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Analysis of factors governing direct electron transfer-type bioelectrocatalysis of bilirubin oxidase at modified electrodes

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ABSTRACT

Direct electron transfer (DET)-type bioelectrocatalysis is an essential technique for constructing simple bioelectrochemical devices such as biosensors, bioreactors, and biofuel cells. Bilirubin oxidase (BOD), a biocatalyst for the four-electron reduction of dioxygen (O_2) into water, is a promising enzyme for powerful DET-type biocathodes. Mesoporous carbon materials are often used in BOD-modified biocathodes. However, the supply of O_2 becomes a key factor governing BOD-catalyzed current densities owing to its low solubility. In this study, we analyzed steady-state rotating disk voltammograms of a BOD-catalyzed reaction in a rigid manner by taking into account the mass-transport as well as the enzymatic and the interfacial electron transfer kinetics. Non-catalytic redox signals of adsorbed BOD were also analyzed to obtain the surface redox properties of BOD. The analysis revealed that modification of electrodes with bilirubin and/or negatively charged carbon nanotubes to improve the DET-type catalytic performance increased the amount of BOD molecules with the proper orientation for bioelectrocatalysis. The interfacial electron transfer kinetic characteristics remained almost unchanged.

1. Introduction

Bioelectrocatalysis, a coupled process involving enzymatic and electrode reactions, is a subject of interest for practical applications of bioelectrochemical devices such as biosensors, bioreactors, and biofuel cells [1–7]. The reactions are classified into two types according to the difference in the electric connection systems between the enzyme and the electrode [1–7]. The first type is mediated electron transfer (MET) in which an artificial redox mediator shuttles electrons to reduce the kinetic hindrance of the interfacial electron transfer. The second type is direct electron transfer (DET) in which an enzyme can directly communicate with an electrode without a mediator. DET-type bioelectrocatalysis is expected to be utilized for advanced applications since it only requires an enzyme and an electrode as structural components [1–7].

In DET-type bioelectrocatalysis, an enzyme is adsorbed on an electrode to catalyze the electron transfer between the enzyme substrate and the electrode [1–7]. In this sense, it is very important to control the orientation of the enzyme on the electrode surface because the interfacial electron transfer rate constant increases exponentially upon decreasing the distance between the electrode surface and the redox site of the enzyme [8–10]. There are two major models for enzyme orientation namely, “simple orientation” [11–13] and “random orientation” (or dispersion model) [4,14,15]. The former model assumes the existence of two types of adsorbed enzymes (i.e., electroactive and inactive) with the electroactive enzyme facing its redox site toward the electrode surface with a uniform orientation [11–13]. On the other hand, the latter model assumes spherical enzymes and considers uniform dispersion of the adsorbed enzymes [4,14,15]. In both cases, favorite (i.e., productive) orientation for DET may result in increasing amounts of electroactive enzymes and higher interfacial electron transfer rate constants. Detailed and rigid analysis of catalytic DET-type voltammograms of enzyme-modified electrodes was shown to provide valuable information on the kinetics and orientation of the enzyme [4,11–15]. Under the limited conditions by which the rate of substrate supply is sufficiently fast compared to that of the catalytic reaction, the increase in the limiting catalytic current density was ascribed to the higher amounts of electroactive enzymes, while the shift of the midpoint potential or the change in the catalytic wave shape reflected a change in the interfacial electron transfer kinetics [4,11–15].

There are several DET-type enzymes including hydrogenases [16–18], glucose oxidase [19], glucose dehydrogenase [20], fructose dehydrogenase [21,22], cellobiose dehydrogenase [23,24], and multi-copper oxidases (MCOs) [25–28]. Among them, hydrogenases and MCOs react with gaseous substrates (i.e., dihydrogen (H_2) and dioxygen (O_2), respectively), and the solubility of the gaseous substrates is very low (i.e., less than sub-mM at atmospheric pressure (1 M = 1 mol dm^{-3})) [16–18,25–28]. MCOs are enzymes that catalyze the four-electron reduction of O_2 into water and are utilized as biocatalysts for biocathodes of DET-

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type biofuel cells using glucose, fructose, or hydrogen as fuels [29–36]. For practical use, mesoporous materials with high specific surface areas are used as electrode material components.

Among MCOs, bilirubin oxidase (BOD) is a promising enzyme for DET-type biocathodes. BOD has a high bioelectrocatalytic activity under mild conditions, and its formal potential is close to that of the H_2O/O_2 redox couple [26,31,35–38]. These features allow high DET-type catalytic current densities with minimum overpotentials. BOD is frequently supported on carbon electrodes to examine its DET-type properties [31,35,39–47]. BOD (and generally MCOs) has four copper atoms that constitute its active site. The copper atoms are classified into three types according to their spectroscopic and magnetic properties namely, type I (T1), type II (T2), and type III (T3) coppers [25,26]. BOD can receive electrons at the T1 copper site from the electrode, and the electrons are transferred to the T2–T3 copper cluster to carry out the reduction of O_2. [25,26] Thus, it is important to control the orientation of adsorbed BOD with the aim to shorten the distance between the T1 copper site and the electrode.

In order to achieve favorite orientation of BOD onto the electrode surface, several approaches have been proposed [31,35,39–47]. Improved DET-type bioelectrocatalysis has been accomplished at negatively charged electrodes constructed by modification with diazonium coupling [39] or amine oxidation [40] reactions. In addition, physical adsorption of bilirubin [31,35,41,42] or other aromatic compounds [43] and utilization of functionalized carbon nanotubes (CNTs) [44,45] have been also shown to be effective for enhancing DET-type bioelectrocatalysis of BOD especially from *Myrothecium verrucaria*. CNTs have been widely used as electrode platforms to achieve favorable orientation of enzymes [48]. Furthermore, the amounts of π-π conjugated systems and carboxy groups on the electrode surface, which can be controlled by the length of the multi-walled CNTs, are considered to be the factors governing the bioelectrocatalytic activity of BOD [46]. It is noted here, however, that not all BODs have similar surface characteristics. For example, for BOD from *Bacillus pumilus*, the above mentioned procedures were not effective to improve the orientation [47].

Enzyme orientation can affect either the amount of effective enzymes or the interfacial electron transfer rate constant, or both [4,11–15]. The detailed reasons of the effectiveness of these modification approaches should be evaluated from robust analysis of catalytic DET-type voltammograms. In this sense, it is noted that catalytic voltammograms have to be carefully analyzed by considering the mass-transport of substrates [4]. In the case of BOD, owing to the low solubility of O_2 and the enlargement of the specific surface areas upon deposition on porous materials, the DET-type bioelectrocatalytic current is likely to be governed by the mass-transport. The diffusion current density in a rotating disk electrode system is ca. -8–12 mA cm^{-2} at 4–25 °C and 4000 rpm [27,46].

In this study, we elucidated the DET-type bioelectrocatalytic properties of BOD by considering the effect of the mass-transport. Ketjen black (KB) and water-dispersed multi-walled CNTs with lengths of 1–4 μm were used as carbon materials to construct the electrodes. Furthermore, bilirubin, a natural substrate of BOD, was supported onto these electrodes. These methods are known to achieve high current densities of the DET-type bioelectrocatalysis (i.e., close to the diffusion current density of O_2) under rotating disk conditions at neutral pH and room temperature [31,41,42,46]. DET-type bioelectrocatalysis consists of three processes namely, heterogeneous electron transfer, enzymatic catalytic reaction, and mass-transport [4]. Under diffusion-controlled conditions, it is impossible (or difficult) to extract the kinetic factors concerning the heterogeneous electron transfer and the enzymatic reaction from the voltammetric characteristics. For the sake of accurate analysis, conditions at which the DET-type bioelectrocatalysis is predominantly governed by the reaction kinetics should be preferably selected. Thus, we carried out measurements at low temperature and high pH conditions at which the activity of BOD decreases while the stability remains unaffected [38]. Under the conditions, we analyzed steady-state rotating disk voltammograms of BOD based on a robust electrochemical model by taking into account of the contribution of the mass-transport. In addition, we evaluated the surface redox properties of adsorbed BOD from non-catalytic Faradaic signals. Combining these data, we discussed the orientation of BOD on the surface of the mesoporous carbon electrodes prepared by the aforementioned methods (utilization of water-dispersed multi-walled CNT with lengths of 1–4 μm and bilirubin modification). The detailed effects of the methods on controlling the enzyme orientation were still unclear [31,35,41,42,46], however the analysis in this study revealed that both methods increased the surface concentration of the effective enzyme, while the interfacial electron transfer kinetics remained almost unchanged.

2. Experimental

2.1. Materials

KB (EC300J) was kindly donated from Lion Co. (Japan). Poly(1,1,2,2-tetrafluoroethylene) (PTFE, 6-J) fine powder was purchased from DuPont-Mitsui Fluorochemicals Co., Ltd., (Japan). Bilirubin was purchased from Wako Pure Chemical (Japan) and dissolved in a 30 mM NaOH aqueous solution. Water-dispersed multi-walled carbon nanotubes (MWCNTs) (outer diameter: 10–15 nm, length: 1–4 μm, without surfactant) were kindly donated from Nitta Co. (Japan). Bilirubin oxidase (BOD; EC 1.3.3.5) from *Myrothecium verrucaria* was donated from Amano Enzyme Inc. (Japan) and used without further purification. The rest of chemicals used in this study were of analytical grade unless otherwise specified, and all aqueous solutions were prepared with distilled water.

2.2. Preparation of the electrodes

KB or MWCNTs were supported on a glassy carbon electrode (GCE for rotating electrode, 3 mm in diameter, BAS). GCE was polished with alumina slurry (Buehler, 1 mm), sonicated, and finally washed with distilled water. KB-modified GCE (KB:PTFE = 8:2 (w/w)) (KB/GCE) and MWCNT-modified GCE (MWCNT/GCE) were prepared according to protocols reported in the literature [31,46]. Note that we sonicated the MWCNT-dispersed solution in an ultrasonic bath for 3 min to completely disperse MWCNT before use. Besides, 10 μL (1 L = 1 dm^3) of a bilirubin solution (3 mM) was applied onto KB/GCE or MWCNT/GCE, dried at room temperature, and subsequently washed with distilled water [31]. The as-prepared electrodes were denoted as BL/KB/GCE and BL/MWCNT/GCE, respectively. Subsequently, 30 μL of a BOD solution (10 mg mL^{-1}) dissolved in a 10 mM phosphate buffer (pH = 7.0) was then applied onto the surface of the prepared electrodes (KB/GCE, BL/KB/GCE, MWCNT/GCE, and BL/MWCNT/GCE), and they were subsequently kept in a water-saturated atmosphere for 1.5 h at 4 °C for slow drying, washed with a buffer solution, and used immediately for electrochemical measurements. In the case of the control experiments carried out to confirm the non-catalytic redox signals of adsorbed BOD, 30 μL of a 10 mM phosphate buffer (pH = 7.0) without BOD were applied onto the surface of the prepared electrodes, and we followed the same procedure as in the case of the BOD-modified electrodes.

2.3. Electrochemical measurements

Linear sweep voltammetry, cyclic voltammetry, and chronoamperometry were conducted on BAS CV50W and ALS 714C electrochemical analyzers. Steady-state measurements were carried out with a rotating disk GCE (RDE-2, BAS Inc.) at 4000 rpm and a scan rate of 5 mV s^{-1} unless otherwise stated. Anaerobic measurements were conducted in a nitrogen (N_2) chamber filled with a mixture of 96% N_2 and 4% H_2. A platinum wire and an Ag|AgCl|sat KCl electrode