IMMOBILIZATION OF MUTANTS OF BLUE COPPER PROTEIN AMICYANIN ON THE MODIFIED GOLD ELECTRODE

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Introduction

Amicyanin (Ami) is an electron-transfer protein which active site constitutes type1 copper, representing distorted tetrahedral geometry formed by Cys92, His53, His95, and Met98[1]. Amicyanin functions as an electron-transfer protein in the periplasmic space of facultative methylotrophic bacteria *Paracoccus denitrificans.* Ami is an electron acceptor of the methylamine dehydrogenase.

In the case of Azurin, which is another blue copper protein, Canters et al. reported that the geometry of type1 copper coordination was restored, when imidazole derivatives were added as an exogenous ligand to a mutant lacking a histidine ligand[2]. In this research, with an exogenous ligand immobilized on a gold electrode, following experiments were carried out to construct strategy of Ami immobilization on the electrode. Two mutants were expressed in *E. coli* system. First the His-Tag oligopeptide was inserted into the C-terminal, and second the His95 ligand of type1 copper site was substituted to Gly. These two mutants were immobilized on the electrode with mutational regions. We expected that the Ami mutants oriented opposite to electrode as a result (Fig. 1).

Experimental

Both His-Tag insertion (C_{His} Ami) and copper ligand substitution (His95Gly) were performed by PCR using wild-type protein expression vector as a template.

Absorption spectra were recorded at r.t. and electron paramagnetic resonance spectra were recorded at 77 K.

A gold electrode which was modified by self-assembled monolayer (SAM) was used as a working electrode for cyclic voltammetry (CV) measurements. For immobilization of C_{His} Ami, the electrode was immersed in 10 mM 3,3'-Dithiobis [N-(5-amino-5-carboxypentyl) propionamide-N',N'-diacetic acid] dihydrochloride (C_2-NTA) methanol solution for 4h, and then in 200 mM nickel sulfate solution for 20 min. The resulting electrode was soaked in C_{His} Ami solution for 30 min. His95Gly Ami was immobilized on the surface of imidazole-mixed SAM[3]. A gold electrode was immersed in the ethanol solution of 20 mM 1-(11-mercaptoundecyl) imidazole and 40 mM 1-octanethiol for 24h, then rinsed and dried at r.t. The electrode modified with imidazole-mixed SAM was soaked in His95Gly mutant solution for 2h. CV measurement was carried out in 10 mM phosphate buffer solution (pH 7.0) free from Ami.

Results and discussion

The UV-vis spectrum of wild-type Ami shows an absorption peak at 595 nm and a shoulder appeared at 464 nm indicating ligand-to-metal charge transfer (LMCT) transition from S of Cys92 to $Cu(d_{x^2-y^2})$. Characteristic blue color was completely disappeared for the His95Gly mutant. It was shown that the copper site exhibits various coordination geometries by EPR spectroscopy. These spectroscopic results suggest that the His95Gly mutant no longer hold type1 copper site. However, absorption band around 600 nm was increased in the UV-vis spectrum, when L-histidine was added as an exogenous ligand.

No redox response of immobilized C_{His} Ami was observed in CV. It suggests that the copper site of Ami could not face to the surface of the electrode by immobilization via the C-terminal region (Fig. 2A). Based on a simple calculation as the sum total of the protein diameter, length of His-tag, and the thickness of SAM layer, copper-electrode distance becomes more than 70 Å. The value seems to be too long for electron transfer.

The apparent redox response was observed in the voltammogram of the His95Gly mutant which immobilized on the electrode coated with imidazole-mixed monolayer (Fig. 2B). Since redox peaks were seen even after 2h potential sweeping, dissociation or denaturation of the His95Gly mutant on the electrode was not occurred. The wild-type Ami immobilized on the imidazole-mixed SAM and the His95Gly mutant on the 1-octanethiol monolayer showed no redox response. It suggests that the imidazole group in SAM contribute to the immobilization of His95Gly amicyanin and as well the electron transfer between the electrode and the active center.

We are now studying on the state of Ami mutants on gold electrode by mean of surface resonance Raman spectroscopy.

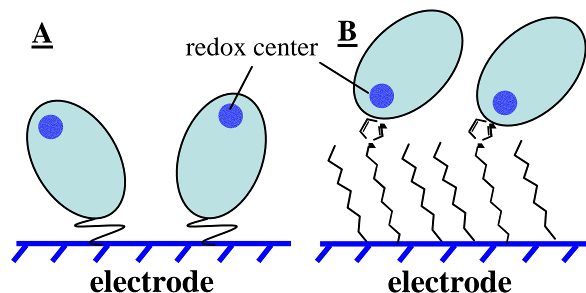

Fig. 1 Schematic diagrams of (A)C_{His} Ami bond to Ni NTA modified electrode with His-Tag and (B)His95Gly Ami immobilized on imidazole mixed SAM.

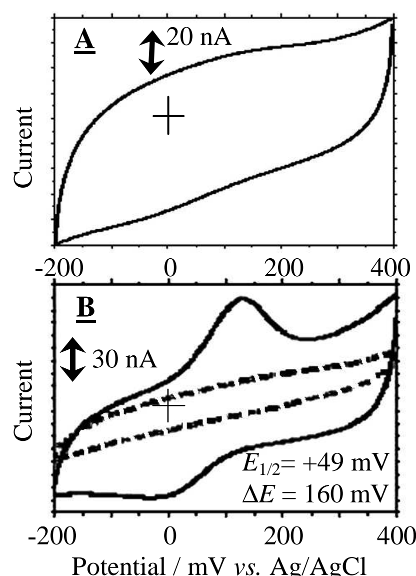

Fig. 2 Cyclic voltammograms of (A)C_{His} AMI on Ni NTA modified electrode and (B)His95Gly on imidazole-mixed SAM (solid line) and the background current (dotted line).

References

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