Electrostatic interaction between an enzyme and electrodes in the electric double layer examined in a view of direct electron transfer-type bioelectrocatalysis

Yu Sugimoto [a], Yuki Kitazumi [a,b], Seiya Tsujimura [c], Osamu Shirai [a], Masahiro Yamamoto [b,d], Kenji Kano [a,b,*]

[a] Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan
[b] Japan Science and Technology Agency, CREST, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan
[c] Faculty of Pure and Applied Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8573, Japan
[d] Department of Chemistry, Konan University, 8-9-1 Okamoto, Higashi-Nada, Kobe, Hyogo 658-8501, Japan

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ABSTRACT

Effects of the electrode poential on the activity of an adsorbed enzyme has been examined by using copper efflux oxidase (CueO) as a model enzyme and by monitoring direct electron transfer (DET)-type bioelectrocatalysis of oxygen reduction. CueO adsorbed on bare Au electrodes at around the point of zero charge (E_{pzc}) shows the highest DET activity, and the activity decreases as the adsorption potential (E_{ad}; at which the enzyme adsorbs) is far from E_{pzc}. We propose a model to explain the phenomena in which the electrostatic interaction between the enzyme and electrodes in the electric double layer affects the orientation and the stability of the adsorbed enzyme. The self-assembled monolayer of butanethiol on Au electrodes decreases the electric field in the outside of the inner Helmholtz plane and drastically diminishes the E_{ad} dependence of the DET activity of CueO. When CueO is adsorbed on bare Au electrodes under open circuit potential and then is held at hold potentials (E_{ho}) more positive than E_{pzc}, the DET activity of the CueO rapidly decreases with the hold time. The strong electric field with positive surface charge density on the metallic electrode (σ_M) leads to fatal denaturation of the adsorbed CueO. Such denaturation effect is not so serious at $E_{ho} \ll E_{pzc}$, but the electric field with negative σ_M induces an orientation inconvenient for the DET reaction during the adsorption process. A positively charged neomycin shows a promoter ability to CueO adsorbed at $E_{ad} \ll E_{pzc}$. The phenomenon is also explained on the proposed model.

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1. Introduction

Direct electron transfer (DET)-type bioelectrocatalysis of redox enzymes adsorbed on electrodes has gained increasing attention in applications as biosensors and biofuel cells (Armstrong, 2005; Bullen et al., 2006; Reda and Hirst, 2006; Sarma et al., 2009). Although some enzymes show outstanding catalytic performance on suitable electrodes (Cracknell et al., 2008; Kamitaka et al., 2007; Murata et al., 2009; So et al., 2014), detailed mechanism of the electron transfer between enzymes and electrodes remain to be elucidated.

In DET-type bioelectrocatalysis, the distance between the active site of the enzyme and the electrode is the most dominant factor for heterogeneous electron transfer (Chi et al., 2001; Feng et al., 1997; Fujita et al., 2004; Léger and Bertrand, 2008; Niki et al., 2003). In order to shorten the distance, the orientation of enzyme on electrodes is very important. From this viewpoint, several electrode materials have been investigated for DET reaction of proteins. For example, nano-structured materials, such as mesoporous materials, nano-particles and nano-fibers, were used for the study of DET-type bioelectrocatalysis (Baghayeri et al., 2014a, 2014b, 2013; Goldstein et al., 2009; Kamitaka et al., 2007; Murata et al., 2009; Sarma et al., 2009; Tsujimura et al., 2007). When these materials are used as electrodes, the total amounts of the adsorbed enzyme per the projected surface area increase due to an increase in the surface area, and the probability of the orientation convenient for DET reactions seems to increase for enzymes adsorbed in a caged space of mesoporous electrodes.

On the other hand, self-assembled monolayer (SAM)-modified electrodes have been widely used for protein electrochemistry

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* Corresponding author. Tel.: +81 75 753 6392; fax: +81 75 753 6456.
E-mail address: kano.kenji.5z@kyoto-u.ac.jp (K. Kano).

(Allen et al., 1984; Gooding et al., 2001; Taniguchi et al., 1982; Willner et al., 1997). In recent years, it has been found that surface functional groups drastically affects the DET-type bioelectrocatalytic reaction (Matsumura et al., 2012; Shleev et al., 2006; Tominaga et al., 2008; Tsujimura et al., 2013, 2008a), in which the authors have suggested that the interaction between the specific surface functional groups and enzyme surface leads to an orientation convenient for the DET reaction. Furthermore, since enzymes have some surface charges in solution, charged functional groups will affect the DET activity due to the electrostatic interaction between the enzymes and the SAM-modified electrodes. However, detailed electrostatic interaction has not been clarified.

The object of this study is to elucidate the electrostatic interaction between an enzyme and electrodes in the electric double layer. We assume here that the DET activity is one of measures of the state of the enzyme on the electrode, and we focus on the effect of the adsorption potential (E_{ad}, at which the enzyme adsorbs) and the hold potential (E_{ho}, at which the adsorbed enzyme is held) on the DET-type bioelectrocatalytic current. In this study, we used copper efflux oxidase (CueO) as a model enzyme with DET activity. CueO is one of the multi-copper oxidase (MCO) family that is a very promising catalyst of DET-type bioelectrocatalytic four-electron reduction of O_2 to H_2O (Miura et al., 2007; Tsujimura et al., 2008b). The active site in the MCO contains four copper atoms classified into three types: type I, type II, and type III, according to their spectroscopic properties; the type I copper site is the inlet of electrons from electron-donating substrates or electrodes and transfers the electrons to the trinuclear center composed of one type II copper and two type III copper atoms. The trinuclear center operates as a catalytic site to reduce O_2 to H_2O. CueO has no polysaccharide chains unlike most of the MCOs and the crystal structure of CueO has been determined (PDB code: 1N68). In addition, CueO shows extremely high DET activity (Miura et al., 2007; Tsujimura et al., 2008b). The substrate of the DET reaction is dissolved O_2, and any specific chemicals (which might disturb the electric double layer) are not required to be added in the test solution. Therefore, CueO is suitable as a model enzyme in this study. CueO was adsorbed on a bare Au electrode at various values of E_{ad} and the DET activity was evaluated from the steady-state catalytic current by rotating disk voltammetry. The effect of E_{ho} on the DET activity of the adsorbed CueO was also examined. Based on the results, we will propose a novel model that the surface charge density and the electric field in the electric double layer affect the enzyme orientation and the stability of CueO on electrodes.

2. Experimental

2.1. Materials

The expression and purification of CueO were carried out as described previously (Miura et al., 2007; Ueki et al., 2006). The coding for the precursor CueO gene was designed to have the *Eco*RI restriction site at the 5′-end and the 6 × His-tag coding sequence with *Bam*HI restriction site at 3′-end. The gene was inserted into pUC18, with which *Escherichia coli* BL21 (DE3) was transformed by the heat shock method. The enzyme concentrations were determined using the molar absorption coefficient at 612 nm ($\varepsilon_{612} = 5800\,M^{-1}\,cm^{-1}$ (M=mol dm^{-3}) (Ueki et al., 2006)). All chemicals used in this study were of analytical reagent grade, and all solutions were prepared with distilled water.

2.2. Quartz crystal microbalance (QCM) measurement

9-MHz At-cut quartz crystal plates coated with Au were used (Seiko EG&G Co., Ltd), the projected surface area of Au being 0.20 cm^2 and the roughness factor being 1.5–1.7 (Seiko EG&G: Technical brochure). The Au-coated quartz crystal resonator was attached to a handmade cell with a solution volume of 1 mL (L=dm^3). Measurements were done on a Seiko EG&G QCA922 QCM analyzer with a handmade software for data acquisition.

2.3. Preparation of electrodes

Polycrystalline Au disk electrodes (3-mm diameter) were obtained from BAS, Inc. The Au electrode was polished to a mirror-like finish with Al_2O_3 powder (0.02-μm particle size), rinsed with distilled water, and sonicated in distilled water. Single crystal Au films were prepared on freshly cleaved mica surface (obtained from Nilaco Co.) by vapor deposition (VPC-260, ULVAC KIKO) at less than 1.3×10^{-3} Pa. The temperature of the mica sheet was maintained at 580 °C during the deposition. The Au-deposited mica sheets were subsequently annealed at 530 °C for 8 h and then quenched in ultrapure water to obtain Au(111) terraces on the sheets (Hobara et al., 1998).

Butanethiol-modified SAM Au(111) electrodes were prepared by immersing the Au-coated mica substrates for at least 24 h in an ethanol solution containing 2 mM butanethiol. Then the electrode was washed with ethanol and ultrapure water before use.

2.4. Electrochemical methods

Almost all electrochemical measurements were performed on a BAS CV 50 W, while impedance measurements were performed on an Als704C. Au disk electrodes, Au(111) and butanethiol-modified Au(111) electrodes were used as working electrodes. In rotating disk electrode measurements, the Au disk electrodes were attached to the shaft of an electrode rotator (RDE-1, BAS). Au(111) and butanethiol-modified Au(111) electrodes were attached to the handmade cell with a solution volume of 1.0 mL. The projected surface area of the electrodes is 0.20 cm^2. Electrochemical quartz crystal microbalance (EQCM) measurements were carried out with the Au electrodes for QCM on the QCM resonator as the working electrodes. A Pt wire and an Ag|AgCl|sat. KCl electrode were used as the counter electrode and the reference electrode, respectively. All potentials are referred to the Ag|AgCl|sat. KCl reference electrode in this paper.

Measurements were carried out in pH 5 McIlvaine buffer. The ionic strength was adjusted to be 0.3 M with K_2SO_4. The temperatures of the measurement solution were maintained at 25 ± 1 °C using a water-jacketed cell at a solution volume of 1.0 mL in the rotating disk electrode measurements. Other measurements were carried out at room temperature (25 ± 5 °C).

CueO was adsorbed on the Au disk electrodes as follows unless otherwise stated. An unmodified electrode was immersed into 1.0 mL of McIlvaine buffer in the electrochemical measurement cell. After the working electrode potential was set at E_{ad}, 10 μL of 57 μM CueO solution was injected in the measurement solution, and the solution was stirred for 5 min. Then rotating disk voltammetry was started from an initial potential of 0.6 V without changing the solution.

2.5. Electric double layer capacitance (C_{dl}) calculation

Impedance of real (Z_r) and imaginary (Z_i) parts were recorded on the Als704C. The impedance measurement was performed with A.C. perturbation spectra of sine waves with 10-mV amplitude and frequencies in the range from 0.1 Hz to 1 kHz with 10 steps per