Bioelectrocatalytic Reduction of O_2 Catalyzed by CueO from *Escherichia coli* Adsorbed on a Highly Oriented Pyrolytic Graphite Electrode

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CueO from *Escherichia coli*, a member of the multi-copper oxidase (MCO) family, was examined as a direct electron transfer-type bioelectrocatalyst for the four-electron reduction of O_2. Although CueO requires the fifth copper located near the type I Cu site to exhibit its oxidase activity (Roberts et al., 2003), it has been found that CueO receives electrons directly from electrodes even in the absence of the fifth copper. The fact indicates that electrons are transferred directly from electrodes to the type I Cu site. Furthermore, the catalytic current density of as large as about $-4\,\mathrm{mA\,cm^{-2}}$ was successfully observed with a rotating pyrolytic graphite electrode at pH 5. CueO is found to be superior to other MCOs in view of the catalytic activity and is an important candidate as a catalyst of the cathode in biofuel cells.

Copper is essential but toxic in mammals and bacteria. CueO (Cu efflux oxidase) is proposed to work for copper homeostasis in *Escherichia coli*, although the details of the mechanism remain unknown.[1] CueO is a monomeric protein with a molecular mass of ca. 53.4 kDa.[2] The enzyme belongs to the family of multi-copper oxidases (MCO)[3] including laccase, bilirubin oxidase (BOD), ascorbate oxidase, ceruloplasmin, CotA, etc. Copper atoms in MCOs are classified into three types based on spectroscopic and magnetic properties: the type I Cu is EPR-detectable and gives a blue color in the oxidized (cupric) state, the type II Cu is also EPR-detectable but colorless, and the type III Cu center composed of two copper ions bridged through hydroxide is EPR-silent and shows an absorption band at 330 nm in the optical spectrum.[3] In MCOs, the type I Cu site is the inlet of electrons from electron-donating substrates and transfers the electrons to the trinuclear center composed of one type II Cu and two type III Cu atoms. The trinuclear center (type II–III Cu cluster) serves as a catalytic site to reduce O_2 into water.

CueO is markedly different from other MCOs in view of the catalytic activities toward electron-donating substrates including 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), *p*-phenylenediamine, and 2,6-dimethoxyphenol.[2] In order to exhibit the oxidase activity, CueO requires excess amount of copper ions in reaction solution.[1] Recent X-ray crystallographic study has revealed that CueO has a fifth copper binding site located near the type I Cu site.[4] The fifth copper would be necessary to exhibit the oxidase activity for electron-donating substrates described above, and the electrons would be transferred from the substrate to type I Cu via the fifth copper.[4]

Some MCOs are promising enzymes as catalysts of direct electron-transfer (DET)-type bioelectrocatalytic four-electron reduction of O_2 to water.[5–10] The catalytic reactions may be utilized in biofuel cells.[5,11] One of the attractive properties of the enzymes as catalysts for the cathode in biofuel cells is that the catalytic reaction does not generate any reactive oxygen species.

In this study, we examined the electrochemical behavior of CueO from the viewpoint of the bioelectrocatalytic activity in the O_2 reduction. To our knowledge, this is the first paper on the electrochemical study on CueO. The catalytic behavior of CueO was compared with other MCOs, such as BOD and fungal laccase.

The gene coding for precursor CueO gene was designed to have sequence coding *Eco*RI restriction site at 5′-site and the 6×His-tag and *Bam*HI restriction site at 3′-end. The gene was inserted into pUC18, with which *E. coli* BL21 (DE3) was transformed by heat shock method. Expression and purification of CueO were carried out as described previously.[12] The enzyme concentrations were determined using a molar absorption coefficient at 612 nm: $\varepsilon_{612} = 5800\,\mathrm{M^{-1}\,cm^{-1}}$ ($\mathrm{M} = \mathrm{mol\,dm^{-3}}$). All chemicals used in this study were of analytical reagent grade, and all solutions were prepared with distilled water.

Voltammetry was performed using a BAS CV-50W electrochemical analyzer. Highly oriented pyrolytic graphite electrodes (edge plane) (HOPGE) were kindly donated by Prof. Abe in Kyoto University, were cut, and set into the PEEK tube (BAS) with insulator. The handmade electrode was polished with an emery paper (#600), rinsed with distilled water, sonicated in distilled water, and was attached to the shaft of an electrode rotator (RDE-2, BAS) for rotating disk electrode (RDE) measurements. The projected surface area of the HOPGE was $0.0337\,\mathrm{cm^2}$. The water-jacketed electrolysis cell had a platinum wire counter electrode and an Ag|AgCl|sat. KCl reference electrode. Measurements were carried at 25 °C under O_2-saturated or deaerated conditions. The electrolyte solution volume was 5 mL, and the enzyme concentration was fixed to be 0.4 μM.

Cyclic voltammogram of CueO under O_2-saturated conditions in the presence of $CuSO_4$ is shown as curve (a) in Figure 1. Clear and large cathodic waves were observed. When CueO was added into the solution, the cathodic current starts to increase gradually with time and reached the plateau. The behavior can be interpreted by model that CueO adsorbs on the electrode as a monolayer and that it catalyses the electrochemical reduction of O_2. The appearance of the peak-shaped wave strongly suggests the depletion of O_2 in the vicinity of the electrode surface and that the mass transfer (diffusion) of dissolved O_2 is the rate determining step at potential more negative than the peak potential. Although voltammograms obtained in the absence of O_2 as controls did not exhibit the faradic current due to the redox of the type I Cu(1+/2+) itself (curve (c) in Figure 1), the wave is ascribed to DET-type catalytic reduction of O_2, in which the electrons are transferred directly from electrode to CueO and CueO reduces O_2 into water.

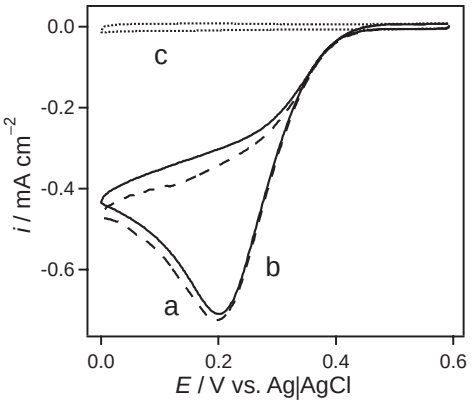

Figure 1. Cyclic voltammograms of CueO (0.4 μM) in a McIlvaine buffer (pH 5.0, 25 °C) under O_2-saturated conditions (O_2 concentration: ca. 1.2 mM) in the presence (a) and absence (b) of 1 mM of $CuSO_4$. The electrode was not rotated. Curve (c) was obtained under deaerated conditions, and the other conditions are same as curve (a). Scan rates are 20 mV s^{-1}.

Surprisingly, similar catalytic wave was observed even in the absence of $CuSO_4$ (curve (b) in Figure 1). The wave is almost identical with that in the presence of $CuSO_4$ (curve (a)) in the shape and location. These results indicate that CueO works as a good catalyst in DET-type catalytic reduction of O_2, in which the electrons are transferred directly from electrode to the type I Cu site. This is in marked contrast with the fact that excess copper ions are necessary for CueO to exhibit or enhance its oxidase activity in solution (e.g., ca. 7.6% of the relative activity toward ABTS in the absence of copper ions[2]). The type I Cu is covered with an α-helix portion, as evidenced by the X-ray crystallographic study.[4] The α-helix would make difficult for substrates to approach the type I Cu site. The fifth copper site would function as a mediator of the electron transfer from substrates to the type I Cu site. In DET-type bioelectrocatalysis, the electron seems to be transferred from electrodes to the type I Cu site in a way that is completely different from that in substrate oxidation.

The formal potential ($E^{\circ\prime}$) of the type I Cu site in CueO was not reported yet. As reported in previous papers on DET-type bioelectrocatalytic reactions of MCOs, the catalytic wave began to increase around $E^{\circ\prime}$ of the type I Cu site. Therefore, the $E^{\circ\prime}$ value of the type I Cu site in CueO may be located around 0.3 V vs. Ag|AgCl, where the catalytic current began to increase (Figure 1). The type I Cu site of CueO is coordinated by two histidine residues, one cysteine residue, and one methionine residue.[4] Most of the other MCOs with the same coordination have $E^{\circ\prime}$ in the range from 0.14 to 0.46 V vs. Ag|AgCl.[5]

The results of RDE measurements are shown in Figure 2. The limiting currents increased with an increase of the electrode rotation rate (ω). The limiting O_2 reduction current showed diffusion-controlled characteristics, as judged from the inset of Figure 2, where the straight broken line represents a theoretical Levich plot for a four-electron transfer process with following parameters: O_2 concentration, 1.2 mM; diffusion coefficient of O_2, 1.7×10^{-5} cm^2 s^{-1}; kinematic viscosity of water, 0.01 cm^2 s^{-1}.

The limiting currents were controlled by the mass transfer of O_2 at ω at least up to 1500 rpm. When BOD or laccase was used

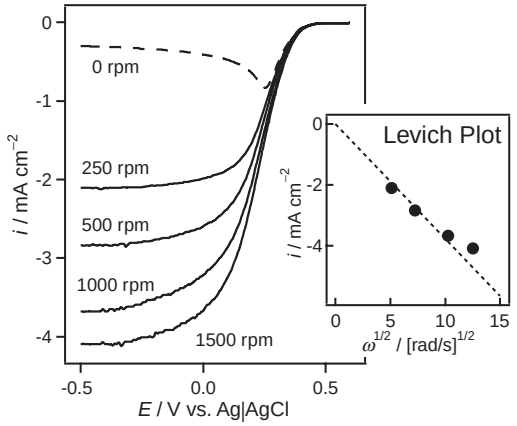

Figure 2. Rotating disk voltammograms of CueO catalyzing reduction of O_2 in a McIlvaine buffer (pH 5.0, 25 °C) under O_2 saturated conditions. Rotating rates (rpm) were listed in the figure. Inset represents the Levich plot of the plateau current in rotating voltammetry for reduction of O_2 on the CueO-modified electrode. Scan rates are 20 mV s^{-1}.

in place of CueO, the limiting current showed characteristics of enzyme-kinetic control at large ω under O_2-saturated conditions. The magnitude of the limiting catalytic current at 250 rpm was about 8 and 9 times as large as that observed using BOD and laccase, respectively.[6,8] These results suggest that the catalytic rate constant of CueO is sufficiently larger than that of other MCOs in DET-type bioelectrocatalysis and that CueO is a great candidate as an electrocatalyst for the cathode in biofuel cells.

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References

1 G. Grass, C. Rensing, *Biochem. Biophys. Res. Commun.* **2001**, *286*, 902.
2 C. Kim, W. W. Lorenz, J. T. Hoopes, J. F. D. Dean, *J. Bacteriol.* **2001**, *183*, 4866.
3 E. I. Solomon, U. M. Sundaram, T. E. Machonkin, *Chem. Rev.* **1996**, *96*, 2563.
4 S. A. Roberts, A. Weichsel, G. Grass, K. Thakali, J. T. Hazzard, G. Tollin, C. Rensing, W. R. Montfort, *Proc. Natl. Acad. Sci.* **2002**, *99*, 2766; S. A. Roberts, G. F. Wildner, G. Grass, A. Weichsel, A. Ambrus, C. Rensing, W. R. Montfort, *J. Biol. Chem.* **2003**, *278*, 31958.
5 S. Shleev, J. Tkac, A. Christenson, T. Ruzgas, A. I. Yaropolov, J. W. Whittaker, L. Gorton, *Biosens. Bioelecrtron.* **2005**, *20*, 2517.
6 S. Tsujimura, T. Nakagawa, K. Kano, T. Ikeda, *Electrochemistry* **2004**, *72*, 437.
7 S. Tsujimura, K. Kano, T. Ikeda, *J. Electroanal. Chem.* **2005**, *576*, 113.
8 C.-H. Lee, H. B. Gray, F. C. Anson, *J. Electroanal. Chem.* **1984**, *172*, 289.
9 S. Shleev, A. E. Kasmi, T. Ruzgas, L. Gorton, *Electrochem. Commun.* **2004**, *6*, 934.
10 Y. Kamitaka, S. Tsujimura, T. Ikeda, K. Kano, *Electrochemistry* **2006**, *74*, 642.
11 S. Tsujimura, K. Kano, T. Ikeda, *Electrochemistry* **2002**, *70*, 940.
12 Y. Ueki, M. Inoue, S. Kurose, K. Kataoka, T. Sakurai, *FEBS Lett.* **2006**, *580*, 4069.

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