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***In situ* regeneration of NADH via lipoamide dehydrogenase-catalyzed electron transfer reaction evidenced by spectroelectrochemistry**

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Abstract

NAD/NADH is a coenzyme found in all living cells, carrying electrons from one reaction to another. We report on characterizations of *in situ* regeneration of NADH via lipoamide dehydrogenase (LD)-catalyzed electron transfer reaction to regenerate NADH using UV-vis spectroelectrochemistry. The Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) of NADH regeneration were measured as 0.80 ± 0.15 mM and $1.91 \pm 0.09 \mu\text{M s}^{-1}$ in a 1-mm thin-layer spectroelectrochemical cell using gold gauze as the working electrode at the applied potential -0.75 V (vs. Ag/AgCl). The electrocatalytic reduction of the NAD system was further coupled with the enzymatic conversion of pyruvate to lactate by lactate dehydrogenase to examine the coenzymatic activity of the regenerated NADH. Although the reproducible electrocatalytic reduction of NAD into NADH is known to be difficult compared to the electrocatalytic oxidation of NADH, our spectroelectrochemical results indicate that the *in situ* regeneration of NADH via LD-catalyzed electron transfer reaction is fast and sustainable and can be potentially applied to many NAD/NADH-dependent enzyme systems.

Keywords

NADH; biocatalytic reduction; electron transfer mediator; spectroelectrochemistry

1. Introduction

The electrochemical reduction and oxidation of enzymatically active NAD/NADH (β -nicotinamide adenine dinucleotide) have received considerable interest because NAD/NADH is a crucial coenzyme couple participating in various enzymatic reactions of NAD/NADH-dependent dehydrogenases and other enzymes in cellular metabolism. In recent years, the regeneration of the bio-electron donor NADH from NAD has been the topic of many studies relevant to biofuels [1], rechargeable batteries [2, 3] and bioreactors [4, 5]. The electrocatalytic reduction of NAD is more difficult than the electrocatalytic oxidation of NADH on the electrode surface [6-8]. Direct electrochemical reduction of NAD by electrolysis on bare or nanomaterial-modified electrodes is known to be a straightforward approach to yield NADH, but this approach would require a large overpotential (>-1.15 V

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vs. Ag/AgCl) and/or lead to the formation of enzymatically inactive dimer NAD₂ [9-14]. With this in mind, lipoamide dehydrogenase (LD) and methyl viologen (MV) [4, 15, 16], poly(neutral red) [2, 17], FAD [18], rhodium(III) complex [19] and ruthenium complex [20] have been used as electron transfer mediators to facilitate the electrocatalytic reduction of NAD. In fact, in many previous reports regarding such indirect reductions of NAD, although the electrocatalytic reduction peak might be observed, presumably ascribable to the oxidation of the electron transfer mediators by NAD (thus the indirect reduction of NAD) [2, 4, 15-17, 19, 20], the kinetics of *in situ* regeneration of the supposed product NADH was rarely monitored or confirmed by the associated spectroscopic method, that is, the specific absorbance of NADH at 340 nm. The FAD-involved NADH regeneration reaction has been studied by spectroelectrochemistry [18], however, the initial rate of the FAD-mediated NADH regeneration was in the order of 0.2 $\mu\text{M s}^{-1}$ [18], which is too slow to be used for practice [18].

The attractive feature of spectroelectrochemistry is to monitor the product resulting from the electrochemical reactions *in situ* by spectroscopic methods. The electrocatalytic reduction of NAD by LD-catalyzed electron transfer reaction using MV as an electron transfer mediator has been studied electrochemically [15, 16]. In this work, we characterized the electrocatalytic reduction of NAD by LD-catalyzed electron transfer reaction via MV using UV-vis spectroelectrochemistry, allowing us to track the kinetics of *in situ* regeneration of NADH from NAD at its characteristic UV-vis peak at 340 nm. Subsequently, the electrocatalytic reduction of the NAD system was further coupled with the enzymatic conversion of pyruvate to lactate biocatalyzed by a lactate dehydrogenase-catalyzed reaction to examine the coenzymatic activity of the regenerated NADH. Our results indicated that as long as the appropriate electrochemical reduction potential was applied, the regeneration of NADH from electrocatalytic reduction of NAD via LD and MV was fast and sustainable, and thus such NADH regeneration systems can be potentially applied to many NAD/NADH-dependent enzymes.

2. Materials and Methods

2.1. Chemicals and supplies

All chemicals were purchased and used as supplied without any further purification. Lactate dehydrogenase (LDH) (EC 1.1.1.27) (source: rabbit muscle), sodium pyruvate, hydrogen peroxide (30% solution), methyl viologen (MV) and β -nicotinamide adenine dinucleotide hydrate (NAD, >98% purity) were purchased from Sigma-Aldrich. Lipoamide dehydrogenase (LD) (EC 1.6.4.3) (source: porcine heart) was purchased from Calzyme. The thin-layer quartz glass spectroelectrochemical cell kit with gold gauze working electrode (EF-1351) was purchased from BASi. Milli-Q ultrapure water (Millipore, 18.2 $\text{M}\Omega\cdot\text{cm}$) was used in all experiments.

2.2. Electrochemical and spectroelectrochemical measurements

All electrochemical experiments were carried out with a potentiostat (Autolab PSTAT12) from Eco Chemie, Netherlands, using the GPES 4.9 (general purpose electrochemical system) software package for cyclic voltammetry and chronoamperometric measurements. The electrochemical measurements were performed with the thin-layer spectroelectrochemical cell kit at ambient temperature ($22\pm 1^\circ\text{C}$) with the gold gauze working electrode (EF-1357, BASi), the Ag/AgCl reference electrode (3 M NaCl) (MF-2078, BASi), and the platinum wire counter electrode (EF-1356, BASi). The light path length for the spectroelectrochemical cell is 1 mm. The gold gauze working electrode was cleaned by immersion in "piranha" solution composed of H_2SO_4 and H_2O_2 in the ratio of 3:1 v/v for 3 min and then rinsed extensively with water prior to use in electrochemical

measurements. Cyclic voltammograms were recorded in the potential range from -0.5 to -0.8 V, and the potential scan rate was 5 mV s⁻¹. Bioelectrocatalytic peak currents for different concentrations of NAD in the calibration plot were obtained from a second scan. The configuration of the three-electrode arrangement in the thin-layer spectroelectrochemical cell for spectroelectrochemical measurements is shown in Scheme 1A. The UV-vis spectra (λ =300-450 nm) in the spectroelectrochemical measurements were recorded with a Diode Array Spectrophotometer (Hewlett Packard 8452A) at a constant potential of -0.75 V. The electrolyte solution was bubbled with nitrogen for 15 min prior to all measurements, and a blanket of nitrogen was maintained over the solution throughout the experiment. The background working electrolyte was 200 μ L of pH 7.0, 100 mM (M= mol L⁻¹) phosphate buffer.

3. Results and Discussion

3.1 Bioelectrocatalytic reduction of NAD

LD is known to catalyze the electron transfer process between NAD and MV, resulting in the bioelectrocatalytic reduction of NAD into NADH (Scheme 1B). This indirect reduction of NAD could produce enzymatically active NADH at a lower overpotential compared to the direct reduction of the NAD [15]. Fig. 1 shows cyclic voltammograms for the gold gauze electrode in the presence of LD (5.0 U mL⁻¹), MV (0.5 mM) and variable concentrations of NAD at a scan rate of 5 mVs⁻¹. The cyclic voltammogram (curve a) obtained in the presence of MV was very similar to that in the presence of MV and LD (curve b), exhibiting the redox peaks of MV. Although the electrolyte solution was bubbled with nitrogen for 15 min prior to all measurements, there might be a small amount of residual oxygen could lead to the oxidized form of MV in the electrolyte, resulting in the increased reduction peak current prior to the addition of NAD (curves a, b). Nevertheless, the cyclic voltammograms (curves c-g) recorded after the addition of various concentrations of NAD clearly show the electrocatalytic reduction wave of MV²⁺ because of the electron transfer reaction between NAD and MV catalyzed by LD (Scheme 1) [21]; that is,

It was noted that this electron transfer reaction cannot occur in the absence of LD. The resulting cathodic current of MV²⁺ at different concentrations of NAD, demonstrating the higher the NAD concentration, the higher the cathodic current, thus the more corresponding NADH regenerated from NAD. The cathodic electrocatalytic current increased continually with the increase of NAD concentration approaching saturation at ~5 mM.

3.2. In situ regeneration of NADH via spectroelectrochemistry at different concentrations of LD

To monitor the *in situ* regeneration of NADH by spectroelectrochemistry, we synchronized the measurement of UV-vis spectra with chronoamperometric measurement of the LD-catalyzed electron transfer reaction between NAD and MV at different concentrations of LD [equation (1)]. Fig. 2 shows the results of spectroelectrochemical measurements performed in the presence of NAD (10.0 mM), MV (0.5 mM) and variable concentrations of LD, synchronized with the chronoamperometric measurement at $E = -0.75\text{V}$ for 1260 s (21 scans). The characteristic UV-vis absorbance of NADH is centered at 340 nm, while the absorbance peak at 400 nm is attributable to MV radical, one of the reduced forms of MV [22]. Fig. 2A shows that, in the absence of LD, there was no increase in the absorbance peak at 340 nm after the stabilization of background because of the electrochemically reduced MV, indicating that the regeneration of NADH cannot occur without LD. Fig. 2B shows that, in the same time window, at a lower concentration of LD (0.25 U mL⁻¹), only a small

part of reduced MV was used by the oxidative LD, and the absorbance peak growth at 340 nm was slow, while the other part of reduced MV could be reflected by the higher absorbance peak at 400 nm. Figs. 2C and 2D show that the characteristic absorbance peak for NADH clearly grew with the electrolysis time at higher concentrations of LD (1.0 and 5.0 U mL⁻¹). The initial regeneration rates of NADH derived from these time-dependent absorbance spectra at 0.25 U mL⁻¹, 1.0 U mL⁻¹ and 5.0 U mL⁻¹ of LD (Fig. 2) were 0.18 $\mu\text{M s}^{-1}$, 1.08 $\mu\text{M s}^{-1}$ and 1.65 $\mu\text{M s}^{-1}$, respectively. The reproducibility of the initial generation rate of NADH was within $\pm 3\%$. These results demonstrate that LD was necessary to catalyze (mediate) the reduction of NAD into NADH by the electrochemically reduced MV. In the presence of 0.5 mM of MV at $E = -0.75\text{ V}$, the higher concentration of LD resulted in the higher availability of reduced LD, and accordingly, the higher regeneration rates of NADH. With the LD concentration higher than 5.0 U mL⁻¹, no obvious increase in the initial regeneration rate of NADH was observed because of the saturation of the enzymatic reaction.

3.3. In situ regeneration of NADH via spectroelectrochemistry at different concentrations of NAD

The effective concentration of NAD on the *in situ* regeneration of NADH by spectroelectrochemistry was measured by the UV-vis spectroelectrochemistry with the fixed concentration of LD and MV. Fig. 3 shows a collection of time-dependent UV-vis absorbance spectra in the presence of MV (0.5 mM) and LD (5 U mL⁻¹) with variable concentrations of NAD, synchronized with the chronoamperometric measurement at $E = -0.75\text{ V}$ for 1260 s (21 scans). Fig. 3A shows that, in the absence of NAD, there was no net growth of the absorbance peak at 340 nm after the slight absorbance change of the background brought by the electrochemically reduced MV. Fig. 3B shows that at a relatively low concentration of NAD (1 mM), the LD-catalyzed reduction of NAD to NADH by the electrochemically reduced MV reached saturation very fast (cal. $\sim 800\text{ s}$). Figs. 3C and 3D show that, at higher concentrations of NAD (5.0 mM and 10.0 mM), the characteristic absorbance peak of NADH at 340 nm was more clearly progressing as a function of electrolysis time, while the absorbance peak at 400 nm for the reduced forms of MV has been decreased significantly. Under the present experimental condition, MV (reduced at $E = -0.75\text{ V}$) was in excess, thus, the initial rate of the LD-catalyzed electron transfer reaction between MV and NAD was depended on the NAD concentration. When LD was saturated by NAD, the maximum initial velocity of NADH regeneration was attained. The Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) were calculated from the plot of the initial rates of the regeneration of NADH as a function of the NAD concentration (Fig. 4). Under the present experimental condition, by using non-linear fitting method, K_m and V_{max} were $0.80 \pm 0.15\text{ mM}$ and $1.91 \pm 0.09\text{ }\mu\text{M s}^{-1}$, respectively. It should be noted that the characteristic absorbance peak of NADH at 340 nm was not increased further after the termination of the potential application, indicating that it was the electrochemical reduction of MV that initiated and kept NADH being regenerated from NAD as long as the potential was applied.

3.4. Confirmation of bioactivity of regenerated NADH

Because both the NAD dimer (NAD₂) that may be produced from the direct reduction of NAD on bare electrodes [9, 10] and NADH have absorbance at 340 nm, and only NADH is enzymatically active, we tested the enzymatic activity of NADH regenerated in our process. The test was carried out with LDH that can convert pyruvate to lactate in the presence of NADH. First, NADH was regenerated via the bioelectrocatalytic reduction of NAD by MV and LD at $E = -0.75\text{ V}$, then we terminated the potential applied and added LDH and pyruvate in the solution. Fig. 5A shows the absorbance spectrum of regenerated NADH after applying the electrochemical potential at $E = -0.75\text{ V}$ for 4800 s in the presence of NAD (2.5

mM), LD (5.0 U mL^{-1}) and MV (0.5 mM), and the subsequent absorbance spectra after adding LDH (10.0 U mL^{-1}) and pyruvate (10.0 mM) for 1800 s (30 scans). The absorbance peak at 340 nm had nearly disappeared at the end of the measurement. The concentration of NADH consumed by the enzymatic reaction was derived from the absorbance peak at 340 nm. Fig. 5B shows the kinetics of the decreasing concentration of NADH caused by the addition of LDH and pyruvate after the termination of the electrolysis. The disappearance of the absorbance peak at 340 nm after the addition of LDH and pyruvate demonstrated that the regenerated NADH from the indirect electrochemical reduction of NAD was consumed and enzymatically active. In the control experiment in the absence of LDH and pyruvate after the application of $E = -0.75 \text{ V}$ for 4800 s, the absorbance peak at 340 nm slowly decayed, which was ~30% of the absorbance change in the presence of LDH and pyruvate at the same time scale. The decay was possibly due to the reversible reaction of the electrochemically regenerated NADH with LD and the oxidized MV in the absence of the applied potential. It should be noted that the possible presence of a small amount of residual oxygen could result in the oxidized form of MV in the electrolyte, causing the conversion of NADH into NAD.

4. Conclusion

In summary, by spectroelectrochemistry, we characterized the electrochemical regeneration of enzymatically active NADH via LD-catalyzed electron transfer reaction via MV. We confirmed that, as long as the electric reduction potential is available, the fast electrochemical way to regenerate the bio-electron donor NADH continuously is feasible. This is especially significant when NAD promotes the unwanted reverse NADH-dependent enzymatic reactions where the regeneration of NADH is needed instantly. Further applications of the electrochemical regeneration of NADH on bioreactors and biosensors are ongoing in this lab.

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Research Highlights

- In situ regeneration of NADH was evidenced by spectroelectrochemistry
- NADH Regeneration via enzymatic electron transfer reaction was confirmed fast and sustainable
- Regenerated NADH was confirmed enzymatically active for NAD/NADH-dependent enzymes

Fig. 1. Cyclic voltammograms of MV in the absence/presence of LD and NAD: (a) in the presence of MV (0.5 mM); (b) in the presence of MV (0.5 mM) and LD (5 U mL⁻¹); and (c)-(g) in the presence of MV (0.5 mM), LD (5 U mL⁻¹) and various concentrations of NAD (0.5, 1, 2, 5, 10 mM). The potential scan rate: 5 mV s⁻¹. Working electrode: gold gauze.

Fig. 2. Time-dependent UV-vis spectroelectrochemical absorbance spectra in the presence of NAD (10 mM) and MV (0.5 mM) at the applied potential of -0.75 V for 1260 s at different concentrations of LD: (A) 0 U mL⁻¹, (B) 0.25 U mL⁻¹, (C) 1.0 U mL⁻¹, (D) 5.0 U mL⁻¹. Working electrode: gold gauze.

Fig. 3. Time-dependent UV-vis spectroelectrochemical absorbance spectra in the presence of MV (0.5 mM) and LD (5 U mL⁻¹) at the applied potential of -0.75 V for 1260 s at different concentrations of NAD: (A) 0 mM, (B) 1 mM, (C) 5 mM, (D) 10mM. Working electrode: gold gauze.

Fig. 4. The initial rate of NADH regeneration derived from the spectroelectrochemical spectra recorded at different initial concentrations of NAD in the presence of MV (0.5 mM) and LD (5 U mL^{-1}) at the applied potential of -0.75 V for 1260 s as a function of the NAD concentration.

Fig. 5. (A) Time-dependent UV-vis absorbance spectra of the regenerated NADH after addition of LDH (10 U mL^{-1}) and pyruvate (10 mM). The NADH was regenerated in the presence of NAD (2.5 mM), LD (5.0 U mL^{-1}) and MV (0.5 mM) at the applied potential of -0.75 V for 4800 s . (B) Time-dependent concentration of NADH derived from the absorbance peak of NADH at 340 nm in (A).

Scheme 1.

(A) Schematic draw of the configuration of the three-electrode system in the thin-layer spectroelectrochemical cell for the spectroelectrochemical measurements; (B) Electrochemical regeneration of NADH by lipoamide dehydrogenase (LD)-catalyzed electron transfer reaction between methyl viologen (MV) and NAD.