Ascorbate Oxidase-catalyzed Electrochemical Reduction of Dioxygen Using 2,6-Dichloroindophenol as an Electron-transfer Mediator

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Electrochemical reduction of dioxygen to water proceeds through the 2,6-dichloroindophenol (DCIP)-mediated and *Acremonium* sp. HI-25 ascorbate oxidase (ASOM)-catalyzed reaction. The optimum pH of the reaction has been proven to be neutral pH 7.5.

Multicopper oxidases such as bilirubin oxidase, laccase, and ascorbate oxidase catalyze the reduction of dioxygen to water with the concomitant oxidation of substrate. They contain four copper binding sites per functional unit, type 1 Cu, type 2 Cu, and two type 3 Cu. Type 1 Cu is involved in electron uptake from the substrate, and the latter three copper binding sites are proximal and constitute a trinuclear center, which is the catalytic dioxygen reduction site. In recent years, mediator-type electrochemical reductions of dioxygen by multicopper oxidases including laccase[1] and bilirubin oxidase[2] have been studied. Understanding the reactions facilitates the practical application of biodevices such as biofuel cells and biosensors. However, to the best of our knowledge, there is no report about the electrochemical catalytic reaction of ascorbate oxidase using an electron-transfer mediator. Although Santucci et al. have reported the direct electron-transfer reaction of ascorbate oxidase,[3] the electrochemical reduction of dioxygen occurs at a slow rate, so far.

Ascorbate oxidase from bacterium *Acremonium* sp. HI-25 (ASOM) is monomeric with a molecular mass of about 80 kDa and is thermostable up to 60 °C,[4] while plant ascorbate oxidases are homo-dimers with one monomer of about 70 kDa and are thermolabile. For applications such as biofuel cells, thermostability is important and required, and therefore we have used ASOM (purchased from Asahi Kasei Co., Japan) in this work. Type 1 Cu of ASOM is expected to be an electron acceptor from a substrate in common with other multicopper oxidases, whereas the redox potential of type 1 Cu has not yet been determined. We first determined the redox potential of type 1 Cu in ASOM by anaerobic potentiometric titration using ferrocyanide in accordance with the method previously reported.[5] The result of the titration at 596 nm is depicted as a Nernst plot in Figure 1, giving a midpoint potential of +197 mV. For many multicopper oxidases, the redox potential of type 1 Cu can be classified by the axial ligand, although not solely.[6] Oxidases which have methionine as an axial ligand for the type 1 site are classified as low redox potential enzymes (340–390 mV vs NHE), whereas middle redox potential enzymes (470–710 mV vs NHE) have leucine, and high-potential oxidases (730–780 mV vs NHE) have phenylalanine, respectively. The potential of type 1 Cu of ASOM (+197 mV, pH 7.0, 25 °C) is slightly higher than that of green zucchini squash (*Cucurbita pepo medullosa*) ascorbate oxidase (+139 mV, pH 6.0, 25 °C)[7] and cucumber (*Cueumis sativus*) ascorbate oxidase (+145 mV, pH 7.0, 25 °C).[5] Based on the results of crystallographic analysis of zucchini ascorbate oxidase,[8] type 1 Cu ligand binding residues have been elucidated to be His^{445}, Cys^{507}, His^{512}, and Met^{517}. It is also expected that cucumber ascorbate oxidase have the same set of ligands, from sequence homology. However, it has been reported that the axial ligand Met^{517} of zucchini ascorbate oxidase is replaced by Lys^{546} in ASOM.[9] Although lysine is not included in the above classification, it seems that the positively shifted potential is related with the replacement of the axial ligand.

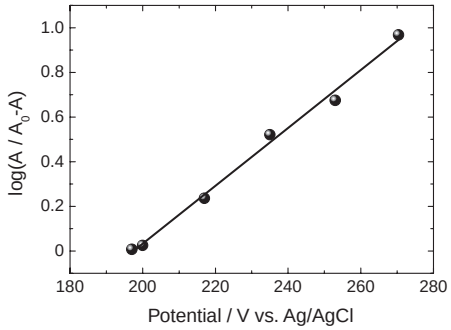

Figure 1. Anaerobic potentiometric titration of type 1 copper in ASOM using hexacyanoferrate(II) ion as a mediator at room temperature.

Here, we demonstrate the ASOM-catalyzed electrochemical reduction of dioxygen using 2,6-dichloroindophenol (DCIP) as an electron-transfer mediator, which has a redox potential close to type 1 Cu of ASOM. Cyclic voltammetry (CV) was performed using a BAS electrochemical analyzer in a three electrode system with a glassy carbon (GC) electrode, Ag/AgCl (3 M NaCl) and a Pt wire as working, reference, and counter electrodes, re-

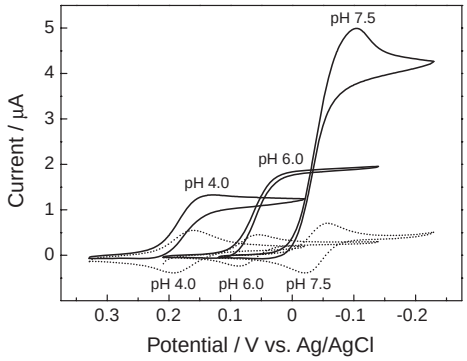

Figure 2. Cyclic voltammograms of 0.1 mM DCIP in an air-saturated buffer solution at pH 7.5, pH 6.0, and pH 4.0 in the absence (dotted lines) and presence of 3 μM ASOM (solid lines). Scan rate: 2 mV/s.

spectively. Voltammetric responses were recorded in 100 μM DCIP and 3 μM ASOM, prepared in 0.1 M buffer solution (pH 4–6; acetate buffer, pH 6–8; phosphate buffer, pH 8–10; boric acid–Na_2CO_3 buffer), which is saturated with dioxygen by bubbling air for 30 min. In this communication, potentials refer to Ag/AgCl (3 M NaCl), whose potential is +205 mV vs NHE, unless otherwise stated.

As shown in Figure 2 (dotted lines), reversible redox responses between DCIP and GC electrode were observed in a wide pH range. The voltammograms of DCIP were independent of the presence or absence of dioxygen in the solution (data not shown). In the presence of ASOM, the redox reaction could be coupled with the ASOM-catalyzed reduction of dioxygen to produce a large cathodic catalytic current as observed in Figure 2 (solid lines). It has been reported that the active site binding pocket of ascorbate oxidase is specific for ascorbate[10] and the electron transfer of ascorbate oxidase is strongly dependent on the nature of the electron donor.[3] In addition to ascorbate, it has been reported that ferrocyanide,[11] cytochrome *c*,[12] organic radicals (such as metronidazole,[11a] lumiflavin,[13] and methyl viologen[13b]) generated by pulse radiolysis and flash photolysis, are also able to act as electron donors for ascorbate oxidase. When the reducing molecule can fit into the cavity near type 1 Cu, e.g. in ascorbate or the metronidazole radical, both electron transfer to type 1 Cu and intramolecular electron transfer between type 1 Cu and trinuclear center are rapid. When the reducing molecule can not fit into the cavity, as in the reaction with ferrocyanide and cytochrome *c*, electron transfer occurs slowly. When we used DCIP as an electron-transfer mediator, it was analogized that DCIP can fit into the cavity and the electron-transfer reaction proceeds at a fast rate; therefore, the electrochemical catalytic reduction of dioxygen was observed by the CV technique.

Figure 3 shows the pH dependence of electrochemical catalysis by ASOM. The catalytic current exhibited a maximum value at neutral pH 7.5. As the redox potential of DCIP was −0.01 V at pH 7.5 and varied linearly with pH (−64 mV/pH) within pH 4.0–10.0, the redox potential difference between DCIP and type 1 Cu of ASOM increased as the pH increased. As a result, the driving force of the reaction increased with increasing pH. This driving force seems to be one of the factors that made the optimum pH 7.5 for the electrochemical catalytic reaction different from the ascorbate oxidation optimum of pH 4.5. It is necessary to perform the cathodic reaction under neutral conditions at which laccase can not catalyze the electrochemical reduction of dioxygen. Tsujimura and co-workers showed that bilirubin

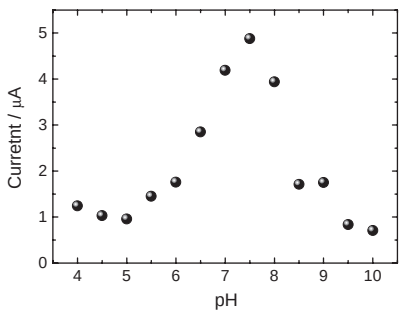

Figure 3. pH Dependence of catalytic current for electrochemical reduction of dioxygen by ASOM.

oxidase can catalyze the electrochemical reduction of dioxygen to water in neutral pH,[2] and actually constructed a one-compartment glucose/dioxygen biofuel cell using a bilirubin oxidase-modified cathode and a glucose dehydrogenase-modified anode, which work at neutral conditions.[14] In this study, we have shown that ASOM is also a useful enzyme which fulfills this pH requirement for a biocathode.

In conclusion, we have demonstrated the electrochemical reduction of dioxygen to water by ASOM using DCIP as an electron-transfer mediator. It has been shown that DCIP is an effective electron-transfer mediator for ASOM, and the optimal pH is proven to be neutral pH 7.5. However, to use ASOM as a biocathode at neutral pH, a mediator with more positive redox potential than DCIP is required. With the use of such mediators, even at neutral pH dioxygen reduction at more positive potentials would be expected. Extension of this study is now under way in this direction.

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References

1 F. Trudeau, F. Daigle, D. Leech, *Anal. Chem.* **1997**, *69*, 882; G. Tayhas, R. Palmore, H.-H. Kim, *J. Electroanal. Chem.* **1999**, *464*, 110; C. Fernández-Sánchez, T. Tzanov, G. M. Gübitz, A. Cavaco-Paulo, *Bioelectrochemistry* **2002**, *58*, 149.
2 S. Tsujimura, H. Tatsumi, J. Ogawa, S. Shimizu, K. Kano, T. Ikeda, *J. Electroanal. Chem.* **2001**, *496*, 69; T. Nakagawa, S. Tsujimura, K. Kano, T. Ikeda, *Chem. Lett.* **2003**, *32*, 54; S. Tsujimura, M. Kawaharada, T. Nakagawa, K. Kano, T. Ikeda, *Electrochem. Commun.* **2003**, *5*, 138.
3 R. Santucci, T. Ferri, L. Morpurgo, I. Savini, L. Avigliano, *Biochem. J.* **1998**, *332*, 611.
4 S. Murao, H. Itoh, T. Yajima, Y. Ozaki, S. Fukuyasu, T. Shin, *Biosci. Biotechnol. Biochem.* **1992**, *56*, 847.
5 K. Kawahara, S. Suzuki, T. Sakurai, A. Nakahara, *Arch. Biochem. Biophys.* **1985**, *241*, 179.
6 S. Shleev, J. Tkac, A. Christenson, T. Ruzgas, A. I. Yaropolov, J. W. Whittaker, L. Gorton, *Biosens. Bioelectron.* **2005**, *20*, 2517.
7 P. M. H. Kroneck, F. A. Armstrong, H. Merkle, A. Marchesine, in *Ascorbic Acid: Chemistry, Metabolism, and Uses, Advances in Chemistry Series*, ed. by P. A. Seib, B. M. Tolbert, Washington, D. C., **1982**, Vol. 200, pp. 223–248.
8 A. Messerschmidt, H. Luecke, R. Huber, *J. Mol. Biol.* **1993**, *230*, 997.
9 K. Takeda, H. Itoh, I. Yoshioka, M. Yamamoto, H. Misaki, S. Kajita, K. Shirai, M. Kato, T. Shin, S. Murao, N. Tsukagoshi, *Biochim. Biophys. Acta* **1998**, *1388*, 444.
10 L. Santagostini, M. Gullotti, L. D. Gioia, P. Fantucci, E. Franzini, A. Marchesini, E. Monzani, L. Casella, *Int. J. Biochem. Cell Biol.* **2004**, *36*, 881.
11 a) P. O'Neill, E. M. Fielden, L. Morpurgo, E. Agostinelli, *Biochem. J.* **1984**, *222*, 71. b) K. Avigliano, A. Finazzi-Agrò, in *Multi-Copper Oxidases*, ed. by A. Messerschmidt, Singapore, **1997**, pp. 265–278.
12 T. Sakurai, *J. Inorg. Biochem.* **1994**, *55*, 193; W. Jin, U. Wollenberger, F. F. Bier, A. Makower, F. W. Scheller, *Bioelectrochem. Bioenerg.* **1996**, *39*, 221.
13 a) T. E. Meyer, A. Marchesini, M. A. Cusanovich, G. Tollin, *Biochemistry* **1991**, *30*, 4619. b) P. Kyritsis, A. Messerschmidt, R. Huber, G. A. Salmon, A. G. Sykes, *J. Chem. Soc., Dalton Trans.* **1993**, 731.
14 S. Tsujimura, K. Kano, T. Ikeda, *Electrochemistry* **2002**, *70*, 940.

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