NAD^+-Dependent Enzyme Electrodes: Electrical Contact of Cofactor-Dependent Enzymes and Electrodes

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Abstract: NAD^+-dependent lactate dehydrogenase (LDH) is assembled onto a pyrroloquinoline quinone-NAD^+ monolayer. The redox active monolayer is assembled *via* covalent attachment of pyrroloquinoline quinone (PQQ) to a cystamine monolayer associated with a Au electrode, followed by covalent linkage of N^6-(2-aminoethyl)-NAD^+ to the monolayer. The surface coverage of PQQ and NAD^+ units is ca. 1.2×10^{-10} mol cm^{-2}. The surface coverage of LDH bound to the redox active monolayer is ca. 3.5×10^{-12} mol cm^{-2}. The assembled LDH monolayer is active in the bioelectrocatalytic oxidation of lactate. The bioelectrocatalyzed process involves the PQQ-mediated oxidation of the immobilized NADH in the presence of Ca^{2+} ions. The LDH associated with the PQQ-NAD^+ monolayer assembled on the electrode surface exhibits moderate stability, and the biocatalyst dissociates to the electrolyte solution. Dissociation of LDH is enhanced in the presence of solubilized NAD^+. Cross-linking of the monolayer-bound LDH with glutaric dialdehyde yields an integrated stable enzyme electrode for the bioelectrocatalyzed oxidation of lactate. The electrode acts as an amperometric biosensor for lactate. Affinity binding of NAD^+-dependent alcohol dehydrogenase to the PQQ-NAD^+-monolayer-modified Au electrode, followed by cross-linking of the enzyme, yields an enzyme electrode for the bioelectrochemical detection of ethanol.

Introduction

Electrical contact of redox enzymes with electrode surfaces is a key process in tailoring of enzyme electrodes for bioelectronics and biosensor applications.[1,2] Diffusional electron transfer mediators were used to electrically contact enzyme redox sites and electrodes.[3] Other methods to generate electrical communication between redox biocatalysts and electrode supports include the chemical modification of the proteins with electron relay groups that mediate electron transfer[4,5] and the immobilization of the proteins in redox polymers associated with electrodes.[6,7] However, in all of these systems the redox biocatalysts are flavoenzymes[6,7] or other cofactor-containing enzymes.[8] That is, the protein-embedded redox active cofactors play a central role as electron relay components in the electron transfer chain between the active site and electrode support. The vast majority of redox enzymes are, however, $NAD(P)^+$/NAD(P)H-dependent biocatalysts, and their electrical contacting with the electrode reveals fundamental difficulties that prevent their broad use in biosensor applications.[9] The $NAD(P)^+$ cofactor is biologically active by a diffusional route, and its reduction to NAD(P)H occurs by the temporary association in the vicinity of the active site.[10] Furthermore, the $NAD(P)^+$/NAD(P)H couple exhibits poor electrochemical reversibility, and hence, the direct electrochemical oxidation of NAD(P)H and the electroreduction of $NAD(P)^+$ are kinetically unfavored.[11,12] Various redox mediators were used to catalyze the oxidation of NAD(P)H,[13−15] i.e., *o*-quinones, *p*-quinones, phenazine, phenoxazine and phenothiazine derivatives, ferrocenes, and Os complexes. Bioelectrocatalytic reduction of

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NAD(P)$^+$ was also achieved.[16] Thus, to tailor integrated, electrically-contacted enzyme electrodes consisting of NAD(P)$^+$-dependent enzymes for biosensor application, the organization of catalyst−NAD(P)$^+$−enzyme assemblies in nondiffusional, electrically-wired configurations is essential.

Modified NAD$^+$ derivatives were linked to polymeric matrices[17] and coupled with NAD$^+$-dependent enzymes.[18] Coimmobilization of the polymeric cofactors and biocatalysts led to the design of bioreactors for biocatalytic transformations[19] and the organization of biosensor devices.[20] Organized ordered assemblies of NAD(P)$^+$- and cofactor-dependent enzymes on an electrode surface (biocatalytic electrodes) exhibiting electrical contact with the electrode surface are presently unknown.[9] Recently, we reported on the preparation of electrically-contacted flavoenzyme electrodes by the reconstitution of apoenzymes onto a redox−relay−FAD monolayer associated with a Au electrode.[21] It was demonstrated that the resulting reconstituted enzyme exhibits most efficient electron transfer turnover with the electrode, resulting in the effective bioelectrocatalyzed oxidation of the enzyme−substrate and unprecedented high current responses. Also, it was demonstrated that Au electrodes modified[22] by the heme-containing polypeptide, microperoxidase-11, form complexes with various hemoproteins at the electrode interface.[23] The complexes exhibit sufficient stability to allow the cross-linkage of the protein layer and the formation of stable electrically-contacted hemoprotein electrodes. Here, we report on a novel method to electrically contact NAD$^+$-dependent enzymes with electrode surfaces. The temporary affinity−associative interactions of the biocatalyst with a catalyst-NAD$^+$ monolayer assembled on an electrode enable the cross-linking of the enzyme layer on the conductive support interface. The resulting integrated enzyme electrodes stimulate the bioelectrocatalyzed oxidation of the respective substrates.

Experimental Section

Pyrroloquinoline quinone (PQQ) and cystamine were purchased from Fluka. Lactate dehydrogenase (LDH, E.C.1.1.1.27 from rabbit muscle, type II), alcohol dehydrogenase (AlcDH, E.C.1.1.1.1), and NAD$^+$ were purchased from Sigma and used without further purification. N^6-(2-Aminoethyl)-NAD$^+$ (amino-NAD$^+$) and N^5-(2-aminoethyl)-FAD (amino-FAD) were synthesized and characterized as reported.[17a,21] Ultrapure water from a Nanopure (Barnstead) source was used throughout this work.

Clean Au electrodes were roughened (roughness coefficient ca. 15−20) and modified with a cystamine monolayer as reported previously.[24] The pyrroloquinoline quinone was covalently linked to the cystamine monolayer as described earlier.[24] The covalent coupling of N^6-(2-aminoethyl)-NAD$^+$ to the PQQ-monolayer-functionalized electrode was performed by incubation of the Au-modified electrodes in a 0.05 M HEPES buffer solution, pH = 7.3, in the presence of the (aminoethyl)-NAD$^+$, 1×10^{-3} M, and 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC), 1×10^{-2} M, for 2 h.

Association and cross-linking of LDH or AlcDH on the functionalized PQQ-NAD$^+$ electrodes was performed by rinsing the PQQ-NAD$^+$-functionalized electrodes three times with water, and their incubation at 0 °C for 20 min in a 0.05 M phosphate buffer solution, pH = 7.0, that includes LDH, 5 mg mL^{-1}, or AlcDH, 5 mg mL^{-1}. The resulting PQQ-NAD$^+$ monolayer electrodes with associated LDH or AlcDH were rinsed for a short time (30 s) with a cold 0.05 M phosphate buffer solution, pH = 7.0, and then immersed into a phosphate buffer solution that includes glutaric dialdehyde, 5% (v/v), for 20 min at 0 °C.

Cyclic voltammetry and steady-state amperometric measurements in nonstirred solutions were performed with the PQQ/NAD$^+$/enzyme electrodes.

Electrochemical measurements were performed using a potentiostat (EG&G VersaStat) connected to a personal computed (EG&G research electrochemistry software model 270/250). All measurements were carried out at 25 ± 0.5 °C in a three-compartment electrochemical cell consisting of the modified electrode as the working electrode, a glassy carbon auxiliary electrode isolated by a glass frit, and a saturated calomel electrode (SCE) connected to the working volume with a Luggin capillary. All potentials are reported with respect to this reference electrode. Ar bubbling was used to remove oxygen from the solution in the electrochemical cell.

Results and Discussion

Pyrroloquinoline quinone was found to act as a catalyst for the oxidation of NAD(P)H.[15] Specifically, the electrooxidation of NAD(P)H was enhanced in the presence of Ca^{2+} ions as cocatalyst.[15a] Accordingly, a PQQ-NAD$^+$ composite monolayer electrode is suggested to be the electrocatalytic interface to assemble an electrically contacted, integrated, NAD$^+$-dependent enzyme electrode. Scheme 1 shows the stepwise assembly of the PQQ-NAD$^+$ monolayer electrode. A cystamine monolayer is assembled on a rough Au electrode (geometrical area 0.2 cm^2, roughness coefficient ca. 15). Pyrroloquinoline quinone that includes three carboxylic acid residues was covalently coupled to the base cystamine monolayer in the presence of 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide. The surface coverage of linked PQQ was determined by coulometric analysis of the reversible redox wave of PQQ at $E^\circ = -0.16$ V, pH = 8.0, to be 1.2×10^{-10} mole cm^{-2}. N^6-(2-Aminoethyl)-NAD$^+$ was covalently linked to vacant carboxylic acid groups associated with PQQ, using EDC as coupling reagent. Since the NAD$^+$ units linked to the PQQ monolayer are electrochemically inactive, their surface coverage cannot be assessed directly. We estimated, indirectly, the surface coverage of the electrode by amino-NAD$^+$ units by the covalent coupling of N^6-(2-aminoethyl)-FAD to the PQQ monolayer treated with the amino-NAD$^+$ for variable reaction times (Scheme 2). A PQQ monolayer electrode that is not treated with amino-NAD$^+$ yields, after the reaction with amino-FAD, a two-wave cyclic voltammogram with the redox waves at $E^\circ = -0.56$ V and E°

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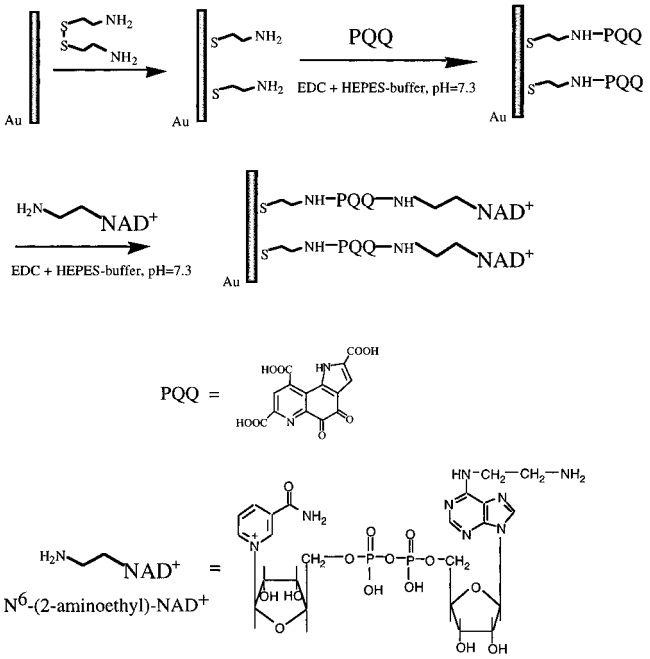
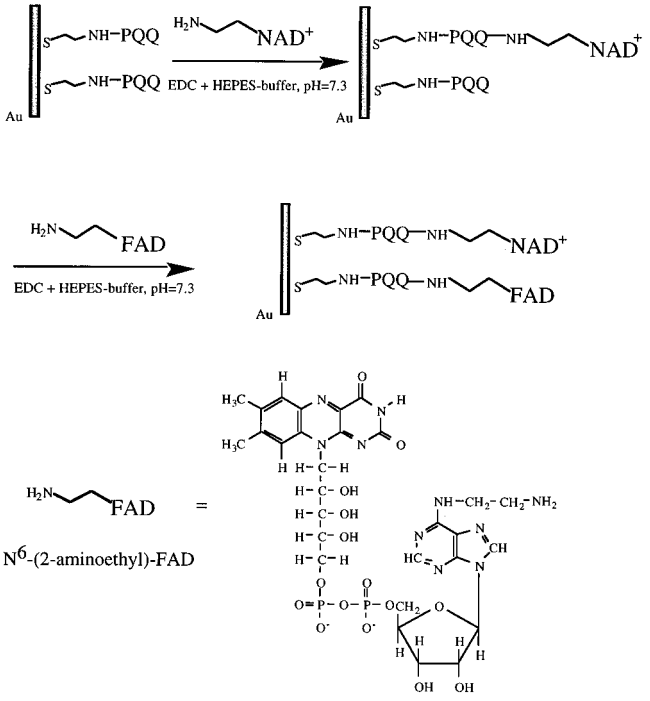
Scheme 1. Stepwise Organization of the PQQ-NAD$^+$-Monolayer-Modified Au Electrode



Scheme 2. Stepwise Organization of the Mixed PQQ-NAD$^+$/FAD-Monolayer-Modified Au Electrode for Evaluation of Binding Sites Occupied by NAD$^+$ Units



$= -0.16$ V, characteristic to the FAD and PQQ components, respectively (curve a, Figure 1A). Coulometric analysis of the two waves, and realizing that both of the redox components exhibit a two-electron redox process, the surface coverage of the FAD sites is identical to that of the PQQ components, ca. 1.2×10^{-10} mol cm^{-1}. That is, one FAD component binds to a PQQ unit in the monolayer assembly as an average stoichiometry. The PQQ monolayer electrode is then reacted with the amino-NAD$^+$ component for variable reaction time intervals and afterward coupled to the amino-FAD redox probe (curves b−g, Figure 1A). The redox wave characteristic to the FAD component, $E^\circ = -0.56$ V, decreases in its intensity as the reaction time of the PQQ monolayer with the amino-NAD$^+$

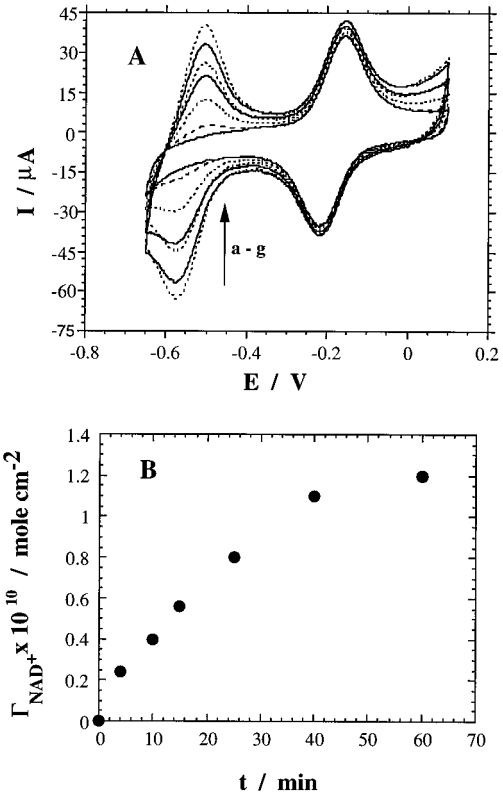
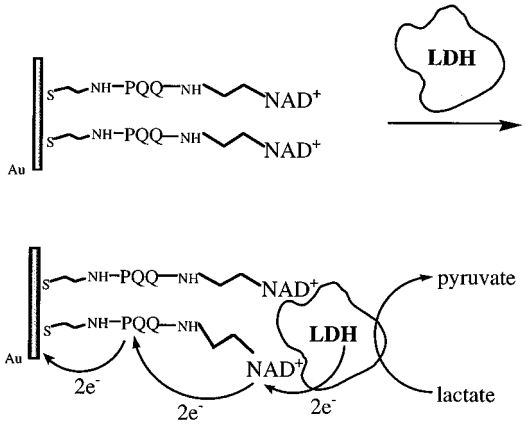


Figure 1. (A) Cyclic voltammograms of the mixed PQQ-NAD$^+$/FAD monolayer-modified Au electrode (roughness factor ca. 15) obtained after different periods of the PQQ monolayer treatment with amino-NAD$^+$: (a) 0, (b) 5, (c) 10, (d) 15, (e) 25, (f) 40, and (g) 60 min. The modification step for the amino-FAD that follows the primary attachment of the amino-NAD$^+$ was always 60 min. Potential scan rate, 100 mV s^{-1}. Background electrolyte, 0.1 M Tris buffer, pH 8.0. (B) Increase of the NAD$^+$ surface density on the PQQ-monolayer-functionalized electrode as a function of the treatment time with the amino-NAD$^+$.

component is prolonged. The redox wave of the PQQ units, $E^\circ = -0.16$ V, is unaffected. After ca. 60 min of reaction between the PQQ monolayer and amino-NAD$^+$, no amino-FAD is coupled to the monolayer interface in the secondary coupling process. This is consistent with the fact that the primary reaction of the PQQ monolayer electrode with amino-NAD$^+$ leads to the covalent coupling of this cofactor to the quinone sites. This blocks the PQQ components for the secondary linkage to the FAD units. As the reaction time of the PQQ monolayer electrode with amino-NAD$^+$ is prolonged, less FAD binds to the monolayer due to enhanced surface coverage of the electrode by NAD$^+$ components. Assuming that the yield of the coupling of amino-FAD to the PQQ layer is quantitative, and unaffected by the presence of NAD$^+$-components, one can use the redox wave of the FAD to assess the surface coverage of the PQQ monolayer by NAD$^+$ upon interaction with amino-NAD$^+$ for variable reaction time intervals (Figure 1B). The monolayer is saturated by NAD$^+$ units after interaction with the PQQ monolayer electrode for ca. 60 min.

The resulting PQQ-NAD$^+$ monolayer-functionalized electrode was interacted with LDH and the electrochemical responses of the rinsed electrode were recorded in the absence and presence of lactate and Ca^{2+} ions as cocatalysts (Scheme 3). Figure 2 shows the cyclic voltammogram of the electrode in the absence (curve a) and presence (curve b) of lactate. The electrode exhibits bioelectrocatalytic activity for the oxidation of lactate. A functionalized PQQ electrode, lacking the NAD$^+$ component, gives, after the treatment with LDH and in the presence of lactate, only the redox wave of the PQQ (curve a, Figure 2).

Scheme 3. Affinity Binding of LDH to a PQQ-NAD⁺-Monolayer-Modified Au Electrode and Bioelectrocatalyzed Oxidation of Lactate



Similarly, the LDH-untreated PQQ-NAD⁺ monolayer electrodes do not yield any electrocatalytic anodic current and only the reversible redox wave of PQQ is detected.[25] These results, and the respective control experiments, clearly imply that LDH is associated with the NAD⁺ cofactor which is a part of the monolayer assembly. Binding of the LDH to the NAD⁺ cofactor originates from noncovalent associative affinity interactions (*vide infra*). Bioelectrocatalyzed oxidation of lactate proceeds only in the presence of the PQQ-NAD⁺ monolayer and associated LDH. That is, LDH-biocatalyzed oxidation of lactate yields immobilized NADH associated with the PQQ units. Oxidized PQQ catalyzes the electro-oxidation of NADH in the presence of Ca^{2+} ions. Note that the electrocatalytic anodic current as a result of lactate oxidation occurs at the PQQ redox potential, consistent with this mediated electron transfer.

Figure 3 (curve a) shows the amperometric response (E = 0.1 V) of the PQQ-NAD⁺/LDH electrode as a function of time in an electrolyte solution that includes lactate, 20 mM. The amperometric response of the electrode decreases with time, and the current drops by ca. 25% within 30 min. Introduction of native NAD⁺ to the electrolyte solution that includes the generated PQQ-NAD⁺/LDH electrode results in the time-dependent amperometric response shown in Figure 3 (curve b). The initial electrocatalytic current, as a result of the oxidation of lactate, increases, but the amperometric response of the electrode decays rapidly to almost zero after ca. 5 min in the NAD⁺-containing electrolyte solution. These results clearly indicate that the biocatalyst, LDH, is bound to the PQQ-NAD⁺ monolayer by associative affinity interactions. The dissociation of the noncovalently bound LDH from the PQQ-NAD⁺ monolayer is relatively slow. Addition of NAD⁺ to the electrolyte causes rapid displacement of LDH from the monolayer due to the high affinity of the native factor for the enzyme. (The origin of the higher current response of the electrode in the presence of native NAD⁺ will be addressed later.) The association of LDH to the PQQ-NAD⁺ monolayer was further supported by the microgravimetric, quartz-crystal-microbalance (QCM) analysis. The PQQ-NAD⁺ was linked to Au electrodes associated with a quartz crystal (9 MHz). Upon interaction of the modified

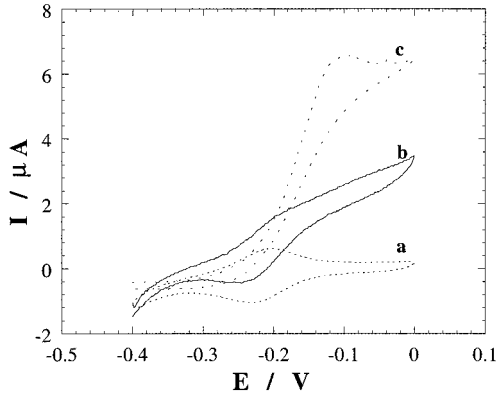


Figure 2. Cyclic voltammograms of the PQQ-NAD⁺/LDH-modified Au electrode (roughness factor ca. 15): (a) in the background solution only, (b) in the presence of 20 mM lactate, (c) cross-linked LDH monolayer in the presence of 20 mM lactate and 10 mM NAD⁺. Background electrolyte: 0.1 M Tris buffer, pH 8.0, and 10 mM $CaCl_2$. Potential scan rate, 2 mV s^{-1}.

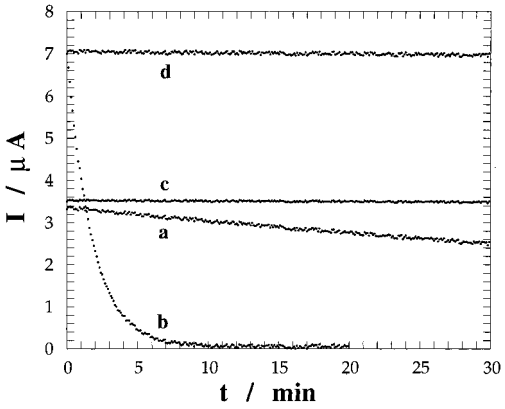


Figure 3. Amperometric responses of the PQQ-NAD⁺/LDH-modified Au electrode (roughness factor ca. 15) under applied potential E = 0.1 V in the presence of 20 mM lactate (nonstirred solution): (a) PQQ-NAD⁺-associated LDH in the absence of added native NAD⁺, (b) PQQ-NAD⁺-associated LDH in the presence of 10 mM NAD⁺, (c) cross-linked LDH monolayer in the absence of added native NAD⁺, (d) cross-linked LDH monolayer in the presence of 10 mM NAD⁺. Background electrolyte: 0.1 M Tris buffer, pH 8.0, and 10 mM $CaCl_2$.

crystal with LDH, 12.5 mg mL^{-1}, a decrease in the crystal resonance frequency corresponding to Δf = 50 Hz was observed. With this experimental value known, with the molecular weight of LDH (MW 140 000), and by the application of the Sauerbrey relation,[26] the surface coverage of the enzyme on the monolayer is estimated to be ca. 3.5×10^{-12} mol cm^{-2}. It should be noted that nonsignificant frequency changes (~ -10 Hz) were observed upon interaction of PQQ-functionalized Au/quartz crystal (lacking the NAD⁺ units) with LDH. Thus, the microgravimetric, QCM measurements support our original conclusion that LDH binds to the PQQ-NAD⁺ monolayer by associative affinity interactions.

The results indicate that the LDH associated with the PQQ-NAD⁺ monolayer exhibits sufficient durable stability for further chemical modification of the assembly that could eventually lead to an integrated, electrically-contacted, stable electrode. For example, the temporary stability of the PQQ-NAD⁺/LDH-layered assembly could enable the cross-linking of the enzyme units. This would generate a stable cross-linked polyenzyme layer with multianchoring sites to the electrode surface. The PQQ-NAD⁺ surface-bound LDH was cross-linked with glutaric

(25) Treatment of the PQQ-NAD⁺-assembled monolayer with glucose oxidase does not yield any enzyme electrode capable of oxidizing glucose in the presence of ferrocenecarboxylic acid as diffusional electron mediator. Also, treatment of the PQQ-NAD⁺ monolayer electrode with butylamine prior to its interaction with LDH, yields a PQQ-NAD⁺/LDH electrode of features similar to those of the amine-untreated electrode. Thus, the possibility of covalent attachment of LDH to EDC-activated carboxylic functions of PQQ can be excluded.

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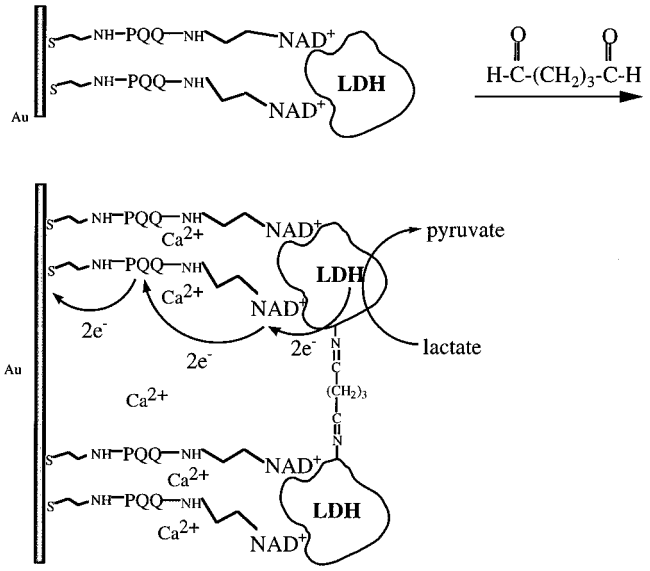
Scheme 4. Cross-linking of the PQQ-NAD^+/LDH-Assembled Monolayer and Bioelectrocatalyzed Oxidation of Lactate Using the Integrated Enzyme Electrode



dialdehyde (Scheme 4). Figure 2 shows the cyclic voltammograms of the resulting cross-linked enzyme electrode in the presence of added lactate and native NAD^+ to the electrolyte solution (curve c). The typical cyclic voltammogram in the presence of lactate and absence of natural NAD^+ is not different from the cyclic voltammogram obtained before LDH cross-linking (curve b). Control experiments show that no electrocatalytic current is obtained in the absence of lactate. Also, attempts to cross-link a PQQ monolayer electrode (lacking the NAD^+ units) in the presence of LDH failed to yield an electroactive electrode for the oxidation of lactate. The time-dependent amperometric response of the cross-linked PQQ-NAD^+/LDH-layered electrode in the presence of lactate (E = 0.1 V) is shown in Figure 3 (curve c). In contrast to non-cross-linked enzyme electrodes that revealed a current decline due to the dissociation of LDH from the monolayer (curve a), the cross-linked enzyme layer exhibits high amperometric stability and no current decrease is observed. It should be noted that the electrocatalytic current of the cross-linked enzyme layer is identical to that for the physically adsorbed, non-cross-linked, enzyme layer. This implies that the cross-linking process does not perturb the bioelectrocatalytic features of the enzyme layer. Even more impressive is the effect of the native NAD^+ added to the cross-linked LDH monolayer electrode (Figure 3, curve d). The resulting amperometric response is ca. 2-fold higher than that observed with the cross-linked enzyme electrode only. The amperometric response of the electrode is almost unaffected by diffusional NAD^+ in contrast to the rapid displacement of non-cross-linked LDH by diffusional NAD^+ (curve b). We thus conclude that the cross-linking of the LDH associated with the PQQ-NAD^+ monolayer by affinity interactions yields a *stable*, *integrated*, and *electrically-contacted*, biocatalytic electrode for the oxidation of lactate (Scheme 4).

The electrocatalytic anodic currents are controlled by the concentration of lactate. As the concentration of lactate increases, the electrocatalytic anodic current is enhanced and levels-off at a lactate concentration above 10 mM. Figure 4A shows the calibration curves of the resulting anodic currents for the PQQ-NAD^+/LDH cross-linked electrode alone (curve a) and in the presence of added native NAD^+ (curve b). The leveling-off of the anodic current (I_{max}) at lactate concentration [S] exceeding ca. 10 mM suggests that the enzyme active site

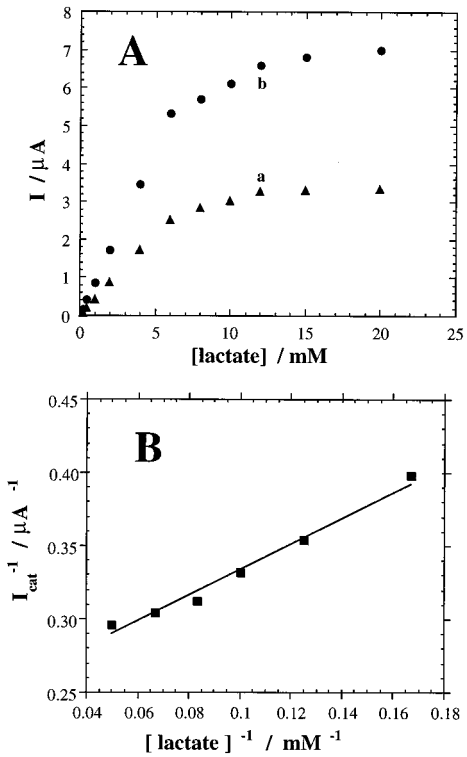


Figure 4. (A) Currents generated by the integrated cross-linked PQQ-NAD^+/LDH-monolayer-modified Au electrode (roughness factor ca. 15) in the presence of different concentrations of lactate: (a) no added NAD^+, (b) in the presence of 10 mM NAD^+. Applied potential E = 0.1 V. Background electrolyte (not stirred): 0.1 M Tris buffer, pH 8.0, and 10 mM $CaCl_2$. (B) Lineweaver−Burk plot of the currents obtained in the absence of solubilized NAD^+ (curve a in Figure 4A).

is saturated by the substrate at this concentration. As the electrocatalytic anodic current reflects the turnover rate of the bioelectrocatalytic oxidation of the substrate, the calibration curve shown in Figure 4A can be analyzed according to the Michaelis−Menten analysis or by the respective Lineweaver−Burk plot (eq 1). Figure 4B shows the graphic analysis of the

$$\frac{1}{I} = \frac{K_m}{I_{max}}\left(\frac{1}{[S]}\right) + \frac{1}{I_{max}} \qquad (1)$$

calibration curve of the cross-linked LDH-layered electrode according to eq 1. The values I_{max} = 4.1 mA and K_m = 3.6 mM (K_m is the Michaelis−Menten constant) are derived. The electrocatalytic anodic currents resulting from the LDH associated to the PQQ- NAD^+ by affinity interactions or the monolayer cross-linked LDH are ca. 2-fold enhanced in the presence of added native NAD^+ as compared to amperometric responses of the PQQ/ NAD^+/enzyme electrodes only. This is attributed to the tetramer structure of LDH. Association of LDH to the two-dimensional PQQ- NAD^+ monolayer proceeds via two units of the enzyme. The remaining two units are not exposed to the monolayer and hence lack electrical contact with the electrode surface. Added native NAD^+ activates these two mute subunits by a diffusional route and yields NADH. Oxidation of the diffusionally generated NADH by PQQ associated with the monolayer results in the increased amperometric response of the functionalized electrode. We thus realize that the primary association of LDH to the PQQ-NAD^+ monolayer via affinity interactions enables the cross-linking of the biocatalyst layer to an integrated, electrically-contacted and stable enzyme electrode. Bioelectrocatalyzed oxidation of lactate proceeds by the biocatalyzed reduction of NAD^+ to NADH in the integrated

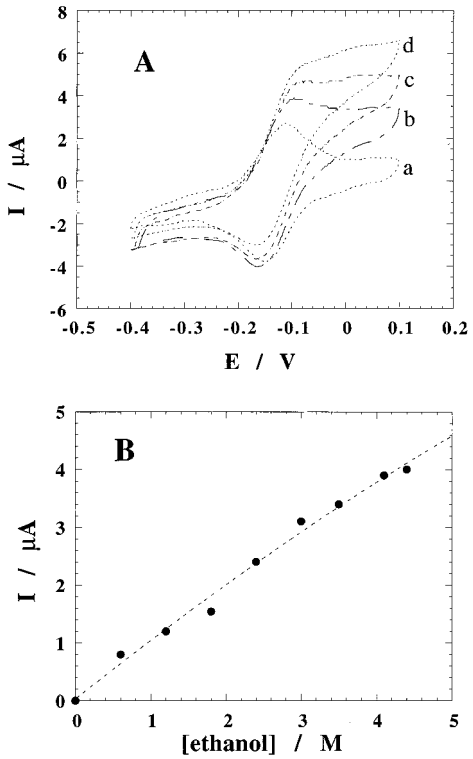


Figure 5. (A) Cyclic voltammograms of the integrated cross-linked PQQ-NAD^+/AlcDH-monolayer-modified Au electrode (roughness factor ca. 15) in the presence of different concentrations of ethanol: (a) no ethanol, (b) 0.6 M, (c) 1.2 M, (d) 1.8 M. Potential scan rate, 2 mV s^{-1}. Background electrolyte: 0.1 M Tris buffer, pH 8.0, and 10 mM $CaCl_2$. (B) Currents generated by the integrated cross-linked PQQ-NAD^+/AlcDH-monolayer-modified Au electrode in the presence of different concentrations of ethanol (evaluated from the cyclic voltammograms at $E = 0.1$ V).

assembly followed by the PQQ-electrocatalyzed oxidation of NADH in the presence of Ca^{2+}.

The approach described for LDH to generate integrated $NAD(P)^+$-dependent enzyme electrodes seems to be a general method. Following a scheme similar to that employed to produce the electrically-contacted LDH electrode, we assembled an integrated alcohol-sensing electrode using AlcDH as bioelectrocatalyst. The biocatalyst, AlcDH, was interacted with the PQQ-NAD^+ monolayer electrode. The resulting enzyme layer associated with the monolayer was cross-linked with glutaric dialdehyde. Figure 5A shows the cyclic voltammogram of the resulting electrode in the absence of ethanol (curve a) and upon increasing the concentration of added ethanol to the system. An electrocatalytic anodic current at the redox potential of PQQ is observed, and its magnitude increases as the ethanol concentration is elevated (curves b−d). Control experiments indicate that the PQQ-NAD^+ monolayer electrode alone does not induce any electrocatalytic oxidation of ethanol. Similarly, the non-cross-linked PQQ-NAD^+/AlcDH-layered electrode loses its electrobiocatalytic activity for the oxidation of ethanol upon addition of diffusional native NAD^+ to the electrolyte solution. These results clearly imply that AlcDH associates to the PQQ-NAD^+ monolayer by affinity interactions and, upon cross-linking, yields an integrated, electrically-contacted enzyme electrode. Biocatalyzed oxidation of ethanol yields the monolayer-bound NADH that is oxidized by PQQ in the presence of Ca^{2+} ions, resulting in the electrocatalytic anodic current. Figure 5B shows the calibration curve of the resulting amperometric responses of the integrated AlcDH electrode at different concentrations of ethanol.

Conclusions

We have demonstrated a novel method to assemble integrated, electrically-contacted, $NAD(P)^+$-dependent enzyme electrodes. The method is based on the assembly of an electrocatalyst (PQQ) and $NAD(P)^+$ cofactor monolayer as an active interface for the electro-oxidation of the reduced dihydronicontinamide cofactor. The second concept involves the assembly of the respective cofactor-dependent enzyme on the NAD^+ unit by associative affinity interactions. The temporary stability of the tailored electrode enables the cross-linking of the enzyme layer to yield a stable, electrically-contacted enzyme electrode. This approach enables the application of a broad class of redox enzymes as active interfaces in biosensor devices. Furthermore, the integrated enzyme electrodes provide a novel means to derive bioelectrocatalyzed chemical transformations with $NAD(P)^+$-dependent enzymes. In the present study, we developed lactate- and ethanol-sensing electrodes. Since L-lactate is a metabolite that reflects oxygen supply to body organs, this sensing electrode could find important clinical applications, e.g., during heart surgery.

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