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FAD-MEDIATED ENZYMATIC CONVERSION OF NAD^+ TO NADH: APPLICATION TO CHIRAL SYNTHESIS OF L-LACTATE

Mihaela Draganoiu Leonida,[a,*] Susan B. Sobolov,[b] and Albert J. Fry[c]

[a]*Fairleigh Dickinson University, Chemistry Department-H444N, Teaneck, NJ 07666, U.S.A.*
[b]*Pfizer Company, Eastern Point Drive, Groton, CT 06340, U.S.A.*
[c]*Hall-Atwater Laboratory of Chemistry, Wesleyan University, Middletown, CT 06459, U.S.A.*

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Abstract: Electroenzymatic reduction of NAD^+ to NADH for subsequent use in enzymatic synthesis has been carried out at carbon electrodes bearing lipoamide dehydrogenase (LiDH) immobilized under a Nafion^R film. The self-mediated electron transfer was made possible by an excess of flavin adenine dinucleotide (FAD) entrapped together with LiDH. Results were compared to those obtained with a similar electrode containing both LiDH and a polymeric form of FAD (pFAD) prepared by anodic polymerization of FAD.

We reported earlier that an electrode coated with a mixture of lipoamide dehydrogenase (LiDH) and the redox mediator methyl viologen (MV^{+2}) under an electrically conductive polymer (Nafion^R) coating readily converts NAD^+ into NADH at the redox potential of the viologen.[1] This electrode can be used for the electrocatalytic synthesis of α-hydroxy and α-amino acids using the electrogenerated NADH.[1]

A source of concern with this system is the slow leakage of the mediator from the electrode during use. MV^{2+} is very toxic and would represent an undesirable contaminant in substances prepared by this method. We subsequently found that leakage of the viologen from the electrode can be prevented either by chemically binding an MV^{+2} derivative to the enzyme[2] or by coating a polymer containing viologen-derivative subunits onto the electrode.[3] However, an alternate solution that would totally avoid the use of the toxic redox mediator would be more attractive. Any such solution must take into account the fact that direct electrochemical reduction of NAD^+ affords an inactive dimer rather than the desired NADH, and that therefore a redox mediator of some sort must be used as the actual electroactive substance.[4,5] We report here the successful application of both monomeric and polymeric forms of the natural redox cofactor FAD for this purpose.

FAD (flavin adenine dinucleotide) is an essential cofactor for LiDH.[6] The electrochemistry of FAD has been the subject of a variety of reports.[7] Durliat et al. reported that FAD used as a mediator can facilitate electron transfer between platinum and several biomacromolecules, including cytochrome c, hemoglobin, myoglobin, and glucose oxidase.[8] The electrode reactions of flavin have been studied on other conducting materials such as mercury and carbon.[9] Zhang et al. recently pointed out several advantages of FAD as a redox mediator: fast electron exchange between various electrodes and FAD, high reaction rates between FAD and biomacromolecules, and, since FAD is naturally associated with some enzymes in electron transfer, a better

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biocompatibility than that observed with most organic and inorganic mediators.[10] Chi and Dong found in 1994 that FAD can be anodically polymerized on the surface of a platinum electrode and the resulting polymerized-FAD (pFAD) electrode was found to catalyze the reduction of oxygen.[11]

There are however no previous reports in the literature on the use of FAD, either polymerized or monomeric, as mediator in an electroenzymatic synthetic process. In order to test the capacity of FAD to function as a mediator, FAD was used in both monomeric and polymeric forms, in L-lactate synthesis coupled with coenzyme regeneration, while immobilized on the electrode together with LiDH.

Experimental Methods

Electropolymerization of FAD. Electropolymerization was carried out on a glassy carbon (GC) electrode (0.07 cm^2), previously polished with Al_2O_3 paste and cleaned ultrasonically in distilled water for 5 min. The polymerized FAD film (pFAD) was prepared by a two-step method.[10] The initiation process was carried out by cyclic scanning of the electrode potential between + 1.6V and – 1.2 V (vs. SCE) at 50 mV s^{-1} for three cycles, in 5 mM FAD in 0.1 M phosphate buffer, pH 7.0. The film growth was then achieved by scanning continuously between + 0.5V and – 0.8V at the same scan rate. The film thickness was controlled by the number of scans. In Figure 1 it is easy to notice the increase in cathodic and anodic currents when the number of scans (layers) increases (together with the number of charge carriers). This shows that a pFAD film has been formed on the GC electrode. The cyclic voltammetry experiments were performed on a Princeton Applied Research (PAR) Versastat using the PAR Model 250 software and a Gateway 386 computer to generate wave forms and acquire data.

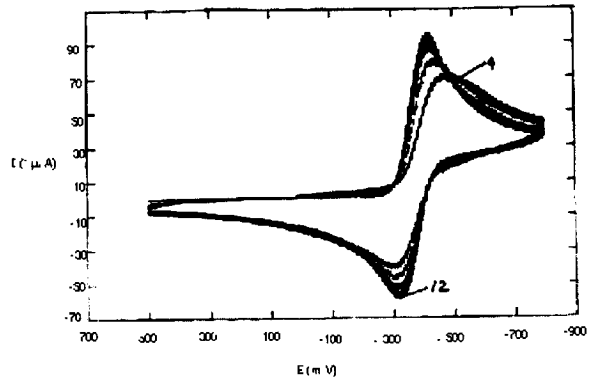

Figure 1. Cyclic voltammograms for different scans (numbers 4–12), in 5 mM FAD in 0.1 M phosphate buffer (pH 7.0), scan rate 50 mV s^{-1}, GC electrode 0.07 cm^2.

The same procedure was used to coat a reticulated vitreous carbon (RVC) electrode (ca. 30 cm^2) to be used subsequently in L-lactate synthesis.

The cell for the synthesis. One hundred milliliters of phosphate buffer (pH 7.0) were used to prepare a 0.145 M solution of pyruvate. The solution also contained 2 mM NAD^+ and 20 U L-lactic dehydrogenase (LDH). The RVC electrode was used as working electrode, coated with pFAD as described above. LiDH was dip-coated on top of the pFAD film, air-dried, and then dip-coated again with a Nafion^R film (from a 0.625% alcoholic solution) to prevent leakage of the enzyme, then air-dried again. The counter electrode was a Pt wire; a calomel electrode (SCE) was used as reference. The synthesis was carried out under a nitrogen blanket, in controlled-potential mode at – 0.5 V.

A variant of this cell used as working electrode monomeric FAD (2 mM) and LiDH (233 U) dip-coated onto an RVC electrode and physically entrapped under a Nafion^R film.

L-lactate analysis. L-lactate production was monitored by proton NMR spectroscopy (following the appearance of the peak at δ = 1.36) and HPLC analysis on a PRP-X100 cationic exchange column from Hamilton, using as mobile phase 0.1 M phosphate buffer (pH 4.5) and acetonitrile in a 2:1 ratio, flow rate 1 mL/min and UV detection at 210 nm.

Assay for LiDH. The activity of LiDH in solution was determined using the Sigma assay:[12] to 1 mL phosphate buffer (0.05 M, pH 6.6), in a quartz cuvette, 30 μL EDTA disodium salt (1 mM aqueous solution containing 2% bovine serum albumin, BSA), 30 μL β-NADH (7 mM), 30 μL NAD^+ (20 mM), 30 μL thioctic amide (28 mM, 20 mg/2mL absolute ethanol and 1.5 mL phosphate buffer pH 6.5), 30 μL LiDH (0.06 U/mL) were added (all the solutions were made up in 0.05 M phosphate buffer pH 6.5). The decrease of optical density was recorded at 340 nm (characteristic for NADH), at 25 °C. The activity of the enzyme was calculated as units per mg enzyme in the reaction mixture. One unit reduces 1.0 μmol of D,L-lipoamide to D,L-dihydrolipoamide per minute, at 25 °C.

The assay for the immobilized LiDH on the RVC electrode (in a batch reactor) used the same reagents. The content of the reactor was circulated to a spectrophotometer, in a flow cell using a ISCO Model 2300 HPLC pump. The change in absorbance at 340 nm was recorded.

Assay for LDH. The activity of LDH in solution was determined in a total volume of 1 mL, at 37 °C using 0.93 mL NADH (0.13 mM in phosphate buffer pH 7.0), 40 μL of LDH (0.25–0.75 U/mL solution in the same buffer containing 1% BSA) and 30 μL of solution 1.1 mM pyruvate (in the same buffer). The decrease in absorbance at 340 nm has been monitored for 5 min. The activity of LDH was calculated as units per mg enzyme in the reaction mixture. One unit reduces 1.0 μmol of pyruvate to L-lactate per minute, at 37 °C.

Results and Discussion

Electropolymerization has been used to prepare electrodes with polymeric coatings.[3,5] Typically a solution of monomer is electrochemically oxidized or reduced to produce a reactive intermediate, which then polymerizes to form a film directly on the electrode. Polymer-modified electrode surfaces contain the equivalent of many molecular layers.[5,11] This is advantageous when the film contains redox centers, since their

concentration can be high, giving higher currents than electrodes with immobilized monolayers. An important consideration in constructing enzyme electrodes is the fabrication of a uniform and reproducible coating containing the active protein. In situ electropolymerization produces uniform multilayer coatings. The immobilized enzyme may be entrapped within a polymer matrix during electropolymerization or simply physically retained under a polymeric film together with the mediator. Immobilization of biological species via electrochemical polymerization of monomers offers a promising approach for the preparation of biochemically active electrodes.

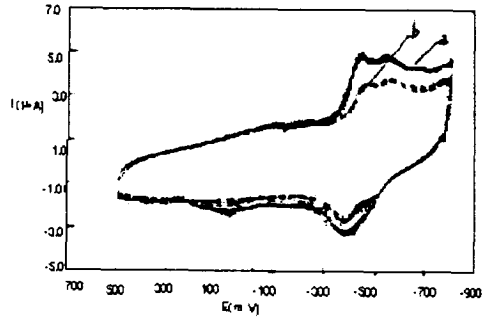

Figure 2. Cyclic voltammograms on the GC electrode, 50 mv s^{-1}, in phosphate buffer (a) (——) pFAD and LiDH coimmobilized on GC, (b) (-------) same as a) with NAD^+ 2 mM.

Electropolymerization of FAD on the GC electrode as well as on the RVC electrode was carried out as described. The amount of pFAD loaded was calculated (using Faraday's law and the fact that it is a two-electron process) as being 0.1 μmol FAD/cm^2.

Both electrodes were characterized by cyclic voltammetry. The cyclic voltammograms for the GC electrode are presented in Figure 2. As in previous experiments, the current was not enhanced by addition of NAD+ in solution.[1] In spite of this apparent lack of catalytic activity, the pFAD-modified electrode was found to catalyze the formation of L-lactate in a preparative experiment (Figure 3). By-product formation, that is, aldol dimerization of pyruvate[1] in the electrolysis cell was negligible.

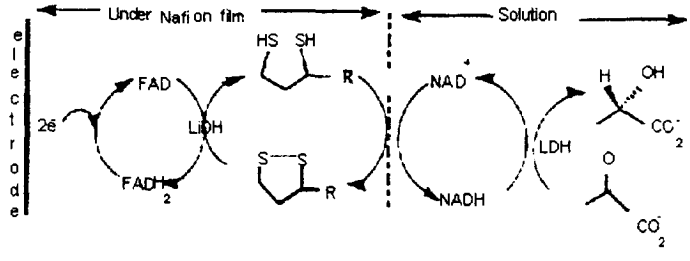

Figure 3. FAD-mediated electroenzymatic synthesis of L-lactate coupled with NADH regeneration.

Table 1 presents a comparison between the efficiency of the pFAD electrode and of that containing monomeric FAD and LiDH physically entrapped under Nafion^{R} when used for L-lactate synthesis. Pyruvate was completely consumed and L-lactate was produced in both cases (at a rate roughly 50% of that for the cell having LiDH and MV^{+2} co-immobilized on the electrode[1]). No consumption of pyruvate took place when FAD was omitted.

Table 1. Efficacy of FAD and pFAD electrodes for L-lactate synthesis

	FAD under Nafion ^{R}	**PFAD under Nafion ^{R}**
Stoichiometric yield (%) of lactate	58	30
Current yield (nmol L-lactate/cm^2/h)	738	620
Total turnover number(TTN), (mol L-lactate/mol NAD^+)	123	43
Turnover number (TN), (mmol L-lactate/ mol NAD^+/s)	41	35

Electrocatalytic processes involving viologen and LiDH film electrodes typically exhibit an induction period of about two days.[1] In the case of the pFAD electrode the induction period was three days (instead of two), probably because there are two polymeric films on the electrode, pFAD and Nafion, respectively. This makes mass transport into and out of the electrode more important in the overall process. Slower conversion of pyruvate into L-lactate was also expected with the pFAD electrode because FAD is a weaker reductant than viologen ($\sim$ 0.4V reduction potential vs. SCE for FAD compared to – 0.66V for viologen).

An important argument in favor of the pFAD electrode relative to that containing monomeric FAD (which is easier to prepare) is its longevity; it functioned for 41 days, with constant performance, in two different cells. When the experiment was finally interrupted (after 30 days of running the second cell), the activity of LiDH on the electrode was still 80% of the initial value. This is the longest-lived LiDH electrode we have prepared.[1–3] It also provides another demonstration of the beneficial effect of FAD on LiDH immobilized on an electrode. It may be that FAD helps maintain the tertiary structure of the enzyme thus maintaining it in active form.

In spite of the higher cost of FAD compared to methyl viologen on a per-mole basis, the present electropolymerization procedure is not more expensive because of (a) the very low amounts of FAD used to prepare the polymer, (b) the elimination of the catalytic amounts of NAD^+ and NADH added when the electrode is constructed,[1–3] and (c) the added benefit of extended operational lifetime of LiDH. The use of FAD as redox mediator represents both a step forward towards increasing the purity of the product and an opportunity to solve environmental problems associated with the use of toxic viologen derivatives as mediators in such processes.

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