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IMMOBILIZATION OF SEVERAL MULTIENZYME SYSTEMS ON POROUS GLASS BEADS

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Equimolar concentrations of malate dehydrogenase (EC 1.1.1.37) and fumarase (EC 4.2.1.2) and equimolar concentrations of malate dehydrogenase and citrate synthase (EC 4.1.3.7) were simultaneously immobilized to alkylamine porous silica beads with gluteraldehyde. The activity of each enzyme in the two-enzyme immobilized systems was determined and exact concentrations of the free nonimmobilized enzymes were prepared. The activities of the coupled free and coupled immobilized systems were measured, and it was observed that there was a 10-fold enhancement in the catalysis of the immobilized enzymes.

INTRODUCTION

Over the past ten years the simultaneous immobilization of several enzymes to a water insoluble matrix, followed by comparisons of the activity to identical nonimmobilized enzyme systems, has been investigated. Mosbach et al. (1,2), using gel type polymers, have shown rate enhancements for immobilized glycolytic and citric acid cycle enzymes. Srere (3) suggested that the inordinately high turnover rates of many mitochondiral enzymes may be due to their being membrane bound within the cell. It was further demonstrated (4,5) that various enzymes are able to associate with one another under hydrophobic conditions. It was later shown in our laboratory (6) that the enhancement effectiveness of such bound enzymes was dependent on the ratio of the enzyme activities within the immobilized complexes.

In order to obtain a clearer picture of the nature of immobilized enzymes, we feel that it is advantageous to investigate as many different types of insoluble matrix surfaces as possible. Therefore we chose porous glass beads, first employed by Weetall (7), as the source of insoluble polymer to be used in these preliminary investigations. The coupled enzyme systems studied were fumarase-malate dehydrogenase and malate dehydrogenase-citrate synthase.

Abbreviations used: NAD^+, nicotinamideadenine dinucleotide; NADH, reduced form of NAD^+; DTNB, dithiobis (2-nitrobenzoic acid); CoA, coenzyme A.

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MATERIALS AND METHODS

Alkylamine silane porous silica beads were a generous gift from H. H. Weetall, Corning Glass Company. Citrate synthase, malate dehydrogenase, fumarase, acetyl CoA, NAD^+ (oxidized and reduced forms) and oxaloacetate were all purchased from the Sigma Chemical Company. Glutaraldehyde and L-malic acid were obtained from Aldrich Chemicals. DTNB was purchased from Eastman Kodak, and other chemicals used were of reagent grade.

Glutaraldehyde activation of the alkylamine beads was accomplished using a method similar to that proposed by Marsh (8). Fifty ml of beads was mixed with 100 ml 2.5% glutaraldehyde solution under vacuum, and the mixture was allowed to stand for 2 h. The supernatant was removed by suction and the activated beads were washed three times with 100 ml 0.1 M phosphate buffer, pH 7.0. Then 250 μg each of 10 mg/ml solutions of malate dehydrogenase and citrate synthase or malate dehydrogenase and fumarase were added to 10 ml of beads and allowed to couple for a period of 48 h at 4°C, although coupling was found to be complete after 24 h under the given conditions. The uncoupled enzyme was washed from the beads with the phosphate buffer, and the protein concentration in the wash was analyzed by the Lowry method (9). This activation and coupling were repeated several times, and the results were reproducible.

The activity of malate dehydrogenase in the presence of either citrate synthase or fumarase was determined by measuring the extinction of NADH in an oxaloacetate : NADH solution (pH 7.0) (10) using a Gilford Model 250 Spectrophotometer coupled to a Model 6051 Recorder. Citrate synthase activity in the presence of malate dehydrogenase was measured by employing an acetyl CoA : oxaloacetate : DTNB mixture (pH 7.0) as described by Srere et al. (2), and recording the rate of production of the thiophenol chromophore ion produced at 412 nm, while fumarase activity in the presence of malate dehydrogenase was determined by recording the rate of fumarate produced at 240 nm from a L-malate solution (pH 7.0) as described by Massey (11). From the above data, the activity of the free nonimmobilized enzymes was adjusted to equal those of the immobilized species.

The activity of the enzymes in the immobilized system was measured by adding a specified amount of beads to 2.5 ml of assay solution in a 1-cm cuvette, into which was placed a magnetic circular stirring bar. The system was mixed by employing a specially adapted stirring apparatus, Gilford Spectro-stir Model 2445, which was attached to the spectrophotometer (6). Due to the weight of the beads, there was no interference seen in the light path during the stirring process. The activity was also observed to be

constant during the inital part of the reaction (5 min). The malate dehydrogenase-fumarase coupled system utilized an assay solution containing 50 μM oxaloacetate and 20 μM NADH 0.1 M phosphate buffer, pH 7.0. The production of fumarate was measured at 240 nm. The coupled malate dehydrogenase-citrate synthase system employed an assay solution containing 200 μM NAD^+, 1 μM acetyl CoA, and 2 μM DTNB in the phosphate buffer. The absorbance of the thiophenol chromophore ion was measured at 412 nm. Units of absorbance are expressed in μmole quantities.

RESULTS AND DISCUSSION

The results of the coupled immobilized versus the free malate-dehydrogenase-citrate synthase enzyme systems are shown in Fig. 1(a). The calculated velocities from the slopes of the curves are 5.5 and 0.60 nmol min for the immobilized and free enzyme systems, respectively. Analogously, for the malate dehydrogenase-fumarase couple, presented in Fig. 1(b), the calculated velocities are 11 and 1.2 nmol min, respectively, for the immobilized and free enzyme systems.

From the protein content of bead washes and individual enzyme measurements, it is observed that 90% of both malate dehydrogenase and

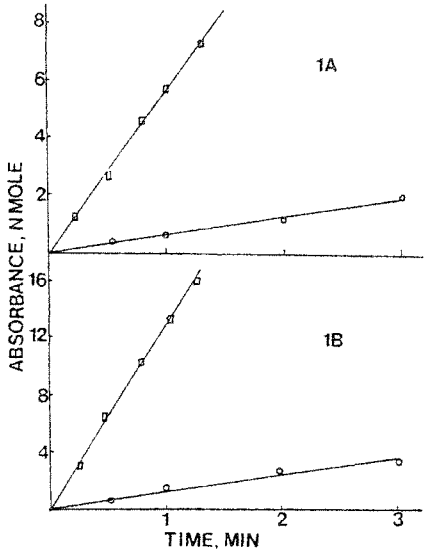

FIG. 1. Measurement of the activity of the free and immobilized enzymes. (a) Malate dehydrogenase-citrate synthase: ○, free; □, immobilized. (b) Malate dehydrogenase-fumarase; ○, free; □, immobilized.

fumarase and 70% of citrate synthase is coupled to the glass beads. The specific activity of the single immobilized relative to the free enzyme is approximately 60, 75, and 90% for malate dehydrogenase, citrate synthase, and fumarase, respectively. Thus immobilization on the glass beads does reflect some loss of each enzyme activity. This is probably due to conformational changes in enzyme structure caused by the binding process. It is also observed that the ratio of activities of the two enzymes is approximately one in each coupled immobilized system.

The above data indicate that for both coupled enzyme systems the activity shown by each one of the three coupled enzymes is less than that seen by an identical amount of enzyme studied free in solution in a nonimmobilized state. Paradoxically, when each of the two coupled systems are studied and compared to the activity shown by identical amounts of both enzymes in true solution phase, we observe that each of the immobilized systems produces an expected rate enhancement of approximately 10-fold over that seen using the identical two enzymes in the nonimmobilized state (Fig. 1). The ten-fold enhancement seen here with malate dehydrogenase-citrate synthase is larger by at least 5–8-fold over enhancements reported using gel type matrices (2,5).

This leads to the view that a different and more favorable surface nature exists on the glass beads for passage of the substrates. It may be that the crevices are larger and thus able to encompass both enzymes more favorably.

This preliminary study strongly suggests the importance of knowing the type of membrane surface in which the enzymes may be embedded or on which they may be located. Such studies may also yield a great deal of information allowing for better prediction of the effectiveness of specific multienzyme systems. Lastly, in some initial studies, we have recently observed that upon coupling all three of the above enzymes together, the rate enhancement increased by another order of magnitude, and we are in the process of exploring that system more fully.

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