Structures of Various Cytochromes *c* Evaluated from the Redox Behaviors Using the Optically Active Co(III) Complex-Modified Au Electrode

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Abstract: Electrochemical studies of three *c*-type cytochromes (cyt *c* from horse heart, cyt c_2 from *Rhodospirillum rubrum*, and cyt c_{553} from *Alcaligenes xylosoxidans* GIFU 1051) were performed by using the optically active Co^{III} complex-modified Au electrode. Three cytochromes gave different redox behaviors reflected from the respective structural information. From relationship between their redox behaviors and their structural characteristics, we evaluated the solution structure around heme center of cyt c_{553}.

Key Words: Cytochrome *c*, modified Au electrode, optically active Co^{III} complex, solution structure, electrochemical measurement, conformational change.

INTRODUCTION

Cytochrome *c* plays a key role in the respiratory electron transport chain inside the mitochondrial membrane [1]. The cytochrome *c* released from membrane binds to Apaf-1, which triggers the apoptosis process [2]. Analyses of the electrochemical response for mitochondrial cytochrome *c* and the environmental structures around the heme centers are important from a viewpoint of clarification and control of the biological metabolic process. Although eukaryotic cytochromes *c* have similar physiological function each other, it is not like that their structures are identical [1]. At present, there is little methodology which can estimate the surface structure of a protein.

We have previously reported the Au electrodes modified with optically active Co^{III} complexes containing (*S*)-/(*R*)-phenylalanine derivatives **1** so as to evaluate the surface structure of metalloproteins through their electrode reactions (Fig. (**1**)) [3-5]. From the electrochemical studies of horse heart cytochrome *c* (cyt *c*) and azurins, the overall electron transfer rate with the modified electrode derived from (*S*)-isomer was faster compared with that from (*R*)-isomer in each case. This is the result that was derived from difference in the association rates between proteins and optically active Co^{III} complexes. This electrochemical analysis made it possible to discriminate the little difference in amino acid side chains (Val and Ala) around the metal center of two azurins [5].

In this study, we evaluated the solution structures of some *c*-type cytochromes from the redox behaviors of proteins using the densely packed monolayer of the negatively charged Co^{III} complex containing the (*S*)-phenylalanine derivative (**1** and **1−Au** shown in Fig. (**1**)). Three cytochromes *c*, cyt *c* from horse heart, cyt c_2 from *Rhodospirillum rubrum*, and cyt c_{553} from *Alcaligenes xylosoxidans* GIFU 1051, are known as similar electron transfer proteins in a mitochondrial respiratory chain (electron donor: cyt bc_1 complex, electron acceptor: cyt *c* oxidase) [1], photosynthetic bacteria (cyt bc_1 complex, reaction center) [1,6], and denitrifying bacteria (cyt bc_1 complex, NO reductase) [7], respectively. X-ray structures of cyt *c* and cyt c_2 have been revealed [8,9]: the sulfur (Met) and nitrogen atoms (His) coordinate to heme Fe in both cases. In addition, the heme of cyt *c* is buried into the interior of the protein and that of cyt c_2 is located near the protein surface. On the contrary, the structural environment of cyt c_{553} is hardly known unless the axial ligands of the heme are His and Met as the cases of cyt *c* and cyt c_2 [7]. Here, we focused on the relationships between their structural differences and their redox behaviors with **1−Au** and evaluated the solution structure of cyt c_{553} from comparison with the electrochemical results of cyt *c* and cyt c_2.

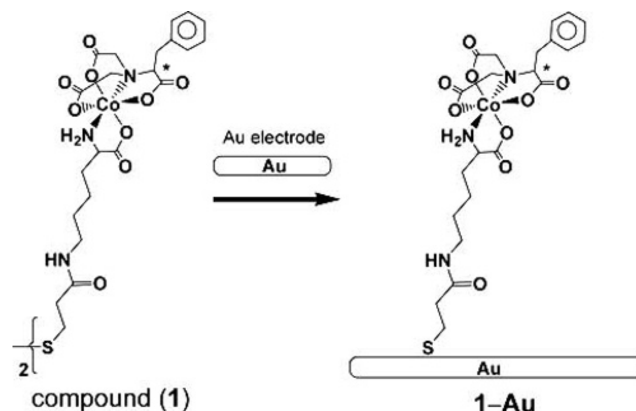

Fig. (1). Schematic view of the optically active Co^{III} complexes (**1**) and the Au electrode modified with **1** (**1−Au**).

Cyt c_2 and cyt c_{553} were obtained from *Rhodospirillum rubrum* and *Alcaligenes xylosoxidans* GIFU 1051 and purified according to the previous literature, respectively [7,10-12]. **1** was synthesized from (*S*)-phenylalanine derivative

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[3,4] and its densely packed monolayer (**1−Au**) was prepared according to the previous methods [5]. All cyclic voltammograms were recorded in a 0.1 M phosphate buffer solution (pH 7.0). Counter and reference electrodes were Pt wire and Ag/AgCl (3 M NaCl), respectively. The respective concentrations of cytochromes were 20 μM.

Fig. (**2**) shows the cyclic voltammograms in the absence and presence of *c*-type cytochromes in a phosphate buffer solution as measured with **1−Au**. In the absence of proteins, no Faradaic currents were observed in this potential region. In the presence of cyt *c*, its redox wave was also not observed (Fig. (**2a**)). However, as clearly seen in Fig. (**2b** and **2c**), cyclic voltammograms of cyt c_2 and cyt c_{553} gave their redox responses. Since their peak currents were dependent upon the square root of scan rates, the observed redox peaks were due to the redox of heme center of cytochromes in solution [13].

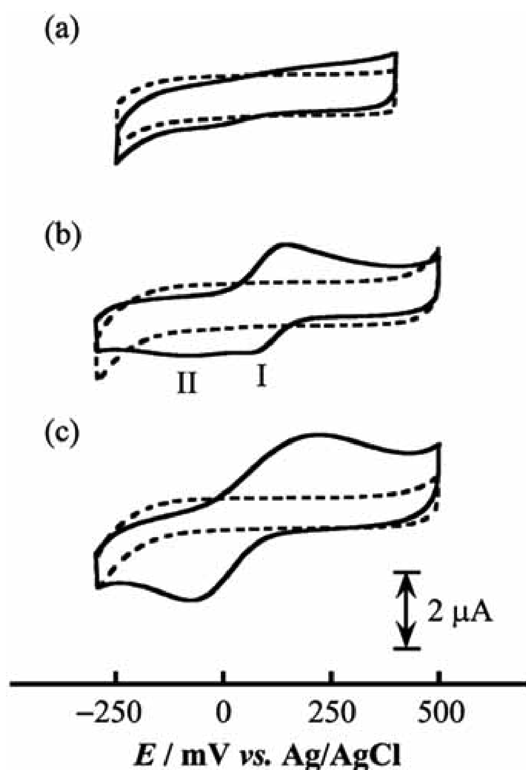

Fig. (2). Cyclic voltammograms of proteins (solid line: cyt *c* (a), cyt c_2 (b), and cyt c_{553} (c)) in a 0.1 M phosphate buffer solution at pH 7.0 using **1−Au**. The dashed line denotes that in the absence of proteins. Scan rate is 50 mV s^{-1}.

We have previously reported that the peak current of cyt *c* gives the largest value when the surface coverage of **1** is *ca.* 4.0×10^{-11} mol cm^{-2} (calculated ideal one: 5.2×10^{-10} mol cm^{-2}) [3,4]. If the coverage was over this value, the peak current of cyt *c* decreased. We concluded that the polypeptide chain around the burial heme interfered with the penetrative association between cyt *c* and Co^{III} complex [4]. Therefore, no redox wave of cyt *c* was observed as measured with densely packed monolayer of **1** (**1−Au** in this manuscript), whose coverage was *ca.* 3.5×10^{-10} mol cm^{-2} [5]. On the contrary, the redox wave of cyt c_2 was obtained, suggesting that cyt c_2 easily associates with Co^{III} complex because its heme lies at the protein surface [9]. These results indicated that the redox response in a protein solution was largely affected by the location of the heme of cytochromes in this system.

Then, we focused on the fact that cyt c_2 gave one oxidation wave and two reduction waves, denoted by "I" and "II" in Fig. (**2b**), respectively. The split of oxidation wave was not detected in all scan rates. The quantity of electricity of the oxidation wave agreed with the sum of those of the reduction waves. This result suggests that both reduction waves are coupled with the oxidation wave. As measured with the electrode modified with 4-mercaptopyridine, which can detect the redox wave of metalloproteins [1], the reduction wave "II" was not detected in cyt c_2 solution. Consequently, two redox waves of cyt c_2 were obtained only in the case of using **1−Au**. Various researchers have pointed out that there are two conformational states of cyt c_2 at neutral pH, which is assigned to the ionization of His42 imidazole [14], and no conformational change of cyt *c* is observed at neutral pH region [15-17]. In fact, in the case of cyt *c*, only a pair of redox wave was detected using low-density monolayer of **1** [3,4]. This suggests that the split of reduction wave of cyt c_2 are attributed to the protonation and deprotonation of His42.

The two conformers of cyt c_2 gave the reversible and irreversible redox waves. This indicates that their electron transfer rates with **1−Au** are quite different. There are two factors contributing to the difference in the rates: the structural change around the electron transfer site and the change in the net charge. These factors probably influence the steric and electrostatic interactions with the Co^{III} complex on Au and negatively charged surface, respectively, resulting in acceleration/deceleration of the electron transfer rates with Au electrode. Thus, the difference in the rates between **1−Au** and two conformers is amplified by either or both protonation effects, which culminates in the split of reduction wave.

From the information obtained in the cases of cyt *c* and cyt c_2, we estimated the structural environment of heme of cyt c_{553}. CV measurement of cyt c_{553} with **1−Au** gave the redox wave without any splitting (Fig. (**2c**)). This finding gives us two important structural information about cyt c_{553}. At first, since the clear redox wave of cyt c_{553} was observed by using **1−Au**, the heme center was probably placed at the neighbor of the protein surface. At second point, no splitting of redox wave of cyt c_{553} was observed, suggesting that no conformational change occurred in neutral solution as the case of cyt *c*. Though three cytochromes play similar role in the respective electron transport chains, their structural characteristics were largely different. The most important point is the fact that the difference among proteins was easily distinguishable by the redox measurements using **1−Au**.

In summary, we evaluated the solution structures of *c*-type cytochromes by analyzing their peak responses and their peak behaviors by using **1−Au**. Three cytochromes gave different redox behaviors reflected from the respective structural information. Using the electrochemical behaviors, we could estimate two structural characteristics of cyt c_{553}: (i) the heme center is located on the protein surface, (ii) there

are no conformational changes in neutral solution. Our optically active Co^{III} complex-modified electrode is very powerful analyzing tool to obtain the solution structures around heme of a series of *c*-type cytochromes by convenient electrochemical measurements.

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