Role of 2-mercaptoethanol in direct electron transfer-type bioelectrocatalysis of fructose dehydrogenase at Au electrodes

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Abbreviation: ME: 2-mercaptoethanol, TX: Triton[®] X-100

Abstract

Effects of the electrode potential on a direct electron transfer (DET)-type bioelectrocatalysis of fructose dehydrogenase (FDH) at Au electrodes were investigated. Adsorbed FDH showed the highest DET activity at an adsorption potential (E_{ad}) around the point of zero charge (E_{pzc}). Since FDH stock solution contains 2-mercaptoethanol (ME) for stabilization, ME is partially bound to the Au electrode. However, the DET activity drastically decreased at $E_{ad} \gg E_{pzc}$. Au oxide layer is formed at positive potential to hinder the interfacial electron transfer. In contrast, only slight decrease in The DET activity was observed at sufficiently negative $E_{ad} (\ll E_{pzc})$, where ME is reductively desorbed from the Au electrode, but co-exists in the solution. In contrast, when FDH and ME were adsorbed on Au electrodes at an open circuit potential and the FDH- and ME-adsorbed Au electrode was held at such a negative hold potential (E_{ho}) in the buffer without ME, the DET activity drastically decreased. An addition of ME in the test solution prevented the decrease in the DET activity at the negative E_{ho} . These results indicate that ME close to adsorbed FDH plays a significant role in the stabilization of FDH adsorbed on Au electrodes.

1. Introduction

In direct electron transfer (DET)-type bioelectrocatalysis, it is important for enzymes to adsorb on electrodes with keeping their functions and with holding an orientation convenient for the DET reaction [1–4]. When enzymes adsorb on a solid electrode, enzymes might tend to denature and to lead to decrease in their catalytic functions due to conformational change [5–8]. Elucidation of the adsorbed enzyme conditions and controlling of the adsorption state will lead to improvement in the DET-type catalytic performance and a wide variety of its applications, such as biosensors and biofuel cells.

Palecek et al. have studied the influence of the electrode potential on the activity or stability of proteins at bare mercury electrodes by chronopotentiometric stripping [9]. They have found that proteins are denatured by the electric fields

generated on negatively charged mercury surface, but are not denatured when they are adsorbed at the mercury electrode at the point of zero charge (E_{pzc}). Furthermore, the denaturation of proteins is strongly decreased at dithiothreitol self-assembled monolayer (SAM)-modified mercury electrode. Previously, we investigated the electrostatic interaction between an enzyme and electrodes using a soluble enzyme Cu efflux oxidase (CueO) capable of a DET-type bioelectrocatalysis for O_2 reduction [10]. In the work, we have assumed that the DET activity is one of measures of the state of the enzyme on the electrode. We have concluded that negative surface charge density on a metallic electrode leads dominantly to an orientation of CueO inconvenient for the DET reaction during the adsorption. We also have found that SAM of butanethiol on Au electrodes decreases the electric field in the outside of the inner Helmholtz plane and drastically diminishes the effect of the electrostatic interaction based on both experimental data and theoretical calculation. However, since the situation might not be common for enzymes and depends on each individual enzyme (and also on each individual electrode), further investigation is required.

In this study, we focus on fructose dehydrogenase (FDH) as a model membrane-bound enzyme with DET activity. Since FDH shows very high DET activity, increased expectations are placed on FDH to develop several bioelectrochemical devices including DET-type high power biofuel cells [11–13] and D-fructose biosensors [14–16]. In order to improve the performance of such bioelectrochemical devices based on the DET activity of FDH, further elucidation is desired on the interaction between FDH and electrodes and on the details of the DET reaction. FDH is a heterotrimer with a molecular mass of *ca.* 140 kDa [17]. Subunits I and II have a covalently bound FAD and three heme C moieties, respectively. FDH catalyzes two-electron oxidation of D-fructose to 5-keto-D-fructose at the FAD and the electrons are transferred to electrodes via the heme C-containing subunit [18]. Some studies of FDH have been reported with mediator-containing polypyrrole matrix modified electrode [19–22], mediator-containing carbon paste [23], membrane mimetic layer on gold electrode [24] and layer-by-layer assembly of polyelectrolytes [25]. In membrane-bound enzymes, a hydrophobic surface region(s) is located at least at the binding portion to membranes. The hydrophobic region may predominantly bind to hydrophobic electrodes, and therefore, membrane bound-enzymes may have a unique orientation on electrodes

compared with soluble enzymes. Furthermore, FDH stock solution contains Triton[®] X-100 (TX) and 2-mercaptoethanol (ME) for solubilization and stabilization, respectively. These molecules must adsorb on electrode and are expected to affect the adsorption state of FDH. For example, ME forms SAM on Au. TX forms bilayer on the ME-SAM-Au electrode and FDH is embedded in the TX bilayer [26]. Furthermore, in our preliminary experiments, we found that when ME-free FDH is adsorbed on Au electrodes, the DET-activity of FDH becomes extremely small. However, clear DET-activity is observed when only small amounts of ME coexist with FDH in the solution during the adsorption process of FDH on Au electrodes. Therefore, we predict that ME bound on Au at a coverage lower than that at densely packed ME-SAM plays a significant function on the DET reaction of FDH.

In this study, FDH 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 with cyclic voltammetry in order to elucidate the roles of ME in the DET reaction of FDH. Furthermore, effects of hold potential (E_{ho} , at which the potential of the FDH-adsorbed electrode was hold) on the DET activity of the adsorbed FDH were also examined in the presence and absence of ME. Based on the results, we will propose a novel model of the adsorption of ME and FDH on Au electrodes.

2. Experimental

2.1. Materials

The expression and purification of FDH were carried out as described previously [27]. FDH stock solution contains 0.1% (w/v) TX and 1 mM ME ($M = \text{mol dm}^{-3}$). A specific activity to oxidize D-fructose of ME-containing FDH is $295 \pm 70 \text{ unit mg}^{-1}$ at pH 5.0 (one FDH unit being defined as the amount of enzyme to oxidize $1 \mu\text{mol}$ of D-fructose per min.). On the other hand, ME-free FDH was also purified without ME, while the ME-free FDH stock solution contained 0.1% TX. The specific activity to oxidize D-fructose of ME-free FDH was $95 \pm 8 \text{ unit mg}^{-1}$ at pH 5.0. 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 0.5 mL ($L = \text{dm}^3$). Measurements were done on a Seiko EG&G QCA922 QCM analyzer with a handmade software for data acquisition.

2.3. Preparation of electrodes

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 (0.05- μm particle size), rinsed with distilled water, and sonicated in distilled water.

ME-modified self-assembled monolayer (SAM) Au electrodes were prepared by immersing the Au disk electrodes treated as described above in an ethanol solution containing 2 mM ME for 1 h. 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 50W. Electrochemical quartz crystal microbalance (EQCM) measurements were carried out with Au electrodes for QCM on the QCM resonator as the working electrodes. A Pt wire and an $\text{Ag}|\text{AgCl}|\text{sat. KCl}$ electrode were used as the counter electrode and the reference electrode, respectively. All potentials are referred to the $\text{Ag}|\text{AgCl}|\text{sat. KCl}$ reference electrode in this paper.

Measurements were carried out in pH 5.0 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 $25 \pm 1\text{ }^\circ\text{C}$ using a water-jacketed cell at a solution volume of 1.0 mL in the Au disk electrode measurements. QCM measurements were carried out at room

temperature (25 ± 5 °C). All experiments were done under air saturated conditions.

FDH was adsorbed on the Au disk electrodes as follows unless otherwise stated. An unmodified electrode was immersed into 900 μL of McIlvaine buffer in the electrochemical measurement cell. After the working electrode potential was set at E_{ad} , 10 μL of 28 μM ME-containing FDH stock solution was injected in the measurement solution, and the solution was stirred for 5 min. Then 100 μL of 1 M D-fructose solution was added and cyclic voltammetry was started from an initial potential of -0.2 V without changing the solution. In this experiment, the final concentrations were 0.28 μM for FDH, 0.001% for TX, 0.01 mM for ME and 0.1 M for D-fructose. Other experimental methods were written in the following sections in each case.

3. Results and Discussion

3.1. Effects of the adsorption potential on the DET activity of FDH at Au electrodes

As shown in Fig. 1, when FDH was adsorbed on Au electrode from ME-containing FDH solution, clear oxidation currents ascribed to DET-type FDH-catalyzed D-fructose oxidation were observed at the FDH-adsorbed electrode (curve (b)) in the presence of D-fructose. In contrast, when ME was completely removed from the FDH stock solution during the FDH purification, the ME-free FDH gave only small DET current at bare Au electrodes (Fig. 1, curve (a)). The difference in the adsorbed amounts of FDH was not so significant as judged from QCM data (0.65 ± 0.03 $\mu\text{g cm}^{-2}$ for ME-free FDH and 0.86 ± 0.07 $\mu\text{g cm}^{-2}$ for ME-containing FDH). On the other hand, the ME-free FDH gave clear DET signal at ME-SAM-Au electrodes (Fig. 1, curve (c)). The current density was about half of that observed for ME-containing FDH sample (curve (a)), but it seems to be reasonable since the specific activity of ME-free FDH is about one-third of that of ME-containing FDH (see Experimental). From these results, we consider that ME plays a significant role in the DET reaction of FDH. We will use ME-containing FDH stock solution in the following electrochemical experiments.

Figure 2A shows cyclic voltammograms at FDH-adsorbed Au electrodes in the buffer containing 0.1 M D-fructose (except curve (a)). FDH was adsorbed for 5 min on

each bare Au electrode at a given adsorption potential (E_{ad}) in the buffer containing 0.28 μ M FDH, 0.001% TX (which is essential to solubilize FDH), and 0.01 mM ME under stirring before addition of D-fructose.

ME in the FDH stock solution is bound on Au electrodes. However, the concentration of ME derived from the FDH stock solution was very low (0.01 mM) and the immersion period was short (5 min). Therefore, the surface coverage (θ) of ME on the Au electrode was small compared with ME-SAM-Au electrode ($\theta=1$) and may be evaluated as $\theta \approx 0.3$ from the non-Faradaic background current (Fig. S1). In the following, the ME-partially-covered gold electrode is called ME-Au electrode to discriminate it from ME-SAM-Au electrode.

In Fig. 2A, the onset potential of the FDH-catalyzed fructose oxidation wave was about -0.05 V and seemed to be independent of E_{ad} , while the E_{ad} value drastically affected the catalytic current density (I). All of the catalytic waves showed practically no hysteresis and were in steady-states (curve (b)-(j)), indicating that the concentration polarization of the substrate does not occur. Furthermore, the I value at 0.5 V got closer to a limiting one on the voltammograms (although the complete limiting condition was not achieved). Therefore, we will use the I value at 0.5 V on the voltammograms as an index for the DET activity of FDH adsorbed on the Au electrode.

Figure 2B shows E_{ad} dependence of the steady-state value of I at 0.5 V on the voltammograms. A maximum plateau of I was observed around $E_{ad} \approx 0.2$ V and the change of E_{ad} to the positive side over 0.4 V caused a drastic decrease in I . The E_{pzc} is about 0 V at a bare Au electrode [10], although some shift in E_{pzc} is expected due to a partial coverage with ME on the Au electrode. At $E_{ad} > 0.4$ V, the DET activity drastically decreased with E_{ad} . At positive potentials above 0.4 V, the formation of Au oxide layer is expected. When a bare Au electrode surface was exposed to 0.8 V, redox peaks of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ as a redox probe were broadened and the peak current densities decreased (Fig. S2). The Au oxide layer hinders the interfacial electron transfer of $[\text{Fe}(\text{CN})_6]^{3-/4-}$. Same phenomenon seems to occur for the electron transfer from FDH to the electrode. Actually, FDH adsorbed on the Au electrode at $E_{ad} = 0.8$ V retained the enzyme activity, as judged from the appearance of clear oxidation current of a mediated electron transfer-type catalytic reaction with $[\text{Fe}(\text{CN})_6]^{3-/4-}$ as a mediator (Fig. S3). The decreased DET activity at positive potentials is mainly ascribed to the hinderance in the

interfacial electron transfer due to the formation of the Au oxide layer.

In contrast, only small decrease was observed in the DET activity at $E_{ad} \ll E_{pzc}$. In order to get some information on the adsorption state of FDH at ME-Au electrodes, bare and ME-SAM-Au electrodes were immersed into the McIlvaine buffer, and a 10- μ L aliquot of the ME-containing FDH stock solution was injected into 900 μ L of the buffer solution at open circuit potential (OCP) under stirring for 5 min to adsorb FDH on the electrodes. During the procedure, ME is bound to bare Au electrode to form ME-Au. Then 100 μ L of 1 M D-fructose solution was added and cyclic voltammetry was performed with the FDH-adsorbed electrodes. After that, the solution was displaced with the buffer containing 0.1 M D-fructose solution without FDH, TX and ME, and voltammograms were repeatedly recorded with the electrodes. In the interval of the voltammetric measurements, the electrode surface was gently washed three times with 0.5 mL of the buffer.

Figure 3 shows the relative value of the catalytic current density after n -times washing (n) against that at $n = 0$ (denoted as $I(n)/I(0)$). At the ME-Au electrode, the current density remained constant, indicating stable adsorption of FDH. In contrast, at the ME-SAM-Au electrode, the $I(n)/I(0)$ value decreased with the washing number (n). TX forms bilayer on the ME-SAM-Au electrode and FDH is embedded in the TX bilayer [26]. Although the concentration of TX in the test solution was 0.001% in this experiment and is lower than the critical micelle concentration (0.02%) [28], similar situation may be expected for the adsorption state of FDH on the TX-adsorbed ME-SAM-Au electrode. The hydrophilic interaction between ME bound to the Au electrode and TX on the ME-SAM is not so strong, and FDH-embedded TX bilayer seems to desorb from the ME-SAM-Au electrode on washing the electrode surface with the buffer. In conclusion, we consider that at the ME-Au, FDH adsorbs directly on the Au electrode in part and the adsorbed FDH does not desorb easily.

3.2. EQCM evaluation of the adsorbed amounts of FDH on Au electrodes

The adsorbed amounts of FDH on the electrode surface were evaluated by EQCM. The E_{ad} value was set at -0.8 V, 0 V or 0.8 V in the following experiments. On the addition of a given amount of ME-containing FDH stock solution ($28 \mu\text{M} \times 5 \mu\text{L}$)

into the solution (500 μL), the resonance frequency decreased with the adsorption time (t_{ad}) due to the adsorption of FDH on the Au electrode. The decreased values of the frequency (Δf) at $t_{\text{ad}} = 5$ min (and the slopes ($d(\Delta f)/dt_{\text{ad}}$)) were almost independent of E_{ad} examined here (Fig. S4(a)). The Δf value was about 140 Hz, which corresponds to a mass density change of $0.76 \mu\text{g cm}^{-2}$ [29]. Since the molecular mass of FDH is 140 kDa [17], the mass density change corresponds to a surface concentration (Γ_{QCM}) of 5.5 pmol cm^{-2} . Assuming an average diameter of FDH as 7 nm [30], the maximum surface concentration (Γ_{cal}) of FDH is calculated to be 5.9 pmol cm^{-2} on a simple model of monolayer packing on a planar electrode with a roughness factor of 1.5. The agreement between Γ_{QCM} and Γ_{cal} suggests that FDH adsorbs on the electrode as a monolayer at all E_{ad} values examined. At $E_{\text{ad}} = -0.8$ V, ME cannot bound to Au electrode. Therefore, Au electrode surface situation at $E_{\text{ad}} = -0.8$ V is not identical with that at $E_{\text{ad}} = 0$ V and 0.8 V. However, there is no significant difference in the adsorbed amounts of FDH. Since only small decrease in the DET-activity is observed at $E_{\text{ad}} = -0.8$ V, ME-Au does not seem to be concerned with the adsorbed amounts and the orientation of FDH.

On the other hand, slight increase in the Δf value was observed with t_{ad} over 5 min. The adsorbed amount exceeded slightly the expected value for monolayer at $t_{\text{ad}} = 20$ min (Fig. S4(b)). Especially at $E_{\text{ad}} = 0.8$ V, partial formation of FDH multilayer is expected from the data. Most probably, denaturation of FDH on the Au electrode induced the multilayer formation.

In addition, when the electrode potential was changed from $E_{\text{ad}} = \text{OCP}$ to a given hold potential (E_{ho}) in the presence of FDH, TX and ME, the Δf remained practically unchanged at $E_{\text{ho}} = -0.8$ V and 0 V. At $E_{\text{ho}} = 0.8$ V. However, the Δf increased with t_{ad} after the potential step. These phenomena support the multilayer formation of FDH at $E_{\text{ad}} = 0.8$ V and rule out the possibility of the desorption of FDH even at high density of the electrode surface charge.

3.3. Effects of the hold potential on the DET activity of adsorbed FDH

In order to consider the role of ME, we also investigated effects of the hold potential (E_{ho}) on the DET activity for a short hold period in the presence and absence of ME. The experimental flow chart is shown in Chart S1. FDH was adsorbed on the

ME-Au electrode at OCP for 5 min from an FDH solution (McIlvaine buffer) of 0.28 μ M containing 0.001% TX and 0.01 mM ME. The electrode was transferred to the McIlvaine buffer containing 0.1 M D-fructose alone, and the voltammogram was recorded for the catalytic oxidation of D-fructose. The FDH-adsorbed electrode was again transferred to the McIlvaine buffer in the presence or absence of ME and the electrode was held for 5 s at a given E_{ho} of -0.8 V, 0 V, or 0.8 V. In the presence of ME, the ME concentration was set at the same value as that for the experiments described in Section 3.1 (i.e. 0.01 mM). The electrode was again transferred to the McIlvaine buffer containing 0.1 M D-fructose alone, and I was periodically checked by cyclic voltammetry.

As shown in Fig. 4, panel B, the I/I_0 value at $E_{ho} = 0$ V were relatively stable regardless of the presence of the ME. As shown in panel C, the I/I_0 decreased by the holding of the electrode at $E_{ho} = 0.8$ V, but after that the current became stable during periodical measurements. The result indicates that the formation of the Au oxide layer occurs only in the period applying 0.8 V. In contrast, at $E_{ho} = -0.8$ V, the I/I_0 value remained unchanged in the presence of ME, but decreased in the absence of ME with the number of the potential scan (panel A).

As a conclusion, we consider that ME-Au is required to keep the DET activity of the adsorbed FDH on Au electrodes. FDH directly adsorbs on ME-Au electrode and is stabilized by the ME on Au. At electrode potentials more negative than the reductive desorption potential of ME-Au, ME bound to Au desorbs reductively and immediately. The desorbed ME diffuses out to the bulk phase and the adsorbed FDH becomes inactive in the absence of ME. The situation is responsible for the decrease in the DET activity given in Fig. 4 (A), closed circle. However, in the presence of ME, FDH remain active in a short period even at negative potentials. ME is re-bound to Au at OCP to stabilize FDH.

4. Conclusion

Electrode potential strongly affects the DET-type bioelectrocatalytic activity of FDH adsorbed at a bare Au electrode. The electrode potentials do not critically affect the total surface concentration of the adsorbed FDH. ME contained in the FDH stock solution binds to the Au electrode at a partial coverage during the FDH adsorption

process and it drastically affects the electrode potential dependence of the DET activity of the adsorbed FDH. We have proposed a model of the FDH adsorption state and also proposed the function of ME: ME on Au can stabilize the adsorbed FDH. The positive potential causes the formation of Au oxide layer which hinders the interfacial electron transfer between FDH and the Au electrode. On the other hand, the electrode potential more negative than the reduction potential of ME induces the reductive desorption of ME-Au and the adsorbed FDH is denatured in the absence of ME. We have also found that FDH adsorbs directly on the Au electrode in part and ME-Au located in the gap of the adsorbed FDH plays an important role in the stabilization of FDH.

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Figure Legends

Fig. 1. Cyclic voltammograms in the buffer containing 0.1 M D-fructose at (a) FDH (derived from ME-free FDH stock solution)-adsorbed Au electrode, (b) FDH (derived from ME-containing FDH stock solution)-adsorbed Au electrode and (c) FDH (derived from ME-free FDH stock solution)-adsorbed ME-SAM-Au electrode. The cyclic voltammograms were recorded at 25 °C and at a scan rate of 10 mV s⁻¹.

Fig. 2. (A) Steady-state voltammograms in the buffer containing 0.1 M D-fructose at (a) the FDH-unmodified Au electrode and (b)–(j) FDH-adsorbed Au electrodes in the presence of 0.28 µM FDH, 0.001% TX and 0.01 mM ME at E_{ad} = (b) –0.8 V, (c) –0.6 V, (d) –0.4 V, (e) –0.2 V, (f) 0 V, (g) 0.2 V, (h) 0.4 V, (i) 0.6 V, and (j) 0.8 V. Time of FDH adsorption is 5 min. The voltammograms were recorded at 25 °C and at a scan rate of 10 mV s⁻¹.

(B) E_{ad} dependence of the I value at 0.5 V of the voltammogram. The error bars were evaluated by the Student t -distribution at 90% confidence level.

Fig. 3. The relative value of the catalytic current density as a function of the number of electrode-washing (n). FDH was adsorbed on (closed circle) an ME-Au and (open circle) an ME-SAM-Au electrodes at $E_{ad} = OCP$ for 5 min. The cyclic voltammograms at $n = 0$ were recorded in the buffer containing 0.28 μ M FDH, 0.001% TX, 0.01 mM ME and 0.1 M D-fructose, and the cyclic voltammograms at $n \geq 1$ were recorded in the buffer containing 0.1 M D-fructose alone at 25 °C and at a scan rate of 10 mV s⁻¹.

Fig. 4. The relative value of the catalytic current density after holding at $E_{ho} =$ (A) -0.8 V, (B) 0 V, and (C) 0.8 V for 5 s in the buffer in the (closed circle) absence and (open circle) presence of 0.01 mM ME. The cyclic voltammograms were recorded in the buffer containing 0.1 M D-fructose alone at 25 °C and at a scan rate of 10 mV s⁻¹. In this figure, I_0 means the value of the catalytic current density before holding at $E_{ho} =$ -0.8 V, 0 V, and 0.8V.

Fig. 1

Fig. 2

Fig. 3

Fig. 4