Flavin-Mediated Two-Way Bioelectrocatalysis of Lactate Dehydrogenase Reaction Combined with Diaphorase Reaction

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(Received September 14, 1995)

FMN(H_2) has been found to work as a two-way mediator of diaphorase to reduce NAD^+ as well as to oxidize NADH. The FMN/diaphorase/NAD system has been successfully combined with lactate dehydrogenase-catalyzed reduction of pyruvate and oxidation of lactate. The thermodynamics of the two-way bioelectrocatalytic system is described.

The NAD-dependent dehydrogenases (E(NAD)) constitute the largest group of redox enzymes and virtually all reactions catalyzed by E(NAD)s are considered to be reversible. Therefore, two-way bioelectrocatalytic systems using E(NAD)s would be one of the ultimate goals in order to develop new electrochemical approaches to biochemistry and to lead to better understanding of catalytic systems useful for sensors and reactors. Since the direct electrochemistry of NAD(H) requires large overpotentials and is accompanied by the side-reactions,[1] several attempts have been devoted to one-way enzyme-catalyzed NAD(H)-regeneration systems.[2-6] Recently we have presented a simple thermodynamic model in which some redox compounds with a formal redox potential ($E^{\circ\prime}$) more positive or negative than that of NAD can function, respectively, as one-way mediators for the bioelectrocatalytic oxidation of NADH and the reduction of NAD^+ using flavoproteins such as diaphorase (DI).[7] NAD^+ and NADH thus produced has been successfully incorporated into E(NAD)-catalyzed one-way reactions. The model may be extended to get a working hypothesis that a certain redox compound with $E^{\circ\prime}$ close to that of the substrates of an E(NAD) can work as a two-way mediator of the E(NAD)-reaction system linked with the DI-reaction. In this work, we utilized FMN and lactate dehydrogenase (LDH) as a promising two-way mediator and an E(NAD), respectively, because $E^{\circ\prime}$ of FMN is close to that of the pyruvate/lactate (Pyv/Lac) couple (Figure 1). In this system, thermodynamically unfavorable (uphill) electron transfers (ET) may be driven by coupling with thermodynamically favorable (downhill) ETs via the NAD redox cycling.

DI (EC. 1.6.99.-; Unitika, 3.5×10^{-10} mole) and LDH (EC. 1.1.1.27; Toyobo, 0.97 U) were co-entrapped on a glassy carbon (GC) electrode surface by covering with a dialysis membrane according to the literature.[7] Cyclic voltammogram (CV) of FMN exhibited one pair of redox waves at a midpoint potential of -0.408 V vs. Ag/AgCl at this electrode (Figure 2, a). Addition of excess amount of NAD^+ resulted in an appearance of only a very small catalytic reduction wave (Figure 2, b). This can be attributable to the uphill ET from $FMNH_2$ to NAD^+ (Figure 1). The generation of NADH was demonstrated more clearly by combination of LDH-catalyzed reduction of Pyv; addition of Pyv enhanced the catalytic current and quenched the reoxidation wave of FMN (Figure 2, c). This proves that $FMNH_2$ works well as a mediator of the DI-catalyzed bioelectrochemical reduction of NAD^+. Although the possibility of such function of flavins has been already pointed out, no clear voltammetric evidence was reported.[5] The increase in the catalytic current indicates that the LDH-catalyzed downhill ET from NADH to Pyv stimulates the bioelectrocatalytic uphill ET from $FMNH_2$ to NAD^+. This implies that the NADH concentration in the enzyme layer would be negligibly small compared with NAD^+ during the two enzyme-linked catalytic reduction.

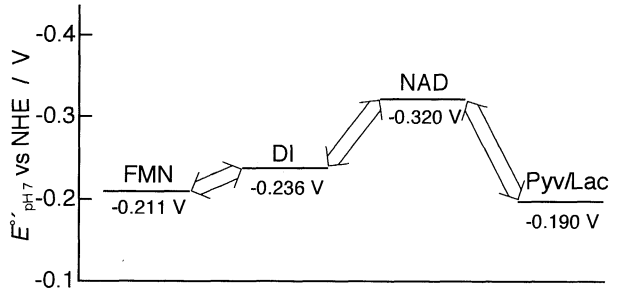

Figure 1. Bioelectrocatalytic reaction scheme with their redox levels.[7b,8]

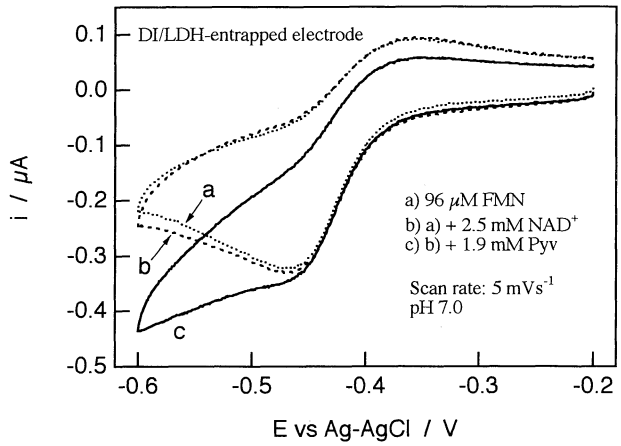

Figure 2. CV evidence of uphill electron transfer from $FMNH_2$ to NAD^+.

With respect to the oxidation of NADH, FMN is known to function as an effective mediator for the bioelectrocatalytic oxidation of NADH.[5-7] Thus we tried to combine this downhill bioelectrocatalytic reaction with the reversed reaction of LDH (i.e. the oxidation of Lac). Addition of Lac enhanced the catalytic oxidation wave as evidenced in Figure 3 (b compared with a). Generation of NAD^+ by the FMN-mediated DI-catalyzed oxidation appears to drive the uphill LDH-catalyzed oxidation of Lac. These results are the first voltammetric evidence demonstrating that FMN(H_2) behaves as a two-way mediator in the DI/LDH-catalyzed bioelectrochemical redox system for Pyv/Lac.

It is important to see what is happening in this two-way bioelectrocatalytic system. The following experiments may give some insight into this question. Figure 3 (b-d) shows changes in voltammetric responses upon successive injection of Pyv at practically constant concentration of Lac. With increasing in the

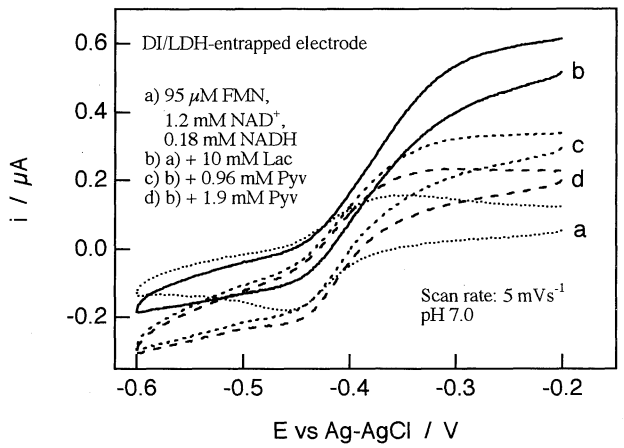

Figure 3. CVs representing bioelectrocatalytic reduction and oxidation of the substrates of LDH.

Pyv concentration, the catalytic oxidation waves decreased and the catalytic reduction waves increased. Because the oxidative and reductive wave heights depend on the effective concentrations of $FMNH_2$ and FMN, respectively, this phenomenon can be ascribed to a decrease of $FMNH_2$ and an increase of FMN in the bulk phase (in the entrapped enzyme layer in this case). This suggests the existence of an redox equilibrium among FMN/$FMNH_2$, NAD^+/NADH, and Lac/Pyv. The reversible nature of DI- and LDH-catalyzed redox reactions seems to be responsible for the redox equilibrium.

The above consideration is strongly supported by potentiometry with a bare GC electrode in the present catalytic system under completely anaerobic conditions, during which the total concentrations of Lac, FMN, and NAD were kept practically constant as 44, 0.17, and 0.20 mM, respectively. The potentiometric response upon successive injection of Pyv gave a linear relationship against log([Pyv]/[Lac]) with a slope of 37 mV/decade and a correlation coefficient of 0.996 in the log([Pyv]/[Lac]) range of -0.8 to 0 (data not shown). The potential at [Pyv]/[Lac] = 1 was -193 mV vs. NHE, which is very close to $E^{\circ\prime}$ of the Pyv/Lac couple in the literature.[8] The fact that the catalytic reduction current was smaller than the oxidation current (Figure 3) seems to reflect difference in the enzyme kinetics between the oxidation and reduction. Detailed kinetic study in the two-enzyme linked system is in progress.

To our knowledge, this is the first report demonstrating that FMN(H_2)/DI system works as an effective two-way NAD(H) regeneration system for the LDH-catalyzed oxidation and reduction of the substrates. In order to realize such two-way catalytic systems, it appears to be very important to select mediators with $E^{\circ\prime}$ close to that of the enzyme substrates. This work has also proved that an uphill catalytic reaction can be accelerated by combination of a downhill one to be followed within voltammetric time windows. Such novel two-way bioelectrocatalytic systems will allow us to understand future electrochemical regulation systems of the E(NAD) reactions and also biological regulation systems.

We thanks to Biochemistry Department, Unitika Ltd. for a kind gift of DI. This work was supported by Grant-in-Aids for Scientific Research on Priority Area No. 236 and General Research from the Ministry of Education, Science and Culture of Japan and by The San-Ei Gen Foundation for Food Chemical Research.

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