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Enhanced direct electron transfer-type bioelectrocatalysis of bilirubin oxidase on negatively charged aromatic compound-modified carbon electrode

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ABSTRACT

Effects of chemical modification of mesoporous Ketjen Black (KB) electrodes on direct electron transfer (DET)-type bioelectrocatalytic reduction of dioxygen by bilirubin oxidase (BOD) were investigated under air-saturated neutral conditions. Several amines were electrochemically oxidized at KB-modified electrode to generate nitrogen–carbon bond. The modification with negatively charged aromatic amines such as 4-aminobenzoic acid (4-ABA) drastically increased the catalytic current density compared with that by positively charged and non-charged aromatic compounds and negatively charged non-aromatic compound. Considering the basic amino acid residues around the type I site of BOD, it can be concluded that weakly negative charge on electrode surface induces a favorable orientation of BOD for the DET-type catalysis via the electrostatic interaction, while the π–π interaction is also essential for effective orientation of BOD on the electrode surface. The 4-ABA-modification leads to an increase in the heterogeneous electron transfer rate constant and a decrease in the randomness of the orientation as well as a slight increase in the surface concentration of BOD.

1. Introduction

Direct electron transfer (DET)-type bioelectrocatalysis has attracted increasing attention in the past decades due to its advantages such as simple construction, the absence of energy loss concerning mediators, and the absence of the problem on the stability of mediators [1–5]. Unfortunately, most of enzymes have been found to be difficult or limited to directly communicate with electrodes, because the active center of enzymes is often embedded deeply in the polypeptides. Therefore, some novel materials and methods have been developed to improve the activity of the DET-type bioelectrocatalysis. Good performance of DET-type reaction can be achieved by using nano-materials, such as carbon nano-materials [6,7], Fe_3O_4 magnetic nanoparticle [8], and Au nanoparticle [9], thanks to increased specific surface areas and high conductivity.

On the other hand, the electron-transfer rate is governed by the potential difference, the reorganization energy, and most importantly the distance between the active center of enzymes and the electrode surface, as described in Marcus theory [10]. Therefore, it is important to control the orientation of enzymes on the electrode surface to shorten the distance from the active center of enzymes to the electrode surface. Several immobilization methods of enzymes on the electrode surface have been reported to control the orientation of enzymes for DET-type bioelectrocatalysis via covalent amide bond and Au–S bond [11–13].

Recently, modification of electrodes with substrate or 'substrate-like linker' with structurally similar to natural substrate has been reported to induce a favorable orientation of the enzyme for the DET-type bioelectrocatalysis [14–20]. The linker is expected to be inserted into the active center 'pocket' of the enzyme and improves the coverage, the orientation, and the stability of the enzyme on the electrode. More recently, the characteristics of the electrode surface, such as positively/negatively charged property, hydrophilic or hydrophobic property, are considered to affect the orientation of enzymes [21,22].

Bilirubin oxidase (BOD) is in a family of multi-copper oxidase (MCO) and our group has first found that BOD can work as a good DET-type bioelectrocatalyst for four-electron reduction of dioxygen in neutral solution [23,24]. The active site of BOD contains four copper atoms which can be divided into three types according to their spectroscopic and magnetic properties [25,26]: type I (T1), type II (T2), and type III (T3) coppers. The T1 copper oxidizes bilirubin to biliverdin and transfers the electron to the trinuclear center composed of one T2 copper and two T3 copper atoms where O_2 is reduced to H_2O. In a DET-type reaction, the T1 site accepts electron from electrodes [27,28]. Recently, higher DET-type bioelectrocatalytic activity of BOD on electrodes modified with several compounds has been reported [14,28–31]. Although the accurate information about the interaction between BOD and the modified electrode surface remains still unclear, DET-type BOD-based biocathodes have been developed for biofuel cells [4,13,14,32–37] in the past decades.

In this work, we focus on effects of chemical modification of carbon electrode surface on the DET-type bioelectrocatalytic activity of

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BOD. Several amines were linked to Ketjen Black (KB) through an electrochemically generated nitrogen–carbon bond. The modification provides charged/non-charged characteristics and aromatic/aliphatic characteristics on the KB surface. We may show that the electrostatic interaction between the active site of BOD and negatively charged aromatic compounds on the modified surface leads to a favorable orientation of BOD.

2. Experimental

2.1. Materials and regents

Ketjen Black EC300J (KB) was kindly donated from Lion Co. (Japan). Poly (tetrafluoroethylene) fine powder (PTFE, 6-J) was obtained from DuPont Mitsui Fluorochemicals (Japan). 4-Aminobenzoic acid (4-ABA), 2-aminobenzoic acid (2-ABA), 4-aminophthalic acid (4-APA), 4-aminobenzenesulfonic acid (4-ABS), 3-aminobenzenesulfonic acid (3-ABS), 2-aminobenzenesulfonic acid (2-ABS), *p*-phenylenediamine (*p*-PDA), methyl 4-aminobenzoate (MABA), and 6-aminohexanoic acid (6-AHA) were purchased from Tokyo Chemical Industry Co. (Japan). Bilirubin oxidase (BOD; EC 1.3.3.5) from *Myrothecium verrucaria* was donated from Amano Enzyme Inc. (Japan) (3.04 unit mg^{-1}) and used without further purification. Copper efflux oxidase (CueO) from *Escherichia coli* was prepared as described previously (187 unit mg^{-1}) [38,39]. All other chemicals used in this study were of analytical grade, and all solutions were prepared with distilled water.

2.2. Electrochemical measurements

All of electrochemical measurements were performed on an electrochemical analyzer ALS 701E with a rotating disk glassy carbon electrode (GCE, 3 mm in diameter, BAS) as a working electrode, a Pt wire as a counter electrode, and an Ag|AgCl|sat. KCl electrode as a reference electrode, respectively. All potentials are referred to the reference electrode in this work. The bioelectrocatalytic activity of BOD for the dioxygen reduction was evaluated by linear scan and cyclic rotating disk voltammetry at a scan rate (v) of 20 mV s^{-1} at 25 °C in 0.1 M air-saturated phosphate buffer (pH 7.0) at a rotating rate (ω) of 2000 rpm to get steady-state voltammograms (1 M = 1 mol dm^{-3}).

2.3. Electrochemical modification of carbon electrodes with amines

GCE was polished with alumina slurry (3 μm and 0.5 μm, in turn), sonicated, and washed with distilled water. The GCEs were chemically modified with several amines according to the literature [40]. In brief, amine was dissolved in 0.1 M KCl at a final concentration of 5 mM. In preliminary experiments, the amine solution was electrolyzed by 5-cycle potential scan in the potential range from 0.5 to 1.4 V at $v = 20$ mV s^{-1}. A large oxidation peak of the amino group of 4-ABA was observed at around 0.95 V in the first cycle, but the current decreased drastically in the subsequent scans (Fig. S1 (A)). The oxidation of the amine on carbon electrodes generates a nitrogen–carbon bond and the carbon electrodes were chemically modified with the amine [40]. In the following, one-step electrochemical oxidation was done at 0.8 V for 40 s to simplify the procedure. The modified electrode was washed with distilled water to remove physically absorbed amines. The expected structure of the covalently bound amines is given in Fig. 1. For example, the modification with 4-ABA provides negatively charged aromatic platform, since the carboxy group is dissociated under neutral pH and the amino group is oxidized and covalently linked to the electrode. The chemical modification of the amines were electrochemically checked with $[Fe(CN)_6]^{3-/4-}$ and $[Ru(NH_3)_6]^{2+/3+}$ as redox probes [40] (Fig. S2).

KB-modified GCE (KB/GCE) was prepared according to the literature [14]. Briefly, KB and PTFE (4:1, w:w) were distributed in 2-propanol and homogenized by ultrasonic disruptor (Heat Systems GmbH & Co.) for 3 min in an ice bath to prepare KB slurry. One microliter of the KB slurry

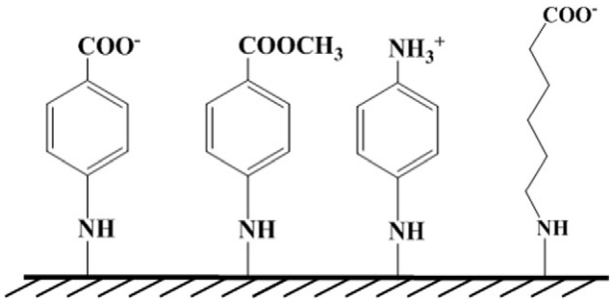

Fig. 1. The expected structures of the covalently bound amines that are electrochemically oxidized on carbon electrodes. The original amines are 4-aminobenzoic acid (4-ABA), methyl-4-aminobenzoate (MABA), *p*-phenylenediamine (*p*-PDA) and 6-aminohexanoic acid (6-AHA) from the left side to the right side.

was dropped on a GCE surface and dried at room temperature (about 25 °C) for 10 min to make a KB/GCE (1 L = 1 dm^{-3}). The oxidation peak of 4-ABA at KB/GCE was observed at around 0.85 V (Fig. S1 (B)). The KB/GCEs were chemically modified with the amines in a manner identical with that in the case of GCE described above. In the following, the chemically modified KB/GCE is called amine/KB/GCE. The surface area of the modified electrode was roughly estimated from the charging current at a potential where faradic processes are minimal [41]. The surface area of KB/GCE was calculated as 6.9 cm^2, which is about 100 times larger than bare GCE (0.07 cm^2). The chemical modification of KB/GCE described here caused practically no change in the surface area.

2.4. Adsorption of BOD on KB/GCEs and amine/KB/GCEs

BOD solution was prepared by dissolving the enzyme into 0.1 M phosphate buffer (pH 7.0) at a concentration of 10 mg mL^{-1}. Ten microliter of the BOD solution was dropped on KB/GCEs or amine/KB/GCEs and dried at room temperature for 1.5 h. The electrode was washed with distilled water and used for electrochemical experiments. In the following, the electrodes are called BOD/KB/GCE and BOD/amine/KB/GCE, respectively. For long time storage, BOD/amine/KB/GCEs were kept in water-saturated atmosphere at 4 °C.

3. Results and discussion

3.1. Effects of the chemical modification of KB/GCE with amines on the bioelectrocatalytic activity of BOD

Fig. 2 shows rotating disk cyclic voltammograms at several BOD/amine/KB/GCEs at $\omega = 2000$ rpm under air-saturated conditions. Well-defined sigmoidal catalytic waves were observed at all of the BOD/amine/KB/GCEs examined. Such catalytic waves were not observed in the absence of BOD or dioxygen (Fig. S3). Thus, the catalytic current is ascribed to the DET-type bioelectrocatalytic reduction of dioxygen by BOD on the electrode.

As comparison, the broken line in Fig. 2 indicates the rotating disk voltammetric response at a BOD/KB/GCE without amine-modification. As judged from Fig. 2, BOD/4-ABA/KB/GCE (panel D) gave much larger density of the limiting current (1.2 ± 0.1 mA cm^{-2} at 0 V) compared with the other electrodes modified with *p*-PDA, MABA, and 6-AHA. In addition, the onset potential of the catalytic wave at the BOD/4-ABA/KB/GCE was about 0.58 V, which is more positive than that at BOD/KB/GCE without amine-modification (0.52 V). Two pairs of peaks at around 0.1 and 0.3 V are surface-confined redox species (most probably quinone species) generated as by-products during the oxidation of 4-ABA, as judged from voltammogram in the absence of BOD (Fig. S3 (A)). Since the redox waves do not change after adsorption of BOD, the by-products on the electrode surface do not affect the BOD activity. Therefore, we can conclude that the aromatic carboxy group generated on the carbon electrode (Fig. 1) enhances the BOD activity.