Catalytic Activity of Native Enzymes during Capillary Electrophoresis: An Enzymatic "Microreactor"[1]

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This work evaluates the use of a plug of enzyme, migrating in an electrophoresis capillary under nondenaturing conditions, to convert substrate (which may be injected onto the capillary as a separate plug or included in the electrophoresis buffer) to product. This concept is demonstrated using two systems: the irreversible oxidation of glucose-6-phosphate (glc-6-P) to 6-phosphogluconate using glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) and either NAD or NADP as cofactor, and the reversible conversion of ethanol to acetaldehyde using yeast alcohol dehydrogenase (YADH, EC 1.1.1.1) and NAD(H) as cofactor. These procedures illustrate the use of the electrophoresis capillary as a microreactor in which reactants and products are moved into and out of contact with one another based on differences in electrophoretic mobilities. It offers a useful approach to the manipulation of enzymes and enzyme-catalyzed reactions on a microscale.

Introduction

We outline experiments in which enzyme-catalyzed reactions occur in an electrophoresis capillary[2] and differential electrophoretic mobilities[3] of reactants and products are used in their identification and quantitation. We describe two types of enzyme-catalyzed reactions using models to illustrate the interplay of the mobilities of the enzyme, substrates, and products in the analysis of electropherograms during the reaction in the capillary.

Plug–Plug Reaction. In the first example (Figure 1), a sample of enzyme and of substrate are injected separately onto a capillary and subjected to electrophoresis; differential mobility in electrophoresis is used to bring them into contact. Since the enzyme and substrate(s) usually have different electrophoretic mobilities, the relative order and timing of injection onto the capillary can be used to ensure contact: in general, the more slowly migrating component is injected onto the capillary first. Differential mobility also separates products from reactants. Since the enzyme migrates (to an approximation) as a plug under conditions of capillary electrophoresis,[4] the confined volume of the enzyme in the capillary can be considered as a microreactor:[5] This type of process in the capillary is a reaction between two independently migrating plugs of reactive species (one of which is the enzyme) (Figure 1).

Continuous Reaction. In a second protocol (Figure 1), a sample of the enzyme is injected onto a capillary that is equilibrated with a buffer containing the substrate(s) for that enzyme. This protocol is the extension of the plug–plug reaction to the reaction of a plug of enzyme with a continuous stream of reactants (essentially an indefinitely long plug). This type of a reaction in the capillary is analogous to a continuous flow reactor. In continuous reaction experiments, we were able to detect 1 pg (10^{-17} mol; 10 atamol) of glucose-6-phosphate dehydrogenase (G6PDH) based on its activity in converting NAD to NADH; with optimization, smaller quantities of enzyme could certainly be detected. Regnier et al., in

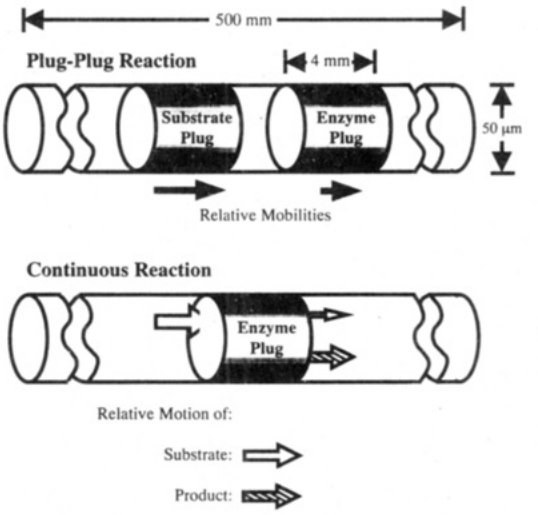

Figure 1. The substrate, of higher electrophoretic mobility than the enzyme, moves into the plug of enzyme and is consumed by enzyme-catalyzed reaction; the remaining unutilized substrate migrates away from this plug (the arrow exiting the plug in the continuous reaction format is narrowed to indicate reduction in concentration). A product (hatched arrow; continuous reaction format) that also has a higher electrophoretic mobility than that of the enzyme migrates from the enzyme-containing plug; in this instance its motion is in the same direction as that of the substrate leaving the plug. Different combinations of relative mobilities of the enzyme, substrates, and products will result in different combinations of relative directions of motion to and from the enzyme-containing region. The average mobility of the enzyme may also be modified on binding a charged substrate.

(1) This research was supported by the National Institutes of Health (Grant GM30367) and the M.I.T. Biotechnology Processing Engineering Center (Cooperative Agreement CDR-88-03014).

(2) For background on capillary electrophoresis (CE) see: Grossman, P. D.; Colburn, J. C. *Capillary Electrophoresis: Theory and Practice*; Academic: San Diego, 1992. Kuhr, W. G.; Monnig, C. A. *Anal. Chem.* **1992**, *64*, 389R–407R. Mazzeo, J. R.; Krull, I. S. *BioTechniques* **1991**, *10*, 638–645. Novotny, M. V.; Cobb, K. A.; Liu, J. *Electrophoresis* **1990**, *11*, 735–749.

(3) For background on electrophoretic mobilities under conditions of CE see: Compton, B. J.; O'Grady, E. A. *Anal. Chem.* **1991**, *63*, 2597–2602. Compton, B. J. *J. Chromatogr.* **1991**, *559*, 357–366. Rickard, E. C.; Strohl, M. M.; Nielsen, R. G. *Anal. Biochem.* **1991**, *197*, 197–207. Grossman, P. D.; Colburn, J. C.; Lauer, H. H. *Anal. Biochem.* **1989**, *179*, 28–33. Deyl, Z.; Rohlicek, V.; Adam, M. *J. Chromatogr.* **1989**, *480*, 371–378.

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(5) Smith, J. M. *Chemical Engineering Kinetics*, 3rd ed.; McGraw Hill: New York, 1981; pp 1–32.

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similar independent experiments with this system, were able to detect 10^{-17} mol of G6PDH by monitoring the conversion of NADP to NADPH.[6]

These methods for using enzyme-catalyzed reactions consume only small quantities of enzymes and substrates. They should be readily amenable to automation. Potential applications of these techniques include analyzing samples for enzymatic activities or concentrations of substrates, evaluating molecules as substrates for enzyme-catalyzed reactions,[7] examining mixtures of proteins for activity against a particular substrate, and studying enzyme–inhibitor interactions.[8]

Results and Discussion

The Electrophoresis Capillary as Microreactor. For a typical sample injection volume of 8 nL, the injected sample (the "microreactor") occupies a space that is 50 μm in diameter (the inner diameter of the capillary) and about 4 mm in length. This total volume represents less than 1% of the total volume of the capillary between the point of injection and the point of detection. If one considers the enzyme-containing plug as a fixed reference point (Figure 1), plug(s) containing the substrates and reaction products (even if neutral) can be considered to sweep through this plug as a consequence of differences in electrophoretic mobilities. As the plug migrates toward the detector, the enzyme contained in the plug catalyzes the conversion of substrate(s) present in the buffer to product(s). The resulting electropherograms provide information about the consumption of the subtrates and generation of products in the buffer.

Enzymes/Cofactors. We used the enzymes G6PDH (EC 1.1.1.49) from *Leuconostoc mesenteroides*,[9] and substrate glucose-6-phosphate (glc-6-P), and yeast alcohol dehydrogenase (E.C. 1.1.1.1)[10] and ethanol or acetaldehyde and NAD(P)(H) as model systems to illustrate the concept of a capillary-contained enzymic microreactor. The G6PDH/NADP(H) system has been examined independently by Regnier.[6] NAD(P)(H) served as chromophoric substrates and products with which to monitor the reaction. The electrophoresis was monitored at two wavelengths: 260 nm to observe the *combined* absorbance of NAD(P) and NAD(P)H, and 340 nm for NAD(P)H alone.

Glucose-6-phosphate Dehydrogenase. We chose G6PDH for its ability to use either NAD or NADP as cofactor in oxidizing substrate glc-6-P to 6-phosphogluconate (eq 1).[9] This flexibility provided two directly

$$\text{glucose-6-phosphate} + \text{NAD(P)} \xrightarrow{\text{G6PDH}} \text{6-phosphogluconate} + \text{NAD(P)H} \quad (1)$$

comparable examples in which the electrophoretic mobilities of the oxidized and reduced forms of the cofactors were different. Comparison of the two absorbances allowed us to separate the profiles of NAD(P) and NAD(P)H.

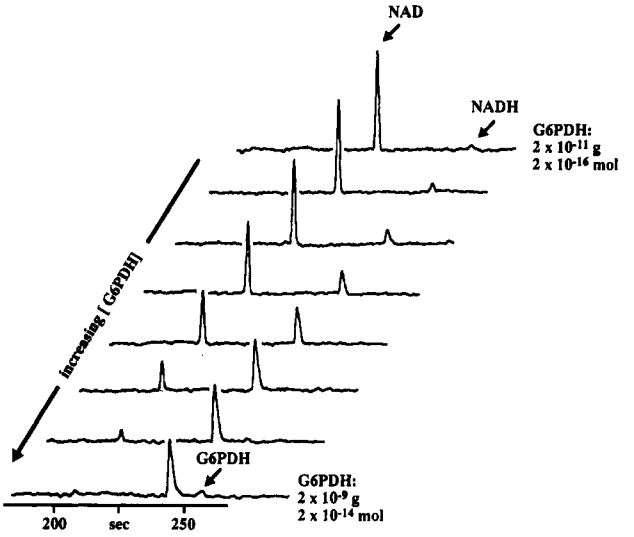

Figure 2. Sequential introduction of plugs of enzyme (G6PDH), buffer, and substrate (NAD, 200 μM) followed by electrophoresis in buffer containing glc-6-P (200 μM) results in enzyme-catalyzed conversion of the substrate to product as the substrate-containing plug migrates through the enzyme-containing plug. The amount of G6PDH in the enzyme plug was serially diluted by a factor of $^1/_2$ in going from one electropherogram to the next.

Yeast Alcohol Dehydrogenase. We also examined the reactions catalyzed by yeast alcohol dehydrogenase (YADH). We chose this enzyme for the reversibility of the oxidation of ethanol and reduction of acetaldehyde using NAD(H) as cofactor (eq 2).[10] Unlike the reactions

$$\text{ethanol} + \text{NAD} \overset{\text{YADH}}{\rightleftharpoons} \text{acetaldehyde} + \text{NADH} \quad (2)$$

catalyzed by G6PDH, the reactions catalyzed by YADH are expected to have the same enzyme-substrate-product complex in either direction. This reversibility means that the migration time of the enzyme plug should be similar in both directions of the enzyme catalyzed reaction; the significance of this condition will be elaborated below.

Plug–Plug Reactions. In an initial experiment, we examined the enzyme-catalyzed reaction when a plug of substrate is introduced into the region occupied by an enzyme. Plugs of NAD and G6PDH were introduced sequentially into the capillary based on initially determined mobilities. In this experiment, the enzyme was injected first. The experiment was designed so that the plug of the NAD was to migrate through the region containing the enzyme (Figure 1) as both moved toward the cathode and the detector (the net movement of ions is determined by electroosmotic flow in these experiments with uncoated capillaries). The second substrate of the reaction, glc-6-P, was present as part of the buffer.

The series of electropherograms shown in Figure 2 shows that the extent of conversion of the substrate NAD to NADH was a function of the amount of enzyme G6PDH in the enzyme plug. The narrow peaks for the product NADH and the unreacted NAD indicate that no dilution has taken place during the mixing of the plug of substrate and the enzyme-containing plug.

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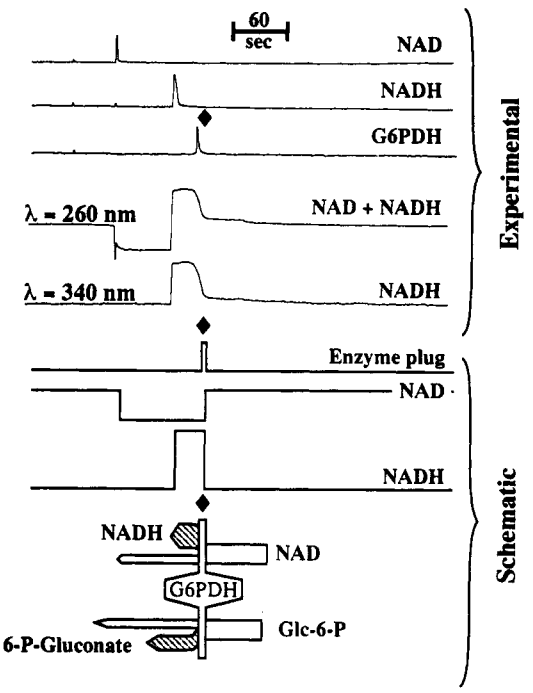

Figure 3. The relative migration times of G6PDH, NAD, and NADH (observed at 200 nm) serve as reference for the remainder of the figure. The experimental electropherograms were obtained at 260 and 340 nm with a plug of G6PDH migrating in a buffer containing NAD (200 μM) and glc-6-P (200 μM). The schematic shows the analysis of the experimental electropherograms in terms of consumption of NAD and generation of NADH. The schematic section also shows the analysis of the experimental electropherograms in terms of the mobilities of the cofactors relative to the plug of enzyme. The arrows indicate substrate flowing through the plug in the direction shown; the narrowed width of the arrow coming out of the plug indicates reduction in the concentration of the substrate. The hatched arrows indicate the flow of the products in the direction indicated. The putative time of arrival of the enzyme at the detector in the presence of the substrates is represented by (♦).

Continuous Reaction. We also examined an approach where a buffer containing substrates passes continuously through a plug of enzyme. We first determined the migration time t_m of NAD, NADH, and G6PDH in buffer (25 mM Tris–192 mM glycine, pH 8.2) when injected separately; the order of migration times was t_m (G6PDH) > t_m (NADH) > t_m (NAD) (Figure 3). In examining enzymatic activities, the capillary was first equilibrated with Tris–glycine buffer containing glc-6-P and NAD; a solution of G6PDH was injected into this buffer and allowed to migrate under an applied potential (30 kV). Conversion of NAD to NADH was easily detected (Figure 3). Examination of these electropherograms showed that one of the boundaries of the profiles for the consumption of NAD and the generation of the NADH coincided with the migration times of the two components in the Tris–glycine buffer alone.[11] Because the electrophoretic mobility of G6PDH is higher (and the elution time correspondingly longer) than that of both NAD and NADH, we visualize the NAD and NADH as being fed into and being swept away from the plug in the direction shown in Figure 3. The G6PDH was not observed directly; the putative migration time of the plug of G6PDH was deduced from the electropherogram. This migration time does not coincide exactly with that observed for the enzyme in the absence of substrates, but appears instead to be increased slightly. This change in the migration time reflects the difference in the electrophoretic mobility of G6PDH and of G6PDH with bound, negatively charged substrates (glc-6-P and NAD) and/or products (6-phosphogluconate and NADH). Interactions between enzyme and substrates and/or products may modify their mobilities relative to those of pure components; we have documented this effect elsewhere.[8] Figure 4 summarizes the response of the electropherograms to changes in the concentration of G6PDH in the plug and to the contact time between enzyme and substrate.[12] Increasing consumption of NAD and generation of NADH correlated with increasing concentration of the enzyme in the plug using the same applied potential (30 kV), and thus, contact time (Figure 4a). The contact time between the substrates and G6PDH increased on decreasing the voltage used in the electrophoresis and on slowing the rate of electrophoretic migration of the various components; more NAD is consumed and more NADH generated (Figure 4b). This latter variation in the procedure allows amplification of

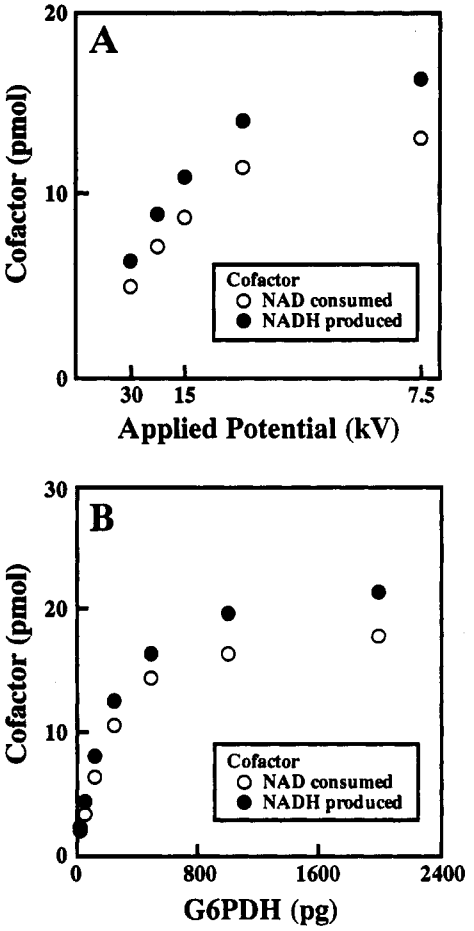

Figure 4. The amounts of NAD consumed and NADH generated during the G6PDH-catalyzed reaction are directly proportional to (a) the duration of the contact of a volume of substrate with a volume of solution containing enzyme (amount of enzyme: 40 pg) and (b) the amount of enzyme in the plug.

(11) Analysis of the concentration profiles for the substrate glc-6-P and product 6-phosphogluconate is not possible because these two molecules lack chromophoric groups.

(12) The volume of the cylinder represented by the two edges of the substrate/product electropherogram was determined from the relative mobilities of the two edges and the radius of the capillary. The product of this volume and the concentration of NAD or NADH (based on absorbance)[13] gave the amount of the cofactors consumed or generated.

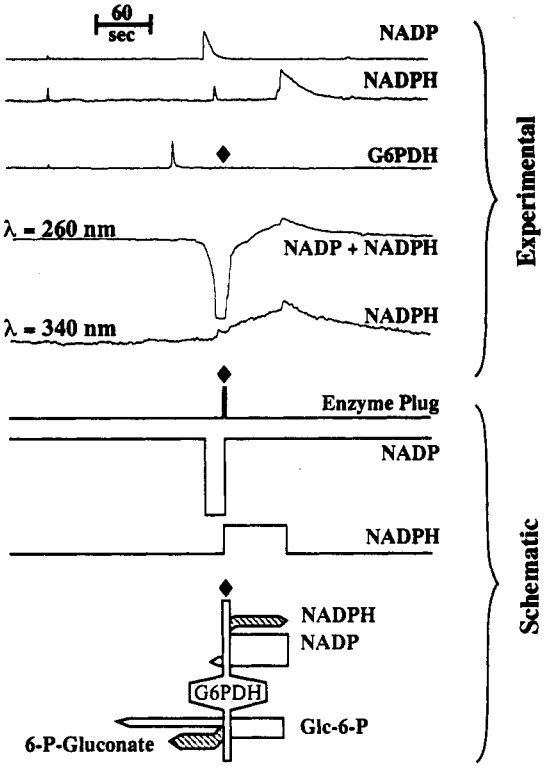

Figure 5. Oxidation of glc-6-P (200 μM) using NADP (200 μM). The full description of the figure is similar to Figure 3.

enzymatic activity in the plug and results in an increase in the detected signal.

In a second example, we examined the oxidation of glc-6-P using NADP. On the basis of similar determination of the migration times of the individual components in the buffer alone, the migration times follow the order t_m (NADPH) > t_m (NADP) > t_m (G6PDH) (Figure 5). The increase in the migration time of the enzyme in the presence of substrate(s) (determined from the edges of the profiles of NADP(H)) compared to that for the pure enzyme can again be explained by the enzyme becoming more negative on forming the ternary complex. The direction of movement of NADP to (and NADPH from) the enzyme-containing volume is shown in Figure 5 after adjusting for this change in mobility of the enzyme.[8,14] The asymmetric, trailing shape of the NADPH profile seems to be a characteristic of this molecule under the conditions used; we observe similar shapes in the electropherogram of pure NADPH. We have not established the origin of this shape.[15]

Yeast Alcohol Dehydrogenase. Figure 6 shows the electropherograms obtained for oxidation of ethanol by NAD and the reduction of acetaldehyde by NADH, both catalyzed by YADH. The putative migration time of the enzyme plug is expected to be the same for both the oxidation of ethanol and the reduction of acetaldehyde

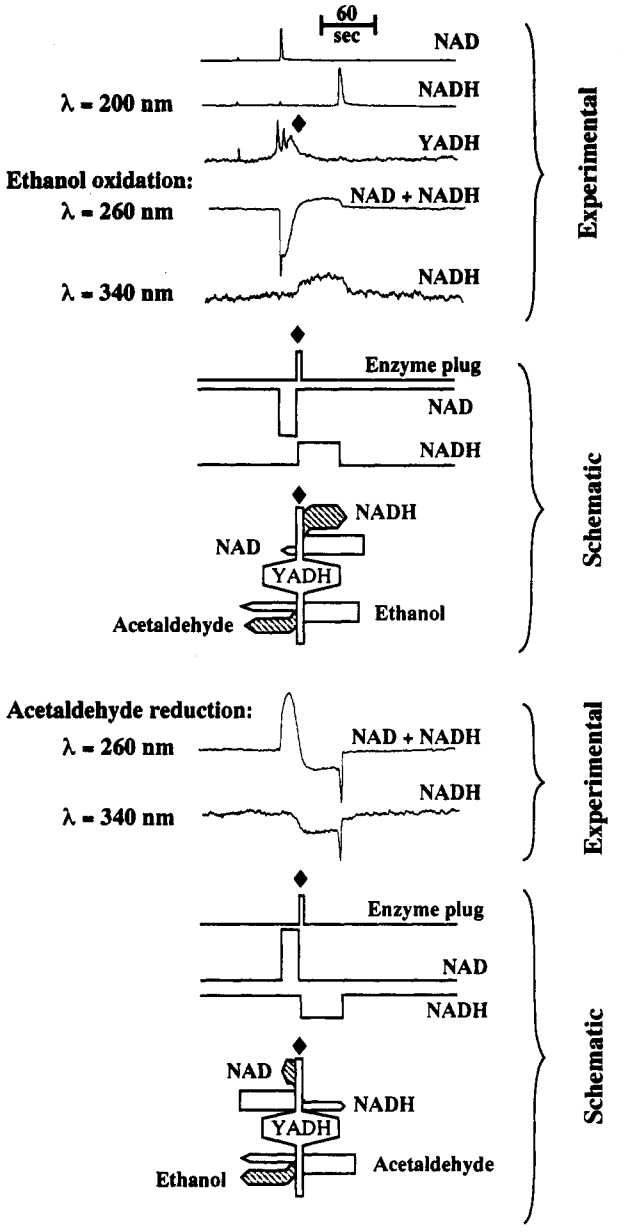

Figure 6. Yeast alcohol dehydrogenase-catalyzed oxidation of ethanol (200 μM) using NAD (200 μM) and reduction of acetaldehyde (200 μM) using NADH (200 μM). The full description of the figure is similar to Figure 3.

and is longer, as anticipated, than the migration time of the free enzyme alone.[8]

Conclusions

This report demonstrates that it is practical to use differential electrophoretic mobilities to "feed" or "pump" substrates through a plug of active enzyme and to remove the products of enzyme-catalyzed reaction from that plug, all under conditions of capillary electrophoresis. The models rationalize the form of the observed electropherograms adequately (if qualitatively). This procedure can be applied most conveniently when there is a difference in the electrophoretic mobility of the substrate and product and when the substrate and the product are UV active.

Experimental Section

Apparatus. The ISCO 3140 capillary electrophoresis apparatus (ISCO, Inc., Lincoln, NE) was used in this study with the anode on the injection side and the cathode on the detection

(13) Extinction coefficients: NAD, λ_{260} 18 ×10⁻³ (cm² mol⁻¹); NADH, λ_{259} 14.4 × 10⁻³, λ_{340} 6.23 × 10⁻³. Campbell, I. D.; Dwek, R. A. *Biological Spectroscopy*; Benjamin/Cummings: Menlo Park, CA, 1984; p 71.

(14) The ternary complex of G6PDH, NADP, and Glc-6-P has a more negative charge than that containing NAD and Glc-6-P; this change is reflected in the relative positions of the solid diamond relative to the enzyme G6PDH in Figures 3 and 5, respectively.

(15) A referee has suggested that the peak shape may reflect mismatches in conductivity between the NADPH zone and the running buffer. Adsorption of protein and cofactor on the capillary wall may also contribute.

side. The capillary tubing (Polymicro Technologies, Inc., Phoenix, AZ) was of uncoated fused silica with an internal diameter of 50 μm and a total length of 75 cm (50 cm from the injection side to the detector). The elution was monitored on-column at 200, 260, or 340 nm as specified in the text. The temperature of the column was maintained at 30 ± °C. The raw data representing the electrophoresis experiments were collected using ICE software (ISCO, Inc.) and later exported as ASCII files for processing and analysis using Kaleidagraph (Synergy Software, Reading, PA).

Chemicals. Glucose-6-phosphate dehydrogenase (*Leuconostoc mesenteroides*, E. C. 1.1.1.49), yeast alcohol dehydrogenase (EC 1.1.1.1), glc-6-P, and cofactors were purchased from Sigma. Stock solutions of glucose-6-phosphate dehydrogenase, yeast alcohol dehydrogenase, substrates, and cofactors were each prepared by dissolving the lyophilized proteins, substrates and cofactors in glycine (192 mM) and Tris (25 mM) buffer.

Procedures. Continuous reaction. Eight nanoliters of a sample solution containing G6PDH or YADH and mesityl oxide (neutral marker) in 192 mM glycine–25 mM Tris buffer (pH 8.3) was introduced onto the capillary by vacuum injection. The electrophoresis was carried out using an electrophoresis buffer (pH 8.2–8.3) consisting of 192 mM glycine, 25 mM Tris, and appropriate concentrations of substrate and cofactor. The electrophoresis was generally carried out under constant voltage of 30 kV generating a current of approximately 10 μA, except in the experiment where contact times between enzyme and substrate(s) were varied by varying the applied voltage across the capillary.

Plug–Plug Reaction. Eight nanoliters of a sample solution containing G6PDH was introduced by vacuum injection onto the capillary equilibrated with Tris (25 mM), glycine (192 mM), and glc-6-P (200 μM). G6PDH was electrophoresed at 30 kV for 20 s to provide for a volume of buffer between the two plugs. Eight nanoliters of NAD was next introduced by vacuum injection and the electrophoresis run at 30 kV to complete elution of all species.

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