Cross-Linked LDH Crystals for Lactate Synthesis Coupled to Electroenzymatic Regeneration of NADH

Susan B. Sobolov,*,§ Mihaela Draganoiu Leonida, Anita Bartoszko-Malik, Kamen I. Voivodov,‡ Frank McKinney, Jason Kim, and Albert J. Fry*,†

Department of Chemistry, Wesleyan University, Middletown, Connecticut 06459

Received July 31, 1995 (Revised Manuscript Received November 27, 1995⊗)

Lactate dehydrogenase (LDH) was crystallized from concentrated ammonium sulfate solution and cross-linked with glutaraldehyde to afford long-lived enzymatically active cross-linked crystals (LDH-CLC). The crystals were employed in an electrolytic cell for lactate production from pyruvate, in which the cathode consisted of a carbon electrode containing a coating of lipoamide dehydrogenase (LiDH) immobilized with methyl viologen under a Nafion membrane. This cell was more effective than a similar cell containing LDH in soluble form. An even greater improvement in performance was achieved by chemically binding a viologen derivative to the LiDH and using an electrode based on this modified enzyme in a cell containing LDH-CLC. The activity of the LDH-CLC is much less sensitive to pH than that of the soluble enzyme.

The high cost[1] and instability of the cofactors required in enzymatic syntheses justifies efforts to regenerate them. Since 1980, a number of experiments have been reported in which NADH has been regenerated using electrochemical methods.[2,3] Direct electrochemical reduction of NAD^+ does not produce NADH; instead, a catalytically inactive dimer is formed.[2] A common solution to this problem is to utilize two enzymes in the electrosynthesis; one enzyme transfers a reducing equivalent from an electrogenerated redox mediator to NAD^+, while a second enzyme carries out the actual synthesis using the NADH produced in the first step.[4,5] The synthetic sequence is illustrated in Figure 1, where the first enzyme is lipoamide dehydrogenase (LiDH), the second enzyme is lactate dehydrogenase (LDH), and the redox mediator is methyl viologen (MV^{2+}).[2] A problem encountered in all such electroenzymatic reactions is the instability of the enzymes in solution. We and other research groups have shown that the first (NADH-regenerating) enzyme can be stabilized by immobilizing it on the electrode surface.[1,6−8] However, the instability of the second enzyme remains a problem. For this reason, it is generally necessary to add fresh enzyme to the electrolysis solution periodically during the course of an extended electrolysis.[1−3,9] It is this problem which we wish to address in the present paper.

Many studies have been reported of ways to immobilize enzymes under a variety of conditions to extend their lifetimes for practical use. There are a number of general approaches to this problem, but each has disadvantages of one sort or another.[10] In general, enzymes are much more stable in the crystalline state than in solution. For this reason, enzyme crystallization or precipitation is useful not only as a method of purification but also for long-term storage. This makes the crystalline form of the enzyme very interesting from a practical point of view. However, even under the most favorable conditions, enzyme crystals remain mechanically fragile and this affects their operational stability. The solution to the fragility problem has been found to be cross-linking of enzyme crystals in an insoluble matrix.[11−14] This is usually carried out by cross-linking amino groups on the enzyme with glutaraldehyde, a practice used by crystallographers as early as the 1960s.[14] Cross-linking stabilizes the crystalline structure of the enzyme by forming immobilized enzyme particles which retain the enzymatic activity of crystals. Recent studies have shown cross-linked enzyme crystals (CLC) to be highly pure, very active, stable, and insoluble biocatalysts.[11−13]

In our previously reported experiments,[6,7] the electroenzymatic regeneration of NADH was coupled with electroenzymatic syntheses of several organic acids. Lactic, malic, and glutamic acids were prepared successfully utilizing the appropriate substrates and redox enzymes. Because of the instability of the soluble enzymes studied, it was necessary to add fresh enzyme to the cell every other day in long-term electrosynthetic reactions. As a solution to this problem, we describe here

† Address correspondence to Department of Chemistry, Wesleyan University, Middletown, CT 06459. Phone (860) 685-2622; FAX (860) 685-2211; e-mail afry@wesleyan.edu.

§ Current address: Pfizer Corp., Eastern Point Dr., Groton, CT 06340. Phone (860) 441-1516; FAX (860) 441-6543.

† Current address: Department of Food Science and Technology, University of California, Davis, CA.

⊗ Abstract published in *Advance ACS Abstracts,* February 15, 1996.

(1) Current prices (Sigma) of NADH are $13 000−$25 000 or more per mole, depending on purity.

(2) Fry, A. J.; Sobolov, S. B.; Leonida, M. D.; Voivodov, K. I. *Tetrahedron Lett.* **1994**, *35*, 5607.

(3) Blaedel, W. J.; Engstrom, R. C. *Anal. Chem.* **1980**, *52*, 1691.

(4) Roerig, P.; Wolff, C. M.; Schwing, J. P. *Anal. Chim. Acta* **1983**, *153*, 181.

(5) DiCosimo, R.; Wong, C.-H.; Daniels, L.; Whitesides, G. M. *J. Org. Chem.* **1981**, *46*, 4622.

(6) Fry, A. J.; ; Sobolov, S. B.; Leonida, M. D.; Voivodov, K. I. *Denki Kagaku* **1994**, *82*, 1260.

(7) Voivodov, K. I.; Sobolov, S. B.; Leonida, M. D.; Fry, A. J. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 681.

(8) Chang, H.; Matsue, T.; Uchida, I. In *Electroorganic Synthesis-Festschrift for Manuel M. Baizer;* Little, R. D., Weinberg, N. L., Eds.; Dekker: New York, 1991, p 281.

(9) Grimes, M. T.; Drueckhammer, D. G. *J. Org. Chem.* **1993**, *58*, 6148.

(10) (a) Zaborsky, O. R. *Immobilized Enzymes,* CRC Press: Cleveland, 1973. (b) Wong, C.-H.; Whitesides, G. M., Elsevier: Tarrytown, NY, 1994.

(11) St. Clair, N. L.; Navia, M. A. *J. Am. Chem. Soc.* **1992**, *114*, 7314.

(12) Navia, M. A.; St. Clair, N. L.; Griffith, J. P. in *Stability and Stabilization of Enzymes (Proceedings of Int. Symposium)*; van den Tweel, W. J. J.; Harder, A., Buitelaar, R. M., Eds.; Elsevier Science Publishers B.V.: Maastricht, The Netherlands, 1993; p 63.

(13) Sobolov, S. B.; Bartoszko-Malik, A.; Oeschger, T. R.; Montelbano, M. M. *Tetrahedron Lett.* **1994**, *35*, 7751.

(14) (a) Quiocho, F. A.; Richards, F. M. *Proc. Natl. Acad. Sci. U.S.A.* **1964**, *52*, 833. (b) Quiocho, F. A.; Richards, F. M. *Biochemistry* **1966**, *5*, 4062. (c) Quiocho, F. A.; Bishop, W. H.; Richards, F. M. *Proc. Natl. Acad. Sci. U.S.A.* **1967**, *57*, 525.

0022-3263/96/1961-2125$12.00/0

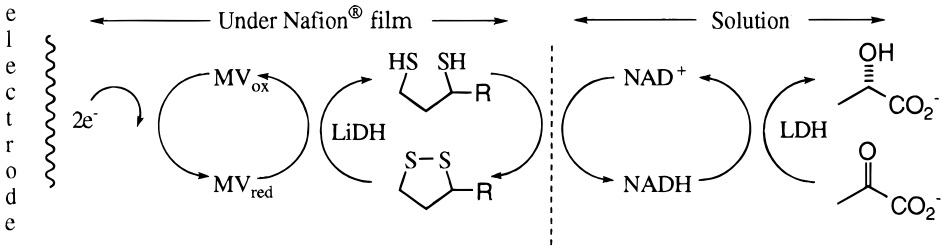

Figure 1. Electron flow in the methyl viologen-mediated electroenzymatic reduction of pyruvate to lactate employing methyl viologen (MV) and lipoamide dehydrogenase (LiDH) immobilized under a Nafion film on a carbon electrode and lactate dehydrogenase (LDH), NAD^+, and pyruvate in solution.

the enzymatic synthesis of L-lactate using a combination of cross-linked crystals of lactate dehydrogenase (LDH-CLC) suspended in solution for synthesis and an NADH-regenerating electrode with lipoamide dehydrogenase (LiDH) and a methyl viologen (MV^{2+}) salt immobilized on the electrode. We also describe a similar cell in which the LiDH is covalently modified with a viologen derivative[7] to enhance the working stability of the enzyme and prevent leakage of the toxic mediator.[15]

Experimental Section

Enzyme assays were carried out at 340 nm using a Perkin-Elmer Lambda 2 UV/vis spectrophotometer and its resident software, using as background the mixture without enzyme and cofactor. Rabbit muscle lactate dehydrogenase (EC 1.1.1.27) and Torula yeast (*Candida*) lipoamide dehydrogenase were obtained from Sigma Biochemicals. The synthesis of lipoamide dehydrogenase covalently modified with an alkyl viologen was described in ref 7.

LiDH Activity Assay. To 1 mL of 0.05 M of pH 6.5 sodium phosphate buffer in a spectrophotometer cuvette was added 30 μL of EDTA disodium salt (50 mM aqueous solution containing 2% bovine serum albumin); 30 μL of β−NADH (7 mM in 0.05 M phosphate buffer, pH 6.5), 30 μL of NAD^+ (20 mM in 0.05 M phosphate buffer, pH 6.5), 30 μL of thioctic amide (28 mM; 20 mg/2 mL of absolute ethanol and 1.5 mL of 0.05 M phosphate buffer, pH 6.5), and 30 μL of LiDH (0.6 U/mL of 0.05 M phosphate buffer). After mixing by inversion, the decrease of optical density was recorded at 340 nm at room temperature. The activity of the enzyme was calculated as units per mg of enzyme in reaction mixture. One unit activity reduces 1.0 μmol of D,L-lipoamide to D,L-dihydrolipoamide per minute at 25 °C at pH 6.5.

Immobilized LiDH Activity Assay. In a batch reactor[6,7] equipped with magnetic stirrer were mixed 15 mL of 0.05 M sodium phosphate buffer, pH 6.5; 0.45 mL of EDTA disodium salt (50 mM aqueous solution containing 2% bovine serum albumin); 0.45 mL of β−NADH (7.0 mM in 0.05 M phosphate buffer, pH 6.5); 0.45 mL of NAD^+ (20 mM in 0.05 M phosphate buffer, pH 6.5); 0.45 mL of thioctic amide (28 mM; 20 mg/2 mL of absolute ethanol and 1.5 mL of 0.05 M phosphate buffer, pH 6.5), and LiDH immobilized on a reticulated vitreous carbon (RVC) electrode under a Nafion membrane.[6,7] The contents of the reactor were circulated to a spectrophotometer flow microcell using an ISCO Model 2300 HPLC pump, and the change of absorbance at 340 nm was recorded.

L-LDH Activity Assay. In a reaction mixture of total volume of 1.0 mL at 37 °C were mixed 0.93 mL of β−NADH (0.13 mM in 0.1 M sodium phosphate buffer, pH 7.0), 40 μL of LDH (0.25−0.75 U/mL of solution in the same buffer containing 1% (w/v) bovine serum albumin (BSA)), and 30 μL of 1.1 mM sodium pyruvate solution in 0.1 M phosphate buffer, pH 7.0). The decrease of optical density at 340 nm for 5 min was monitored. Activity of the enzyme was calculated as units per mg of enzyme in reaction mixture. One unit activity reduces 1.0 μmol of pyruvate to L-lactate per minute at pH 7.0 at 37 °C.

Crystallization of L-Lactic Dehydrogenase (EC 1.1.1.27) (Type XI: from Rabbit Muscle, Sigma). Lyophilized L-lactic dehydrogenase (0.05 g) was dissolved in 10 mL of 0.1 M phosphate buffer pH 7.5 containing 0.1 mM EDTA. Solid ammonium sulfate was added to the mixture over a period of 1 h until a faint turbidity appeared (a total of 3.5 g), and 0.5 mL aliquots were dispensed into 22 test tubes. After approximately 1 h the enzyme began to crystallize. The crystals were allowed to form for several days at 25 °C. They were dissolved for activity measurements and were found to retain 70% of the original soluble enzyme activity.

Cross-Linking of LDH with Glutaraldehyde. Solutions of glutaraldehyde of different concentration (5.0, 7.5, 10.0, 12 .5, and 15 mM) were made up from a 25% aqueous solution of glutaraldehyde and used to determine conditions for obtaining the maximum activity of final product. The best results were obtained by using the 15 mM solution. Crystalline LDH (2.5 mg) was placed in 1 mL of ice-cold 0.5 M triethanolamine buffer (pH 7.5) containing 0.05 mL of 25% aqueous glutaraldehyde (in phosphate buffer no solid product was formed). The reaction mixture was shaken overnight at 4 °C. After centrifuging, the solid product was washed with water and then lyophilized before use in the electrolysis cell.

Assay of the LDH-CLCs. The lyophilized solid (50 mg) was placed in the reaction vessel equipped with a magnetic stirrer together with 3.7 mL of NADH (13 mM in 0.1 M phosphate buffer, pH 7.5) and 120 μL of sodium pyruvate (34 mM in 0.1 M phosphate buffer, pH 7.5) and connected through a Teflon filter to a spectrophotometer flow microcell. The change in absorbance at 340 nm was monitored for 10 min, using as the background correction all the reagents except the cofactor and the enzyme. The activity of the LDH-CLC was calculated as units per mg of solid; 30% of the original lyophilized LDH activity and approximately 40% of that of the crystals was retained.

Synthesis of L-Lactate by Preparative Electrolysis. We have described in detail the procedure used for these electrolyses in our previous publications.[1,,6,7] However, at the request of a reviewer of the paper, we repeat some of the details here. A 2 mg sample of LiDH is dissolved in 2 mL of 0.6 mM FAD^+/2 mM NAD(H) solution, and the entire solution is evaporated onto the electrode by alternate syringe injection and evaporation by air drying. The RVC electrode is then dipped once into an 0.63% aqueous alcohol solution of Nafion and air-dried. In order to avoid possible leakage of toxic viologen derivatives, the electrode is rinsed repeatedly with phosphate buffer before use. The enzyme electrode is placed in a solution containing 0.145 M sodium pyruvate, 2 mM NAD^+, and the cross-linked crystals of LDH (20 U) in a total volume of 100 mL of 0.2 M pH 7.0 sodium phosphate buffer. The LDH-CLCs were either added directly to the solution or enclosed in dialysis tubing. Electrolysis was carried out at pH 7.0. The cell is constructed with a platinum wire counterelectrode and a calomel reference electrode. The cell is degassed by nitrogen flow for 30 min, and the electrolysis is run under nitrogen. The cathode potential is maintained at −0.9V vs SCE during the course of the reaction. Lactate

(15) Armstrong, F. A.; Hill, H. A. O.; Walton, N. J. *Accounts Chem. Res.*, **1988**, *21*, 407.

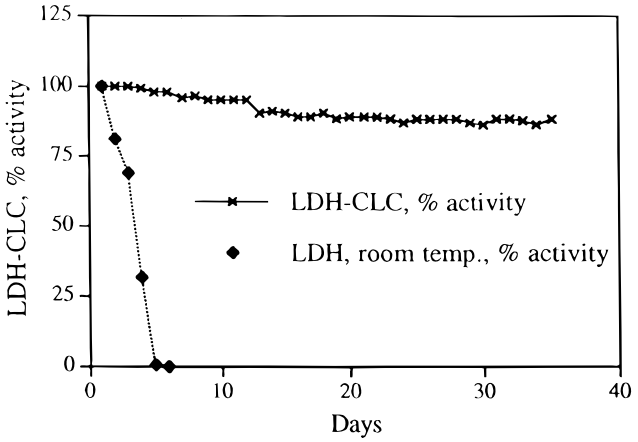

Figure 2. Activity−time relationships for lactate dehydrogenase in solution (LDH) and cross-linked crystals of lactate dehydrogenase (LDH-CLC), respectively. Activities are expressed as a percent of the initial activity of each form of the enzyme. The LDH-CLCs were stored at 25 °C between measurements.

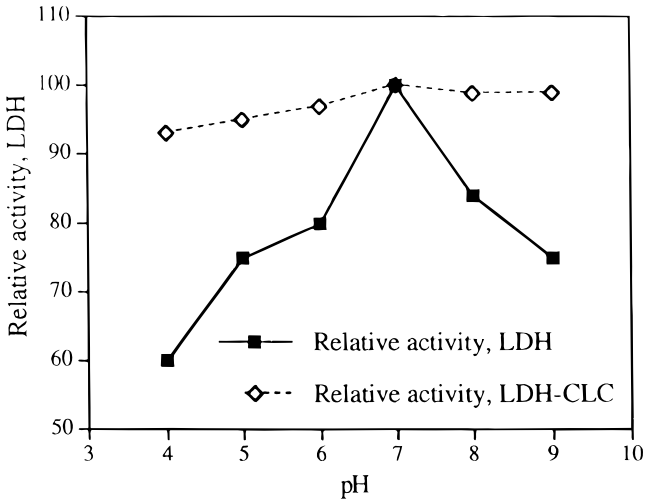

Figure 3. Activity−pH relationships for lactate dehydrogenase in solution (LDH) and cross-linked crystals of lactate dehydrogenase (LDH-CLC), respectively. Activities are expressed as a percent of the initial activity of each form of the enzyme.

production begins after an induction period of up to 2 days (see Discussion). The progress of lactate synthesis was monitored by NMR and reverse-phase HPLC. HPLC analyses were carried out using a Hamilton PRP-X100 anion-exchange column; the mobile phase was 1:2 acetonitrile:0.1 M pH 4.5 phosphate buffer at a flow rate of 1.5 mL/min.

Results and Discussion

Lactate dehydrogenase has been crystallized under a variety of conditions.[16] We obtained the best results when the enzyme was crystallized from 35% ammonium sulfate. The crystals obtained retained 70% of the dry enzyme activity. The crystals were cross-linked chemically with 15 mM glutaraldehyde in order to achieve greater operational stability of the enzyme. After varying the conditions for cross-linking the crystals using different concentrations of glutaradehyde and different buffers, we found our stable new biocatalyst to have 30% of the original activity overall. As we expected, LDH-CLCs are much more stable than the soluble enzyme (Figure 2). The half-life for the soluble enzyme in both storage and under electrolysis conditions is 2.5 days. However, after a decline in activity of about 10% in the first 10 days, the LDH-CLCs retained constant activity over the next 25 days. It is likely that the initial decline is the result of a small amount of dissolution of the crystals during treatment with glutaraldehyde, followed by cross-linking of the soluble enzyme. It is known that the amorphous precipitates produced by cross-linking enzymes from solution lose their activity much faster than do the corresponding cross-linked crystals.[11−14] After lyophilization the LDH-CLCs can be stored for a long period of time at room temperature or used in extended electroenzymatic experiments while retaining their catalytic activity.

We made a surprising and potentially quite useful discovery in the course of these experiments. The pH optima for LiDH and LDH in solution differ by one unit (6.5 and 7.5, respectively). In previous experiments,[6,7] the pH of the electrolysis solution was buffered at 7.0, a compromise betwwen the optimal pH of each enzyme. As shown in Figure 3, the activity of cross-linked lactate dehydrogenase in 0.1 M phosphorous buffer is relatively constant throughout the pH range tested (4.0−9.0), which would allow the LDH crystals to be used over a much wider range of conditions than are normally possible with lactate dehydrogenase. For example, the electrolysis could be run at the pH for maximum activity for lipoamide dehydrogenase if so desired.

We used the LDH-CLC to effect the electroenzymatic synthesis of lactate and compared the results with those obtained from earlier experiments with soluble LDH.[6,7] The latter were carried out as follows: a modified reticulated vitreous carbon (RVC) working electrode was employed onto which LiDH and a viologen salt had been previously deposited, after which the deposit was covered with an outer layer of Nafion.[2,6] The water-insoluble Nafion layer prevents either LiDH or the mediator from dissolving in the electrolysis solution and extends the lifetime of the immobilized enzyme. When this electrode was immersed in a buffer solution containing pyruvate, NAD^+, and LDH-CLC, and polarized at a potential corresponding to the first voltammetric wave of viologen, lactate was produced after an induction period of up to 2 days. The yield of lactate was higher when the LDH-CLCs were used (70% compared to 50% for the standard cell containing soluble LDH), while the current yield (yield based on current consumed, assuming consumption of 2 Faradays per mol of pyruvate) improved by a factor of 3 as compared with the synthesis utilizing soluble LDH (2115 nmol/cm^2/h).[1] While in the standard cell a new sample of soluble lactate dehydrogenase was added every other day (23 U), in the present cell we only use one batch of crystals (approximately 20 U). In the latter case a more appropriate term is *apparent activity* because when we calculate the units used, we use the gross weight of the sample with no correction for the glutaraldehyde (assuming this weight to be negligible as compared with the enzyme); the specific activity is therefore actually higher.

After 25 days the cell was disconnected because of the decrease in the rate of lactate formation as a result of the diminishing activity of the immobilized LiDH during this time.[2,6] Only 50% of the original activity of the LiDH remained after 25 days, and at this low activity, self-

(16) (a) Pickles, P.; Jeffery, B. A.; Rossmann, M. G. *J. Mol. Biol.* **1964**, *9*, 598. (b) Schär, H.-P.; Zuber, H.; Rossmann, M. G. *J. Mol. Biol.* **1982**, *154*, 349.

condensation of pyruvate is faster than lactate formation.[2] Only 20% of the initial LDH-CLC weight was retrieved from the electrolysis in our initial experiments. The loss of material was due to (a) losses during daily sampling and (b) mechanical destruction of the crystals by the magnetic stirring bar (conversion to a fine powder) during the extended period of time required for the electrolysis. We therefore carried out the experiment with the LDH-CLC enclosed in dialysis tubing during the electrolysis. From this cell we were able to recover 70% of the initial LDH-CLC crystals. They had retained complete activity and could have been reused if so desired. (Note that LDH in solution, with a half-life of under 2 days, would not have survived for the 25 days which this electrolysis was carried out.) The turnover number (TN; 64 mol of lactate/mol of NAD^+/s) and the total turnover number (TTN; 87 mol of lactate/mol of NAD^+) represent a 2-fold increase in comparison to the cell which utilized soluble LDH.[1,2]

The LDH-CLCs were also tested in a cell in which LiDH was covalently bound to a viologen derivative before immobilization in the Nafion film.[7] Attachment of the redox mediator to the enzyme enhances communication between the redox centers of the enzyme and the electrode[17,18] and appears to cause relatively little alteration of the enzyme structure. The result is a dramatic decrease in the leakage of the mediator together with an extended life of LiDH.[7] In all cases, FAD^+ (1 mg/mL) was added (to compensate for any which might have been lost from LiDH during purification and handling); this was found to increase enzyme activity.[7] The results obtained by coupling the enzymatic action of LDH-CLC with the regeneration performed by the modified enzyme electrode were even better than those obtained with the cell containing unmodified LiDH (79% yield in lactate, 4242 nmol/cm^2/h current yield, TN = 237 mol of lactate/mol of NAD^+/s and TTN = 368 mol of lactate/mol of NAD^+), corresponding to a 4-fold enhancement in efficiency over our previous electrolyses.[2,6] This is consistent with our earlier experiments using the chemically modified enzyme and soluble LDH.[7]

As we have pointed out earlier, an induction period is observed in all electrolyses which we have carried out involving LiDH immobilized in a polymer film on the electrode surface.[2,6,7] We have previously reported experiments designed to probe the origin of this induction period.[6,7] In the course of the present work, we tested the hypothesis that it takes some time for the Nafion film to become permeable to NAD^+ and NADH. To do this, a cell was assembled in the usual fashion but through which no current was passed for 3 days. As expected, no lactate was formed during this time. At this point current was passed through the solution. After only 6 h lactate had already been formed in sizeable amounts (NMR). Thus it appears that the induction period arises in part because the Nafion film, as initially prepared (dry), is not fully permeable and that it is immersion in the electrolysis solution, not passage of current, which is necessary to activate the film for NADH production. However, this is probably not the only factor involved. An electrolysis was carried out using the modified enzyme electrode and LDH in solution, but only half the usual amount of NAD^+ (1 mM). The induction period was longer in this case (4 days instead of 2). This supports the idea that the rate of NAD^+/NADH diffusion through the Nafion layer (especially NAD^+) is part of the reason for the induction time observed, together with polymer film conditioning. Subsequent increases in NAD^+ concentration by factors of 2, 3, and 4, respectively, did not result in commensurate increases in the rate of lactate formation. This suggests that the rate-determining step in the synthesis is a combination of both slow NAD^+/NADH diffusion through the Nafion film and conditioning of the film. We also noted that the induction period is shorter when the LDH-CLCs were crushed before use. This is consistent with the suggestion that diffusion is faster in smaller crystals.[11,12]

Practicality. A reviewer disputed the value of this approach using cross-linked crystals (CLCs) and asked that their practicality be addressed. We point out a number of advantages deriving from the use of CLCs in this work: electrolyses can go unattended without the need to constantly add LDH, with its short solution half-life of less than 2 days (LDH-CLCs produced in this laboratory 15 months ago still retain their activity); the CLCs can be retrieved and reused when desired,[13] thus reducing the cost of carrying out the electrolysis. We should note however that, unlike an earlier series of experiments involving the enzyme aldolase,[13] the conditions for crystallization and cross-linking of the enzyme were not optimized in the present work. The yield of CLCs could undoubtedly be improved in future work. We also note that although the use of cross-linked enzyme crystals dates to only 1992, these materials have already spawned a new industry.[19]

Summary. We have shown that cross-linked lactate dehydrogenase crystals (LDH-CLC) have high stability and retain their enzymatic activity as biocatalysts during electroenzymatic electrolyses for long times. This new form of LDH[20] will be an attractive component in schemes for recycling redox cofactors during electroenzymatic synthetic reactions.

Acknowledgment. Financial support was generously supplied by the National Science Foundation and the Electric Power Research Foundation through Grant CHE-9208562. Frank McKinney of Prairie View A&M University was the recipient of a summer undergraduate research fellowship at Wesleyan University from the Howard Hughes Foundation.

JO951408V

(17) Marcus, R. A.; Sutin, N. *Biochim. Biophys. Acta* **1985**, *811*, 265.
(18) (a) Heller, A. *J. Phys. Chem.* **1992**, *96*, 3579. (b) Heller, A. *Acc. Chem. Res.* **1990**, *23*, 128.
(19) Altus Biologics, Inc., Cambridge, MA, now supplies CLCs for a variety of synthetic purposes.
(20) LDH has previously been cross-linked using using diimido esters; this procedure also increases its active lifetime: Bayne, S.; Ottesen, M. *Carlsberg Res. Commun.* **1976**, *41*, 211. (b) Bayne, S.; Ottesen, M. *Carlsberg Res. Commun.* **1977**, *42*, 465.