Journal of Solid-Phase Biochemistry, Vol. 1, No. 4, 1976

NONENZYMATIC REGENERATION OF NADPH FROM NADP IN WATER-SOLUBLE POLYMER MATRIX-BOUND FORM

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Accepted December 13, 1976

In order to inhibit the dimerization during the electrolytic reduction of nicotinamide adenine dinucleotide phosphate (NADP), NADP was covalently attached to a water-soluble polymer at proper intervals. Matrix-bound NADP was found to exhibit coenzymatic functions comparable to that of free NADP when it was coupled with glucose-6-phosphate dehydrogenase. According to the polarographic behaviors, matrix-bound NADP was electrolytically reduced at a controlled potential of −1.8 V vs. saturated calomel electrode (SCE). The electrolytically reduced product was identified as the enzymatically active NADPH by means of spectrophotometric and enzymatic assays. The dimerization of radical (NADP ·) during the electrolytic reduction might be significantly retarded by immobilizing NADP to a water-soluble polymer matrix.

INTRODUCTION

Nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) were electrolytically regenerated from their correspondingly reduced forms with retention of coenzymatic function (1–3). These electrolytic processes were found to be applicable to a cofactor regenerator. The electrolytic regeneration of cofactors has the following advantages: When an electron transfers directly from a reduced form of cofactor to an electrode, the electrolytic regeneration requires no additives except a pair of electrodes. In addition, an oxidized form of cofactor can be regenerated in a continuous flow system by applying electric power.

Very special advantages will be realized if the reduced form of nicotinamide cofactors (NADH and NADPH) is electrolytically regenerated from the corresponding oxidized form, because few nonenzymatic regeneration processes have been proposed for them. The electrolytic reduction of NAD and NADP has been studied extensively by means of polarography (4–9) and voltammetry (10,11). These investigations have revealed that NAD and NADP make a dimer through radical formation

during the electrolytic reduction, as shown schematically in Fig. 1 (12,13). If the NAD(P) dimerizes it will lose coenzymatic function.

Two novel methods have been applied to the electrolytic reduction of NAD in order to obtain NADH with retention of coenzymatic function. One method involves the use of a liquid crystal membrane electrode, which is made by coating the surface of a mercury electrode with a thin liquid crystal membrane, for the reduction of free NAD (14). The other method is characterized by the covalent immobilization of NAD to a water-soluble polymer matrix. NAD is reduced in matrix-bound form using a conventional electrode (15,16). Both methods have been found to be effective in regenerating NADH with retention of the coenzymatic function. The dimerization is considered to be mostly inhibited throughout the electrolysis.

The purpose of the present paper is to describe the applicability of the electrolytic process to the regeneration of NADPH from the corresponding oxidized form. NADP was covalently immobilized to alginic acid using γ-aminopropyl triethoxysilane and carbodiimide (3). The electrolytic reduction was performed for NADP in matrix-bound form.

MATERIALS AND METHODS

Materials

NADP, NADPH, and glucose-6-phosphate dehydrogenase (G-6-PDH) (EC 1.1.1.49) were purchased from the Oriental Yeast Company

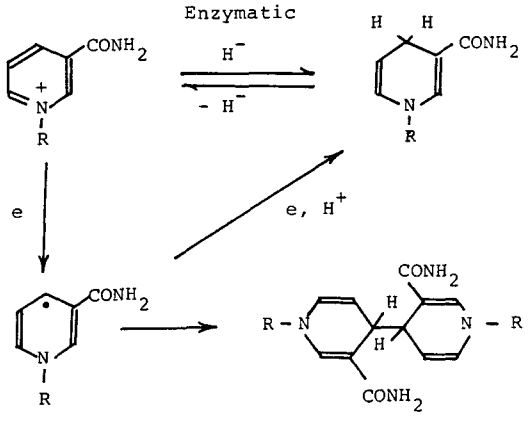


FIG. 1. Enzymatic and electrolytic reduction of $NAD(P)^+$.

(Tokyo). Glutathione (oxidized form) (GSSG) and glutathione reductase (GR) from yeast (EC 1.6.4.2) were obtained from Boehringer Mannheim GmbH. Diepoxybutane, γ-aminopropyltriethoxysilane, 1-cyclohexyl-3-(2-morpholinomethyl) carbodiimide metho-*p*-toluene sulfonate (morpho CDI), and alginic acid were obtained from the Tokyo Kasei Company (Tokyo).

Immobilization of NADP to Alginic Acid

NADP was immobilized to alginic acid mainly composed of mannuronic acid by the same procedure described in the previous paper (3). NADP was silanized with γ-aminopropyltriethoxysilane, and then coupled with alginic acid through morpho CDI. The amount of bound NADP was estimated as 190 μmol/g dry alginic acid, which indicated that NADP was immobilized to alginic acid at intervals of 25 mannuronic acid units.

Polarography

Reduction potentials of free and bound NADP were determined by means of polarography, in which a dropping mercury electrode and a mercury pool electrode were used as the working electrode and counterelectrode, respectively. The electrode potential was scanned at a rate of 1 mV/min with a Hokuto Denko Model 107A function generator attached to a Model 101 potentiostat, referring to a saturated calomel electrode (SCE).

Potential-Controlled Electrolysis of Bound NADP

The electrolytic cell used had two compartments separated with fritted glass. The mercury pool (1.8 cm ϕ) and a platinum plate (1×4 cm^2) were used as the working electrode and counterelectrode, respectively. The electrode potential of the mercury pool was controlled at -1.8 V vs. SCE with the potentiostat.

Photometric Measurements

Ultraviolet spectra and fluorescence spectra were obtained with a Shimadzu Model UV-200 double beam spectrophotometer and a JASCO Model EP-4 fluorescence spectrophotometer, respectively.

Enzymatic Reduction of Bound NADP

G-6-PDH immobilized to alumina particles, prepared according to the procedure described in the previous paper (3), was packed in a column type of reactor. A feed solution containing bound NADP and G-6-P at pH 8.3 flowed in the reactor with a peristaltic pump. The solution was circulated until complete reduction of bound NADP was attained. The reduced form of bound NADP was separated from unreacted G-6-P and the product with a hollow fiber dialyzer.

RESULTS

Coenzymatic Function of Bound NADP

As described in the previous paper (3), bound NADP was found to retain the coenzymatic function of free NADP. The coenzymatic function of bound NADP was assayed at pH 8.0 and 30°C using native G-6-PDH and G-6-P. The V_{max} of G-6-PDH coupled with bound NADP was determined as 1.5×10^{-4} M/min, while G-6-PDH coupled with free NADP showed a V_{max} of 1.6×10^{-4} M/min. There was no photometric difference between bound and free NADP.

Polarographic Behavior of Bound NADP

Free NADP gives a two-step reduction wave in alkali above pH 9 (13). Tetraalkylammonium salt and carbonate buffer are commonly used to obtain these waves. At pH 9.3 NADP in a carbonate buffer and 0.4 M tetrabutylammonium bromide gives a two-step wave (waves I and II) in a polarogram as presented in Fig. 2. Waves I and II have been attributed as follows:

$$\text{Wave I:} \quad NADP^+ + e \rightarrow NADP\cdot \tag{1}$$

$$\text{Wave II:} \quad NADP\cdot + e + H^+ \rightarrow NADPH \tag{2}$$

In contrast, wave II disappears at the pH range below 9 due to the disturbance of background discharge. Figure 2 shows a polarogram obtained at pH 8.3. Only wave I is given in the polarogram. These polarograms were obtained for 0.1 mM free NADP.

Polarography was performed for bound NADP under the same conditions mentioned above. The concentration of bound NADP was assayed as 0.49 mg/ml, which was comparable to 0.09 mM free NADP. As Fig. 2 shows, the polarograms of bound NADP at pH 9.3 and 8.3 were essentially

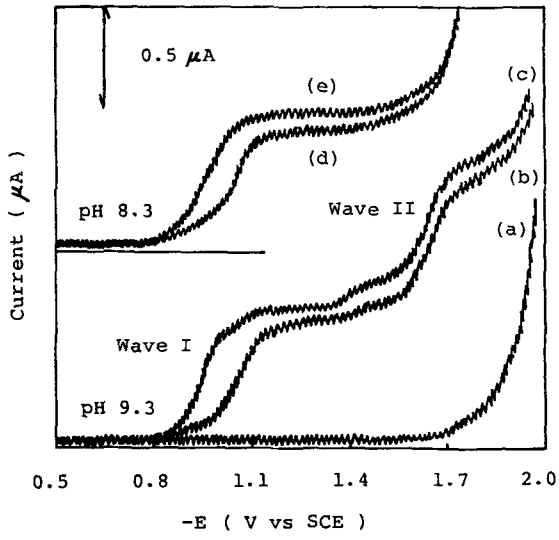


FIG. 2. Polarograms of free and bound $NADP^+$. (a) 0.4 M tetrabutylammonium bromide, carbonate buffer, pH 9.3; (b) and (d) 0.49 mg/ml bound $NADP^+$, 0.4 M tetrabutylammonium bromide, carbonate buffer, pH 9.3 and pH 8.3, respectively; (c) and (e) 0.1 mM $NADP^+$, 0.4 M tetrabutylammonium bromide, carbonate buffer, pH 9.3 and pH 8.3, respectively.

comparable to those of free NADP, except for a slightly negative shift in the half-wave potentials of waves I and II. No appreciable depression of cathodic current was observed for bound NADP as compared with free NADP. Half-wave potentials of bound NADP at pH 9.3 were determined as −1.08 and −1.65 V vs. SCE for waves I and II, respectively. At pH 8.3, however, the half-wave potential of wave II could not be determined because of background discharge. From the polarogram at pH 9.3, wave II is estimated to appear around −1.7 V vs. SCE at pH 8.3. Therefore, the electrode potential must be controlled around −1.8 V vs. SCE at pH 8.3, which is negative enough to reduce NADP into NADPH.

Potential-Controlled Electrolysis of Bound NADP

According to the polarographic behavior of bound NADP, potential-controlled electrolysis was performed to reduce bound NADP into NADPH. The catholyte was made by dissolving bound NADP in pH 8.3 tris-HCl buffer containing 0.4 M tetrabutylammonium bromide. The initial

concentration of bound NADP was 0.49 mg/ml, which was comparable to 0.09 mM free NADP. The anolyte contained pH 8.3 tris-HCl buffer and 0.4 M tetrabutylammonium bromide. Twenty milliliters of the anolyte and the catholyte was applied to the electrolytic cell. The cathode potential was controlled at −1.80 V vs. SCE.

The current was recorded continuously during the electrolysis. The concentrations of oxidized and reduced forms of NADP were assayed for the catholyte at a fixed time by means of spectrophotometry at 260 and 340 nm. The conversion ratio of NADP to NADPH ([NADPH]/[NADP+NADPH]) in the electrolysis is presented in Fig. 3. The conversion ratio increased with time. The electrolysis was stopped 2 h after starting. The 2-h electrolysis converted 79% of the NADP to NADPH.

Characterization of Electrolytically Produced NADPH

Spectrophotometric and enzymatic assays were carried out for the electrolytically produced NADPH. Figure 4 shows the ultraviolet spectrum of the electrolytically produced NADPH. The electrolytic product contains

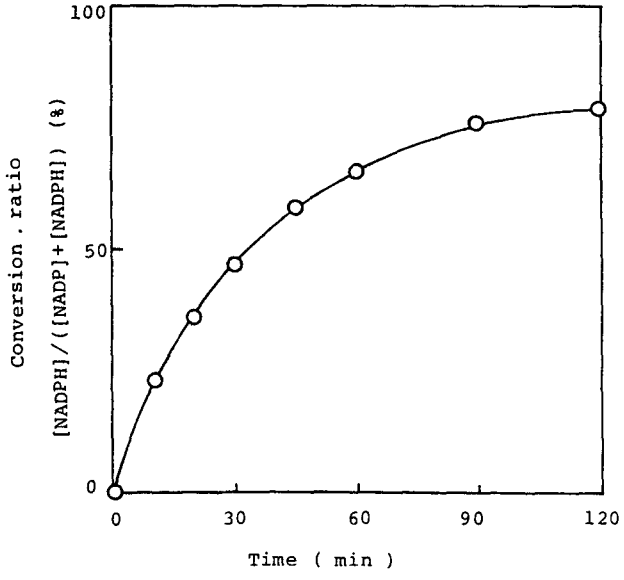


FIG. 3. Potential-controlled electrolytic reduction of bound $NADP^+$. The electrode potential was controlled at −1.8 V vs. SCE. The catholyte (20 ml) contained 0.49 mg/ml bound $NADP^+$, pH 8.3 tris-HCl buffer, and 0.4 M tetrabutylammonium bromide.

a small amount of the oxidized form (approximately 1%). Absorption maxima appeared at 260 and 340 nm, which agreed with those of free NADPH and enzymatically produced bound NADPH. The spectrum of free NADPH is presented for comparison in Fig. 4. The absorbance ratio, A_{340}/A_{260}, is slightly lower than that of free NADPH.

Fluorescence spectra are depicted in Fig. 5. The excitation spectrum was taken with emission at 458 nm and the emission spectrum obtained by exciting at 340 nm. The electrolytically produced NADPH exhibited at 458 nm in the emission spectrum when it was excited at 340 nm. These results essentially agree with those for free NADPH.

The coenzymatic function of the electrolytically produced bound NADPH was determined as V_{max} of free glutathione reductase. Glutathione reductase was coupled with the electrolytically produced bound NADPH at various concentrations under conditions of excess glutathione (oxidized form) at 30°C. V_{max} and K_m were determined from the Lineweaver–Burk plots, as shown in Fig. 6. Free NADPH (enzymatically reduced) exhibited V_{max} and K_m of 1.9×10^{-5} each. Bound NADPH (enzymatically and

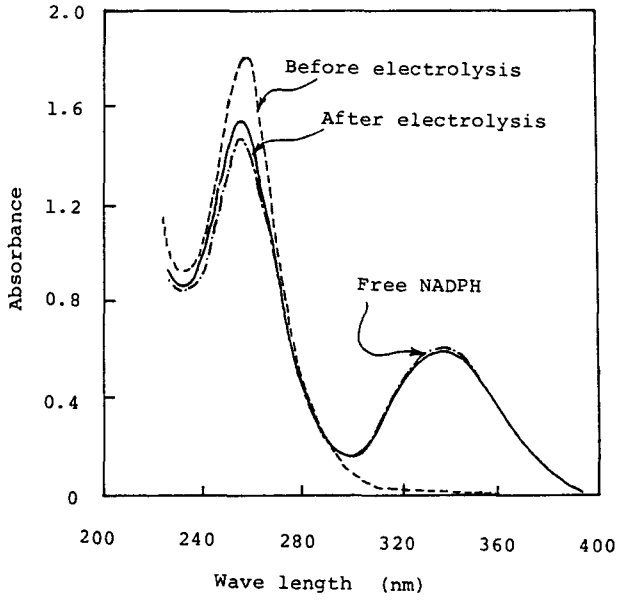


FIG. 4. Ultraviolet spectra of the catholyte before (– – –) and after (——) the potential-controlled electrolysis. The catholyte contains 1.0×10^{-4} M bound $NADP^+$. The spectrum of free NADPH (1.0×10^{-4} M) (— · —) is presented for comparison.

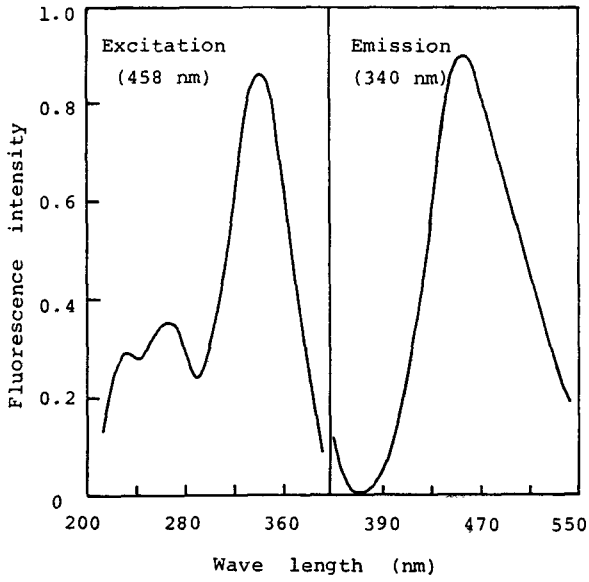
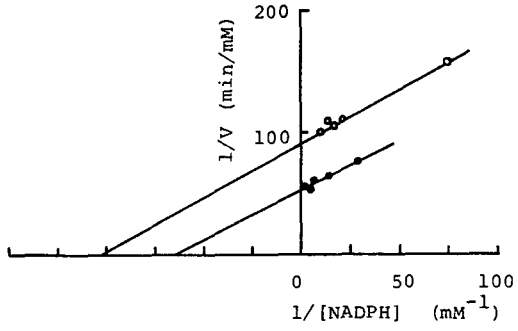


FIG. 5. Fluorescence spectra of the electrolytically produced bound NADPH. The excitation spectrum was taken with emission at 458 nm and the emission spectrum obtained by exciting at 340 nm.



	K_m (M)	V_{max} (M/min)
Free NADPH	1.9×10^{-5}	1.9×10^{-5}
Bound NADPH	0.7×10^{-5}	1.1×10^{-5}

FIG. 6. Coenzymatic function of the electrolytically produced bound NADPH.

electrolytically reduced) exhibited V_{max} and K_m of 1.1×10^{-5} and 0.7×10^{-5}, respectively. It is to be noted that both enzymatically and electrolytically produced NADPH in bound form are comparable in V_{max} and K_m. This indicates that the electrolytically produced NADPH in bound form retained complete coenzymatic function during the electrolytic reduction.

The conversion of NADP to NADPH depended on such conditions as electrolysis time. It was found that 100% of NADP could be converted to NADPH. The electrolytic product was enzymatically oxidized using glutathione reductase and glutathione (oxidized form). Glutathione reductase was insolubilized and packed in a column reactor. The reaction solution was circulated through the reactor until the NADPH was completely oxidized. All the NADPH was enzymatically oxidized to NADP. No evidence was found that decomposition and loss of the coenzyme accompanied the electrolysis.

DISCUSSION

The definite silanization position of $NADP^+$ has not been determined. In view of the following facts the most reasonable coupling site of $NADP^+$ for reaction with γ-aminopropyltriethoxysilane is considered to be the hydroxyl groups of the ribose residue.

Kaplan and his co-workers (17) reported that hydroxyethylation of the adenine amino group of NAD had relatively little effect on the ability of the coenzyme to react with dehydrogenases, and that hydroxymethylation of the N_1 of the adenine in NAD greatly reduced the efficiency of the coenzyme. Furthermore, [N]-6-alkylated and [N]-6-hydroxylated adenine derivatives of NAD showed absorbance maxima at longer wavelengths than 260 nm. On the other hand, no appreciable shift in absorbance maximum resulted through modification of the N_1 of the adenine in NAD.

No significant difference was found between bound and free $NADP^+$ in enzymatic, photometric, and electrochemical properties. The bound $NADP^+$ gave an absorbance maximum at 260 nm because of the adenine moiety, and retained complete coenzymatic function. These results support the hypothesis that the ribose moiety of $NADP^+$ should be linked with the carboxyl group of the alginic acid.

Half-wave potentials of bound $NADP^+$ in the cathodic polarography were slightly more negative than those of free $NADP^+$: $E_{1/2} = -1.08$ and -1.65 V vs. SCE at pH 9.3 for bound $NADP^+$; $E_{1/2} = -0.95$ and -1.62 V vs. SCE at pH 9.3 for free $NADP^+$. The negative shift of $E_{1/2}$ might be caused by the polymer matrix, which induces environmental change at the electrode surface. Since bound $NADP^+$ has a similar polarographic behavior

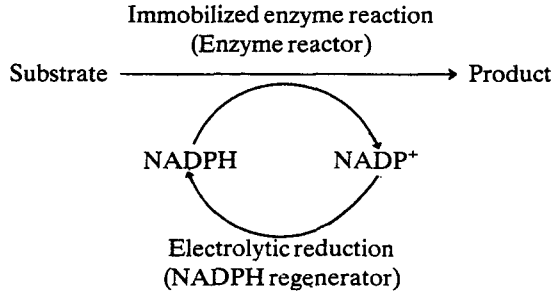
to free $NADP^+$, the electrochemical process of bound $NADP^+$ would be as follows:

$$\text{Bound } NADP^+ + e \rightarrow \text{Bound } NADP\cdot$$

$$\text{Bound } NADP\cdot + e + H^+ \rightarrow \text{Bound } NADP$$

The absorption spectrum of the electrolytically reduced product was identical to that of the enzymatically produced free NADPH. Furthermore, the electrolytically reduced product was found to retain complete coenzymatic function. These results indicate that dimerization of NADP radicals should be significantly inhibited by attaching $NADP^+$ to a water-soluble polymer matrix.

Since the electrolytic regeneration of NADPH from $NADP^+$ requires no additives, the electrolytic regenerator can be directly coupled with an enzyme reactor in either a continuous-flow or a batch system. One possible system composed of an enzyme reactor and a NADPH regenerator is schematically shown as follows:



As an example, glutathione reductase (GR) was immobilized to alumina particles activated by diepoxybutane (3). The immobilized GR was packed in a fluidized bed type of reactor. The electrolytic NADPH regenerator was attached to the enzyme reactor. Then the feed solution containing GSSG and NADPH in pH 7.2 tris-HCl buffer was circulated through the enzyme reactor and the NADPH regenerator at a constant flow rate. The enzyme reactor may convert GSSG and NADPH to GSH and $NADP^+$, respectively. After the separation of GSH, $NADP^+$ was continuously reduced to NADPH in the regenerator.

CONCLUSION

Enzymatically active NADPH was electrolytically regenerated from its oxidized form in a soluble polymer matrix-bound form. No appreciable loss

of the coenzyme was detected in the electrolytic regeneration. The dimerization of NADP radicals during the electrolytic regeneration might be extremely retarded by immobilizing $NADP^+$ to a water-soluble polymer matrix.

ACKNOWLEDGMENT

The authors wish to thank Prof. R. W. Coughlin (Lehigh University, Bethlehem, Pennsylvania 18015) for his continuing interest and encouragement.

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