Electrochemistry of D-Gluconate 2-Dehydrogenase from *Gluconobacter frateurii* on Indium Tin Oxide Electrode Surface

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Direct electron-transfer-type bioelectrocatalytic oxidation of D-gluconate is studied with D-gluconate dehydrogenase (GADH, EC 1.1.99.3, from *Gluconobacter frateurii*) at indium tin oxide electrodes. The adsorbed GADH gives a surface-redox wave attributable to the heme *c* site in the absence of the substrate and clear catalytic oxidation current in the presence of the substrate. The enzyme kinetics, the surface electron transfer kinetics, and the adsorption characteristics of GADH have been analyzed by voltammetry and quartz crystal microbalance measurements.

Great attention has been paid to direct electron transfer (DET) between enzymes and electrodes for construction of simplified and downscaled biosensors and biofuel cells.[1–4] However, DET reactions occur in some limited combinations of enzymes and electrodes. Electro-enzymatic reactions on the electrode surface strongly depend on the chemical and structural properties of electrode surface, but the factors governing DET reactions have not yet been clarified.

We have previously proposed a model to express voltammograms observed in DET-type catalytic reactions and applied it to quantitative analyses of several DET-type reactions.[5–7] This analysis allows rough evaluation of catalytic kinetics and electrode kinetics of enzymes adsorbed on electrode surfaces, but some other analytical methods are required for more detailed characteristics of DET-type catalytic reactions. Recently, we also tried to monitor the adsorption behavior of enzymes on Au electrodes with quartz crystal microbalance (QCM) measurements.[8] Combination of two measurements would provide information on the active and total enzymes adsorbed on electrodes, and may be utilized to further characterization of DET-type catalytic reactions.

In this study, we focused on the DET-type catalytic oxidation of D-gluconate with D-gluconate 2-dehydrogenase (GADH; EC 1.1.99.3) from *Gluconobacter frateurii*. GADH is a membrane-bound enzyme catalyzing the oxidation of D-gluconate (GlcA) to 2-keto-D-gluconate. This enzyme has three subunits, and contains 1 mol of flavin and 2 mols of heme *c* per mol of the enzyme.[9,10] GlcA is oxidized at the flavin and the electrons are transferred to the heme *c* site, which is believed to react with ubiquinones in membrane. Based on our knowledge on electrode materials in preliminary experiments, indium tin oxide (ITO) electrode was selected for characterization of adsorption behavior and electro-enzymatic kinetics of GADH.

GADH was purified from *Gluconobacter frateurii* NBRC 3271 (formerly called as *Gluconobacter dioxyacetonicus* IFO 3271) as described previously.[9,10] QCM measurements were performed on a Seiko EG&G QCA917 QCM analyzer. 9-MHz At-cut quartz crystal plates coated with ITO (Seiko EG&G Co., Ltd.) were used, the projected surface area being 0.196 cm^2. The ITO electrodes were sonicated in an ionic detergent (New Vista) for 15 min and repeated this procedure at least twice, and the electrodes were washed by sonication in distilled water.[11] Cyclic voltammetry was performed on a BAS CV50W electrochemical analyzer. A platinum wire and an Ag|AgCl|KCl (sat.) were used as counter and reference electrodes, respectively. All potentials are referred to the Ag|AgCl|KCl (sat.) electrode. Electrolyte solution was 30 mM acetate buffer (pH 5.0).

GADH gave a pair of surface redox wave at an ITO electrode in the buffer solution containing 8 nM GADH and 0.005% triton X-100 (Figure 1A). The peak current was linearly proportional to the scan rate (inset in Figure 1A), indicating that GADH adsorbed to the electrode surface communicated with electrode directly. To our best knowledge, this is the first observation of the redox responses of GADH in the absence of substrates. The mid-potential of the anodic and cathodic potentials was 0.05 V. This suggests that the redox signal is attributed to the heme *c* site in GADH. The surface concentration of the electro-active GADH was evaluated to be ca. 4 pmol cm^{-2} from the electricity of the peak attributed to the redox reaction.

The adsorption behavior was also observed by QCM measurements. An ITO electrode was dipped in the acetate buffer. The resonance frequency decreased with time after GADH was added into the solution (Figure 1B). The total frequency shift at 4000 s corresponded to 16 pmol cm^{-2} as a surface concentration of the adsorbed GADH (Γ_{GADH}) by using Sauerbrey equation[12] and regarding the molecular mass of GADH as 130,000 g. This value is larger than the one evaluated from the

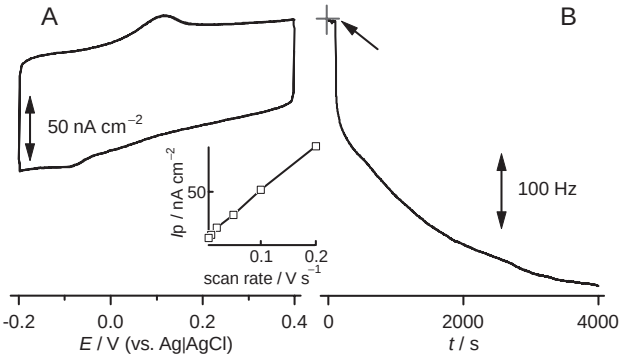

Figure 1. (A) CV after adsorption of GADH onto an ITO electrode in acetate buffer (pH 5) containing 8 nM GADH in the absence GlcA. Scan rate was 0.02 V s^{-1}. Inset shows the scan rate dependence of the anodic peak current. (B) Time dependence of the resonance frequency shift in QCM measurements. GADH was added into acetate buffer (pH 5) at the point indicated by the arrow.

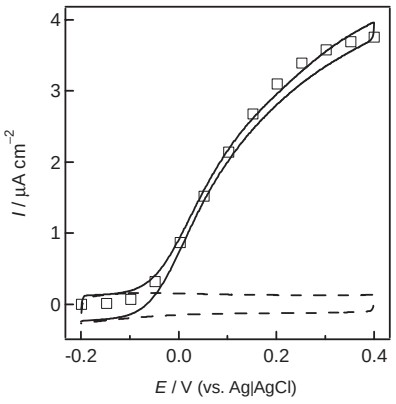

Figure 2. CVs at a GADH-adsorbed ITO electrode (for 1 h) and at pH 5 in the absence (broken curve) and the presence (solid curve) of 50 mM GlcA, at a scan rate $0.02\,V\,s^{-1}$. The open squares represent the regression curve obtained based on eqs. 1–3.

CV. The difference suggests that some GADHs would be electro-inactive, most probably due to multilayered adsorption, denaturalization, or random orientation on the electrode surface. In this report, we introduce an efficiency parameter (β), which denotes the ratio of the amount of the enzyme actually involved in the DET reaction to the total amount of the enzyme on the electrode surface. The β value can be calculated as 0.25.

Figure 2 shows cyclic voltammograms (CV) of the catalytic oxidation of GlcA (100 mM) at a GADH-adsorbed ITO electrode. This is the first report of DET-type bioelectrocatalysis at ITO electrodes. When the electrode was removed from the GADH solution and rinsed with distilled water, the catalytic current remained almost unchanged in a fresh 100 mM GlcA solution without GADH. The results indicate that GADH is adsorbed irreversibly on the electrode surface and works as a catalyst in the DET-type electrochemical oxidation of GlcA. In separated experiments, the catalytic current at a fresh ITO electrode increased gradually with time after addition of GADH into the GlcA solution. The catalytic current densities were not affected by stirring of the solution and did not have any property due to substrate depletion. This means that the enzyme kinetics is the rate-determining step during the GADH-catalyzed electrochemical oxidation of GlcA (100 mM).

The DET-type catalytic reaction of GADH has been reported by Ikeda et al. by using GADH from other species (*Pseudomonas fluorescens* FM-1) at carbon paste, Au and other metal electrodes (except ITO).[13] In contrast with the present results, the voltammograms of GADH from *P. fluorescens* showed peak-shaped property, indicating potential-dependent enzyme inhibition and activation.

We attempted to analyze the enzymatic kinetics-controlled current–potential curves by considering the enzyme catalytic constant (k_c), surface ET kinetics (k°), and the formal potential of enzyme ($E^{\circ'}$). The current density (I) can be expressed by the following equation, which is close to that reported in the literature[6] with small modification:

$$I = \frac{nFk_c\beta\Gamma_{GADH}}{1 + k_c/k_f + k_b/k_f}, \quad (1)$$

where n and F are the number of electrons (=1 for the heme c of GADH) and the Faraday constant, respectively. We considered that k_c is independent of the electrode potential, as judged from the typical sigmoidal catalytic wave (Figure 2), and is a function of the intermolecular ET rate constant for GlcA oxidation at the flavin site[10] and the intramolecular ET rate constant from the flavin to the heme c. The surface ET rate constants k_f and k_b (f: forward, b: backward) are expressed by Butler–Volmer-type equations as follows:

$$k_f = k^\circ \exp[(1-\alpha)(nF/RT)(E - E^{\circ'})], \quad (2)$$

$$k_b = k^\circ \exp[-\alpha(nF/RT)(E - E^{\circ'})], \quad (3)$$

where k° and α are the standard surface ET rate constant and the transfer coefficient, respectively. $E^{\circ'}$ was set as 0.05 V as judged from the mid-potential of the CV (Figure 1A) Γ_{GADH} was obtained from the QCM measurements as described above. The regression curves were shown in Figure 2 as open squares, which reproduce the experimental curves well. Three parameters (α, k°/k_c, and βk_c) were refined as 0.7, 0.3, and $2.5\,s^{-1}$, respectively. Considering the β value evaluated above, the k° and k_c values can be calculated as 3 and $10\,s^{-1}$, respectively. Small k° value would be responsible for the large peak separation between the oxidation and reduction peak potentials.

The present analytical method can be applied to the analysis of voltammograms at functionalized electrodes, such as thiol-modified Au, and the data would be very important to elucidate factors affecting interactions between GADH and electrodes.

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