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Effect of Viologen Structure on Electroreduction of NAD^+ Catalyzed by Diaphorase Immobilized on Electrodes

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The bioelectrocatalytic reduction of NAD^+ to NADH on diaphorase immobilized electrodes was investigated using seven viologen derivatives with various side chains. The hydrophobic interaction between the enzyme and mediator has been shown to promote the reduction.

Biochemical reactions with NAD(H)-dependent dehydrogenases have been recognized as a useful approach for chiral syntheses, since these enzymes are ubiquitous and are able to transfer a hydride stereospecifically to substrate. Commonly, the bioreactors based on the dehydrogenases consist of two enzyme reactions; one is for the production of the desired chemicals and the other for regeneration of the coenzyme. Several approaches to the regeneration of NADH have been developed; however, in general these have problems, such as high cost and low chemical yield.[1] Whitesides and coworkers,[2] using electron transfer mediators, reported the regeneration of NADH catalyzed by lipoamide dehydrogenase entrapped in polyacrylamide gel, but the conversion rate was not satisfactory. To promote the reduction, the enzyme should be present at the electrode surface where the reduction of mediators occurs.

We previously reported a smooth bioelectrocatalytic reduction of NAD^+ by N,N'-dimethyl-1,4-dipyridinium salt (Methyl viologen, 1) on a diaphorase immobilized electrode.[3] In this paper, we describe the effect of the structure of the mediator on the bioelectrocatalytic reduction of NAD^+ on a diaphorase immobilized electrode.

Cyclic voltammetry (CV) and controlled potential electrolysis (CPE) were performed at 30 °C in 7 cm^3 of a solution of 0.05 M (=mol dm^{-3}) phosphate-NaOH buffer (pH=7.5). The working electrode used for CV was a 3-mm-diameter glassy carbon disc (Tokai Carbon, GC-20) mounted in a Teflon rod. A glassy carbon plate (area, 6.0 cm^2) was used for CPE. Preparation of the immobilized electrode was carried out by mixing equal volumes of 0.44 mM diaphorase solution (NADH: lipoamide oxidoredutase from pig heart, EC 1.6.4.3, Boehringer Mannheim) and 4% (v/v) glutaraldehyde on the electrode surfaces. The concentration of diaphorase was determined by absorption at 455 nm using an extinction coefficient of 11.3 mM^{-1} cm^{-1}.[4] The surface concentrations of the immobilized diaphorase were 2.3 nmol cm^{-2} for CV and 0.7 nmol cm^{-2} for CPE. The electrodes were kept at room

temperature for two hours to allow the polymerization at the surfaces to be completed. The immobilized electrodes were kept in the buffer solution at 4 °C for at least two days before the measurements. The electrolysis was carried out in N_2 atmosphere. Four symmetric (1, 5, 6, 7) and three asymmetric (2, 3, 4) dipyridinium salts (viologens) were synthesized[5-7] and used as electron mediators (structures and abbreviations are shown in Table 1). The concentrations of NAD^+ and NADH were analyzed on HPLC using a Develosil ODS-5 column (Nomura Chemical Co.) with a phosphate buffer solution (pH=6.4) containing 1.5% methanol as the mobile phase.

Figure 1 depicts the cyclic voltammograms for 0.25 mM viologens on the diaphorase immobilized electrode. In the absence of NAD^+, the voltammogram for 1 shows two well-defined, reversible peaks at ca.-0.70 V and ca.-1.1 V vs. SCE. The addition of 2 mM NAD^+ to the solution caused an obvious increase in the first and second reduction peaks and an oxidation peak for NADH appeared at +0.6 V vs. SCE on the reverse scan. This result indicates that the diaphorase on the electrode surface acts effectively as a catalyst for electron transfer between the reduced viologen and NAD^+. Asymmetric viologens also show considerable electrocatalytic ability as is evident from the first reduction peaks. On the other hand, symmetric viologens except 1 show poor ability as mediators. All mediators used in the present study show significant catalytic ability at the second reaction potentials. Judging from the first peak, viologens having hydrophilic side chains seem to be unsuitable as mediators in the reduction of NAD^+ catalyzed by diaphorase. In particular, mediator 7 shows no increase in the first reduction peak at 20 mV s^{-1}.

The CPE experiments were carried out at -0.85 V, where the viologens are reduced to the corresponding radical cations. The results using seven viologens are shown in Fig. 2. The desired product, NADH, was formed while NAD^+ disappeared, and no other product was found in the solution. The reduction rate depends greatly on the structure of the viologens. CPE was also performed with identical amounts of diaphorase in solution to clarify the advantage of diaphorase immobilized on the electrode (Fig. 2-1'). The initial rate for the conversion of

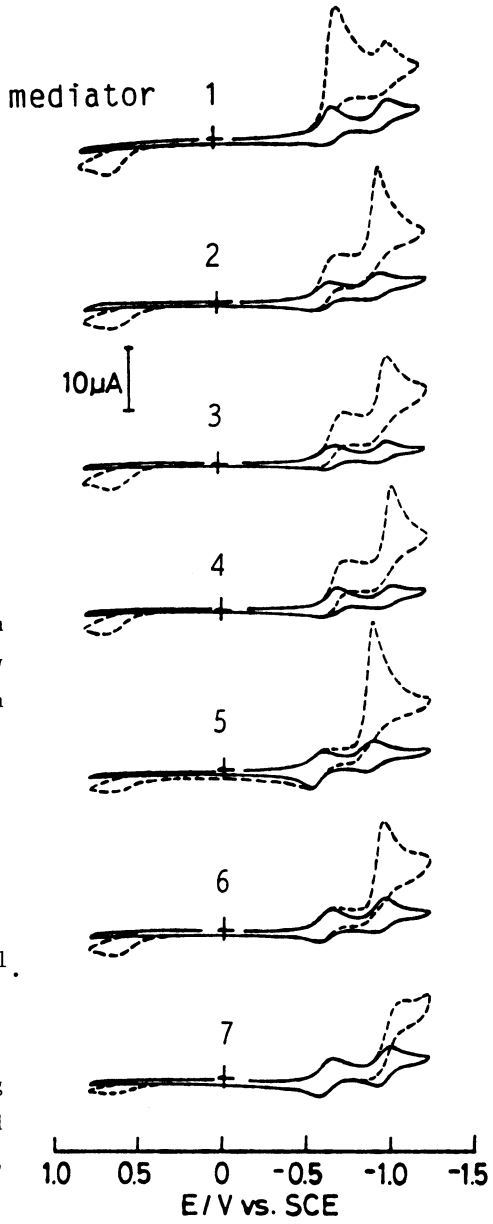


Fig.1. Cyclic voltammograms for 0.25 mM viologen derivatives on the diaphorase immobilized electrode without (——) and with 2.0 mM NAD^+ (-----) in 0.05 M phosphate buffer (pH=7.5). Scan rate: 20 mV s^{-1}. Temperature: 30 °C.

NAD^+ on the diaphorase immobilized electrode was 4 times than that on the bare electrode when 1 was used as the mediator.

The results obtained in the present study are summarized in Table 1. Free energy values (ΔG) were calculated from the difference in the formal potentials of mediator and coenzyme. There is no obvious relationship between the catalytic ability of the mediator and the ΔG values. The initial rates or turnover numbers listed in Table 1 allow an important inference; the more hydrophilic the side chain is, the slower the reaction proceeds.

It can be deduced that the reduction pocket, where electron-transfer from the reduced viologen to the flavin takes place, is hydrophobic. This deduction is consistent with previous findings.[8] Although the whole primary structure of diaphorase from pig heart has not been determined, amino acid residues around the active site are relatively hydrophobic.[8] Ethyl viologen also showed a catalytic ability comparable to 1. Therefore, we believe that the bioelectrocatalytic reduction of NAD^+ is promoted effectively by the hydrophobic interaction between enzyme and mediator, and that this effect is more important than that of bulkiness of mediator. The diaphorase immobilized electrode was quite stable; no obvious decrease in the catalytic ability was observed on repeated use. The viologens used here are also stable; no decomposition seemed to occur during the electrolysis. High turnover numbers for viologens were observed when the electrolyses were conducted at low concentrations of the mediators on the diaphorase immobilized electrode.

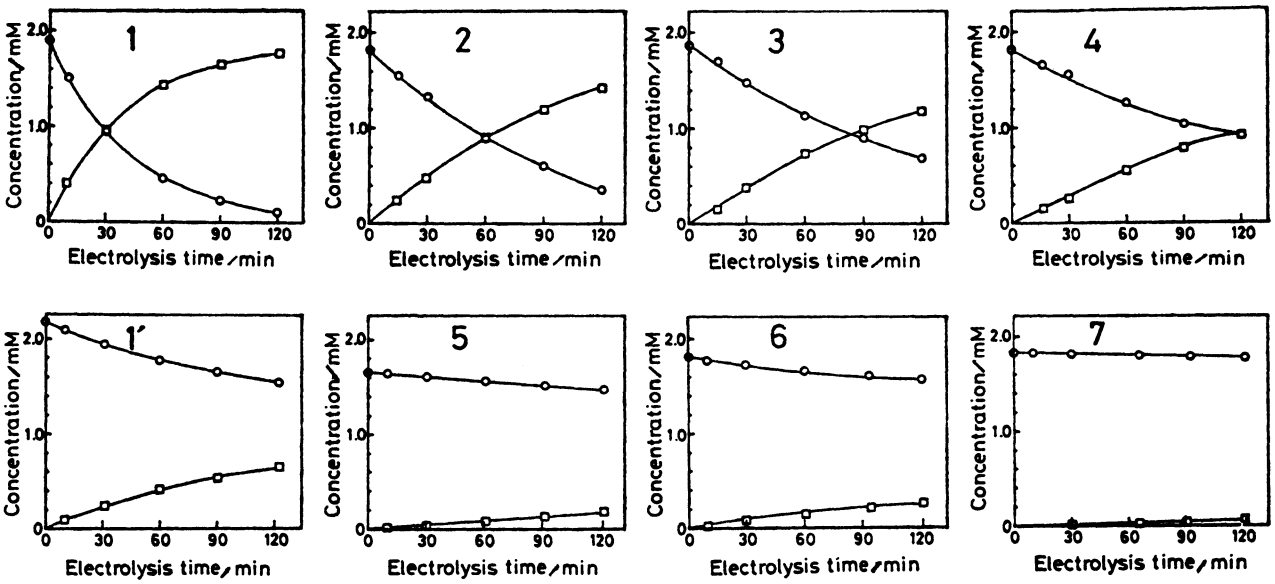
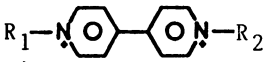


Fig.2. Time dependence of NAD^+ (○) and NADH (□) concentration in CPE on the diaphorase immobilized electrode with 0.25 mM viologen derivatives (1—7). Fig.2-1' shows the result on the bare electrode with 0.25 mM methyl viologen and 600 nM diaphorase. Potential: -0.85 V vs. SCE. Electrolyte: 0.05 M phosphate buffer (pH=7.5). Temperature: 30 °C.

Table 1 Summary of CV and CPE results

Mediator	R_1	R_2	$E_{1/2}$ / V vs. SCE	$\Delta G(=-nF\Delta E)$ / kcal mol^{-1}	v_o [a] $\times 10^6$ / mol h^{-1}	TN_M [b]	TN_D [c]
1	CH_3	CH_3	-0.68	-2.42	17.5	14.0	3080
2	CH_3	CH_2CONH_2	-0.62	-1.04	8.4	11.5	2520
3	CH_3	$(CH_2)_3NH_3^+$	-0.65	-1.73	5.3	9.5	2065
4	CH_3	$(CH_2)_2SO_3^-$	-0.67	-2.19	4.3	7.3	1592
5	CH_2CONH_2	CH_2CONH_2	-0.55	+0.58	0.63	1.5	367
6	$(CH_2)_3NH_3^+$	$(CH_2)_3NH_3^+$	-0.59	-0.35	1.47	2.1	455
7	$(CH_2)_2SO_3^-$	$(CH_2)_2SO_3^-$	-0.60	-0.58	0.21	0.5	114



a) Initial reaction rate.

b) Turnover number for mediator after 2 hours.

c) Turnover number for diaphorase after 2 hours.

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