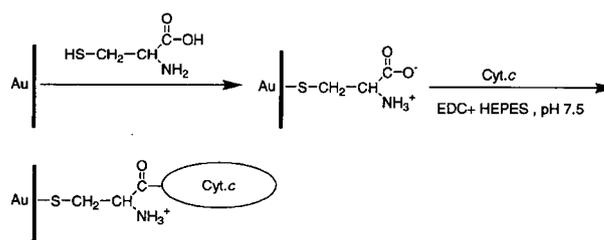

Fig. 2. Stepwise organization of the covalently modified Cyt. c/L-Cysteine monolayer Au electrode.
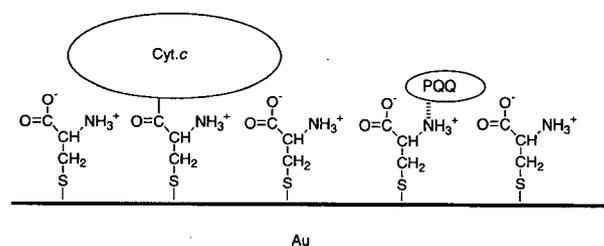

Fig. 3. Schematic diagram of Cyt. c and PQQ/L-Cysteine-modified Au electrode.

mV (scan rate; 20 mV/s) respectively. Hill and coworkers have investigated the redox properties of cytochrome c on the gold electrode modified with mercaptopropionic acid and reported that the separation on this modified electrode was 170 mV (sweep rate : 50 mV/s). Our result was similar to that. As compared with electrochemical responses of cyt. c on 3-mercaptopropinic acid monolayer, the peak current of cyt. c on L-cysteine monolayer was decreased. The decreased peak current reflects the incomplete monolayer or the presence of nonelectroactive cytochrome c. Denaturation or nonoptimal electron transfer orientations via covalent attachement to backside lysing groups on cytochrome c are possible. Also EDC can make the covalent bonding between amino and carboxyl groups within L-cysteine and these bonding intercept the bond formation of cyt. c and monolayer. The chemical modification of a gold electrode with a monolayer of chemisorbed L-cystein can enable immobilized PQQ to
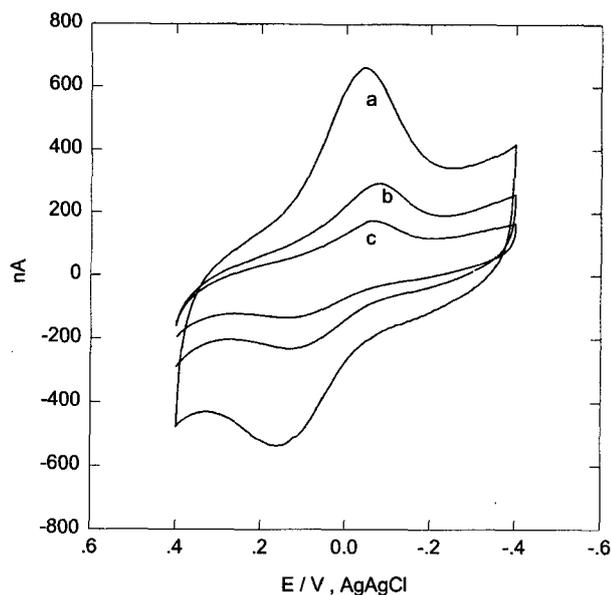
Fig. 4. Cyclic voltammograms of Cyt. c covalently modified L-Cysteine monolayer in 20 mM phosphate buffer (pH 7.0) at different scan rate; (a) 50 mV/s (b) 20 mV/s (c) 10 mV/s.

Fig. 6. Cyclic voltammograms of (a) Cyt. c covalently immobilized on L-Cysteine monolayer, (b) PQQ electrostatically adsorbed on L-Cysteine monolayer, (c) Cyt. c covalently immobilized and PQQ electrostatically adsorbed on same monolayer.

at pH 7.0 is very different from the redox potential in this report. It was the negative charged of carboxylate group that electrochemical reaction of PQQ was suppressed on an L-cysteine electrode surface. Also, the E is rather large (200 mV).

Fig. 6 shows cyclic voltammograms of L-cysteine monolayer which has both cyt. c and PQQ. As compared with Fig. 4 and Fig. 5, it shows nearly similar peak potentials. It means that positively charged cyt. c connects carboxylic group on monolayer and negative charged PQQ is electrostatically bound on same monolayer simultaneously. Therefore, carboxylic and amino group on Au electrode does not present a barrier seriously to electron transfer through zwitterion (i.e. cyt. c vs amino group, PQQ vs carboxylic group).

As the size of cyt. c is very larger than that of PQQ, previous adsorption of cyt. c can lower the spaces which PQQ will be located. The proper exposed time of adsorption for each molecules may be used to control the ratio.
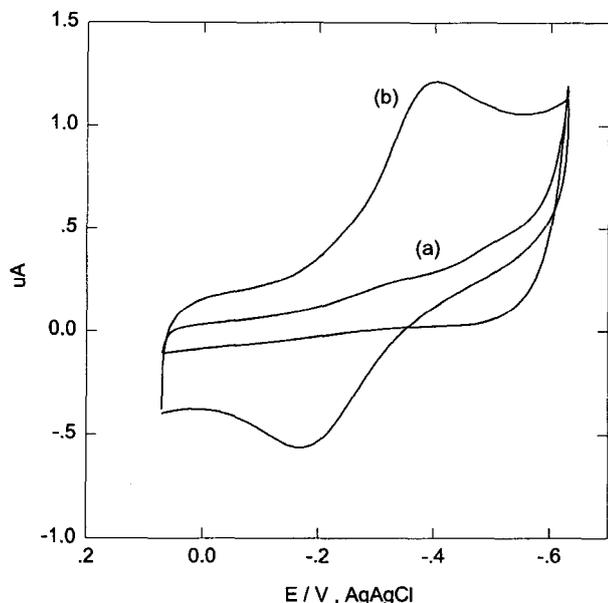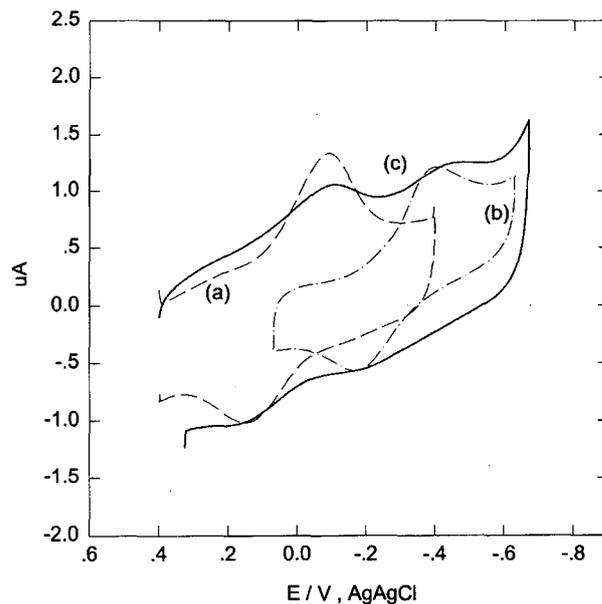

Fig. 5. Cyclic voltammograms of (a) L-Cysteine monolayer covered Au electrode (b) PQQ electrostatically adsorbed on this monolayer. scan rate; 100 mV/s, solution; 20 mM phosphate buffer (pH 7.0).

4. Conclusion

The monolayer of chemisorbed L-cysteine on a gold electrode can enable cytochrome c and PQQ to undergo reversible electrochemical reactions. The negatively charged carboxylic groups on the electrode surface are principally involved in the promoting effect for cyt. c. The positively charged amino groups have same effect on the PQQ. It was shown that L-cysteine monolayer can be used simultaneously as a mediator for covalently immobilized of cyt. c and electrostatically adsorbed of PQQ. In conclusion, carboxylic groups do not hinder the electron transfer of the negatively charged PQQ and amino groups do not hinder the electron transfer of the positively charged cyt. c.

undergo an electrochemical reaction (Fig. 5). The positively charged amino groups on the electrode surface are principally involved in the promoting effect. The redox reaction of PQQ is known as a one-step two-electron transfer mechanism.[13,14] This means that the PQQ semiquinone is very unstable (at least at neutral pH in aqueous solution), when PQQ can be reduced to the hydroquinone from (PQQH$_2$) and oxidized back with two electrons like the majority of quinones in aqueous solution.[15]

The measured formal potential $E^\circ = -0.125 \pm 0.003$ V/SCE

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