Proc. KSAM Fall Meeting
October 28, 2000, Chonju, Korea

Electrochemical Evaluation of the Reaction Rate and Electrochemical Optimization of the Mediated Electrochemical Reduction of NAD^+

Young Wan Kang[1], So Hyoung Kim[2], Chan Kang[2] and Sei Eok Yun[1]
[1]Dept. of Food Sci. and Technol. and [2]Dept. of Chem., Chonbuk National University

Abstract

The electrocatalytic reduction of NAD^+ using diaphorase was studied. methyl viologen (MV^{2+}) mediator between an electrode and the enzyme. Steady-state currents could be obtained under the conditions of slow scan rate, low MV^{2+} concentration, and high NAD^+ concentration as the electrode reaction was converted to an electrochemical-catalytic (EC') reaction. The bimoecular rate constant for the reaction of the reduced methyl viologen with the oxidized diaphorase was estimated as 7.5×10^3 $M^{-1}s^{-1}$ from the slope of the current versus [MV^{2+}] plot. And the optimal concentrations of diaphorase, MV^{2+} and NAD^+ in the mediated electrocatalytic reduction of NAD^+ were decided by applying the cyclic voltammetry. The optimal concentrations of the species were obtained by finding the conditions which gave the highest and steady-state current at a gold-amalgam electrode. The highest and steady-state catalytic current was achieved under the conditions of 1.5 U/ml diaphorase, 0.2 mM MV^{2+}, and 4.8 mM NAD^+ at the scan rate of 2 mV s^{-1}, suggesting that the rate of the electrocatalytic reation is the higest under the former conditions. The electrochemical procedure under the conditions of 1.5 U/ml diaphorase, 0.2 mM MV^{2+}, and 4.8 mM NAD^+ was used favorably to drive an enzymatic reduction of pyruvate to D-lactate.

Introduction

Recently the mediated electrocatalytic reduction of $NAD(P)^+$, in which one enzyme transfers a reducing equivalent from an electrogenerated redox mediator to $NAD(P)^+$, has received increasing attention in the construction of NAD(P)H regenerating bioreactors and biosensors. The electrochemical procedure is known to be an efficient means because no stoichiometric byproduct from which the product must be separated is produced and the process can be monitored easily by the electrical current. The electrochemical regeneration of NAD(P)H has been usually performed by

indirect electrochemical method, because the direct electrochemical reduction of $NAD(P)^+$ leads to yield enzymatically inactive NAD(P)H or NAD(P)-dimers, although efforts to improve the selective reduction of $NAD(P)^+$ to enzymatically active NAD(P)H have been made by the use of polymer-supported cofactor[1]or coated electrode[2].

Despite the importance of the study of the system, relatively fewer reports about this system exist. The difficulty of observing voltammeric responses for the catalytic NAD^+ reduction, especially when the enzyme molecules are in the dissolved state in solution, is due to slowness in the rate determining step. In the present study, by controlling the experimental variables carefully, a much clear steady-state catalytic current could be obtained at a gold-amalgam electrode at the potential where the MV^{2+} was reduced to MV^+. Based on the voltammetric measurement of the electrocatalytic reduction of NAD^+, an electrochemical-catalytic (EC') reaction model[3] was applied to evaluate a rate constant between the MV^{2+} and diaphorase. And we tried to apply the cyclic voltammetry to determine the optimal concentrations of diaphorase, MV^{2+}, and NAD^+ for the electrocatalytic reduction of NAD^+. The optimal concentrations of the concentrations of diaphorase, MV^{2+}, and NAD^+ were thought to be obtained by finding the conditions which give the highest and steady-state current. The electrocatalytic reduction of NAD^+ was coupled with the enzymatic conversion of pyruvate to D-lactate by D-lactate dehydrogenase in order to examine whether the electrochemical procedure works satisfactorily as an NADH regenerator where the reaction species, diaphorase, MV^{2+}, and NAD^+ are present in the solution at the concentrations determined voltammetrically.

Materials and methods

Preparation of the gold-amalgam working electrode

A gold plate(area, 0.32 cm^2) was dipped into mercury for a short time and a gold-amalgam surface was formed.

Linear sweep voltammetric measurements

Electrochemical experiments were performed in a two compartment cell closed with a Teflon cap. A platinum auxiliary electrode, the gold-amalgam working electrode(area, 0.32 cm^2), and a gas bubbling tube was immersed in another compartment separated by a glass frit. A BAS 50 electrochemical analyzer(Bioanalytical Systems Ins., USA) connected to a personal computer was used to perform

electrochemical measurements. Potentials were measured and quoted with respect to an Ag/AgCl reference electrode with a potential of 0.22 V versus SHE. All experiments were conducted at a temperature of 25 ℃ with the cell immersed in a thermostat.

Procedure for NADH regenrating reaction

Electrochemical reduction of NAD^+ was performed at 25 ℃ in a cell which was divided into two compartments(each volume : 50 cm^3) with a sulfonated membrane of styrene-divinyl copolymer(an anion-charged membrane). A plate(6 cm^2) of gold amalgam was used as a working electrode and a platinum plate as a counter electrode. Ag/AgCl reference electrode was immersed into the cathode compartment filled with 0.5 M phosphate(pH 7.0) electrolyte containing NAD^+(4.8 mM), MV^{2+}(0.2 mM), diaphorase(1.5 U/ml), D-lactic dehydrogenase(3.0 U/ml), and sodium pyruvate(50.0 mM). After the electrolyte was purged with N_2 gas for 30 min, the electrolysis was started using a potentiostat(HA-501, Hokuto Denko Co., Japan). Electrolysis was carried out at the constant potential of – 0.7 V vs. Ag/AgCl electrode at 25 ℃.

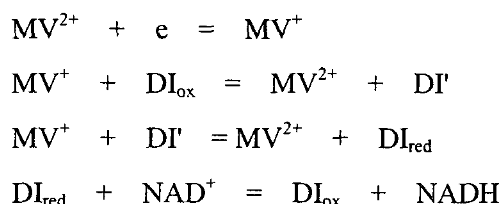
Assays

D-lactate and pyruvate were analyzed with an HPLC, using an Aminex HPX-87H column with an UV detector(210 nm). A mobile phase of 0.3 % H_3PO_4 (v/v) was used.

Results and discussion

Kinetic measurements for the mediated electrocatalytic reduction

The mediated electrocatalytic reduction of NAD^+ by diaphorase(DI) was proposed as follows[4, 5]:



$$MV^{2+} + e = MV^{+}$$
$$MV^{+} + DI_{ox} = MV^{2+} + DI'$$
$$MV^{+} + DI' = MV^{2+} + DI_{red}$$
$$DI_{red} + NAD^{+} = DI_{ox} + NADH$$

Since two electrons are required for the reduction of one diaphorase enzyme molecule, two reduced mediators are used and DI' was assigned as the one-electron reduced form of the enzyme.

The reduced form of the enzyme (DI_{red}) will be consumed rapidly by the substrate and the enzyme will remain in its steady oxidized state when the substrate concentration is high[6]. Under these conditions, the enzyme concentration for the bimolecular reaction with the mediator stays at constant level and the reaction of the enzyme with the MV^{2+} follows a pseudo-first-order in the mediator. This system is equal to the EC' system which gives a steady-state voltammetric response under the conditions in which the substrate concentration is high and the scan rate is low[3].

To check the scan rate showing a steady-state limiting current, cyclic voltammetric measurement was performed at various scan rates in the presence of excess NAD^+. current. In the scan rate range under 2 mV s^{-1}, no more current change was observable and the reaction was considered to follow the EC' case which is purely kinetically controlled by the reaction step between the mediator and the enzyme[3].

The steady-state limiting current in cyclic voltammetry is expressed as:

$$I_{\lim} = nFAc^*_{med}(D_{med}\,kc^*_{enz})^{1/2}$$

where c^*_{med} and c^*_{enz} are the bulk concentrations of the mediator and the enzyem, respectively, D_{med} is the diffusion coefficient of the constant between the mediator and the enzyme, and other symbols have their usual meaning [7]. The current is scan rate independent and proportional to the mediator concentration or the square root of the enzyme concentration . The rate constant can be determined from the slope of the plot of the liming current versus c^*_{med} or another plot against $c^*_{enz}{}^{1/2}$. The rate constant k was calculated as 7.5×10^3 M^{-1} s^{-1}.

Optimization of the concentration of the species for electrocatalytic NAD^+ reduction.

The catalytic currents at various concentrations of diaphorase were measured to determine the effective concentration of the enzyme for the electrocatalytic reduction of NAD^+ by finding the enzyme concentration which gives the highest and steady-state cerrent. As shown in Fig. 1, the enhancement of current was observed with the increase of the concentration of diaphorase. In the concentration range of diaphorase under 1.5 U/ml, the cathodic wave grew continually with the increase of diaphorase concentration while small current change was observed with more addition of the enzyme after 1.5 U/ml. Therefore, it was assumed that 1.5 U/ml is the amount close to the concentration to enable the bimolecular reaction with the mediator MV^{2+} to stay at constant level.

The voltammetric response was checked at various concentrations of MV^{2+} while the concentrations of diaphorase and NAD^+ were kept constant as 1.5 U/ml and 5 mM, respectively, at a scan rate of

2 mV s^{-1}. As was presented in Fig. 2, the cathodic wave grew continually with the increase of MV^{2+} concentration without reaching any saturation. The voltammograms showed steady-state wave shapes at low values, but peak shaped voltammograms were recorded as MV^{2+} was increased because all of the reduced MV^{+} could not be consumed by the enzyme and some of it remained unreacted in the diffusion layer. The linear relationship between the mediator concentration and the current height was examined. The cathodic steady-state currents of peak currents measured from the voltammograms of Fig. 2 were plotted against their corresponding concentrations of MV^{2+} to present the Fig. 3. Linearity was obtained when the concentration of MV^{2+} was as low as 0.15 mM although the current started to deviate at concentrations higher than 0.20 mM, which suggests that 0.20 mM of methyl viologen is sufficient to transfer the electrons to diaphorase.

Fig. 4 is a collection of cyclic voltammograms obtained at different NAD^{+} concentrations while the concentrations of diaphorase and MV^{2+} were kept at 1.5 U/ml and 0.2 mM, respectively at a scan rate of 2 mVs^{-1}. The increase of the cathodic current was saturated at about 4.8 mM of NAD^{+} and the voltammogram lost the steady-state wave shape as the NAD^{+} concentration decreased, suggesting that NAD^{+} is present in large excess in the solution and its concentration is essentially unchanged during the voltammetric measurement. The result confirms that the 4.8 Mm of NAD^{+} is an optimal concentration with 1.5 U/ml of diaphorase and 0.2 Mm of MV^{2+} . In a given concentration 4.8 Mm of NAD^{+}, the concentratioas of the diaphorase and MV^{2+} were decided as 1.5 U/ml are 0.2 mM , respectively. This is one optimal point and other optimal sets will be also deside as the concentration of was varied.

Production of D-lactate by enzymatic reaction coupled with the electrocatalytic reduction of NAD^{+}

In the previous sections, the mediated electrocatalytic reduction of NAD^{+} was considered to be optimal under the conditions of 1.5 U/ml diaphorase, 0.2 mM MV^{2+}, and 4.8 mM NAD^{+}. The supposed optimal conditions of the three species were confirmed by performing a coupled reaction between the mediated electrocatalytic reduction of NAD^{+} and an enzymatic reduction in the presence of D-lactate dehydrogenase and pyruvate. The result was compared with that from the same reaction except that the conditions of 0.3 U/ml diaphorase, 2 mM MV^{2+}, and 0.05 mM NAD^{+} which were dertermined by investigating the amount of product and proposed to be effective in the enzymatic production of malic acid combined with electrochemical regeneration of NADH using a glassy carbon

bead electrode[8]

The conversion yield of D-lactate from 50 mM pyruvate was higher under the conditions of 1.5 U/ml diaphorase, 0.2 mM MV^{2+}, 4.8 mM NAD^{+} than under the other conditions of the species. The conversion yield of D-lactate from 50 mM pyruvate was about 90% at 120 h of total reaction time. These results suggest that under the conditions of 1.5 U/ml diaphorase, 0.2 mM MV^{2+}, and 4.8 mM NAD^{+} the electrolysis worked satisfactorily as an NADH regenerator.

References

1. Aizawa M, Coughlin RW, Charles M (1976) Biotechnol. Bioeng. 18: 209-215
2. Baik SH, Kang C, Jeon IC, Yun SE (1999) Biotechnol. Techniq. 13 : 1-5.
   Kim SH, Yun SE, Kang C (1999) J. Electroanal. Chem. 465 : 163-159.
3. Bard AJ, Falkner LR (1980) Electrochemical Methods, Wiley, New York, p.457.
4. DiCosimo R, Wong C-H, Daniel L. Whitesides GM (1981) J. Org. Chem. 46 : 4622-4623
5. Wong C-H, Whitesides GM (1981) J. Am. Chem. Soc. 103 : 4890-
6. Coury Jr LA, Murray RW, Johnson JL, Rajagopalan KV (1991) J. Phys. Chem. 95 : 6034-6040.
7. Coury Jr LA, Oliver BN, Egekeze JO, Sosnoff CS, Brumfield JC, Buck RP. Murray RW (1990) Anal.Chem. 62 : 452-458.

.8. Maeda H, Kajiwara S (1985) Biotechnol. Bioeng. 27 : 596-602.

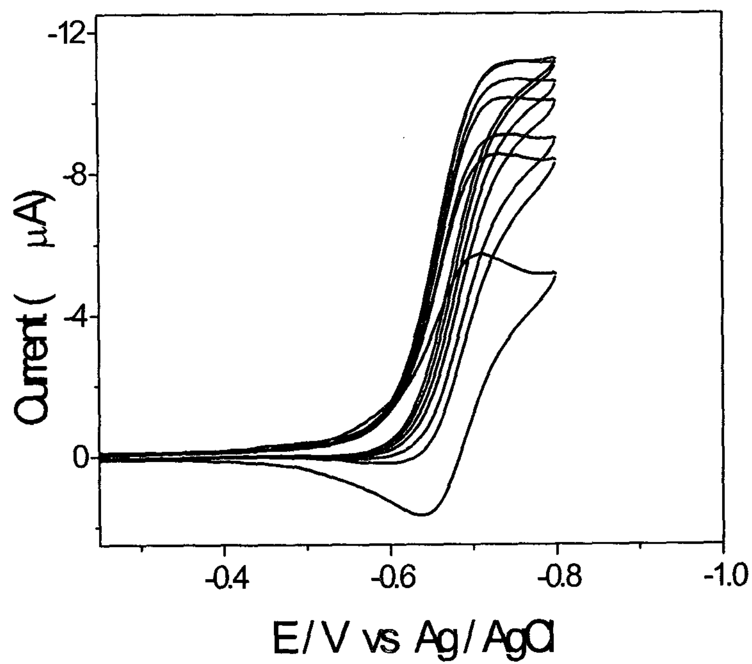


Fig. 1. Cyclic voltammograms at various NAD^+ concentrations

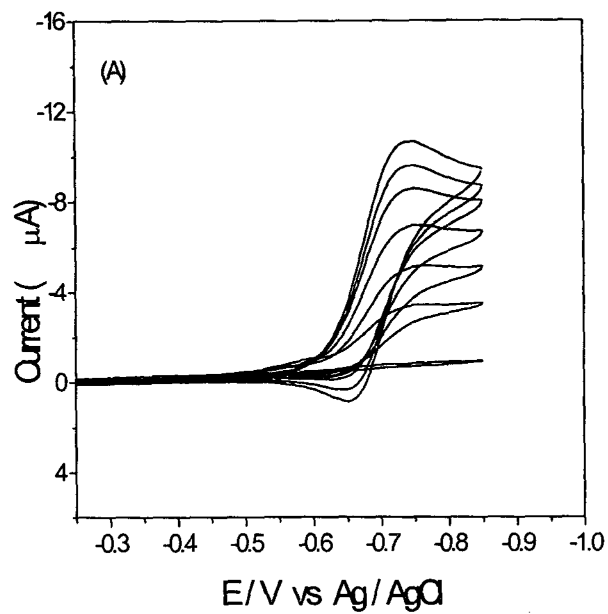


Fig. 2.Cyclic voltammograms at various NAD^+ concentrations.

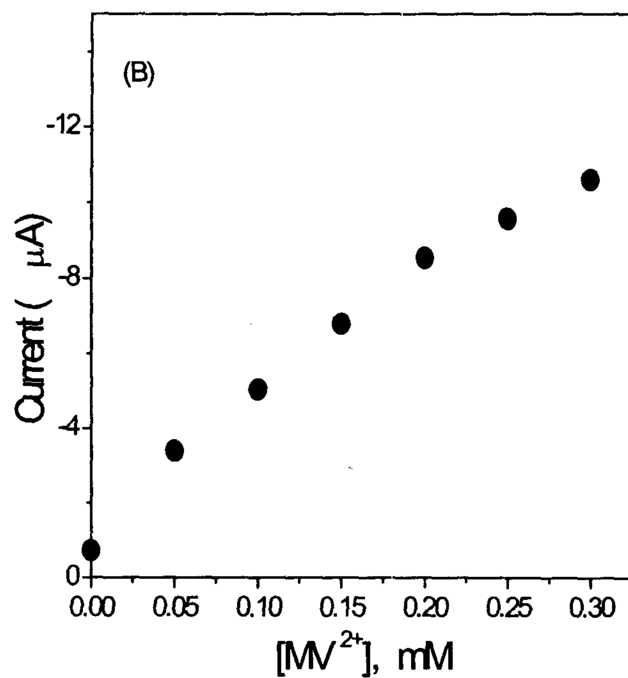


Fig. 3. Dependence of the peak currents of the voltammograms on the MV2+ concentrations.

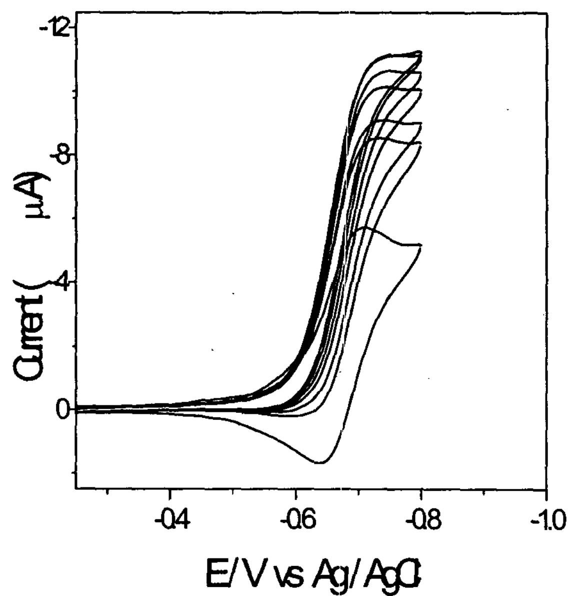


Fig. 4. Cyclic voltammograms at various diaphorase concentrations