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IMMOBILIZATION OF FUMARASE AND MALATE DEHYDROGENASE ON AGAROSE EFFICIENCY OF ACTIVITY AS A FUNCTION OF THE ENZYME RATIO[1]

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Two enzymes, L-malate dehydrogenase, L-malate : NAD^+ oxidoreductase (EC 1.1.1.37) and fumarase, L-malate hydrolyase (EC 4.2.1.2) were immobilized on a (Sepharose 4B) resin by the cyanogen bromide method. Studies showed that the matrix-immobilized fumarase retains the same characteristics as the free enzyme, while the matrix-immobilized malate dehydrogenase has reduced activity. The activity of the coupled enzymes is more enzyme-concentration dependent than the free enzymes, and at a ratio of 0.3 (fumarase : malate dehydrogenase) the simultaneously coupled immobilized enzymes become a better catalytic system. Individually immobilized enzymes, mixed to form a coupled system, yielded the poorest catalytic action.

INTRODUCTION

It has been previously shown that the immobilization of several enzymes, in either a polyacryamide matrix or by coupling to a polymer with cyanogen bromide, has caused an enhancement of catalytic activity in comparison to the same enzymes in the free state (1,2). Furthermore, it has been proposed that enzymes in the tricarboxylic acid cycle, such as L-malate dehydrogenase and citrate synthase, must be near the mitochondrial membranes in order to account for their high turnover in the cell relative to their calculated high K_m values (3). To support this hypothesis it has been further shown (4,5) that physical interaction between aspartate aminotransferase and L-malate dehydrogenase, as well as between L-malate dehydrogenase and citrate synthase, takes place in a low ionic strength–high polymeric aqueous media. Therefore, it is suggested that many of the easily removable enzymes in the tricarboxylic cycle may be loosely associated with the mitochondrial membrane (3) by exclusion of the protein from the solvent (6,7).

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This study will describe the catalysis of two Sepharose-4B-immobilized mitochondrial enzymes, L-malate dehydrogenase and fumarase, as a function of their relative concentrations, in order to see if there is any relationship between the concentration and catalysis in a free versus an immobilized system. The catalysis has been recorded in a direction opposite to the mitochondrial pathway in order to obtain measurable kinetic data (Eq. 1).

$$\text{oxaloacetate} \xrightarrow[\text{dehydrogenase,+NADH}]{\text{L-malate}} \text{L-malate} \xrightarrow[-H_2O]{\text{fumarase}} \text{fumarate} \qquad (1)$$

EXPERIMENTAL

Materials

Pig heart mitochondrial-L-malate dehydrogenase, fumarase, L-malic acid, oxaloacetic acid, fumaric acid, nicotinamideadenine dinucleotide (NAD^+) and reduced NAD^+ (NADH) were all purchased from the Sigma Chemical Company. Sepharose 4B was obtained from the Pharmacia Chemical Company, while CNBr was purchased from the Eastman Chemical Company. All other chemicals were of reagent grade.

Methods

CNBr Activation of Sepharose 4B. The method of Srere, Mattiasson, and Mosbach (2) was used with slight modification. Approximately 8–10 g of Sepharose 4B was allowed to react with 0.4–0.6 g of CNBr, while maintaining the pH between 10.5 and 11.5 with 5 M KOH first and then with 1 M KOH. The reaction was completed after 15 min (no observable change in pH) and the CNBr-activated resin was rapidly (approx. 5 min) washed with 500 ml of 0.1 M $NaHCO_3$, pH 8.0.

Coupling of Enzymes to Activated Sepharose. Between 2.5 and 100 μl of enzyme (2.5–100 μg) per g activated resin were mixed in a beaker containing 10 ml 0.1 M $NaHCO_3$, pH 8.0, and allowed to couple over a period of 24 h at 4°C. The suspension was continually mixed using a glass rod attached to a motor-driven stirrer or a magnetic stirrer. After coupling was completed, the enzyme-bound resin was washed first with 1.0 l of 0.1 M $NaHCO_3$ containing 0.5 M NaCl, pH 8.0, and then with 100 ml of 0.1 M phosphate buffer, pH 7.0. The washings were saved for activity and protein determinations. Controls, using nonactivated Sepharose, were performed along with the tests.

Simultaneous Coupling of L-*Malate Dehydrogenase and Fumarase to CNBr-Activated Sepharose 4B.* The procedure as outlined in the previous section was followed. Various ratios of the enzymes were employed, and they were both added at the same time.

Determination of Enzymatic Activity. L-malate dehydrogenase was assayed according to the procedure of Friedman et al. (8). The assay solution contained 3 ml of 10 mM L-malate, 100 mM glycine, and 0.3 mM NAD^+, pH 9.5, or 0.5 mM oxaloacetate, 100 mM phosphate buffer, and 0.2 mM NADH, pH 7.0. The change in absorbance was read at 340 nm on a Gilford Recording Spectrophotometer, Model No. 250, using 1-cm quartz cells. Fumarase was assayed according to the method of Massey (9) with some modification. The assay solution contained 20 mM L-malate in 100 mM phosphate buffer, pH 7.0. The change in absorbance at 235 or 250 mm was read on the Gilford Spectrophotometer. In all experiments 5 or 10 μl of enzyme solution was removed for activity measurement.

Determination of the Activity of the Individual Matrix-Bound Enzymes. Two methods were employed. The earlier experiments involved the addition of 3 ml of substrate solution to preweighed amounts (15 to 25 mg added directly into the cuvette) of resin-bound enzyme, and then dispersing the solid in the solution by manual shaking. The activity was immediately recorded in the spectrophotometer. After a few minutes the cuvette was removed, reshaken, and inserted for another measurement. This process was repeated several times, and it was observed that the linearity of the kinetics was constant over the entire removal and insertion process. Later a Gilford Spectro-Stir, Model No. 2445, was attached to the spectrophotometer, and the activity was measured while the resin particles were continuously stirred within the cuvette. Scattering at any wavelength, by use of this stirring device, was controlled by letting the resin completely disperse throughout the cell before any readings were taken. The best straight line was used for the activity calculations. The data recorded from this later technique were in excellent agreement with that obtained from the shaking method.

Protein Determination. These determinations were performed according to the method of Lowry et al. (10).

Determination of K_m. The K_m values for both the free and immobilized enzymes were computed from Lineweaver–Burke double reciprocal plots. For L-malate dehydrogenase, the K_m was determined for both L-malate and oxaloacetate. The concentration of the former varied from 0.6 to 8.0 mM with the NAD^+ concentration maintained at 0.2 mM, while the concentration of the latter varied from 5 to 50 μM with the NADH concentration maintained at 0.2 mM. The K_m for L-malate, using fumarase, was calculated by employing a concentration range of 4–40 μM L-malate.

Determination of the Activity of the Two Enzyme Systems. The activities of the simultaneously Sepharose-coupled enzymes, the individually Sepharose-coupled enzymes (which were mixed together in specific proportions), and the free enzymes were determined by the addition of the substrates, oxaloacetate and NADH, and measurement of the fumarate produced. The reaction was run in the direction opposite to that of the tricarboxylic acid due to the poor kinetics obtained when the reaction is run in the forward direction at pH 7.0. The assay solution contained 3 ml of 0.5 mM oxaloacetate, 0.2 mM NADH in a 0.1 M phosphate buffer, pH 7.0. The fumarate concentration was recorded at 235 or 250 nm. The individual activity of the enzymes in the presence of one another was determined by measurement of the decrease in NADH absorption in the presence of 0.5 mM oxaloacetate in the case of L-malate dehydrogenase and by measurement of the fumarate produced when 20 mM L-malate was added to the enzyme mixture. In this manner a ratio of the two enzymes, in both the free and immobilized systems, could be determined.

RESULTS

In the immobilization experiments, different amounts of enzymes were added to approximately 1.0 g of Sepharose. After measurement of the activity of the washings and determination of the amount of activity that was destroyed by interaction with the nonactivated resin, calculation of the activity on the resin was determined. Protein determinations of the washings verified the activity findings. A typical set of data is shown in Table 1. The theoretical yield was obtained from the difference between the initial

TABLE 1. **Determination of the Percentage of Activities of Sepharose-Bound L-Malate Dehydrogenase (MDH) and Fumarase (F) Relative to the Activities of the Enzymes in the Free State**

Amt. Enzyme[a] Immobilized (μl)	Activity (μmol l^{-1} min^{-1})					
	Total	Washings	Destroyed by resin	Theoretical yield	Experimental yield	% Activity
25 MDH	11.1	1.5	4.2	5.4	2.8	52
50 MDH	22.0	6.0	7.7	8.3	5.1	61
25 F	17.0	5.1	4.0	7.9	8.4	106
50 F	34.0	10.6	12.0	11.4	12.0	105

[a] All tests were run at least in duplicate.

TABLE 2. **K_m Values of Free and Sepharose-Bound L-Malate Dehydrogenase (MDH) and Fumarase (F)**[a]

Enzyme state	Substrate	K_m (mM)	Value from literature
Free MDH	L-malate	1.10	0.99 (11)
Bound MDH	L-malate	1.58	
Free MDH	oxaloacetate	0.40	0.3 (12)
Bound MDH	oxaloacetate	2.15	
Free F	L-malate	0.24	0.25 (13)
Bound F	L-malate	0.23	

[a] All experiments were run in duplicate.

amount of enzyme added to the resin and the loss due to destruction on the resin along with the portion that was not immobilized. The experimental yield was obtained from the direct activity measurements of the resin-bound enzyme. The percent activity on the resin was calculated from the ratio of the experimental and theoretical yields. It was observed that malate dehydrogenase retained only about 50–60% of its original activity when bound to the resin, while fumarase retained its full activity. All values obtained were good to within 10% of the reported values. The measurement of the K_m (Table 2) values for these enzymes also reflected the changes in activity of the molecules when bound to the Sepharose 4B.

The activities of mixtures of the two enzymes were then measured, and the results are presented in Table 3 and Fig. 1. Table 3 illustrates a representative sample of the activities of the free enzyme systems and the simultaneously coupled enzyme systems. The ratios of the fumarase to L-malate dehydrogenase in the free systems were chosen to correspond to those ratios that were calculated for the two enzymes in a series of coupled systems. In all experiments the amount of L-malate dehydrogenase was kept constant and the amount of fumarase was varied. The last column in Table 3 presents the normalization of the concentration of L-malate dehydrogenase so that the production of the fumarate will be reflected in the difference in the ratios of the two enzymes and not in their absolute amounts.

Figure 1 contains the curves that were obtained when the activities of the two-enzyme system were plotted against the ratio of the two enzymes in that system. The individually coupled enzyme resins were first mixed before they were weighed into the sample cuvette. The slopes of the curves were obtained by using least-square analysis, and the error for any experiment was found to be no greater than 5%, which is within the 10% activity variation for any experiment.

TABLE 3. **Measurement of the Activity of the Free L-Malate Dehydrogenase (MDH)–Fumarase (F) Systems and the Activity when both Enzymes were Simultaneously Coupled to Sepharose 4B**

	Activity (μmol l^{-1} min^{-1})				
System	Separate F	Separate MDH	Activity of system	Ratio of F to MDH in systems	Ratio of activity of systems to separate MDH activity
Free	0.023	0.72	0.079	0.035	0.110
	0.071	0.64	0.090	0.111	0.141
	0.170	0.66	0.109	0.257	0.166
	0.302	0.67	0.113	0.450	0.169
Coupled	0.070	0.66	0.056	0.106	0.085
	0.112	0.70	0.084	0.161	0.121
	0.216	0.70	0.112	0.309	0.162
	0.284	0.71	0.143	0.400	0.202

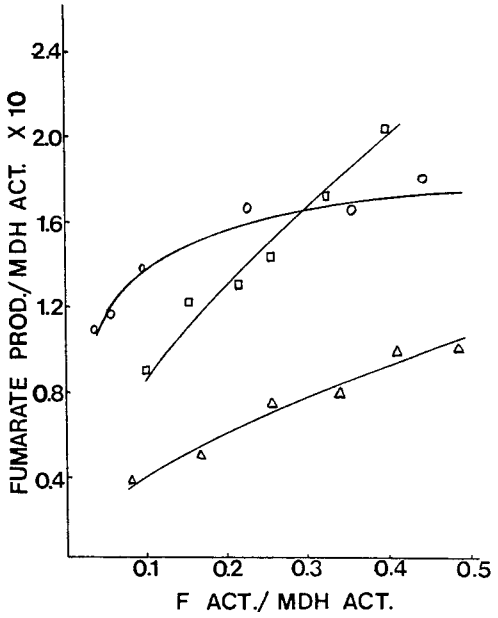

FIG. 1. Fumarate production obtained from the fumarase (F)–L-malate dehydrogenase (MDH) systems that are normalized for L-malate dehydrogenase activity is plotted against the ratio of the activities of fumarase to malate dehydrogenase. Some of these data are given in the last two columns of Table 3. The free enzyme system is given by open circles, the simultaneously immobilized enzyme system by open squares, and the individually immobilized (and then mixed) enzyme system by open triangle.

DISCUSSION

It was observed that the immobilization of L-malate dehydrogenase by the cyanogen bromide method lowers the activity by about 40–50% of its nonimmobilized value. This reduced activity is also reflected in the higher K_m values of immobilized L-malate dehydrogenase for both L-malate and oxaloacetate. In comparison, there was little change in the K_m of fumarase using L-malate as the substrate. Thus, there was some conformational change that took place when L-malate dehydrogenase was immobilized, which caused it to lose some of its effectiveness as a catalyst. However, both enzymes were observed to be more stable as immobilized adducts. Thermal denaturation studies (14) in our laboratory have shown both enzymes to be less heat labile, and the halflife of both enzymes increased from days to weeks upon immobilization.

It was observed (see Fig. 1) that when the ratio of fumarase to L-malate dehydrogenase was small, the free enzyme system catalyzed the two-step reaction more effectively. Since the magnitude of the activity from the free enzyme system did not increase greatly, it is suggested that the rate-controlling step in the production of fumarate from oxaloacetate was the first reaction in the sequence. When both enzymes were immobilized on the resin it was observed that at a low ratio of fumarase to L-malate dehydrogenase, the net activity of the coupled enzymes was lower than that of the free enzyme system. This was probably due primarily to the fact that L-malate dehydrogenase is a poorer catalyst when immobilized, and thus the turnover of L-malate was significantly lowered. However, as the ratio of the two enzymes was increased, it was now observed that the activity increased more rapidly than in the free system, which suggested that the product (L-malate) of the first reaction was being more effectively transported to the second enzyme (fumarase) due to the close proximity of the two enzymes on the resin particle. Thus, the microenvironment of the substrates, as had been previously suggested (2), plays a major role in the effectiveness of a series of enzyme-catalyzed reactions. In order to further test this concept, the two enzymes were immobilized separately, and then mixed together, so that their distances should be separated, on the average, by at least the radius of the resin bead. As can be seen from curve 3 in Fig. 1, the activity of this type of system was not only poorer than that of the free system, but it did not increase at nearly the same rate as the simultaneously immobilized system. Thus, the poorer L-malate dehydrogenase activity and the lack of an effective transport of product yielded a poorer catalytic system.

The crossover activity for the free and simultaneously immobilized enzyme systems corresponded to a ratio of 0.30 (fumarase to L-malate dehydrogenase). The absolute value of this number is probably not significant in a theoretical sense, since it represented the characteristics of

the two enzymes chosen for the study; yet, it did not show that when the enzyme concentrations began to approach one another and were brought into close proximity, they would produce a more effective catalysis in comparison with the freely moving species. This appeared to be true even if the individual effectiveness of each as a catalyst was lowered, as was observed for the case of L-malate dehydrogenase.

It appears, therefore, that the association of enzymes on a surface, or with one another as has been previously suggested (5), is a major condition for the effective catalytic action for many linked processes in the cell. In our own laboratory we are at present investigating different types of immobilization, both covalent and noncovalent, and also expanding the number of linked enzymes under study, in order to obtain a fuller picture of these processes.

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