Membrane-Enclosed Electroenzymatic Catalysis with a Low Molecular Weight Electron-Transfer Mediator

Michael T. Grimes[1] and Dale G. Drueckhammer*

Department of Chemistry, Stanford University, Stanford, California 94305

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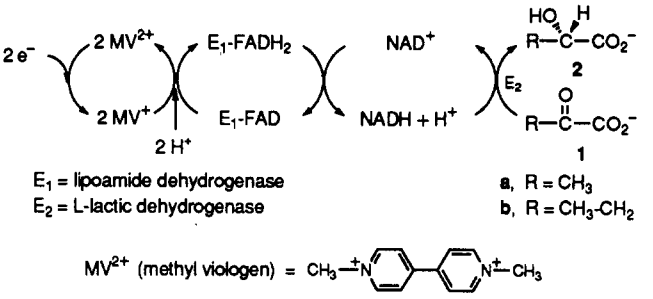
The coupling of enzyme catalyzed reactions to electrochemistry has been demonstrated in a number of synthetic applications.[2-4] Diffusible mediators, usually 4,4′-bipyridyl (viologen) derivatives, are generally used to facilitate electron transfer between electrodes and enzyme-bound cofactors. Nonenzymatic reaction between reduced mediator and typical ketone substrates has been perceived to not generally be a problem. In initial efforts to extend the scope of electroenzymatic synthesis to new enzymes, we have observed that while nonenzymatic decomposition of substrates by reduced methyl viologen is certainly very slow, it can become significant with a 2 mM or greater concentration of reduced methyl viologen over a reaction time of several days. Simon and co-workers have demonstrated apparent oxidation of reduced benzyl viologen by α-keto acids, though the products were not identified.[5] Previous work has found optical purities in electroenzymatic processes[3] significantly lower than the optical purities using the same enzyme in a nonelectrochemical process.[6] The decreased optical purity is likely due to a small degree of competing nonenzymatic reduction of substrate by reduced mediator to form racemic product.

Membrane-enclosed enzyme catalysis has also been well demonstrated.[7] A membrane-enclosed reactor has recently been reported for electroenzymatic oxidation reactions in which a ferrocene derivative attached to poly(ethylene glycol) is used as mediator.[8] This high molecular weight mediator is retained, along with enzyme, inside the membrane with substrates and products diffusing between the membrane-enclosed volume and a much larger volume outer solution. Studies were initiated in this lab to develop simple laboratory procedures for using a similar membrane-enclosed reactor to perform electroenzymatic reduction reactions, for which a more reducing mediator such as a viologen derivative would be required. While high molecular weight viologen derivatives have been prepared by attachment to a protein,[9] we reasoned that confinement of the mediator inside the membrane might not be necessary and that a low molecular weight mediator could be used. With a relatively low concentration of mediator, the quantity of reduced mediator permeating through the membrane would be small and thus only the contents of the membrane would be in contact with high concentrations of reduced mediator. Here, we report a membrane-enclosed electroenzymatic process using methyl viologen as the electron-transfer mediator.

Scheme I



Results and Discussion

A coupled reaction sequence shown in Scheme I was chosen as a simple model for these studies. This consists of an NADH-dependent enzymatic reduction reaction coupled with enzyme-mediated electrochemical cofactor regeneration and is similar to previously reported electroenzymatic processes.[3,4] In electrochemical NADH regeneration by lipoamide dehydrogenase, reduction of the lipoamide dehydrogenase by mediator is the rate-limiting step as indicated by an increase in rate with increasing concentration of mediator.[3] A fairly high concentration of reduced mediator is thus required to achieve an acceptable rate with a minimal amount of enzyme. This increases the tendency for nonenzymatic reaction between substrate and reduced mediator. This is in contrast to some other enzymatic processes involving mediated electron transfer in which reduction of oxidized mediator is the slow step and the steady-state concentration of reduced mediator is thus very low.[5]

For membrane enclosed electroenzymatic catalysis to be successful, permeation of substrate through the membrane should be sufficiently rapid to not limit the rate of the reaction. In preliminary experiments, the rate of permeation of α-ketobutyrate **1b** through the dialysis membrane was measured. With a membrane containing an internal volume (V) of 1.0 mL of a 0.2 M α-ketobutyrate solution and a membrane surface area (A) of 8.0 cm^2, a first-order rate constant (k) of 3.8×10^{-4} s^{-1} was determined for permeation of α-ketobutyrate out of the membrane into a 0.2 M NaCl solution. This gives a permeation coefficient (P) of 4.8×10^{-5} cm s^{-1} using eq 1.[10]

$$P = k(V/A) \quad (1)$$

The rate of permeation of methyl viologen was also measured and was identical to that of α-ketobutyrate within experimental accuracy. While permeation of the reduced form of methyl viologen was not measured directly, it is expected to also be very similar.

The reactor was constructed as shown in Figure 1 using a 2.7-cm i.d. × 6.0-cm high glass TLC jar. A platinum gauze cathode, a platinum wire anode, and a Ag/AgCl reference electrode were inserted through openings in the lid. The anode was separated from the reactor solution by a fritted glass disk. The cathode was covered by a cellulose membrane (dialysis tubing, molecular weight

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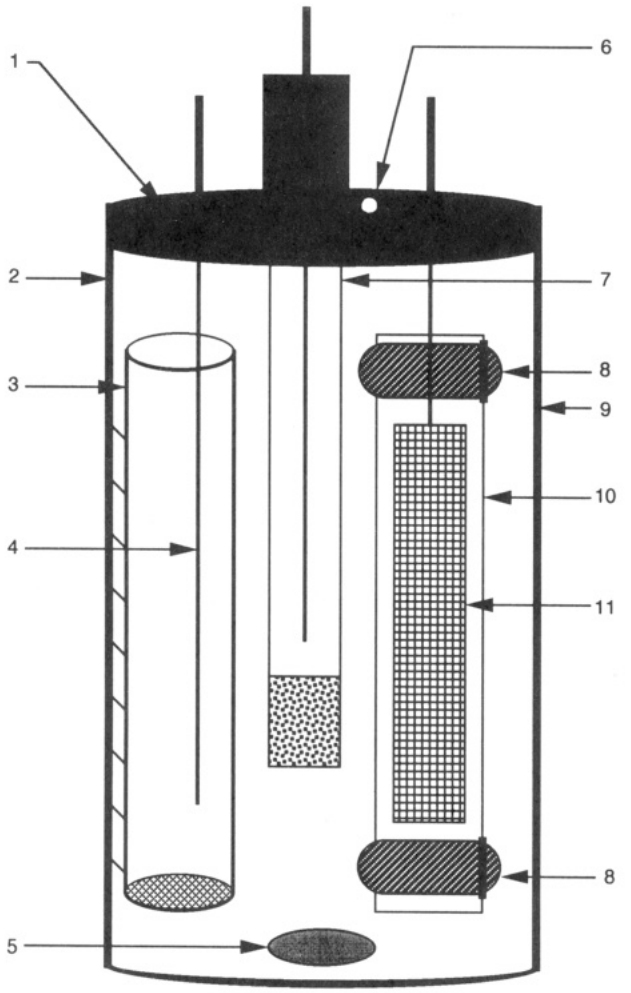

Figure 1. Membrane reactor design: (1) electrochemical cell cover with attached platinum wire auxiliary electrode; (2) glass TLC developing jar (2.7-cm i.d. × 6-cm h); (3) 12-mm o.d. tube with fritted glass disk (4–8 μm pore size, cut to 4-cm length); (4) platinum wire anode (inside fritted tube 3); (5) stir bar; (6) opening for inert gas tube; (7) Ag/AgCl reference electrode; (8) membrane clamps; (9) liquid level; (10) cellulose membrane; (11) platinum mesh cathode.

cutoff 12 000–14 000, 1.0 cm flat width) sealed at the top and bottom by plastic dialysis tubing clamps. The enzymes L-lactate dehydrogenase and lipoamide dehydrogenase were contained in the membrane (inner volume 0.20 mL) with the α-keto acid (2.5 mmol), NAD (3.0 μmol), and methyl viologen (75 μmol) freely diffusing through the membrane between the inner and outer (volume 15 mL) solutions. The outer solution was purged with nitrogen for 0.5 h before applying a potential at the cathode. The cathode potential was set at −740 mV vs Ag/AgCl, and the contents of the membrane quickly developed the blue color of reduced methyl viologen. An initial current of about 1.2 mA was observed. In a control without enzyme the current was about 0.2 mA. Samples were removed periodically from both the inner and outer solutions and assayed for α-keto acid concentration. An initial rate of disappearance of α-keto acid of about 0.30 μmol min^{-1} was observed, which is consistent with the observed current. The inner solution was also assayed for remaining enzyme activities, and more lipoamide dehydrogenase was added every 2 days. The reaction was >95% complete after 8 days.

The measured permeation coefficient (P) of 4.8×10^{-5} cm s^{-1} and a membrane of surface area (A) 8 cm^2 predicts

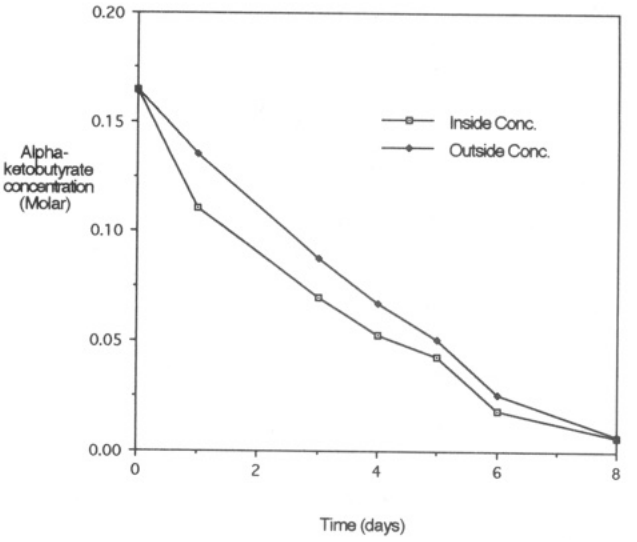

Figure 2. Plot of concentration of α-ketobutyrate inside the dialysis membrane and in the outer solution vs time over the course of the electroenzymatic reduction.

a quantity (Q) permeating through the membrane of 3.8 μmol min^{-1} for α-ketobutyrate and 0.11 μmol min^{-1} for methyl viologen at their initial concentrations (C) using eq 4, derived from eqs 2 and 3.

$$Q = kVC \tag{2}$$

$$k = P(A/V) \tag{3}$$

$$Q = PAC \tag{4}$$

This indicates that permeation of α-keto acid into the membrane-enclosed reactor solution should not have been rate limiting. The concentration vs time profile for the inner and outer solutions in the reduction of α-ketobutyrate is shown in Figure 2. The α-ketobutyrate concentration of the inner solution remained at >70% that of the outer solution throughout the reaction. This indicates that as expected, the reaction rate was not limited by permeation of substrate into the membrane enclosed solution. In a control experiment without enzyme, no measurable change in α-keto acid concentration was observed after 1 day. In a similar setup without enzyme and without the membrane, α-keto acid concentration decreased about 5% per day over 3 days.

The predicted rate of permeation of reduced methyl viologen is in fact quite significant relative to the rate of the reaction. However, the outer solution remained colorless throughout the course of the reaction. The goal of having only the small membrane enclosed volume (1.3% of total volume) in contact with reduced mediator was thus achieved. The reduced viologen permeating through the membrane was perhaps oxidized by residual oxygen present in the outer solution, even after purging with nitrogen. The products (L)-lactic acid **(2a)** and (L)-2-hydroxybutyric acid **(2b)** were obtained in >98% ee as indicated by ^{1}H-NMR analysis of the (*R*)-MTPA derivatives.[6,11,12] This contrasts the nonmembrane reactor which was reported to give 94% ee in the reduction of pyruvate **1a** to (L)-lactic acid **(2a)**.[3]

The application of membrane-enclosed catalysis to electroenzymatic catalysis, as demonstrated here, may be

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useful for minimization of nonenzymatic reactions thereby increasing optical purity of product. This work has shown that a readily available low molecular weight electron transfer mediator may be employed, avoiding the synthesis of more complex mediators. This technique may be useful in laboratory scale-preparative electroenzymatic reactions and in research directed at further developments in electroenzymatic synthesis. A membrane reactor strategy may be essential in electroenzymatic reduction reactions of substrates such as imines which are more easily reduced electrochemically and would thus be more susceptible to nonenzymatic reduction.[13] A disadvantage of membrane-enclosed catalysis is that in larger scale processes the rate of the reaction may ultimately be limited by diffusion of substrate through the membrane.

Experimental Section

General Procedures. L-Lactic dehydrogenase (rabbit muscle), lipoamide dehydrogenase (porcine heart), other biochemicals, methyl viologen, and cellulose dialysis tubing were obtained from Sigma. (*R*)-(+)-MTPA-Cl was obtained from Aldrich. NMR spectra were obtained at 400 MHz in $CDCl_3$.

Measurement of Rates of Diffusion through a Cellulose Membrane. A membrane with surface area of 8.0 cm^2 containing 1.0 mL of 0.2 M sodium α-ketobutyrate **1b** was immersed in 100 mL of 0.2 M aqueous NaCl. The solution was mixed with a magnetic stirrer. Aliquots of the outer solution were removed every 5 min and assayed for α-ketobutyrate by enzymatic assay using L-lactate dehydrogenase. A first order-rate constant of 3.8×10^{-4} sec^{-1} was found. Diffusion of methyl viologen was measured similarly with methyl viologen appearance in the outer solution quantitated by measurement of absorbance at 257 nm using a molar absorbtivity of 17 700. Again, a first-order rate constant of 3.8×10^{-4} s^{-1} was found.

Electroenzymatic Reduction of α-Ketoacids. The reaction was performed in the reactor described in the Results and Discussion. The outer solution contained sodium phosphate (50 mM, pH 8.0), K_2SO_4 (50 mM), methyl viologen (5 mM), β-mercaptoethanol (2 mM), pyruvate **1a** or α-ketobutyrate **1b** (0.15 M), and NAD (3.0 μmol) in 15 mL of H_2O. A solution of the enzymes L-lactate dehydrogenase (10 units) and lipoamide dehydrogenase (100 units) in 200 μL of the outer solution was pipetted into the membrane which was then submerged in the outer solution. The anode chamber was filled with 50 mM aqueous K_2SO_4 solution. The outer solution was purged with nitrogen for 0.5 hour to remove oxygen. The cathode potential was set at −740 mV vs Ag/AgCl. Samples were removed daily from both the inner and outer solutions and assayed for α-keto acid concentration using L-lactic dehydrogenase. The inner solution was also assayed for remaining enzyme activities, and more lipoamide dehydrogenase was added every 2 days. The reaction was complete in 8 days. The product was isolated by acidification of the reactor solution followed by extraction with ethyl acetate (5 x 40 mL).

Optical Purity Determination. Optical purity was determined by conversion of the α-hydroxy acid to the methyl ester with diazomethane followed by conversion to the MTPA ester as described previously.[6] The 400-MHz ^{1}H-NMR spectrum showed >98% ee for both compounds, key resonances for the major diastereomers being the methyl ester at 3.8 ppm and the methyl ether at 3.65 ppm.

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