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ELECTROCHEMICAL CHARACTERIZATION OF IMMOBILIZED NAD^+

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SUMMARY

Nicotinamide adenine dinucleotide (NAD^+) has been covalently attached to alginic acid using carbodiimide coupling, thereby producing a macromolecular adduct of NAD, which can be rendered either soluble or insoluble by adjustment of pH. It was found that this NAD^+ · alginic acid complex was enzymatically active, and also that the oxidized form could be electrochemically reduced without loss in enzymatic activity. This NAD^+ adduct has now also been polarographically characterized as to its two-step reduction waves, which are slightly shifted toward more cathodic potential as compared to free NAD^+. When controlled electrolysis was conducted to reduce the bound NAD^+ at the cathode, the NADH so formed by electrochemical action was found to be again oxidizable either enzymatically or electrochemically without loss in co-enzymic function. The NADH adduct produced by electrochemical reduction of the NAD^+ adduct has also been characterized by voltammetry.

INTRODUCTION

Great advantages in stability, regenerability and separability can be realized by immobilizing cofactors to various matrices. Nicotinamide adenine dinucleotide (NAD^+), for instance, has been attached covalently to a Sepharose gel [1], dextran [2], glass beads [3] and alginic acid [4, 5]. The last-mentioned NAD^+ · alginic acid conjugate can be rendered soluble or insoluble by simply adjusting the pH. We reported previously that this matrix-bound NAD^+ could offer desirable properties in electrolytic regeneration of the reduced form from the oxidized form of NAD^+, because immobilization of NAD^+ to polymer matrices could prevent intermolecular coupling of the NAD radical produced as an intermediate during electrolytic reduction at a cathode.

In this paper not only reductive regeneration of NADH from NAD^+ but also oxidative regeneration of NAD^+ from NADH was carried out by electrolyzing alginic acid · NAD^+ and alginic acid · NADH, respectively.

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EXPERIMENTAL

Materials. Alcohol dehydrogenase (EC 1.1.1.1) from yeast, diaphorase from *Clostridium kluyveri* (Sigma, Type II), β-nicotinamide adenine dinucleotide (β-NAD^+), and alginic acid from *Macrocystis pyrifera* were purchased from Sigma Chemical Co. From Aldrich Chemical Co. were obtained 2,6-dichlorophenolindophenol (sodium salt) and 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (CMC metho-*p*-toluenesulfonate).

Preparation of matrix-bound NAD^+. 4 g of alginic acid was dissolved in 80 ml of water and the final pH was adjusted to a pH of 4.7 with 1 M NaOH. To this solution was added 1 g of NAD^+ and 1.1 g of CMC metho-*p*-toluenesulfonate. After the pH was adjusted again to 4.7 with 0.1 M HCl, the resulting solution was extensively stirred at room temperature for 21 days. After this reaction period acetone was added to the solution to precipitate the NAD^+ · alginic acid conjugate and the precipitate was filtered off, washed extensively with acetone/water and 0.01 M HCl and dried at 5 °C.

Voltammetric measurements. Polarography was carried out in an H-type cell (E. H. Sargent Co., No. S-29392) at pH 9.4 (carbonate buffer) with drop times between 3 and 6 s. The same cell, which was utilized for polarographic experiments, was also employed in stationary electrode voltammetry. For this work, the working electrode was a platinum wire (1.0 mm external diameter × 10 mm) and platinum mesh (30 × 40 mm) was used for the counter electrode.

The electrode potential was controlled and scanned continuously with a Heath Model EUA-19-2 and Model EUW-19B Operational Amplifier.

Controlled-potential electrolysis. A two-compartment type of cell was used in controlled-potential electrolysis for oxidation and for reduction of the cofactor complex. Electrolytic reduction was carried out over a mercury pool electrode (18 mm external diameter) in the cell compartment (30 ml capacity), which was fitted with a rubber stopper. The stopper was drilled to permit introduction of sample and to accommodate a salt bridge and de-aeration tubing. Platinum mesh was used for the counter electrode.

Electrolytic oxidation was conducted using the same apparatus except that both the working and counter electrodes were platinum mesh (30 × 40 mm).

Assay of co-enzymic function. Co-enzymic function was assayed for the reduced form of the NAD · alginic acid conjugate using diaphorase with the electron acceptor 2,6-dichlorophenolindophenol. Ethanol and the corresponding dehydrogenase were used to assay the oxidized form of the complex.

RESULTS AND DISCUSSION

Co-enzymatic function of the immobilized NAD^+

The NAD^+ · alginic acid complex was readily soluble in water above a pH of 3 at room temperature but could be precipitated by lowering the pH or adding an organic solvent such as acetone. The ultraviolet spectrum, which was obtained for this bound NAD^+ complex dissolved in a pyrophosphate buffer (pH 9.0), was identical to that of free NAD^+ with $\lambda_{max} = 260$ nm. The absorbance measured at 260 nm varied linearly with concentration. The extent of binding of NAD^+ to alginic

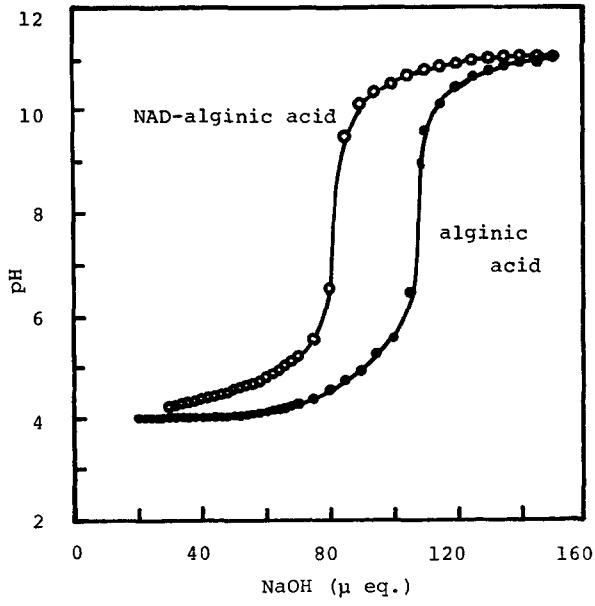

Fig. 1. Titration curves for uncoupled alginic acid and NAD^+ · alginic acid. 20 mg of the sample dissolved in 20 ml of deionized water were titrated with 0.01 M NaOH.

acid was determined to be about 100 μmol NAD^+/g of dry complex by a hydrolysis method described elsewhere [4]. Fig. 1 shows the titration curves for uncoupled alginic acid and NAD^+-coupled alginic acid (20 mg of the sample dissolved in 20 ml of deionized water was titrated with 0.01 M NaOH). The titration curves suggest that native alginic acid has one free carboxyl group per mannose unit, and that about 25 % of these carboxyl groups were coupled with CMC metho-*p*-toluenesulfonate. From the extent of bound NAD^+ it is estimated that one NAD^+ molecule was immobilized for about every 54 mannuronic acid units.

The matrix-bound NAD^+ was found to be enzymatically reducible using alcohol dehydrogenase and ethanol, and its reduced form gave an ultraviolet spectrum identical to that of free NADH with absorbance maximum at 340 nm [5].

Polarographic behavior

100 mg of the NAD^+ · alginic acid complex were dissolved in 30 ml of a carbonate buffer with 0.4 M tetraethylammonium chloride (pH 9.45). Potential sweep started at −0.5 V vs. the saturated calomel electrode with a rate of 0.1 V/min. Fig. 2 shows polarograms for the NAD^+ · alginic acid complex and free NAD^+ (0.4 mM). For free NAD^+ a two-step reduction wave was obtained at half wave potentials of −0.89 and −1.67 V vs. saturated calomel electrode. The bound NAD^+ gave a similar two-step reduction wave in which the half wave potentials were slightly shifted to more cathodic values. The first step of the reduction wave for bound NAD^+ was found to be deformed, resulting perhaps from some interaction with the polymer matrix.

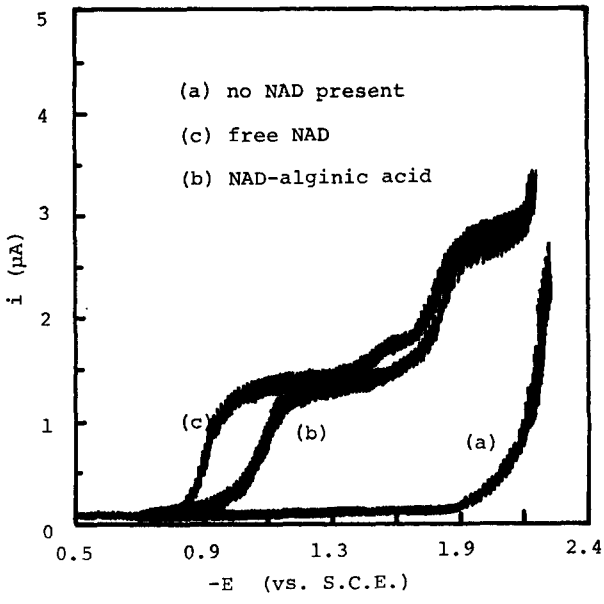

Fig. 2. Polarograms for the NAD^+ · alginic acid complex and free NAD^+. Carbonate buffer (pH 9.45) containing 0.4 M tetraethylammonium chloride was used as supporting electrolyte. Concentration of NAD^+ was 0.4 mM for both samples; (a) without NAD^+, (b) NAD^+ · alginic acid complex, and (c) free NAD^+. S.C.E., saturated calomel electrode.

Reductive electrochemical regeneration of the bound NADH

Based on the above-described polarographic studies for the NAD^+ complex a cathode potential of −1.75 V vs. the saturated calomel electrode should be suitable for the reduction of the bound NAD^+. Accordingly, electrolysis of the NAD^+ · alginic acid complex dissolved in pH 9.45 carbonate buffer with 0.4 M tetraethylammonium chloride was carried out over a mercury-pool cathode controlled at a potential of −1.75 V vs. saturated calomel electrode. The volume of the catholyte was about 30 ml; it contained 9 mg/ml of bound NAD^+ (comparable to 0.9 mM free NAD^+) and was agitated during the electrolysis by bubbling N_2. The electrolytic production of NADH in the catholyte was monitored by continuous measurement of absorbance at the wavelength of 340 nm characteristic of the reduced form. This macro-electrolysis was continued under oxygen-free conditions for 20 h at room temperature while the cathode potential was controlled at −1.75 V vs. the saturated calomel electrode and the current was maintained at approximately 300 μA after an initial drop. Absorbance of the catholyte at 340 nm increased gradually during the electrolysis as shown in Fig. 3. Fig. 3 also shows the time dependence of current during the electrolysis. After 20 h electrolysis the ultraviolet spectrum of the catholyte displayed two maxima at 260 and 340 nm but with the size of the 260 nm peak sufficiently large to suggest the continued presence of some NAD^+ in the mixture. Neither of these absorbance peaks appeared to be shifted from wavelengths corresponding to free NAD^+ and NADH. From the absorbances of the catholyte at these wave lengths, $A_{260} = 1.42$ and $A_{340} = 0.30$, the concentration of the oxidized and reduced forms of NAD were estimated to be 0.4 and 0.49 mM, respectively

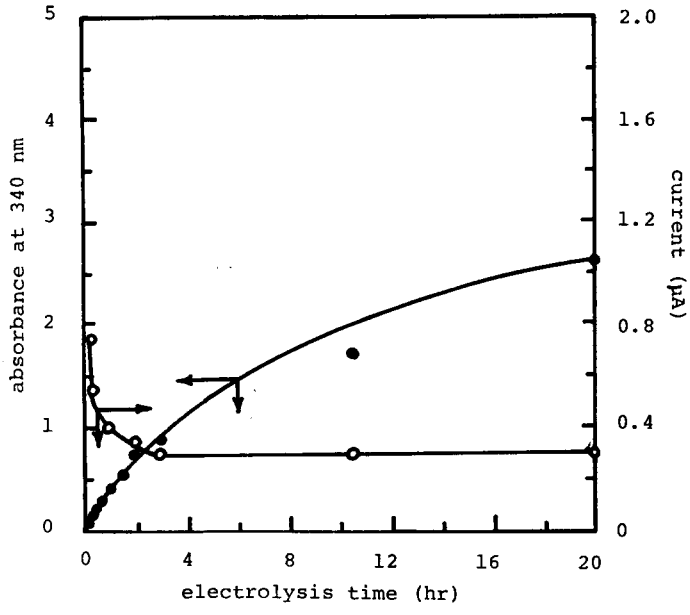

Fig. 3. Absorbance at 340 nm for the catholyte and current during reductive electrolysis of the NAD^+ · alginic acid conjugate. The 30 ml of catholyte contained 9 mg/ml NAD^+ · alginic acid conjugate (comparable to 0.9 mM free NAD^+) in pH 9.45 carbonate buffer containing 0.4 M tetraethylammonium chloride. The cathode, a platinum screen 30 mm × 40 mm, was controlled at −1.75 V vs. saturated calomel electrode.

by assuming the contributions to absorbance at 260 nm from NAD^+ and NADH are additive, employing $A_{260} = 1.42$ for the mixture and assuming the following molar extinction coefficients: $\varepsilon_{260} = 14.4 \cdot 10^3$ l/mol and $\varepsilon_{340} = 6.1 \cdot 10^3$ l/mol for NADH; $\varepsilon_{260} = 18.0 \cdot 10^3$ l/mol for NAD^+.

The products contained in the electrolyzed catholyte were separated by combined ion exchange and gel filtration into two main components, which were identified as the oxidized and reduced forms of the NAD complex. On the basis of this separation it was estimated that 61 % of the initial NAD^+ complex was reduced to the NADH complex during the electrolysis.

In order to determine the extent of coenzymatic function of the electrolytically reduced form of the NAD · alginic acid adduct separated by ion exchange and gel filtration as just mentioned, the reduced fraction was dissolved in pH 7.5 phosphate buffer previously deaerated by bubbling with N_2. To the resulting solution 2,6-dichlorophenolindophenol solution (also de-aerated) together with diaphorase was added and the decrease in absorbance at 620 nm was monitored. The decrease in absorbance indicated that the dichlorophenolindophenol was readily reduced by enzymatic reaction with the NADH · alginic acid complex.

These results indicate that enzymatically active matrix-bound NADH can be produced electrolytically from matrix-bound NAD^+ with retention of coenzymatic function.

Anodic voltammetric studies of the electrolytically regenerated matrix-bound NADH

We reported previously [6] that it is possible to produce enzymatically active

NAD^+ by electrolytic oxidation of the reduced form, NADH, of the free coenzyme. Very few investigations of the direct electrochemical oxidation have been reported, although two such papers [11, 12] have been published very recently. The feasibility of such oxidative electrolytic regeneration has now also been confirmed for the NADH complex formed by electrolytic reduction of the corresponding NAD^+ · alginic acid conjugate. In order to characterize the NADH · alginic acid conjugate by anodic voltammetry the bound NADH was dissolved in pH 9.45 carbonate buffer containing 0.4 M tetraethylammonium chloride and the resulting solution was used as anolyte with a platinum electrode as described elsewhere [6]. The anolyte concentration of the NADH conjugate was 5.4 mg/ml, which was comparable to 0.54 μmol free NADH/ml. Carbonate buffer (pH 0.45) with 0.4 M tetraethylammonium chloride was used as catholyte. Potential scanning was performed at 1 V/min starting at 0.3 V vs. the saturated calomel electrode.

Typical voltammograms for oxidation of bound and free NADH are shown in Fig. 4 where the current (i) is plotted in normalized form as the ratio to the peak current (i_p). The voltammograms presented in Fig. 4 are those corresponding to the 5th scan of a multiscanning sequence; as described previously [6], steady-state voltammograms are not obtained until about the fifth scan, presumably due to a slow approach to adsorption equilibrium. Reproducible voltammograms could not be obtained for either free NADH or its complex with alginic acid, presumably attributable to adsorption of either form on the electrode. The peak potentials were reproducible, however, and that for the alginic acid complex of NADH is slightly shifted to less positive potential compared to free NADH. The shape of the voltammogram for the NADH complex was quite deformed but that of free NADH fit Delahay's function [7] $\chi(\beta t)$ reasonably well. The anomalous behavior of the NAD conjugate might be attributed to adsorption phenomena involving the anionic polymer matrix, alginic acid. The peak current was proportional to the root square of scanning rate ($v^{\frac{1}{2}}$). It is very difficult to calculate a definite diffusion constant from these data, but it is estimated to be about 10^{-6} ($cm^2 \cdot s^{-1}$), which is only slightly less than that of free NADH: $2.4 \cdot 10^{-6}$ $cm^2 \cdot s^{-1}$.

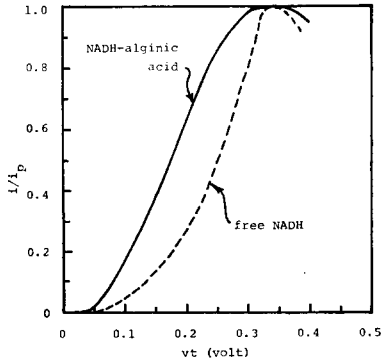

Fig. 4. Anodic voltammograms for bound and free NADH on a platinum electrode at 25 °C. Pc [illegible] scanning started at 0.3 V vs. saturated calomel electrode with a rate of 1 V/min. The ratio of current (i) to peak current (i_p) is plotted at each potential, where v is the rate of potential scanning and t is time elapsed from the start. The test electrolyte contained 5.4 mg/ml of bound NADH comparable to 0.54 mM free NADH (—) or 0.5 mM free NADH (– – –) in pH 9.45 carbonate buffer with 0.4 M tetraethylammonium chloride.

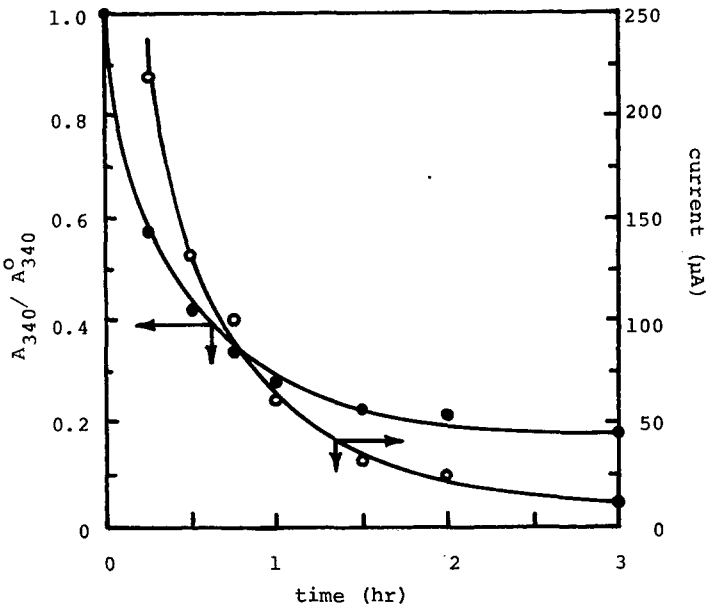

Fig. 5. Current and absorbance at 340 nm during the electrolytic oxidation of bound NADH. Anode potential was controlled at 0.7 V vs. saturated calomel electrode. The ratio of absorbance during electrolysis (A_{340}) to that before electrolysis ($A°_{340}$) is plotted.

Electrolytic oxidation of matrix-bound NAD at constant controlled potential

A complete cycle of reduction and oxidation is attained by re-oxidation of the alginic acid · NADH conjugate originally prepared by electrolytic reduction of the oxidized form. Accordingly, 20 mg of bound NADH dissolved in 30 ml of pH 9.45 carbonate buffer containing 0.4 M KCl was made the anolyte and agitated by bubbling N_2 during the electrolysis with anode potential controlled at 0.7 V vs. the saturated calomel electrode. The absorbance at 340 nm due to NADH was monitored during electrolysis and the results are shown in Fig. 5 as current and absorbance at 340 nm plotted vs. time. After electrolysis for 3 h at room temperature the ultraviolet spectrum of the anolyte was identical to that of free NAD except for the presence of some superimposed turbidity attributable to the macromolecular alginic acid matrix. Furthermore, the electrolytically oxidized NAD^+ · alginic acid complex was found to be enzymatically reducible by ethanol using the corresponding dehydrogenase. During this electrolysis about 95 % of bound NADH appeared to be oxidized to NAD^+, but such a conclusion cannot be based on spectroscopic and enzymological results alone. For example, Braun et al. [11] report that direct protonation of NADH at the anode can lead to some decomposition, the amount of which is pH dependent; the high pH (9.45) employed in the present work may be expected to minimize such protonation.

CONCLUSIONS

It has been shown that matrix-bound NAD^+ is electrolytically reducible to a corresponding form of NADH. Further oxidation of this matrix-bound NADH so

formed is possible both electrolytically and enzymatically. These results suggest that the intermolecular coupling of NAD to form a dimer during electrochemical reduction in free solution as previously reported [9, 13] can be prevented or at least substantially repressed by immobilizing the coenzyme to polymer matrices such as alginic acid. It appears that dimerization is retarded by simple steric hindrance of the mutual approach of NAD free radical intermediates and similar effects can be expected for immobilization of NAD on solid surfaces as well.

Electrolytic reduction was observed to proceed at a slower rate than electrolytic oxidation and increasing the rate of reductive regeneration is an attractive goal for subsequent investigations. Nevertheless, electrolytic regeneration of either NADH from NAD^+ or NAD^+ from NADH is feasible when the coenzyme is attached to polymer matrices. These regeneration processes can be coupled with immobilized or free enzymes for various uses [5, 8, 10] in enzymatic reactors.

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