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STUDIES ON pH–ACTIVITY PROFILES OF AN IMMOBILIZED TWO-ENZYME SYSTEM

STINA GESTRELIUS, BO MATTIASSON AND KLAUS MOSBACH
Biochemical Division, Chemical Centre, University of Lund, P.O. Box 740, S-220 07 Lund (Sweden)

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SUMMARY

1. The pH–activity profile of a two-enzyme system, with both enzymes immobilized together by CNBr coupling to a neutral support (Sepharose), has been studied and compared with that of an analogous system consisting of the two enzymes unbound and in solution.

2. The enzymes studied were amylo-α-1,4-α-1,6-glucosidase and glucose oxidase (β-D-glucose:O_2 oxidoreductase, EC 1.1.3.4). All measurements were carried out in a buffer solution of high ionic strength so that the influence of charged products could be neglected.

3. Immobilization of the enzymes together does not change the pH optimum for either enzyme.

4. The pH optimum for the sequential two-enzyme reaction could be displaced towards the alkaline side in one case by a factor of about 0.75 pH unit compared to the system in solution. Such a displacement is a function of the ratio of the bound enzymic activities.

INTRODUCTION

A literature search revealed that studies of pH–activity profiles of soluble enzymes working in sequence appear to have been neglected. Information on this subject may contribute to better understanding of the overall metabolic events in living cells. However, care should be taken when interpreting such data because most enzymes appear to be associated with particulate fractions of the cells[1].

An increase in efficiency during the initial phase of sequences catalyzed by matrix-bound multi-enzyme systems relative to analogous systems in solution has already been demonstrated[2,3]. This effect is ascribed to an increased concentration of intermediates in the microenvironment of the matrix. Such enrichment of a rate-limiting enzyme–substrate in the initial phase of the reaction is reflected in increased final product formation relative to the soluble system. Since it might also result in an apparent displacement of the pH optimum, when dealing with a two-enzyme system in which the individual enzymes have different pH optima, we chose to study the

enzymes amylo-α-1,4-α-1,6-glucosidase and glucose oxidase (β-D-glucose:O_2 oxidoreductase, EC 1.1.3.4) with pH optima of 4.8 and 6.4, respectively.

Displacement of pH optima of individual enzymes when immobilized has already been reported in a number of cases and shown to be caused by increased concentrations of pH-active (acidic or basic) products in the microenvironment and/or by charged matrices[4–8]. In this study these influences were undesirable and were obviated by making all measurements in a high ionic strength buffer and by choosing an uncharged matrix.

The significance of the type of information gained from such model studies for understanding *in vivo* situations has been elegantly demonstrated in the interpretation of the different pH–activity profiles of acetylcholinesterase (EC 3.1.1.7) in its natural membrane-bound state and free in solution[9].

MATERIALS

Amylo-α-1,4-α-1,6-glucosidase (*Aspergillus niger*; 14 units/mg) was purchased from Boehringer, Mannheim, Germany, glucose oxidase (*Aspergillus niger*; 200 units/mg) from Sigma, St. Louis, Mo., U.S.A., and peroxidase (EC 1.11.1.7, horseradish) from Merck, Darmstadt, Germany. Sepharose 4B was obtained from Pharmacia, Uppsala, Sweden. β-D(+)-Glucose was obtained from Sigma. Commercially available maltose had to be purified prior to use by acetylation to β-maltose octaacetate, which was recrystallized to constant rotation $\alpha_D = +62.5°$ (*c* 1.0; chloroform), m.p. 159–160 °C and deacetylated[10].

METHODS

Amyloglucosidase (1–3 mg) and glucose oxidase (0.05–0.25 mg), were bound to Sepharose 4B (corresponding to 400 mg dry weight) by the CNBr method[11]. Coupling proceeded in a rotating test tube for 12 h. at 4 °C. The immobilized enzyme preparation, with the different enzymes bound probably at random, was washed for 30 min each with the following solutions to ensure removal of enzymes not covalently bound: 0.5 M $NaHCO_3$, 0.1 M glucose, 1 M NaCl and 0.05 M sodium maleate–acetate buffer (pH 5.3), 0.25 M in NaCl. In the assay method a flow cuvette was used which had previously been found suitable for continuous spectrophotometric assays of particle-bound enzymes[2]. The incubation proceeded in a 25-ml Erlenmeyer flask with enzyme–matrix (30 mg) suspended in 0.05 M sodium maleate–acetate buffer (11.4 ml) 0.25 M in NaCl, and peroxidase (100 units). To this suspension were added ethanolic Triton X-100 (100 μl, 80:20, v/v), *o*-dianisidine (4 μmoles) in ethanol (100 μl) and finally a solution of maltose (220 μmoles) in the above buffer (400 μl).

The enzymic reaction of the two-enzyme system

$$\beta\text{-D-Maltose} \xrightarrow{H_2O} 2\ \beta\text{-D-glucose} \xrightarrow{2O_2} 2\ \text{D-glucono-}\delta\text{-lactone} + 2\ H_2O_2$$

was carried out with stirring (120 rev./min, dimensions of Teflon bar used: 0.4 cm × 1.5 cm) at 25 °C and assayed at 450 nm as *o*-dianisidine is oxidized by the peroxidase reaction on the hydrogen peroxide formed[12].

We define the total activities of the two different enzymes of the immobilized

system as those determined separately at their respective optimal pH. The above buffer was used over the whole pH range investigated. Amyloglucosidase activity was determined by addition of excess soluble glucose oxidase (110 units) to the above incubation mixture at pH 4.8, and glucose oxidase activity was determined at pH 6.4 by substituting maltose with excess D-glucose (312 μmoles). The activities of the soluble enzymes, separate or in the two-enzyme reaction, were determined as above except for assaying in a normal cuvette in a total volume of 3 ml.

RESULTS AND DISCUSSION

In Fig. 1 (lower part) the pH–activity profiles of the two immobilized enzymes are plotted. They were shown to be identical with those of the corresponding soluble enzymes.

On carrying out the two-enzyme reaction with either immobilized or soluble enzymes under comparable conditions, the pH–activity profiles depicted in the same figure (upper part) are obtained. As expected, their pH optima lie between those of the two different enzymes. The precise position on the pH scale is dependent upon the ratio of the total activity of amyloglucosidase to total activity of glucose oxidase, *i.e.* the "activity quotient", as is more readily seen from Fig. 2. Here the activity quotient of the enzymes are plotted against each pH optimum found for the matrix-bound and the soluble two-enzyme systems, respectively. Both curves show the pH optimum to be a function of the activity quotient. Thus when the ratio is changed by increasing the amyloglucosidase proportion the optimum pH becomes higher, whereas on increasing the proportion of glucose oxidase the pH optimum is lowered. In the latter case the limiting value of pH is 4.8 at which the pH optimum of the system and of amyloglucosidase alone are the same. Then the rate of the coupled reaction is determined only by the rate of formation of glucose, the substrate for the second step.

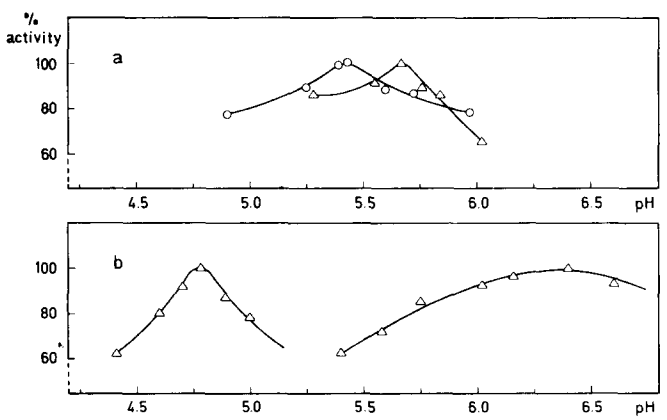

Fig. 1. Graphical illustration of pH–activity profiles. Upper part: profiles for the two-enzyme system in the coupled reaction; △, Sepharose-bound (pH optimum 5.7); and ○, in solution (pH optimum 5.4). The activity at the pH optima (0.024 ΔA/min for both systems) has arbitrarily been set at 100%. Lower part: profiles of the above Sepharose-bound system for the separate enzyme activities: *i.e.* amyloglucosidase (pH optimum 4.8) and glucose oxidase (pH optimum 6.4). 100% activity corresponds to 0.30 ΔA/min for amyloglucosidase and 0.49 ΔA/min for glucose oxidase. Thus the ratio between the total enzyme activities in this case is 0.6:1.

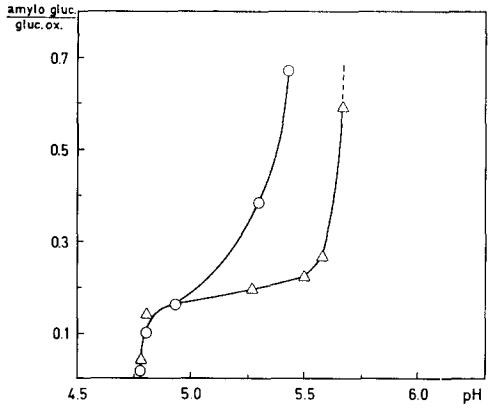

Fig. 2. Graphical illustration of the optimal pH for the two-enzyme reaction as a function of the ratio of the total enzymic activities (amyloglucosidase/glucose oxidase), when the enzyme system is Sepharose-bound (△) and in solution (○).

A comparison of the pH optima of the matrix-bound and soluble enzyme systems at a given ratio of total enzyme activities reveals a shift towards the alkaline side in the case of the immobilized system (Fig. 1 upper part, Fig. 2), in one case by as much as 0.4 pH units. We interpret this observation to be the result of increasing the concentration of the intermediate glucose in the microenvironment of the bound system during the initial phase of the two-enzyme reaction, as discussed below. The activity ratio used in Fig. 2 is derived from the total enzymic activities determined at respective pH optima. A more meaningful representation of these results, however, is given in Fig. 3, in which the substrate concentration in the microenvironment has been taken into consideration, thus giving a ratio of the actual enzymic activities. In other words, at the pH optimum of a particular two-enzyme preparation the actual activity of amyloglucosidase at that pH is determined in the presence of excess of substrate and divided by the measured activity of the two-enzyme system at the same pH, which is equivalent to that of glucose oxidase.

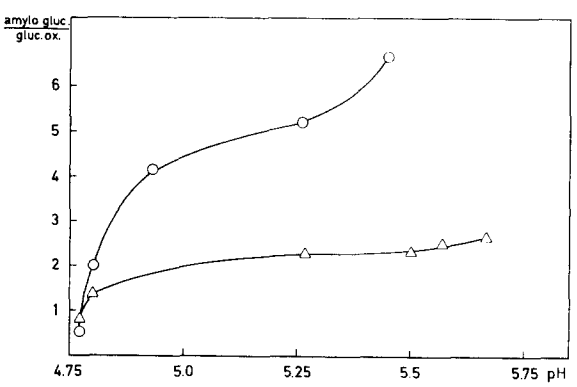

Fig. 3. Graphical illustration of the optimal pH for the two-enzyme reaction as a function of the ratio of the actual enzymic activities (amyloglucosidase/glucose oxidase) at the actual pH and substrate concentration for the enzyme system, Sepharose-bound (△) and in solution (○).

(Since the latter enzyme operates at less than substrate saturation, its activity cannot be obtained from its pH–activity profile.) A plot of these quotients *versus* the pH optima of the two-enzyme systems illustrates that lower quotients occur with the immobilized systems than with the soluble at the same pH optimum. This is because, although the amyloglucosidase activities are the same for both systems, the higher concentration of glucose in the microenvironment of the immobilized enzymes will result in a higher glucose oxidase activity and hence lower quotients. As before at identical ratios the pH optimum is higher for the immobilized system than for the same system in solution. Further the observed alkaline shift is even more pronounced when the actual activity ratio is used rather than the apparent. For instance, at a quotient of 2.5, the shift is 0.75 in the former case as opposed to 0.4 in the latter.

In summarizing, the results lead us to conclude that a pH optimum observed for a sequential two-enzyme system in solution cannot simply be transferred to *in vivo* conditions. In the latter situation, in which the enzymes are associated with particulate fractions of the cell, an enrichment of intermediate substrate concentration can lead to a different pH optimum as the next enzyme in the sequence obtains more favourable substrate conditions, resulting in a new pH optimization of the whole system. These results are also consistent with our previous findings of increased efficiency in the initial phase of other sequential reactions catalyzed by matrix-bound multi-enzyme systems as compared to the corresponding soluble system[2,3].

Information of this nature may also have practical implications. Thus on using sequential enzyme systems in future enzyme replacement therapy, where the pH of the external medium, *e.g.* blood, cannot be changed arbitrarily, optimal pH–activity conditions can be obtained by changing the enzyme activity quotient. Also, adjustments of the ratio of the participating enzyme activities or of the pH of the medium with such systems can be made to yield optimal product formation on a technical scale.

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