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Oxygen reduction reactions of the thermostable bilirubin oxidase from Bacillus pumilus on mesoporous carbon-cryogel electrodes

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ABSTRACT

This study demonstrates the bioelectrocatalytic reactions of a new bilirubin oxidase (BOD) from Bacillus pumilus on a mesoporous carbon cryogel (CCG) electrode, in the presence and absence of a mediator. BOD, physically adsorbed on the mesoporous matrix of a CCG electrode, allowed a direct electron transfer (DET) from the carbon electrode to the type I copper site of the enzyme. The current from the dioxygen reduction reaction (ORR), catalyzed by BOD, depended on the temperature and pH of the electrolyte. The mediated ORR catalyzed by BOD on CCG electrode was also investigated using osmium based redox polymers. The catalytic current on the CCG electrode modified with 0.2 mg cm^{-2} of hydrogel consisting of an enzyme, a redox polymer and a cross linker, was 1.8 mA cm^{-2}, which was almost five times higher than that on a flat glassy carbon electrode for the same hydrogel composition and loading. The catalytic current linearly increased with the total amount of hydrogel on the porous carbon electrode while the catalytic current on the flat electrode was indifferent to the loading.

1. Introduction

The efficient enzymatic 4e$^-$/4H$^+$ reduction of O_2 to water under physiological conditions is of particular importance for the elaboration of cathodes in enzymatic biofuel cells (BFCs) [1–5]. Recently, we identified and reported a new bilirubin oxidase (BOD) from *Bacillus pumilus* as a promising thermostable bioelectrocatalyst for BFCs [6]. This new BOD displayed excellent catalytic activity and stability for the electrochemical O_2 reduction under physiological conditions (phosphate buffer at pH 7.4, 0.15 M NaCl, and 37.5 °C) as well as in serum [7]. We also demonstrated that it could work as an electrocatalyst over broad ranges of pH (4-7) and temperature (30-70 °C) [8].

It is essential to increase the specific surface area of the electrodes in BFCs to improve the output power density per unit of the geometric surface area [9–13]. To achieve this, porous carbon electrodes with large specific surface area are relevant [3,14]. Previously, we reported two efficient biocathodes, which had uniform and size-controlled mesopores, made of carbon gel using either laccase from *Trametes* sp. or BOD from *Myrothecium verrucaria* in the absence of any redox mediators [15,16]. They demonstrated diffusion-controlled oxygen reduction currents.

In this study, the BOD from *B. pumilus* was immobilized in a mesoporous matrix of the carbon cryogel (CCG) with an average radius of 20 nm [17]. We examined and compared two different kinds of electron transfer reactions between the BOD active site and electrode: direct electron transfer (DET), and mediated electron transfer (MET). This is the first report of a more efficient production of O_2 reduction current based on the MET reaction, involving a monolith-type mesoporous biocathode containing a redox hydrogel, compared to those based on the MET reactions involving a flat electrode.

2. Experimental

2.1. Chemicals

The BOD from *B. pumilus* was produced and purified as described earlier [6]. PAA-PVI-[Os(4,4′-dichloro-2,2′-bipyridine)$_2$Cl$^-$]$^{+/2+}$ was synthesized as previously described [18]. Poly(ethylene glycol) diglycidyl ether (PEGDGE 400) was purchased from Sigma-Aldrich. All other chemicals used were of reagent grade. CCG with an average pore radius of 20 nm was prepared according to reported procedures [17]. Ketjen black (KB, EC300 J) and poly(vinylidene difluoride) (PVDF, 5% (w/w) dissolved in *N*-methyl-2-pyrrolidone

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(NMP)) were kindly donated by Lion Co. (Japan) and Kureha Co. (Japan), respectively.

2.2. Preparation of BOD-modified CCG electrodes for DET and MET reactions

CCG powder (25 mg), PVDF solution (100 mg), and NMP (150 μL) were mixed thoroughly by sonication to obtain the CCG slurry. Five microliters of the CCG slurry was applied to the surface of a glassy carbon (GC) disk (geometric surface area: 0.20 cm^2; Pine Instruments, USA) and dried in an oven at 60 °C for more than 12 h. KB-modified electrodes were prepared as the CCG electrode using 25 mg KB powder, 100 mg PVDF solution, and 400 μL NMP. The DET electrode was prepared by applying 5 μL of the BOD solution (5.0 mg mL^{-1} (85 μM) in 50 mM Tris-H_2SO_4 at pH 7.4, (M = mol dm^{-3})) to the CCG electrode, which was pretreated with O_2 plasma (1 Torr) for 10 min. The electrode was dried in air at room temperature for 10 min.

The MET electrodes were prepared by the following procedure. A deposition solution was prepared by mixing 3 μL of 10 mg mL^{-1} aqueous PAA-PVI-[Os(4,4′-dichloro-2,2′-bipyridine)$_2$$Cl^-$]$^{+/2+}$ solution, 2.9 μL of milli-Q water, 1.2 μL of 5.0 mg mL^{-1} BOD in 50 mM Tris-H_2SO_4 at pH 7.4, and 0.9 μL of 3 mg mL^{-1} PEGDGE in water for a total hydrogel loading of 0.2 mg cm^{-2}. The electrodes with different hydrogel loadings were simply prepared by changing the volume of the deposition solution while maintaining a constant composition. The mixed solution was pipetted onto the mounted hydrophilic CCG electrode after treatment with O_2 plasma (1 Torr) for 10 min. The electrodes were cured for 18 h at room temperature before use.

2.3. Electrochemical measurements

Rotating disc cyclic voltammetry was performed using an electrochemical analyzer (CHI 842; CH-Instruments, USA). The electrode was rotated using a rotator from Pine Instruments (USA). The counter and reference electrodes were a platinum wire and an Ag|AgCl|sat. KCl electrode, respectively. The measurements were performed in an electrochemical cell provided with a thermostat jacket. The electrolyte solution was either 0.1 M citrate buffer (pH 3–5) or 0.05 M phosphate buffer (pH 6–7) saturated with O_2 by continuous bubbling. The scan rate was 10 mV s^{-1}. All potentials in this paper are referred to the Ag|AgCl|sat. KCl electrode.

3. Results and Discussion

3.1. DET of BOD on CCG electrode

The solid curves in Fig. 1(A) show the temperature dependence of the cyclic voltammograms for ORR at pH 5, catalyzed by BOD on a rotating CCG electrode (3000 rpm) in the absence of a redox mediator. The catalytic current increased with the increasing temperature of the electrolyte from 30 °C to 60 °C (Fig. 1(A)). In this study, a limited amount of enzymes (25 μg per electrode) was applied on the CCG electrode to characterize the enzymatic reaction rate under various conditions of pH and temperature. Thus, the catalytic current was determined by the enzymatic reaction rate, and not by the mass transfer rate [16]. The maximum catalytic current controlled by the enzyme kinetics could be multiplied by increasing the enzyme loading. For example, at 37 °C and pH 7, ORR currents of 2 mA cm^{-2} and 3 mA cm^{-2} at −0.1 V vs. Ag|AgCl were obtained for enzyme loadings of 50 μg and 75 μg, respectively.

The onset potential for O_2 reduction was approximately 0.4 V, which was close to that observed at electrodes modified with a multi-copper oxidase (CueO) from *Escherichia coli* [19] but was 0.1 V lower than that of the electrode modified with BOD from *M. verrucaria* [16]. Although no redox signal attributed to type 1 (T1) copper (Cu) site was observed under anaerobic conditions, we estimated the formal potential of the T1 Cu site (0.38 V) from the ORR catalytic wave observed under aerobic conditions (*vide infra*) [20]. The analysis of the amino acid sequence of the enzyme showed that the T1 Cu was surrounded by residues of one cysteine (Cys), two histidine (His), and one methionine (Met) residues [21]. As in the case of other blue multi-copper oxidases, the equatorial ligands (two His and one Cys residues), which were highly conserved among the blue multi-copper oxidases from different organisms, directly coordinated to the Cu atom. The S atom in the axial Met ligand weakly coordinated to the T1 Cu. The formal potentials of the T1 Cu site with trigonal bipyramidal geometry were reported to be in the range of 0.2–0.4 V [22–24], which were in good agreement with the observed electrochemical responses.

The voltammograms did not show the typical sigmoidal catalytic wave shapes, with limiting currents, at potentials more negative than 0.3 V under any pH condition. This catalytic behavior was attributed to the slow interfacial electron transfer from the electrode to T1 Cu site of the BOD rather than the enzymatic turnover [25]. The T1 Cu site of the enzyme was buried within the protein; and/or the enzyme was not properly orientated on the electrode surface, thereby avoiding a direct electronic connection with the electrode. The crystallographic study of the BOD is currently in progress, and may provide a better understanding of its behavior on the electrode surface.

The BOD immobilized on a porous KB electrode also showed catalytic wave shapes for ORR (Fig. 1 (A), dashed wave shape). Fig. 1 (B) shows the comparison of the background subtracted voltammograms for the modified CCG (red curve) and KB (dashed curve) electrodes at 30 °C. The onset potentials and maximum catalytic current densities at −0.1 V on both the electrodes were almost comparable. However, there was a clear difference in the slope of the catalytic current at approximately 0.3 V, which depended on the interfacial electron transfer rate between the T1 Cu and electrode surface. The cathodic catalytic current on KB-electrode showed a sharp increase and reached the maximum steady state. To evaluate the DET-type BOD catalysis quantitatively, the current-potential curves were analyzed with a theoretical equation [20]. The dashed curves represent the regression curves based on Eqs. (1)–(3) in the reference 20 with the parameters; $k_c \times \Gamma_t = 2.8 \times 10^{-8}$ mol cm^{-2} s^{-1}, k_{cat}/k°=2.1 for KB electrode and 4.8 for CCG electrode, and $E^{\circ\prime}$ = 0.38 V vs. Ag|AgCl respectively. The surface electron transfer kinetic (k° value) of BOD on KB electrode was almost twice as large was that on CCG electrode. The chemical and nano-structural characteristics of the surface affected the heterogeneous electron transfer rate, which decreased exponentially as a function of the distance of electron transfer.

Fig. 1 (C) shows the temperature dependence of the catalytic current density at −0.1 V measured for pH values ranging from 4 to 7. In acidic conditions (pH 4 and 5), the current response at −0.1 V depended on the temperature, as shown in Fig. 1(A). The limitations created by O_2 mass-transfer were only addressed for those conditions of acidic pH and high temperature wherein this thermophilic enzyme (new BOD) was more efficient. On the other hand, the current response at pH 7 as a function of the electrode potential was linear over the potential range of 0.6 V to −0.1 V, which suggested that the interfacial electron transfer rate between the T1 Cu site and electrode was much lower than the rate of enzymatic catalysis. The stability of the BOD-modified electrode on CCG and KB electrodes was also examined by continuously monitoring the current with the modified electrode rotating at 2000 rpm, pH 5, and 50 °C (Fig. 1 (D)). Although the catalytic current on the KB electrode decreased by more than 20% of the initial value after 5000 s, only a 5% decrease in the catalytic current on the CCG electrode was