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Studies on a matrix-bound three-enzyme system

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SUMMARY

The kinetic behaviour of a matrix-bound three-enzyme system has been studied and compared with that of an analogous system consisting of the three enzymes unbound and in solution. The enzymes chosen were β-galactosidase, hexokinase and glucose-6-phosphate dehydrogenase which carry out three consecutive reactions. It was found that the efficiency of the coupled reaction catalyzed by the matrix-bound three-enzyme system was higher prior to reaching steady state than that catalyzed by the corresponding soluble system.

Although a higher efficiency than that of the corresponding soluble system was also found for the coupled reaction when only the two last enzymes of the matrix-bound system were implicated, the measured increase in efficiency between the complete three-enzyme systems was even more pronounced, indicating a cumulative efficiency effect.

A common feature of metabolic pathways in intermediary metabolism is that the product of one enzyme in sequence is the substrate for the next and so forth. Free diffusion in the interior of the cell would, of course, lead to a chaotic situation, *e.g.* when the enzymes operating in different pathways compete for the same substrate. It is now understood, however, that we find within a cell a highly organized compartmentalization of different enzymes. This has recently been elegantly demonstrated by Kempner and Miller[1] who used centrifugal stratification to show that all the intracellular enzymes of the alga *Euglena gracilis* are associated with particulate fractions of the cell. One of the advantages of such an arrangement is that a favourable high local concentration of intermediates in the microenvironment of an enzyme system can thus be created[2].

Recently we demonstrated, using a two-enzyme model system, hexokinase–glucose-6-phosphate dehydrogenase, bound covalently to a polymer matrix, the increased efficiency of such a spatially concentrated enzyme system over a corresponding system with the enzymes free in solution[3]. We hoped that extension to a three-enzyme system would enable us to observe a cumulative efficiency effect. The system studied was: β-galactosidase–hexokinase–glucose-6-phosphate dehydrogenase (Fig. 1). We chose the enzyme β-galactosidase since it catalyzes the reaction immediately preceding that already studied using the two-enzyme sequence. In addition, studying the proper binding conditions of this enzyme to a matrix is of interest *per se*, since matrix-bound β-galactosidase preparations have great potential

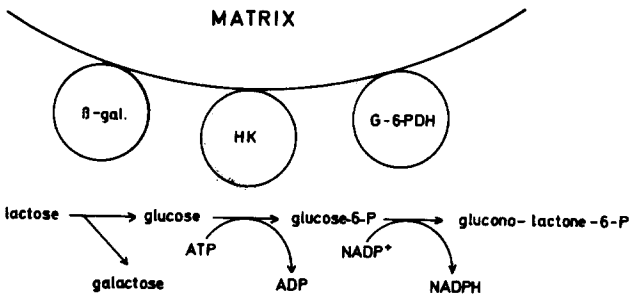

Fig. 1. Schematic presentation of the matrix-bound three-enzyme system (β-galactosidase (β-gal.)–hexokinase (HK)–glucose-6-phosphate dehydrogenase (G-6-PDH) with the reactants.

in the treatment[4] of the often occurring lactase-deficiency disease found in humans[5]. Only one report of such a coupling has hitherto appeared[6].

The three enzymes were surface bound to a highly cross-linked matrix since binding of the enzymes within the interior of a gel matrix would have complicated the interpretation of the obtained data. The coupling procedure chosen was the CNBr method[7]. Following the activation of 100 mg of Sephadex G–50 C (Pharmacia, Uppsala, Sweden), 1.0 ml of a 0.1 M $NaHCO_3$ solution of the three enzymes were added in the following proportions: 0.75 unit of β-galactosidase (EC 3.2.1.23; *Escherichia coli*, Boehringer, Mannheim, Germany), 2.0 units of hexokinase (EC 2.7.1.1c; Bakers yeast, Sigma, St. Louis, Mo., U.S.A.) and 20 units of glucose-6-phosphate dehydrogenase (EC 1.1.1.49; Torula yeast, Sigma).

Coupling proceeded in a rotating test tube for 12 h at 4°. The obtained Sephadex matrix, with the three enzymes bound probably *at random*, was subsequently washed for 30 min each at 4° in the following solutions to assure removal of enzymes not covalently bound: 0.1 M $NaHCO_3$, 0.1 M acetate buffer (pH 5.0) (twice), 0.5 M NaCl and 0.05 M triethanolamine–HCl with 0.07 M $MgCl_2$ (pH 7.6).

The enzymic activity of the bound three-enzyme system in the coupled reaction was then determined by monitoring the absorbance at 340 nm following a simple method found suitable for continuous spectrophotometric assays with particle-bound enzymes[3]. The assay proceeded in a 25-ml erlenmeyer flask with the matrix–enzyme system suspended in 10.0 ml of 0.05 M triethanolamine–HCl, 0.07 M $MgCl_2$ (pH 7.6) containing 5.23 μmoles of $NADP^+$ (Sigma) and 7.26 μmoles of ATP (Sigma). The reaction was carried out with stirring at 25° and was initiated by addition of a solution of 466 μmoles of lactose in 2.0 ml of the above buffer.

The rate of reaction of this system was then compared with that of the three participating enzymes in free solution. To this end a solution was prepared containing the equivalent number of enzyme units per volume as measured from the matrix-bound

preparation. The individual enzymic activity of each bound enzyme was determined from the rate of production of NADPH.

The incubation mixture, 12.0 ml, was modified from that of the coupled system described above in the following manner: β-galactosidase, addition of excess of soluble hexokinase (0.5 unit) and glucose-6-phosphate dehydrogenase (0.5 unit); hexokinase, omission of lactose, addition of glucose (26.64 μmoles) as well as excess of glucose-6-phosphate dehydrogenase (0.5 unit); glucose-6-phosphate dehydrogenase, omission of lactose and ATP, addition of glucose-6-phosphate (11.18 μmoles).

As can be seen from Fig. 2a, the lag phase of the soluble system in the coupled reaction catalyzed by the three enzymes, β-galactosidase, hexokinase and glucose-6-phosphate dehydrogenase, is longer than that of the corresponding matrix-bound system. That is to say, the efficiency of the matrix-bound three-enzyme system is, before reaching steady state, higher than that of the soluble system. This difference is observed, but to a markedly lesser degree, in a system in which only two enzymes participate (see Fig. 2b). Here the above matrix–enzyme system was assayed in a coupled reaction with the last two enzymes in the sequence. The incubation procedure was as for the complete coupled reaction except for the substitution of glucose (26.64 μmoles) for lactose. The reaction rate after reaching steady state was found to be equal to that attained by the complete system after addition of glucose (Fig. 2a, dotted line), indicating that the enzymic activities in the two lots are comparable.

On plotting the quotient $V_{matrix}/V_{soluble}$ found for the β-galactosidase–hexokinase–glucose-6-phosphate dehydrogenase system (Fig. 2a) and for hexokinase–glucose-6-phosphate dehydrogenase (Fig. 2b) against time, the graph given in Fig. 3 was obtained. As can be seen, the time required for the three-enzyme system approaching a situation with $V_{soluble} = V_{matrix}$ was

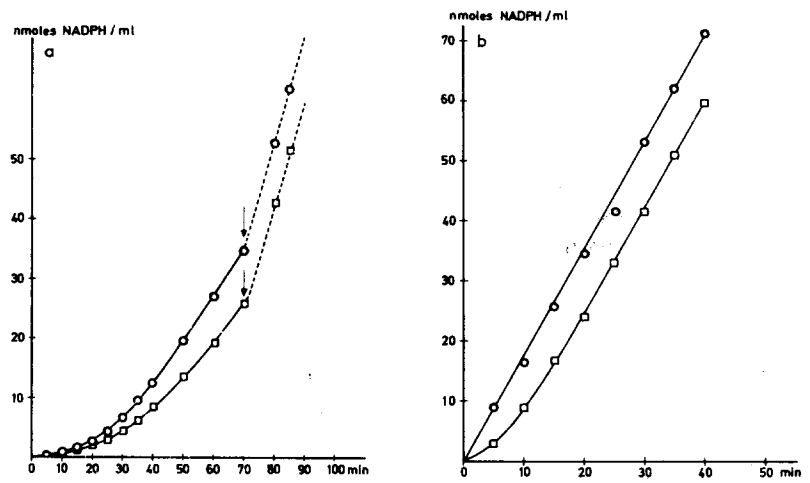

Fig. 2. a. Graphical illustration of the formation of NADPH per ml against time for the matrix-bound (O) three-enzyme system (β-galactosidase–hexokinase–glucose-6-phosphate dehydrogenase) and the corresponding soluble (□) system in the coupled reaction. Addition of excess of the substrate glucose for the enzyme hexokinase is indicated by the arrows given. b. The same system (a) in the coupled reaction over only the last two enzymes in the sequence.

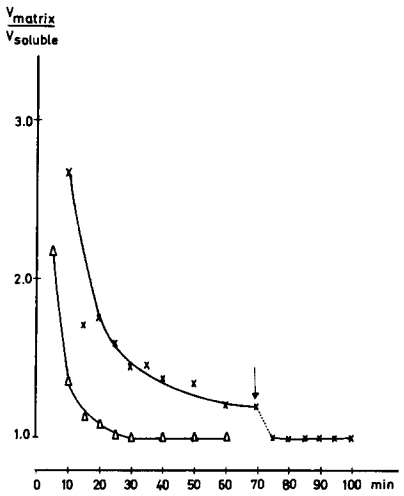

Fig. 3. Graphical illustration of the change of the ratio $V_{matrix}/V_{soluble}$ with time obtained from the reaction course taken by the coupled reaction with the three-enzyme systems (x) given in Fig. 2a. The corresponding change of the ratio for the two-enzyme systems (△) as taken from Fig. 2b. The arrow indicates the effect of the addition of excess of glucose on the ratio.

far longer than that for the two-enzyme system. This implies that on extending the number of enzymes participating in a consecutive set of reactions and in a situation where the concentration of intermediates formed is rate-limiting, binding of the different enzymes to the same mat has an cumulative effect on the overall rate of reaction of the system in its initial phase. Such an effect is not surprising assuming the build-up of an increased concentration of the intermediates in the microenvironment of the matrix-bound enzymes in contrast to the situation for the soluble system in which the intermediates immediately diffuse into the bulk of the solution. Whether the effect is caused by the proximity of the bound enzymes to one another and/or the existence of a diffusion layer around the gel particles impeding diffusion, remains to be investigated.

A theoretical analysis based on the effect of the latter on the kinetic behaviour of a somewhat similar system, a two-enzyme membrane, has recently come to our attention[8]. The possibility of adsorption of the reactants to the matrix-bound enzyme preparation giving rise to local concentration gradients and thus accounting for the effect measured, can be disregarded, since such adsorption was not observed in blank reactions run.

It should be pointed out that the data given for the bound three-enzyme system emanate from a typical preparation obtained according to the procedure given with a particular measured ratio of individual bound enzymic activities of 1.0 (β-galactosidase) : 2.1 (hexokinase) : 3.5 (glucose-6-phosphate dehydrogenase). Needless to say, on changing the internal activity ratio of the three enzymes, the initial lag phase can be varied at will.

Finally, we would like to point to the fact that in the living cell the metabolic events are continuously being switched on and off: thus the effectiveness of the different metabolic pathways is the result of the rate of reaction during rather short intervals. In other words, a short lag phase, as observed with the matrix-bound three-enzyme

system here presented as a model, is advantageous over a soluble system, the effect increasing with the number of different enzymes participating.

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