Electrochemical Evaluation of the Structural Difference in Azurins-1 and -2 from *Alcaligenes xylosoxidans* GIFU 1051 in Aqueous Solution Using Self-assembled Monolayers of Optically Active Co^{III} Complexes

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Two types of azurins (azurins-1 and -2), derived from *Alcaligenes xylosoxidans* GIFU 1051, were first discriminated electrochemically using Au electrode modified with the optically active Co^{III} complexes with (*S*)-/(*R*)-phenylalanine derivatives. They indicated the different redox behaviors induced by small difference in the structural environment around type-1 Cu.

The non-covalent interactions, such as steric, electrostatic, and hydrogen-bonding interactions, at the multiple sites are one of the key points in the molecular recognition process between electron-transfer proteins. To understand the redox partners and the interaction sites with the proteins, the effect of electrostatic interaction has often been reported.[1] However, the association and dissociation processes between proteins could not be completely explained by only electrostatic one. In fact, it is difficult to examine the detailed role of non-covalent interactions except for electrostatic one by current methodologies.

Previously, we have studied the self-assembled monolayer of the optically active Co^{III} complexes modified on Au electrode (**1–Au**, Figure 1a) and indicated that the chirality of (*S*)-/(*R*)-phenylalanine ((*S*)-/(*R*)-Phe) residues on the complex affected the electron-transfer reactions between horse heart cytochrome *c* (cyt *c*) and **1–Au** (**1*S*–/1*R*–Au**, respectively).[2] From the analysis of peak separations of the redox waves, it was demonstrated that **1*S*–Au** associated with cyt *c* faster than **1*R*–Au**. This difference was explained in terms of slight difference in the associative interactions between the chiral Phe residues of Co^{III} units and the heme crevice of cyt *c*.

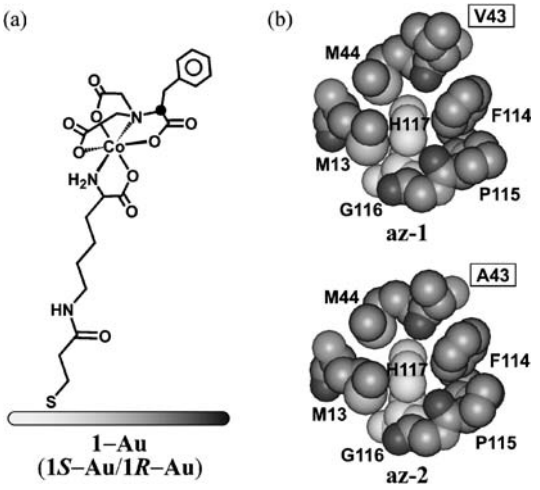

Figure 1. (a) Schematic view of **1–Au**. (b) Local structures around His117 side chain of az-1 and az-2 from *Alcaligenes xylosoxidans* NCIMB 11015.[6]

In order to demonstrate that **1–Au** is effective also for various proteins, we performed the heterogeneous electron-transfer reaction between **1–Au** and two types of azurins, azurin-1 (az-1) and azurin-2 (az-2) isolated from *Alcaligenes xylosoxidans* GIFU 1051,[3] in which the specific electron-transfer site involving His117 around the north pole of azurin, so-called "hydrophobic patch," plays an important role in the physiological electron-transport systems.[4] The difference in their local structures around His117 is only the 43rd residue, Val (az-1) and Ala (az-2), as shown in Figure 1b.[5,6] Herein, we first report that the slight difference around the 43rd residues has electrochemically been discriminated through the association behaviors between two azurins and **1–Au**.

Az-1 and az-2 were obtained from *Alcaligenes xylosoxidans* GIFU 1051 and purified according to the literature.[7] Optically active Co^{III} complexes **1** were prepared according to the previous methods.[2] **1–Au** was obtained by dipping a polycrystalline Au flag electrode[8] in an aqueous solution of **1** (1 mM) for 3 days at 30 °C. The modification of **1** was checked by observation of the redox waves of $Co^{II/III}$ at ca. −360 mV vs Ag/AgCl.[2] Surface coverage of **1–Au** was $(3.5 \pm 1.5) \times 10^{-10}$ mol cm^{-2},[9] which indicates preparation of densely packed monolayer compared to the calculated ideal one (5.2×10^{-10} mol cm^{-2}).[2]

Figure 2a shows the cyclic voltammograms in the absence and presence of az-1 (upper) and az-2 (lower) as measured with **1*S*–Au**.[10] In the absence of azurins, no redox wave was detected

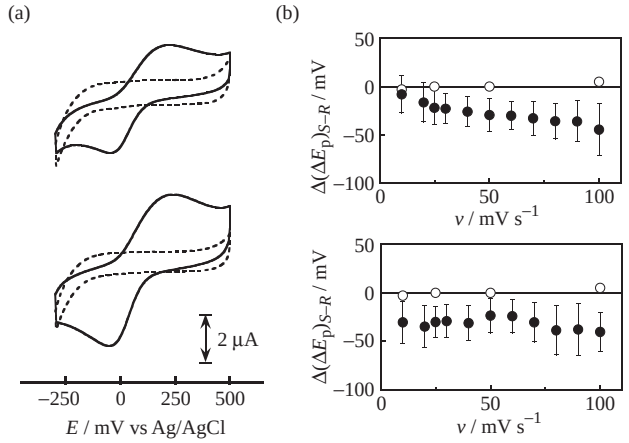

Figure 2. (a) Cyclic voltammograms of azurins (solid line; az-1 (upper) and az-2 (lower)) in a phosphate buffer solution using **1*S*–Au**. The waves recorded with dashed line are in the absence of azurins. Scan rate is 50 mV s^{-1}. (b) The relationship between scan rates and $\Delta(\Delta E_p)_{S-R}$ for $[Ru(NH_3)_6]^{3+}$ (open circle) and azurins (filled circle; az-1 (upper) and az-2 (lower)) using **1–Au**. Error bars are the standard deviation.

in this region, indicating that Co^{III} units cannot mediate electron-transfer reactions with azurins. The redox waves of azurins were clearly observed at +267 (az-1) and +275 (az-2) mV vs NHE, respectively, whose potentials were almost the same between **1*S*–Au** and **1*R*–Au**, and were quite similar to the previously reported one (az-1: +267, az-2: +274 mV vs NHE).[3] Therefore, these redox waves were assigned to the $Cu^{I/II}$ couple of azurins in the solution.

Cyclic voltammograms of az-1, az-2, and $[Ru(NH_3)_6]^{3+}$ were recorded at several scan rates using **1–Au**, respectively (see ESI for details).[11] To estimate the effect of chiral Phe residue of Co^{III} units in **1–Au**, we defined $\Delta(\Delta E_p)_{S-R}$ ($=\Delta E_{p,S} - \Delta E_{p,R}$), which is the difference in the peak separations of redox waves of azurin between **1*S*–Au** and **1*R*–Au**. This is the parameter for the overall electron-transfer rate (k) between each azurin and **1–Au**. The $\Delta(\Delta E_p)_{S-R}$ values were plotted against scan rates (Figure 2b). In the case of $[Ru(NH_3)_6]^{3+}$ (open circle; Figure 2b), the $\Delta(\Delta E_p)_{S-R}$ values were almost zero at 10–2000 mV s^{-1}, indicating that the overall electron-transfer rates (k) estimated from the ΔE_p values are almost the same between **1*S*–Au** and **1*R*–Au**. Thus, it is clear that the chirality of the Phe residue of Co^{III} units does not influence the k values for $[Ru(NH_3)_6]^{3+}$.

For az-1, the $\Delta(\Delta E_p)_{S-R}$ value was almost zero at 10 mV s^{-1}. However, it decreased with increase in the scan rate (filled circle, upper; Figure 2b), whose electrochemical behaviors are quite similar to the case of cyt *c*.[2] Therefore, the electron-transfer process between **1–Au** and az-1 is also controlled by diffusion (k_D), association (k_A), and electron-transfer rate (k_{ET}). Only the scan rate affected the association rate (k_A). Thus, the behavior in $\Delta(\Delta E_p)_{S-R}$ values indicates that the association rate between az-1 and **1*S*–Au** is faster than the case of **1*R*–Au**. These results suggest that az-1 has recognized the chirality of Phe residue of Co^{III} units on the Au surface.

On the other hand, the $\Delta(\Delta E_p)_{S-R}$ values for az-2 was almost constant at all scan rates (filled circle, lower; Figure 2b). This behavior is quite different from the case of az-1 and cyt *c*,[2] which indicates that the k values are different between **1*S*–Au** and **1*R*–Au**. However, the $\Delta(\Delta E_p)_{S-R}$ value was independent on the scan rates. Thus, the rate determining step of electron-transfer reaction between az-2 and **1–Au** is not the association process, although az-2 is able to recognize the chirality of Phe residue of Co^{III} units on the surface. Since the k values were different between **1*S*–Au** and **1*R*–Au** even at 10 mV s^{-1}, the minus constant values of $\Delta(\Delta E_p)_{S-R}$ may suggest that the different k_{ET} values were induced by the chiralities.

These results suggest that **1–Au** interacts in different association manners between az-1 and az-2. As mentioned above, the local structures around His117 of azurins are almost the same each other, except for the 43rd residue, Val (az-1) and Ala (az-2). The isopropyl group of Val is bulkier than the methyl group of Ala. Therefore, the steric effects induced by the 43rd residues are likely to affect their electrochemical behaviors. That is, the protein having more crowded structural environment gave great difference against the k_A values in this system, while such a significant difference was not observed against the flatter surface of protein. Generally, the protein–protein interaction is a reaction between huge molecules, so the small structural differences may not be effective on their electron-transfer reactions. However, our probe electrode, **1–Au**, recognized the slight distinctions through the interaction between small molecule (Co^{III} complexes on Au surface) and huge protein (azurin).

In summary, we focused on the discrimination of slight structural difference between az-1 and az-2, and studied their electrochemical behaviors using **1–Au**. They gave the different dependence in $\Delta(\Delta E_p)_{S-R}$ values against the scan rates. This indicates that two azurins have discriminated the chiral ligands of Co^{III} units on **1–Au**, and that the recognition in az-2 is not due to the associative interactions. We concluded that these recognitions were raised by the structural difference in the 43rd residues around His117, Val for az-1 and Ala for az-2. This is the first report that two different azurins have been discriminated using electrochemical technique. Further experiments and analyses are in progress.

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6 The homologies of primary structures of azurins between from GIFU 1051 and from NCIMB 11015 are 96 (az-1) and 99% (az-2), and local structures around His117 are almost the same.
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8 A 0.1-mm-thickness-round Au plate (ϕ 6 mm) spot-welding Au wire was prepared as a polycrystalline Au flag electrode.
9 Surface coverage was estimated from the reductive cleavage wave of Au–S bonds in a 0.5 M KOH aqueous solution.
10 All cyclic voltammograms were recorded in 0.1 M phosphate buffer (pH 7.0). Counter and reference electrodes were Pt wire and Ag/AgCl (3 M NaCl), respectively. The concentration of $[Ru(NH_3)_6]Cl_3$ and azurins were 1 mM and 100 μM, respectively.
11 Supporting Information is available electronically on the CSJ-Journal Web site, http://www.csj.jp/journals/chem-lett/index.html.

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