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Electron Transfer Reactions of *C*-type Cytochromes with the Self-Assembled Monolayer of the Optically Active Co^{III} Complex on Au

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In order to obtain the structural information around the active site 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) in aqueous solution, we studied their redox behaviors by use of the densely packed monolayer of the (*S*)-phenylalanine-containing Co^{III} complex. In the case of cyt *c*, no redox wave was observed, which agrees with the previous report that the heme is buried inside of the protein. In contrast, the redox wave of cyt c_2 was clearly observed, which coincides with the fact that the heme positions at the protein surface. Interestingly, in the cyclic voltammogram of cyt c_2, a splitting of the wave was detected, which was elucidated to be attributable to the protonation/deprotonation of His42 imidazole of the protein. Cyt c_{553}, of which the structural information has still not been revealed, gave the redox wave without splitting. In the light of the results of cyt *c* and cyt c_2, this redox behavior indicates the existence of two structural characteristics for cyt c_{553}: (i) the heme is probably located near the protein surface, and (ii) there is no conformational change caused by the protonation.

Key words: electron transfer reaction, *c*-type cytochrome, self-assembled monolayer, conformational change, chiral surface

1. INTRODUCTION

Electron transfer reaction plays an important role in the biological systems, for example respiration and photosynthesis [1-3]. Electron transfer proteins recognize their redox partners through non-covalent interactions, such as steric, electrostatic, and hydrogen bonding interactions, and proceed their electron transfer reactions. Although *c*-type cytochromes have similar biological functions, their structural characteristics are not the same [1]. However, it is quite difficult to investigate the relationship between their structures and recognition mechanisms in detail, because there is no methodology to easily estimate their structural information in solution.

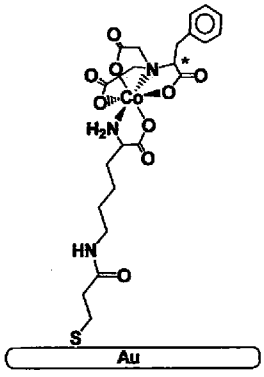

Fig. 1 Schematic view of the Au electrode modified with **1** (**1**–**Au**).

To evaluate the surface structure around the active site of metalloproteins through their redox behaviors, we have prepared self-assembled monolayers (SAMs) of the optically active Co^{III} complexes (**1**) containing (*S*)-/(*R*)-phenylalanine derivatives (Fig. 1) [4-6]. These SAMs can promote the electron transfer reactions between Au electrode and metalloproteins, such as horse heart cytochrome *c* (cyt *c*) and azurins. Moreover, the (*S*)-isomer-containing SAM showed faster electron transfer rate with proteins than (*R*)-isomer-containing one, which reflected the different association rates between proteins and the respective Co^{III} complexes. From such an analytical method, the difference in one amino acid side chain, isobutyl (Val) and methyl residues (Ala), around the metal centers of two azurins could be discriminated [6].

In this research, we examined the redox behaviors of three *c*-type cytochromes by use of the densely packed monolayer of the Co^{III} complex derived from (*S*)-phenylalanine (**1**, **1**–**Au** in Fig. 1) to obtain their structural information in solution. All three cytochromes *c* (cyt *c* from horse heart, cyt c_2 from *Rhodospirillum rubrum*, and cyt c_{553} from *Alcaligenes xylosoxidans* GIFU 1051) act as an electron transfer protein in a mitochondrial respiratory chain (electron donor: cyt bc_1 complex, electron acceptor: cyt oxidase) [1], photosynthetic bacteria (cyt bc_1 complex, reaction center) [7], and denitrifying bacteria (cyt bc_1 complex, NO reductase) [8], respectively. From the X-ray structure analyses, it has been known that the heme center of cyt *c* is buried inside of the protein [9] and that of cyt c_2 is located at the protein surface [10]. However, the

structural information of electron transfer site of cyt c_{553} has not been revealed, except for its axial ligands (His/Met) [8]. Here, we noted the relationship between their structural characteristics and their redox behaviors with **1–Au** and evaluated the surface structure around heme of cyt c_{553} from the results of cyt *c* and cyt c_2.

2. EXPERIMENTAL

All the chemicals and solvents were purchased from Wako Pure Chemical Industries, Tokyo Chemical Industry, Nacalai Tesque and Peptide Institute. All reagents were used without further purification. Milli-Q water was prepared by using a Milli-Q biocel A (Millipore). Co^{III} complex (**1**) was synthesized from (*S*)-phenylalanine and its densely packed monolayer of **1** (**1–Au**) was prepared according to the previous methods, respectively [4,5].

Horse heart cytochrome *c* was purchased from Nacalai Tesque. Cyt c_2 and cyt c_{553} were obtained from *Rhodospirillum rubrum* and *Alcaligenes xylosoxidans* GIFU 1051 and purified according to the previous literature [8,11-13], respectively.

Electrochemical measurements were performed by using a HZ-5000 automatic polarization system (HOKUTO DENKO). The cyclic voltammetry was recorded by the use of each SAM as a working electrode, Pt wire as a counter electrode and Ag/AgCl (3.0 M NaCl) as a reference electrode. Ar gas was purged through the electrolyte solution at least 15 minutes before each measurement.

The voltammetry measurements were carried out in a 0.1 M phosphate buffer solution (pH 7.0). Aqueous solution containing protein (20 μM) was prepared by dialyzing in the same buffer solution a few times before use and its concentration was checked by absorbance spectrum [8,14].

3. RESULTS AND DISCUSSION

3.1 Redox behavior of cyt *c*

Fig. 2 shows cyclic voltammograms of cyt *c* solution as measured with **1–Au**. In the absence of cyt *c*, no redox wave was observed in this potential region [4-6]. On the other hand, in cyt *c* solution, the redox wave was not observed in all scan rates. In the case of low density monolayer of **1**, whose coverage was about one-tenth of that of **1–Au**, cyt *c* gave clear redox wave [4,5]. These results were affected by its structural environment that the heme of cyt *c* was buried inside of the protein [9]. That is, the association between cyt *c* and Co^{III} complex was inhibited by the polypeptide chain around the heme, which resulted in the inefficient redox reaction.

3.2 Redox behavior of cyt c_2

Cyclic voltammograms of cyt c_2 solution were recorded by using **1–Au**, which was shown in Fig. 3. Voltammogram of cyt c_2 exhibited clear redox waves. Relationships between its peak currents and scan rates were plotted (Fig. 4). Anodic and cathodic peak currents showed the linear dependences on the square root of scan rates, indicating that the observed redox waves come from the oxidation/reduction of heme Fe of cyt c_2 in solution [15]. The clear peak responses demonstrated that the redox reactions between Au electrode and cyt c_2 were much effective compared to the case of cyt *c*, because the heme is situated at the protein surface [10]. These observations suggest that peak responses of cytochromes are reflected by the position of the heme center in proteins in this system.

Cyclic voltammograms of cyt c_2 exhibited one oxidation wave and two reduction waves, denoted by "I" and "II" (Fig. 3). The reduction wave "II" was largely shifted with increase in scan rates and disappeared in the faster scan rates over 200 mV s^{-1}. However, a split of the oxidation wave was not detected in all scan rates. At the scan rate of 10 mV s^{-1}, the quantity of electricity of oxidation wave was identical with the sum of that of two reduction waves. These results indicate that these two reduction waves couple with the oxidation wave, that is two different redox responses have been observed.

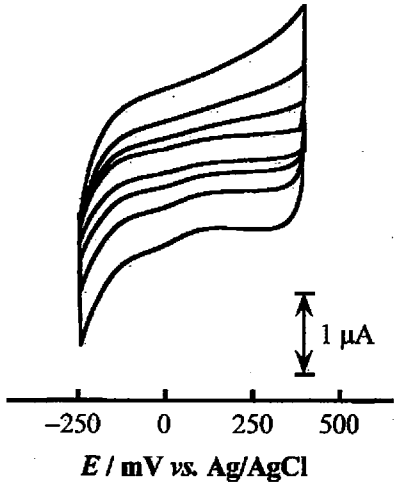

Fig. 2 Cyclic voltammograms of cyt *c* using **1–Au** at the scan rates of 10, 25, 50, and 100 mV s^{-1}.

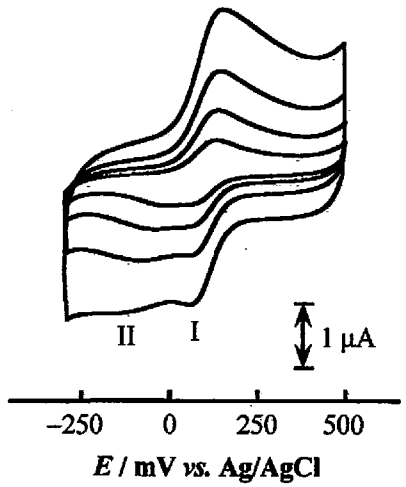

Fig. 3 Cyclic voltammograms of cyt c_2 using **1–Au** at the scan rates of 10, 25, 50, and 100 mV s^{-1}.

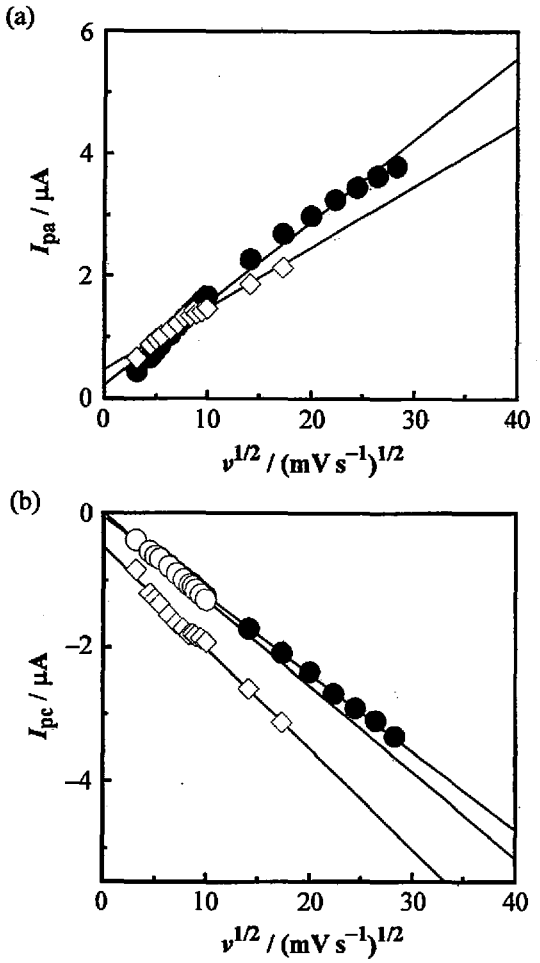

Fig. 4 Relationship between the square root of scan rates and the anodic (a) and cathodic peak currents (b) of cyt c_2 (circle) and cyt c_{553} (diamond) as measured with **1–Au**, respectively. Filled and open circles represent the cathodic waves "I" and "II," respectively.

It has been reported that two conformational states of cyt c_2 at neutral pH were confirmed by ESR spectra [16], redox titration [17], and ^{1}H-NMR spectra [18]. However, cyt *c* did not show such a conformational change in this pH region [16-18]. Smith *et al.* have clarified that this conformational change was due to the ionization of His42 [19]. As measured with a low density monolayer of **1**, cyt *c* gave only a pair of redox wave [4,5]. Therefore, this suggests that the two redox waves have resulted from the protonation/deprotonation of His42.

In the case of the SAM of 4-mercaptopyridine, which has sometimes been employed to detect redox waves of metalloproteins [1,20], the reduction wave "II" was not observed. This finding demonstrates that **1–Au** can detect the two conformational states of cyt c_2. The observed reversible (I: ΔE_p = 74 mV) and irreversible redox waves (II: ΔE_p = 134 mV) mean that the electron transfer rates with **1–Au** are obviously different between two conformers of cyt c_2 [15]. It implies that the difference in the rates has been influenced by the structural change around the electron transfer site or the change in net charges of proteins.

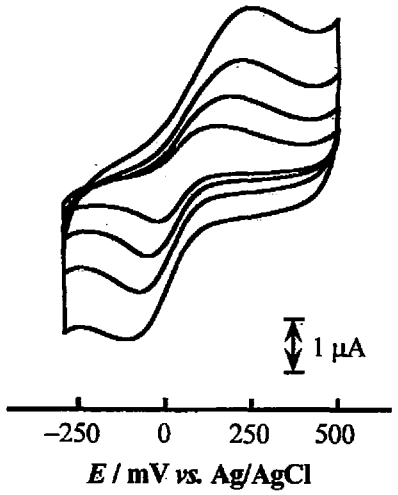

Fig. 5 Cyclic voltammograms of cyt c_{553} using **1–Au** at the scan rates of 10, 25, 50, and 100 mV s^{-1}.

3.3 Redox behavior of cyt c_{553} and its estimated structural characteristics

Fig. 5 shows cyclic voltammograms of cyt c_{553} solution using **1–Au**. Cyt c_{553} gave redox wave, which is due to the redox of cyt c_{553} in solution because of the linear dependence with the square root of scan rates (Fig. 4). From the results of cyt *c* and cyt c_2 in this system, it is clear that their peak responses are largely affected by the location of the heme center of proteins. These observations are likely to indicate that the heme of cyt c_{553} is located near the protein surface.

In addition, the split of redox wave was not observed for cyt c_{553} in all scan rates (Fig. 5). The two redox waves observed in the case of cyt c_2 show the existence of two conformational states at neutral pH, while a pair of redox wave was observed only in the case of cyt *c* [4,5] of which the conformational change does not occur at neutral pH region. This fact suggests that the conformation of cyt c_{553} also does not change in neutral solution as well as the case of cyt *c*.

4. CONCLUSION

We electrochemically evaluated the solution structures around the heme centers of three *c*-type cytochromes through their peak responses and their peak behaviors with **1–Au**. Cyclic voltammograms of cyt *c* did not show the redox wave because of its burial heme. On the other hand, cyt c_2, whose heme is positioned at the protein surface, gave the redox wave. From these findings, the clear redox wave observed for cyt c_{553} means that the heme is located near the protein surface.

Cyt c_2 exhibited two redox waves assigned to two conformational states (the protonation/deprotonation of His42) in neutral solution. However, cyt c_{553} gave only a pair of redox wave, indicating that cyt c_{553} does not occur a conformational change.

Although three cytochromes *c* have similar functions in the electron transport chain, their structural characteristics are quite different. In this system, their

differences were detected as the different redox behaviors through simple electrochemical measurements.

Acknowledgments

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