Flavoenzyme-Catalyzed Electrochemical Oxidation of NADH and NADPH in the Absence of External Mediators

Daisuke KOBAYASHI, Shogo OZAWA, Tatsuya MIHARA[†] and Tokuji IKEDA*

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Diaphorase from *Bacillus stearotheophilus* (EC 1.1.99-) was entrapped on the surfaces of carbon paste and glassy carbon electrodes by covering with dialysis membranes. Ferredoxin-NADP⁺ reductase (EC 1.18.1.2) was adsorbed on the surfaces of glassy carbon, gold and silver electrodes by a dip coating method. Both electrodes modified with these enzymes produced anodic currents due to the enzyme-catalyzed electrolytic oxidation of their substrates in the absence of both electron transfer mediators and promoters. The electrocatalysis behavior was analyzed by an equation for the electrocatalytic oxidation of a substrate at the electrode modified with an adsorbed enzyme, using the electrode as an electron acceptor.

1 Introduction

In recent years increasing attention has been paid to the electrocatalysis based on oxidoreductases, bioelectrocatalysis, and to the enzyme electrodes relying upon bioelectrocatalysis.¹⁾ To achieve the enzyme-electrochemical oxidation of the substrates, redox compounds serving as electron transfer mediators between the oxidoreductases and the electrodes have usually been employed because of sluggish electrode reactions of oxidoreductases.^{1,2)} More recently, however, bioelectrocatalysis based on direct electron transfer of oxidoreductases at electrodes have been realized with several kinds of oxidoreductases in the presence of

promoters such as aminoglycosides^{3,4)} and even in the absence of promoters.^{4c-8)} Interestingly, all of the oxidoreductases employed contain heme group and/or Fe-S clusters, which are also contained in electrochemically active redox proteins such as cytochromes c and ferredoxins. We have interested in the oxidoreductases containing only flavins as redox groups. We employ here diaphorase and ferredoxin-NADP⁺ reductase, FNR, both of which can be obtained in highly purified states. The electrocatalysis behavior of the electrodes modified with these enzymes have been studied in the absence of external mediators.

2 Experimental

Diaphorase (EC 1.6.99-, from *Bacillus stearotheophilus*; molecular weight ca. 30000, one flavine mononucleotide, FMN, per molecule)⁹⁾ was donated from Unitika Ltd. The purity of the lyophilized preparation was checked by SDS-polyacrylamide gel electrophoresis. FNR, (EC 1.18.1.2, from spinach leaves, molecular weight 35000, one flavine

Department of Agricultural Chemistry,
Faculty of Agriculture, Kyoto University,
Sakyo-ku, Kyoto 606

[†]Present address: Research and Development
Center, Unitika Ltd. Kyoto.

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adenine dinucleotide, FAD, per molecule) was donated from Prof. Asada as crystalline suspension in ammonium sulfate.¹⁰⁾ The FNR suspension was dialyzed against 50 mM Tris-HCl buffer of pH 7.5 before use, and the concentration of FNR was determined spectrophotometrically using the absorption coefficient of $10300 \text{ M}^{-1}\text{cm}^{-1}$ at 458 nm.¹⁰⁾

Carbon paste electrodes (CPE, geometrical surface area: 0.09 cm^2) were prepared as described previously.¹¹⁾ A glassy carbon electrode (GCE, 3 mm diameter) and a gold electrode (AuE, 1.6 mm diameter) were obtained from BAS Corp. A disk of silver (2 mm diameter) sealed to the end of glass tubing with epoxy cement was used as a silver electrode (AgE). The GCE, AuE, and AgE were polished to a mirror-like finish with $0.05 \mu\text{m}$ alumina, followed by rinsing with deionized water in a ultrasonic bath. Film-covered enzyme-entrapped electrodes were prepared according to the method described previously.¹¹⁾ Thus the enzymes were entrapped on the electrode surfaces by covering with dialysis membranes. Electrodes with adsorbed enzyme were prepared by dipping the electrodes in an enzyme solution for a given period of time, followed by rinsing with deionized water.

Electrochemical measurements were done at 25°C in a buffer solution deaerated with argon. The solution was stirred at the rate of 400 rpm with a magnetic stirrer, unless stated otherwise. At this stirring rate, the concentration polarization of the substrates in solution was negligibly small. Three electrode system was employed; an Ag/AgCl(saturated KCl) electrode and a platinum coil as a reference electrode and a counter electrode, respectively.

3 Results and Discussion

3.1 Diaphorase

Figure 1 shows cyclic voltammograms in pH 8.5 Tris buffer solution recorded with

Fig. 1 Cyclic voltammograms of (A) a film-Diaph-CPE and (B) a bare CPE with a dialysis membrane in pH 8.5 Tris buffer in the absence (broken lines) and presence of $65 \mu\text{M}$ NADH (solid lines). Scan rate 10 mV/s .

(A) a dialysis membrane-covered diaphorase($1.1 \mu\text{g}/\text{cm}^2$)-entrapped CPE, a film-Diaph($1.1 \mu\text{g}/\text{cm}^2$)-CPE, and with (B) a bare CPE with a dialysis membrane. In the presence of $65 \mu\text{mol}/\text{dm}^3$ (μM) NADH, an anodic current started to appear at -0.2 V at the film-Diaph-CPE, (Fig.1A), whereas at the potential more positive than 0.35 V at the bare CPE with a dialysis membrane (Fig.1B). When the current was measured with the film-Diaph-CPE at a fixed electrode potential, a steady-state current, I , was obtained. I increased with increasing concentration of NADH to approach a saturation value as shown in Fig. 2, which was obtained with film-Diaph ($11 \mu\text{g}/\text{cm}^2$)-CPEs at 0 V . The results indicate that the steady-state current is due to the diaphorase-catalyzed

Fig. 2 Dependence of the steady-state current, I , at 0 V vs. Ag/AgCl on the concentration of NADH, c_{NADH} . $\circ$, $\bullet$, and Δ are the data measured with three different film-Diaph-CPEs prepared by the same procedure.

electrochemical oxidation of NADH. I measured at 0 V and at 100 μM NADH was independent of pH of the solution in the range from 7.5 to 10.5, and I at pH 10.5 increased with the increase in temperature from 25 to 50 $^{\circ}\text{C}$; the Arrhenius activation energy was determined to be 46 kJ/mol from the temperature dependence. These results are in line with the fact that the diaphorase employed here is thermostable and stable in alkaline solution.^{9a)} We have added neither mediators nor promoters to the solution in the electrochemical measurements. Therefore, the direct electron transfer reaction of the enzyme at the electrode is necessary for the enzyme-catalyzed electrochemical oxidation. It should be noted, however, that diaphorase employed in this study can use FMN as an electron acceptor to mediate the electron transfer reaction between the enzyme reaction and the electrode reaction.¹²⁾ Therefore, a small amount of free FMN dissociated from the enzyme, if present in solution, may function as a mediator to produce a catalytic current for the oxidation of NADH. We have shown that the catalytic current due to the mediation of FMN begins to appear at -0.5

V with a mid potential of -0.47 V, the potential at the half height of the limiting current.¹²⁾ Figure 3 shows the dependence of the steady-state current on the potential obtained with a film-Diaph(22 $\mu\text{g}/\text{cm}^2$)-CPE. The current starts to appear at -0.2 V and the mid potential is -0.07 V. This is evidently different from the potential dependence of the current due to the FMN-mediated electrocatalysis. The results support the idea that the direct electron transfer of diaphorase is occurring at the diaphorase-modified electrode during the electrocatalysis, though the wave clearly attributable to the redox reaction of the active site of the enzyme is not observed in the present experimental conditions. When GCE was used in place of CPE, the film-Diaph-GCE also produced the catalytic current, and the potential dependence of the steady-state current was very similar to that obtained with the film-Diaph-CPE (Fig. 3). However when AuE was used, the film-Diaph-AuE showed no detectable current response to NADH. The electrode reaction of diaphorase may be sluggish at a gold electrode.

The dependence of the steady-state

Fig. 3 Dependence of the steady-state current on the electrode potential. The steady-state currents were measured ($\circ$) with film-Diaph(22 $\mu\text{g}/\text{cm}^2$)-CPE and (Δ) with film-Diaph(22 $\mu\text{g}/\text{cm}^2$)-GCE in pH 8.5 Tris buffer containing 82 μM NADH. I : $\circ$ 0.11 $\mu\text{A}/\text{cm}^2$, and Δ 0.055 $\mu\text{A}/\text{cm}^2$.

Fig. 4 Dependence of the steady-state current at 0 V on the amount of entrapped diaphorase, (Diaph). The concentration of NADH is 82 μM .

current on the amount of entrapped diaphorase of film-Diaph-CPE is shown in Fig. 4. The current increased with the increase in the amount of entrapped enzyme to approach a saturation value. The step determining the magnitude of the saturation current should be the process of the permeation of NADH into the dialysis membrane covering the enzyme layer or the process of the enzymatic reaction. If we assume that the permeation process is the limiting step, we can calculate the limiting current to be 0.49 μA from an equation, $I = nFA(D_m/d)(\text{NADH})$, where n , F , A are the number of electrons, the Faraday constant, and the electrode surface area, respectively; D_m is the diffusion coefficient of NADH in the membrane, d the thickness of the membrane, and (NADH) the concentration of NADH at the surface of the membrane layer in the solution, which can be equated to the bulk concentration of NADH, since the concentration polarization of NADH is negligibly small in the present experimental conditions. In the calculation, the D_m and d values of $1 \times 10^{-6} \text{ cm}^2/\text{s}$ and 30 μm , respectively, were used.¹³⁾ The calculated value of 0.49 μA is about ten fold large compared with the saturation value in Fig. 4. Therefore, the saturation tendency should be ascribed to another process, the process of the enzymatic reaction.

When an adsorbed enzyme is taking part in the enzymatic reaction as written by

and

an equation for the steady-state limiting current is given by¹⁴⁾

$$I = I_{\text{max}}(\text{S})/(\text{K}_m + (\text{S})) \quad (3)$$

with

$$I_{\text{max}} = nFAk_2(\text{E})_{\text{ad}} \quad (4)$$

Where $[\text{Eox}]_{\text{ad}}$, $[\text{Ered}]_{\text{ad}}$, and $[\text{ES}]_{\text{ad}}$ are the adsorbed enzyme in the states of oxidized and reduced forms and an enzyme-substrate complex, respectively; S and P the substrate and product, respectively; k_1 , k_{-1} , and k_2 the rate constants of the steps in eq. 1; (S) the concentration of the substrate at the electrode surface, and K_m and $(\text{E})_{\text{ad}}$ the Michaelis constant of the enzyme reaction, eq. 1, and the total concentration of the adsorbed enzyme, respectively.

An alternative to the above scheme of the enzymatic reaction is that the solubilized enzyme in the vicinity of the electrode surface takes part in the enzyme-electrochemical reaction. In this case, $[\text{Eox}]_{\text{ad}}$, $[\text{Ered}]_{\text{ad}}$, and $[\text{ES}]_{\text{ad}}$ in eqs. 1 and 2 should be replaced by the respective states of the enzyme in solution, and an expression for the steady-state limiting current is given by

$$I = I_{\text{max}}((\text{S})/(\text{K}_m + (\text{S})))^{1/2} \quad (5)$$

with

$$I_{\text{max}} = nFA(Dk_{2b})^{1/2}(\text{E}) \quad (6)$$

where D and (E) , respectively, are the diffusion coefficient and the concentration of the enzyme; k_{2b} is the rate constant of the enzyme reaction in solution.

Equations 5 and 6 predict that I

increases linearly with (E), which is not consistent with the nonlinear dependence of I on the concentration of entrapped enzyme in Fig. 4. Equations 3 and 4 show that I depends on $(E)_{ad}$, that is, a quantity nonlinearly related to (E). Thus eqs. 3 and 4 may explain the results in Fig. 4. The adsorbability of diaphorase was examined by a dip coating method. A CPE was dipped into a diaphorase solution for 60 s, then the electrode was rinsed with water. The CPE treated with the diaphorase solution showed current response to the addition of NADH when measured at 0 V in a pH 8.5 buffer solution, but the current magnitude tended to decrease during continuous measurements of the current for 10 min, indicating that the adsorbability of CPE to the enzyme is weak. Accordingly, an explanation of the result in Fig. 4 is that there exists an adsorption equilibrium between the adsorbed enzyme and the enzyme in solution, and that the maximum adsorption is attained at the concentration, 5×10^{-10} mol/cm², of entrapped diaphorase. This quantity can be converted to the bulk concentration of 0.5 mM, if the thickness of the layer of the entrapped enzyme between the electrode and the dialysis membrane is assumed to be 1 μ m. The adsorbability of GCE was even weaker than CPE when examined in the same manner as CPE.

Although we are unable to measure the amount of the adsorbed diaphorase on CPE, we can analyze the dependence of I on the concentration of NADH, c_{NADH} , (Fig. 2) by eq. 3. The plot of c_{NADH}/I against c_{NADH} of the data in Fig. 2 gave linear lines as expected from eq. 3, from which the I_{max}/A and K_m values were determined to be 1.0 ± 0.2 μ A/cm² and 0.17 ± 0.02 mM, respectively. The K_m value agrees broadly with results from the measurements in solution using artificial dyes as electron acceptors.^{9a)} If we use the k_{2b} value, 500 s⁻¹, calculated from the specific activity of the enzyme,^{9a)} we can calculate from eq. 4 with the I_{max} value the amount of

adsorbed enzyme as 1×10^{-14} mol/cm². This is too small compared with the monomolecular surface coverage, 4×10^{-12} mol/cm² of the molecules with the molecular weight ca. 30000.¹⁵⁾ The k_2 value of the adsorbed diaphorase might be very small or only very small part of the adsorbed molecules might be catalytically active.

3.2 FNR

FNR were adsorbed more strongly than diaphorase on a number of electrode materials. Thus, a gold electrode was dipped into an FNR solution for 60 s, then rinsed with water. The AuE with the adsorbed FNR, FNR-AuE, was immersed in a pH 7.5 Tris buffer solution, and the current response to NADPH was recorded at a fixed electrode potential. Figure 5 shows the results measured with the FNR-AuE at 0 V. As shown in the inset, an anodic current was obtained by the addition of NADPH, and a steady-state current was attained in seconds. The current magnitude remained almost constant during the continuous measurements for more than 30 min, indicating that FNR was

Fig. 5 Dependence of the steady-state current on the concentration of NADPH. The steady-state currents were measured at 0 V in pH 7.5 Tris buffer solution with an FNR-adsorbed AuE. The inset shows the current response of the electrode to the addition (indicated by the arrows) of 2 μ M NADPH to the buffer solution.

Fig. 6 Plot of c_{NADPH}/I against c_{NADPH} .

adsorbed on an AuE rather strongly. The steady-state current, I , increased with increasing concentration of NADPH, c_{NADPH} , to approach a saturation value. Figure 6 shows the plot of c_{NADPH}/I against c_{NADPH} of the data in Fig. 5. A linear relationship was obtained as expected from eq. 3, and the I_{max}/A and K_m values were determined from the slope and intercept. The anodic current response was also obtained at more negative potentials up to -0.5 V. FNR could also be adsorbed on GCE and AgE. The FNR-GCE and FNR-AgE prepared by the same method as the FNR-AuE produced anodic currents when NADPH was added to the solution. Similar dependences of I on c_{NADPH} to that in Fig. 5 were obtained with these electrodes, from which the I_{max}/A and K_m values could be calculated by using eq. 3. The I_{max}/A values are 0.91 ± 0.19 , 0.24 ± 0.06 , and 0.86 ± 0.48 $\mu\text{A}/\text{cm}^2$, and the K_m values are 22 ± 1.8 , 17 ± 2.1 , and 93 ± 19 μM for FNR-AuE, FNR-GCE, and FNR-AgE, respectively. Each data is the means of two measurements. There are differences in both the I_{max}/A and K_m values obtained with the three kinds of electrodes, though these K_m values are in the same order of magnitude as those reported for FNR reactions using several kinds of electron acceptors.¹⁶⁾ The results may indicate that small conformational changes of FNR occur by adsorption on electrode surfaces. The

catalytic constant, k_2 , which is related to I_{max}/A by eq. 4, and the affinity to the substrate, K_m , of the adsorbed enzyme should be sensitive to the conformational state of the enzyme adsorbed on electrodes. The amount of adsorbed enzyme, $(E)_{\text{ad}}$, may also depend on the kind of electrodes on which the enzyme is adsorbed. If we use the k_2 value, 60 s^{-1} , calculated from the specific activity of the native enzyme,¹⁰⁾ we can calculate the amount of adsorbed enzyme from the I_{max}/A values 0.91 to $0.24 \mu\text{A}/\text{cm}^2$, by eq. 4 to be 7.8 to $2.1 \times 10^{-14} \text{ mol}/\text{cm}^2$. The actual amount of adsorbed enzyme will be more large, because the k_2 value will be smaller than 60 s^{-1} owing to the expected conformational change of the adsorbed enzyme. Although we tried to record the redox wave for the adsorbed FNR itself and the catalytic current for the reduction of NADP^+ , we were unable to obtain the wave and the current clearly distinguishable from the base current under the present experimental conditions. In spite of this, the observation of the catalytic currents in the absence of mediators show the electron transfer reaction of the adsorbed FNR at the electrode, by which the enzyme-electrochemical oxidation of NADPH is allowed to occur. FNR has different sites to react with the cofactor and an acceptor (or donor).¹⁷⁾ Accordingly, if the adsorbed FNR is appropriately oriented with the reaction site for an acceptor toward the electrode surface, the electrocatalysis using the electrode as an electron acceptor may become possible. The electrocatalysis observed with adsorbed diaphorase might be occurring in a similar way, though the three dimensional structure of the molecule is not yet known.

4 Conclusion

It has been shown that diaphorase and FNR catalyze the electrochemical oxidation of NADH and NADPH, respectively, in the absence of both electron transfer mediators and promoters. The electrocatalytic behavior could be explained by an equation

for the catalysis of an adsorbed enzyme, using the electrode as an electron acceptor. Diaphorase was adsorbed on CPE and GCE rather weakly; on the other hand, FNR was adsorbed more strongly on GCE, AuE and AgE.

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5 References

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