Effects of Elimination of α Helix Regions on Direct Electron Transfer-type Bioelectrocatalytic Properties of Copper Efflux Oxidase

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ABSTRACT

We investigated properties of direct electron transfer (DET)-type bioelectrocatalysis of recombinant native Copper efflux oxidase (rCueO) and its variants which lack α helices covering the electron-donating substrate-binding site (Δα5–7CueO, Δα5CueO, Δα6–7CueO, and Δα5–7+1/2α5CueO) at mesoporous carbon electrodes without pretreatment and modified with positively or negatively charged aromatic amines. Kinetic and thermodynamic parameters of the electrode reaction were obtained by analysis of steady-state catalytic waves based on a random orientation model and examined the results on the basis of the structural information of the enzymes. The data suggested that the electron transfer pathway is different from that in solution; electrons are transferred from an electrode to the T1 Cu site through the negatively charged position near the T1 Cu site in rCueO without passing through the α helix region in DET-type bioelectrocatalysis. Positively charged electrode was a suitable scaffold for DET-type reaction of rCueO. The T1 Cu site in Δα5CueO became somewhat hydrophobic and hydrophobic electrode worked as a suitable scaffold for the variant. Negatively charged electrode seems to induce unfavorable attractive orientation for DET-type reaction between the electrode and positively charged region of the CueOs on the opposite side of the T1 Cu site.

1. Introduction

Multi-copper oxidases (MCOs) such as laccase, bilirubin oxidase, ascorbate oxidase, and copper efflux oxidase (CueO) are redox enzymes catalyzing a four-electron reduction of dioxygen (O2) to water. The enzyme in the family contains four copper (Cu) atoms as components of their active sites, which are classified into three types according to their spectroscopic and magnetic properties: type I (T1), type II (T2) and type III (T3) Cu atoms, and communicates with an electron-donating substrate at the T1 Cu site and with O2 at the T2/3 Cu site.1,2 One of notable characteristics of MCOs is the ability to directly communicate with electrodess thanks to the low substrate specificity for electron donors.3,3 Such direct coupling of enzymatic redox reactions and electrode reactions is called direct electron transfer (DET)-type bioelectrocatalysis, and MCOs are frequently utilized as cathode catalysts of biofuel cells.6

CueO is known as a DET-type bioelectrocatalyst with a high activity thanks to the T1 Cu site with a rather negative redox potential,5 since the intramolecular electron transfer obeys a linear free energy relationship.7 The characteristic structure of CueO is that the enzyme has a large segment including α helices (helices 5, 6, and 7 from N-terminus) covering the electron-donating substrate-binding T1 Cu site, as shown in Fig. S1.8,9 In addition, a regulatory Cu ion is bound to helix 5 of CueO in the presence of excess Cu2+,5 and enhances the enzymatic activity in solution by mediating the electron transfer from an electron-donating substrate to the T1 Cu site.10 In previous studies, our group designed several CueO variants that lack parts of the α helix regions, which were replaced with peptide linkers, and revealed that the variants showed drastically improved activity of an oxidation of steric electron-donating substrates such as 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS).10,11 However, bioelectrocatalytic properties of the variants are not yet examined well.

On the other hand, mesoporous carbon material (Ketjen Black; KB) is frequently utilized as a scaffold for DET-type bioelectrocatalysis. The porous structure of KB-modified electrodes is suggested to improve DET-type reactions by increasing the amount of enzymes immobilized on the enlarged electrode surface,11 adsorbing enzymes in orientations favorable for DET-type reactions in mesoporous structures,12,13 and strengthening the electric field at the top edge of microporous structures by the expansion of the electric double layer.14 Furthermore, we electrochemically introduce positive or negative charge on the electrode surface with charged aromatic amines in order to control the electrostatic interaction between enzymes and the electrodes and to change the orientation of the enzymes.15,16

In this study, we investigated properties of DET-type bioelectrocatalysis of the CueO variants that lack parts of the α helix regions at hydrophobic or charged porous electrodes. The steady-state limiting current of O2 reduction catalyzed by CueO on electrodes is often limited by the mass-transfer of O2 due to its very high DET-type bioelectrocatalytic activity.17 Under such conditions, it is difficult to correctly characterize electro-enzymatic (bioelectrocatalytic) properties. Thus, we performed electrochemical measurements at a low temperature and at a high speed rotation in rotating disk voltammetry in order to decrease the enzyme-kinetic controlled catalytic current density compared with the diffusion-controlled current density. In addition, we analyzed the steady-state catalytic wave on a model of random orientation of enzymes.18 Judging from the evaluated parameters of the thermodynamics and kinetics, the hydrophobic and electrostatic interaction between CueOs and electrodes was discussed by considering the estimated crystal structures of the enzymes. The electron transfer pathway in the DET-type reaction was also discussed by comparing it with that of the enzymatic reaction in solution.
2. Experimental

2.1 Materials and chemicals

The recombinant native CueO (rCueO) and its variants (Δα5–7CueO, Δα5CueO, Δα6–7CueO and Δα5–7–I/α5CueO, named from the deleted α helices) were expressed in *Escherichia coli* and purified according to the literature procedure.\(^5\)\(^6\) KB (EC300J) and poly(1,1,2,2-tetrafluoroethylene) (PTFE, 6-J) fine powder were obtained from Lion Corp. (Japan) and DuPont-Mitsui Fluorochemicals Co., Ltd. (Japan), respectively. 4-Aminobenzoic acid (ABA) and p-phenylenediamine (PDA) dihydrochloride were purchased from Tokyo Chemical Industry Co., Ltd. (Japan). Other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Japan). All solutions were prepared with ultrapure water.

2.2 Electrochemical measurements

All electrochemical measurements were performed using a voltammetric analyzer (ALS 714C, BAS Inc., Japan) and a rotating disk electrode instrument (RRDE-3, BAS Inc., Japan). A Pt wire and a homemade Ag/AgCl sat. KCl electrode were used as counter and reference electrodes, respectively. All of the potentials in this paper are referred to the reference electrode.

2.3 Preparation of amine-functionalized electrodes

Glassy carbon (GC) electrodes (3 mm in diameter, BAS, Japan) were polished with 1.0- and 0.05-µm alumina slurry, sonicated and washed with distilled water. The mixture of KB powder (40 mg) and PTFE (40 mg) was homogenized in 3.5 mL of 2-propanol for 3 min on ice to prepare KB slurry (L = dm\(^3\)). A 3-µL aliquot of KB slurry was applied on GC electrodes and dried at room temperature. This electrode is called KB/GC electrode. The KB/GC electrodes were electrochemically modified with ABA or PDA according to the literature.\(^5\)\(^6\) In brief, the KB/GC electrodes were immersed into a 5 mM ABA or PDA dihydrochloride solution containing 0.1 M KCl, and one-step electrochemical oxidation was done at 0.8 V for 40 s at 25°C under quiescent conditions (M = mol dm\(^{-3}\)). These electrodes were called ABA/KB/GC and PDA/KB/GC electrodes, respectively.

2.4 Preparation of CueO-adsorbed electrodes

After washing the untreated and amine-modified KB/GC electrodes (KB/GC, ABA/KB/GC, and PDA/KB/GC electrodes) with distilled water, a 20-µL aliquot of 1 mg mL\(^{-1}\) CueO variant solution dissolved in a 0.1 M phosphate buffer (pH 7.0) was applied on the surface of the electrodes. The electrodes were placed in a water-saturated atmosphere for 1.5 h at 4°C. The enzyme-adsorbed electrodes were washed with the buffer solution before electrochemical measurements. The graphical views of these electrodes are shown in Fig. S2.

3. Results and Discussion

3.1 DET-type bioelectrocatalysis of O\(_2\) reduction at CueO variant-adsorbed KB/GC electrodes without pretreatment or with amine-modifications

Before rotating disk voltammetric measurements of bioelectrocatalytic reactions at CueO variant-adsorbed electrodes, we investigated the dependence of the limiting current density of the oxidation of 1,1'-ferrocenedimethanol on the rotating speed (ω) of a bare GC electrode. The current linearly increased with the square root of ω up to ω = 9000 rpm (Fig. S3). Thus, we conducted disk voltammetric experiments at ω = 9000 rpm to maximize the diffusion-controlled current density. Figure 1 shows rotating disk cyclic voltammograms (RDCVs) of the CueO variant-adsorbed electrodes recorded in a 0.1 M phosphate buffer (pH 7.0) at ω = 9000 rpm and at a scan rate (v) of 10 mV s\(^{-1}\) under O\(_2\)-saturated conditions. Untreated KB/GC, ABA/KB/GC, and PDA/KB/GC electrodes were used as working electrodes. Each of the enzyme-adsorbed electrodes provided a clear sigmoidal steady-state wave. The sigmoidal waves are ascribed to the DET-type bioelectrocatalytic O\(_2\) reduction by the CueO variants adsorbed on the mesoporous KB/GC electrodes. Small non-catalytic redox waves were also observed around 0–0.2 V at the CueO variant-adsorbed ABA/KB/GC and PDA/KB/GC electrodes. They are ascribed to by-products on the electrodes generated during the electrochemical oxidation of the amines, but the by-products do not participate in the bioelectrocatalytic reaction.\(^5\)

On the other hand, the limiting current densities of the catalytic O\(_2\) reduction reached approximately −10 mA cm\(^{-2}\), which are somewhat smaller than the value of the O\(_2\) diffusion-controlled limiting current density \(j_{\text{lim}} = −15.4 \text{ mA cm}^{-2} \text{ at } 4^\circ\text{C} \text{ and at } \omega = 9000 \text{ rpm}\) calculated by the Levich equation:

\[
j_{\text{lim}} = -0.62n_0F D_{\text{O}_2}^{\frac{1}{2}} v^{-1/2} c_{\text{O}_2} \omega^{1/2}
\]

where \(n_0\), \(F\), \(D_{\text{O}_2}\), \(v\), and \(c_{\text{O}_2}\) are the number of electrons of O\(_2\) reduction (≡ 4), the Faraday constant, the diffusion constant of O\(_2\) (\(= 1.2 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1} \text{ at } 4^\circ\text{C}\)), the kinematic viscosity of the buffer (\(= 0.0156 \text{ cm}^2 \text{ s}^{-1} \text{ at } 4^\circ\text{C}\)),\(^20\)\(^21\)) and the bulk concentration of O\(_2\) (\(= 2.0 \text{ mM at } 4^\circ\text{C} \text{ under an } \text{O}_2\text{-saturated condition}\)),\(^22\) respectively. However, under the present conditions, we have to still consider the effect of \(j_{\text{lim}}\) on the current density in quantitative analysis of kinetics and thermodynamics of the steady-state voltammograms, since the difference between the limiting current densities and \(j_{\text{lim}}\) is not so large.

3.2 Kinetic analysis of DET-type bioelectrocatalysis of CueOs

The steady-state current density (\(j\)) at a rotating disk electrode is expressed by a Koutecky–Levich type equation:

\[
j = \frac{1}{j_{\text{lim}}} + \frac{1}{j_{\text{electro-enz}}} + \frac{1}{\beta ADA}\]

where \(j_{\text{electro-enz}}\) is the steady-state catalytic current density controlled by DET-type bioelectrocatalysis (electro-enzymatic reaction). The limiting value of \(j_{\text{electro-enz}}\) is referred to as \(j_{\text{cat}}\) and defined as follows:

\[
j_{\text{cat}} = -n_0 F k_c I_{\text{eff}}\]

where \(k_c\) and \(I_{\text{eff}}\) are the catalytic constant and the surface concentration of the effective enzyme immobilized on the electrode, respectively. Note here that \(k_c\) is not equal to the catalytic constant of the enzyme in solution. We attempted to estimate thermodynamic and kinetic parameters of DET-type bioelectrocatalysis at the CueO variant-adsorbed electrodes on the basis of a partially random orientation model.\(^5\)\(^6\)\(^15\)\(^16\)\(^18\) In our random orientation model, \(j_{\text{electro-enz}}\) is expressed by the following equation:\(^16\)

\[
j_{\text{electro-enz}} = \frac{j_{\text{cat}}}{\beta ADA(1 + \eta)} \ln \left(\frac{k_c^{\max} (1 + \eta) + \eta^\phi}{k_c^{\max} (1 + \eta) \exp(-\beta ADA + \eta^\phi)}\right)
\]

where \(k_c^{\max}\) is the standard rate constant of the heterogeneous electron transfer at the closest approach in the best orientation of the enzyme, \(\Delta d\) is the difference in the distance between the closest and farthest approaches of the redox center of the enzyme that electrochemically communicates with an electrode, \(\alpha\) is the transfer coefficient and was assumed to be 0.5 in this case, and \(\beta\) is the decay coefficient of the long-range electron transfer and was assumed to be 1.4 Å\(^{-1}\) for proteins.\(^23\) \(\eta\) is defined as follows:

\[
\eta = \exp\left(\frac{n^\prime E^F (E - E^\prime \eta)}{RT}\right)
\]

where \(E\), \(E^\prime \eta\), \(n^\prime\) \(E\) and \(T\) are the electrode potential, the formal potential of the redox center of CueO, the number of electrons in the
rate-determining step of the heterogeneous electron transfer (= 1 in general), the gas constant, and the absolute temperature, respectively. Using $E^{\prime\prime}_{\text{BE,}}$, $k^\circ_{\text{max}}/k_c$, $\Delta d$, and $j^\text{cat}$ as adjustable parameters, Eq. (2) was fitted to the steady-state catalytic waves using non-linear regression analysis by Gnuplot. The background currents were subtracted from the total currents before the analysis. The refined data are graphically compared with each other in Fig. 2 and the refined numerical data are given in Table S1. The fitting results are shown in Fig. 3.

The evaluated values of $E^{\prime\prime}_{\text{BE,}}$ remained almost unchanged by the mutation and the introduction of the ionic charge on the KB/GC electrode surface (Fig. 2, Panels A and B). Focusing on the values of $k^\circ_{\text{max}}/k_c$ with an assumption that $k_c$ of the variants is not significantly different from each other, we may conclude that the $k^\circ_{\text{max}}$ value of rCueO and its variants is also almost equal with each other. This is opposite to our expectation that $k^\circ_{\text{max}}$ would be increased by the deletion of parts of the A helix region covering the electron-donating substrate-binding site, because the distance between the T1 Cu site and an electrode might be shortened, as long as we assume that electrons are transferred from an electrode to the T1 Cu site via the A helix region, just like in the case of the enzymatic substrate oxidation in solution. Therefore, we can conclude that the pathway of the electron transfer from electrodes to CueOs in DET-type bioelectrocatalysis is different from that from electron-donating substrates to CueOs in solution.
Interestingly, a drastic increase in $-j_{\text{cat}}$ was observed by the deletion of helix 5, as shown in Fig. 2. Panel D; the $\Delta$5CueO-adsorbed electrode gave a higher value of $-j_{\text{cat}}$ (90 ± 30 mA cm$^{-2}$) than the rCueO-adsorbed one (40 ± 20 mA cm$^{-2}$), when untreated KB/GC electrodes without amine-modification were used. However, the introduction of the positive and negative charge on the KB/GC electrode surface with PDA- and ABA-modification, respectively, caused significant decreases in $-j_{\text{cat}}$ at the $\Delta$5CueO-adsorbed electrodes. In contrast, a drastic increase in $-j_{\text{cat}}$ was observed at the rCueO-adsorbed electrodes by the introduction of the positive charge on the KB/GC electrode surface with PDA (90 ± 40 mA cm$^{-2}$).

Figure 4 shows the structures and the surface charges of rCueO and its variants estimated on SWISS-MODEL and calculated by PyMOL, respectively. The T1 Cu site and the $\alpha$ helix region are negatively charged in rCueO. The negative charge near the T1 Cu site and the $\alpha$ helix region in rCueO may cause a strong attractive interaction between the negatively charged part(s) and the positively charged PDA/KB/GC electrode, most probably resulting in an increase in $j_{\text{cat}}$. The $-j_{\text{cat}}$ value at the rCueO-adsorbed KB/GC electrode decreased on the introduction of the negative charge with ABA on the electrode. The result also supports our conclusion. As described above, the electron seems to be transferred from an electrode to the T1 Cu site without passing through the $\alpha$ helix region in DET-type bioelectrocatalysis. In the first place, it would be difficult to transfer electrons at quite high speeds over a long distance from the T1 Cu site to electrodes via the large $\alpha$ helix region in rCueO. Note here that rCueO undergoes diffusion-controlled DET-type bioelectrocatalysis in rotating disk voltammetry. Therefore, the most potential electron-accepting site of rCueO is the negatively charged position circled with a solid line in Fig. 4A. There is another negatively charged part located at the opposite site of the $\alpha$ helix region (the lower right of rCueO in Fig. 4A). However, the distance between the part and the T1 Cu site seems to be too long to realize fast interfacial electron transfer.

In the case of $\Delta$5CueO, characteristic localization of charges was not observed near the T1 Cu site in Fig. 4C; rather, some hydrophobic parts locate around the T1 Cu site. Therefore, hydrophobic scaffolds such as untreated KB/GC seem to be better for $\Delta$5CueO to communicate with electrodes for DET-type bioelectrocatalysis than charged hydrophilic scaffolds (ABA- and PDA-modified KB/GCs).

However, the $-j_{\text{cat}}$ values of the other variants ($\Delta$5–7CueO, $\Delta$6–7CueO, and $\Delta$5–7–1/2$\alpha$5CueO) adsorbed on both untreated hydrophobic and ionically charged hydrophilic electrodes were much lower than that of rCueO on PDA/KB/GC and $\Delta$5CueO on hydrophobic KB/GC. In the case of the variants, characteristic localization of charges was not observed near the T1 Cu site circled with a solid line in Figs. 4B, D, and E, as in the case of $\Delta$5CueO. The data in Fig. 2D did not support any hydrophobic interaction between the T1 Cu site of the variants and untreated KB/GC electrodes. The reason remains unknown, but the deletion of helices 6 and 7 might cause some conformation change of the enzymes, which might lead to unfavorable orientation for DET-type reaction.

By the way, for rCueO and all its variants, the introduction of the negative charge on KB/GC electrodes with ABA led to a decrease in the $-j_{\text{cat}}$ values compared with hydrophobic KB/GC and positively charged PDA/KB/GC electrodes. The effect seems to be ascribed to a decrease in $\Gamma_{\alpha}$ by unfavorable attractive electrostatic interaction between the positively charged region of the CueOs on the opposite side of the T1 Cu site (the right sides of the enzymes in Fig. 4) and the negatively charged electrode surface. This orientation increases the population of ineffectively adsorbed enzymes.

4. Conclusion

DET-type bioelectrocatalysis of rCueO and the $\alpha$ helix-lacking variants at hydrophobic and charged amine-modified mesoporous carbon electrodes was kinetically and thermodynamically analyzed. The results mainly suggested the following points. The mutation did not cause significant effect on $k_{\text{max}}^+/k_{\text{cat}}$ as well as $E_{\text{f}}^{\prime\prime}$ and $\Delta d$. On our assumption that $k_{\text{cat}}$ remains unchanged by the mutation, we may conclude that the $k_{\text{max}}^+$ values of the enzymes are almost identical with each other in spite of that several deletion trials of the $\alpha$ helix region covering the substrate-binding site. Therefore, it is suggested that electrons are transferred from an electrode to the T1 Cu site through the negatively charged position near the T1 Cu site in rCueO without passing through the $\alpha$ helix region in DET-type bioelectrocatalysis. The electron transfer pathway is different from that in the enzymatic reaction in solution. Such a difference in the electron transfer pathway between DET-type bioelectrocatalysis and enzymatic reaction in solution is also reported in a membrane-bound flavohemoprotein fructose dehydrogenase (FDH), all three hemes.
$c$ in FDH are essential in the electron transfer from the enzyme to its natural electron acceptor, ubiquinone, while electrons are transferred from the substrate-reduced FDH to an electrode without passing through the first heme $c$ from N-terminus.

Positively charged PDA/KB/GC behaves as a scaffold very suitable for DET-type bioelectrocatalysis of rCueO, in which the T1 Cu site locates close to strongly negatively charged part. The attractive electrostatic interaction between the electrode and the T1 Cu site of rCueO seems to increase $I_{\text{eff}}$. On the other hand, some hydrophobic parts locate around the T1 Cu site in $\Delta \alpha_{55}$CueO. Therefore, hydrophobic and untreated KB/GC electrode works as a scaffold suitable for DET-type bioelectrocatalysis of $\Delta \alpha_{55}$CueO. The hydrophobic parts locating around the T1 Cu site in the variant play an important role in the hydrophobic interaction between the electrode and the variant and to increase $I_{\text{eff}}$. Negatively charged KB/GC surface seems to induce unfavorable attractive orientation for DET-type reaction between the negatively charged electrode and the positively charged region of the CueOs on the opposite side of the T1 Cu site. The present work suggests the importance and possibility to prepare tailor-made scaffolds suitable for DET-type bioelectrocatalysis of redox enzymes based on the information of the protein structure and the surface charge. In addition, we expect that such CueO variants with high bioelectrocatalytic activities would be useful for practical use for biosensing of dissolved O$_2$.

Supporting Information

The Supplementary Information is available on the website at DOI: https://doi.org/10.5796/electrochemistry.20-00015.

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