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THESIS OF DISSERTATION FOR CANDIDATE DEGREE

PREPARATION OF GLYCOLYTIC ENZYMES BOUND TO SOLID SUPPORTS, STUDIES OF THEIR PROPERTIES AND THEIR APPLICATION

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Introduction

During the 1970-s, there was significant progress in the study of immobilized enzymes: their application seemed to be promising, since they have several advantageous properties compared with the free enzymes. The product is free from the enzyme catalysing its formation, because the enzyme can easily be separated. Following removal of the products, the immobilized enzymes can be employed repeatedly, they are applicable in continuous operation and these factors decrease the costs of operation.

The immobilized enzymes are at the centre of attention as concerns both practical importance and basic research. One of the most interesting fields of interest in biochemistry is that of cellular component organization. The cell is currently envisaged as a highly organized system. Previously, cytosol was generally considered to be a well-mixed but concentrated solution of various enzymes, the latter being located in an ordered form bound to the membranes or coupled to each other to give a multi-enzyme system. It is possible that the enzymes of the glycolytic pathway can be found in complexes associated with the ultra-structure of the cell. Hexokinase was considered to be a soluble enzyme in vivo, but it turned out that it binds selectively to an integral membrane protein in the outer mitochondrial membrane. It has recently been reported that glycolytic enzymes of rat skeletal muscle associate with actin filaments. For study of the operation of enzymes catalysing consecutive reactions, one posibility is the attachment of these enzymes to different supports, the preparation of immobilized enzymes and the investigation of their properties.

The immobilized enzyme systems have been used as a model to study how the organization and the structural environment may affect the activity of an enzyme system. For the investigation of the properties of membrane-bound enzymes, supports possessing a negative charge are applicable, because the membranes are abundant in phospholipids and their natural microenvironment will be negatively charged.

Owing to their acid component, cell membranes, are hydrophobic too. In hydrophobic methyl acrylate and other synthetic gels, the hydrophobic microenvironmental effects can be studied.

It is recognized that the covalent binding of a homogeneous enzyme to a supporting matrix can give rise to a highly heterogeneous preparation. The enzymes can be bound differently to the matrix, but the enzymes are also associated with the surfaces in different ways in the cell. The enzyme activity is influenced by the surrounding microenvironment both in vivo and in vitro.

THE AIMS OF THIS WORK WERE:

A) to compare the efficiencies of different covalent immobilization methods in the case of glycolytic enzymes;

B) to study the catalytic properties and stabilities of immobilized enzymes prepared by the most efficient method, and to demonstrate whether there are general consequences involved in the differences measured between soluble and immobilized enzymes; and

C) to study the practical applications of immobilized enzymes having appropriate stability, primarily for analytical purposes, for the quantitative analysis of intermediates.

Materials and Methods

Enzymes were prepared and purified according to the literature procedures. The protein determinations were carried out by the method of LOWRY and BRADFORD.

For the preparation of immobilized enzymes, Sepharose 4B, silica-based Silochrome aldehyde and the polyacrylamides Akrilex P 100 and Akrilex C 100 were used. The Sepharose 4B was activated with cyanogen bromide, and the enzymes were coupled to the activated gel in 0.1 M $NaHCO_3$ solution, pH 8.0. The immobilization on Silochrom aldehyde was performed according to RYAN and FOTTRELL at pH 8.0. The enzymes were attached to Akrilex P 100 after activation with p-benzoquinone.The activation method was developed in our Deparment. The carboxyl groups of Akrilex C 100 were activated with water-soluble carbodiimide and the enzymes were coupled to the activated support. The non-covalently bound protein molecules were removed from the support by washing with buffer containing 1.0 M sodium chloride. The enzymes were generally stored in 0.1 M triethanolamine buffer (pH 7.6) until use.

The enzyme activities were determined with a Varian spectrophotometer (DMS 70). The activities of the immobilized enzymes were measured in the same reaction mixture as used for the activity determinations of the soluble enzymes. The reaction mixture was stirred for an appropriate time, the enzyme was filtered off quickly and the changes in the filtrate were determined.

The pH-dependence of the enzyme activities was studied in 0.1 M buffers between pH 5 and 12. The temperature-dependence of the enzyme activities was measured in the range 25—65°C.

The heat-inactivation of the enzymes was followed between 35 and 60°C, in temperature steps of 5°C.

The application of immobilized enzymes for analytical purposes was studied with enzymes bound to Akrilex C, both in batch and in flow injection system.

Summary of the new scientific results

Active immobilized forms of five glycolytic enzymes were prepared using different methods. The enzymes were bound to the support through nucleophilic amino groups. The activities of the enzymes coupled to different polymers showed diffe-

rences, but there was a definite sequence as regards the efficiency of the various methods.

The enzymes bound to Silochrome aldehyde had the lowest activity, which meant that the Silochrome support was not suitable for the immobilization of these enzymes.

Better results were achieved when agarose and Akrilex P activated with p-benzoquinone were used for the immobilization of the enzymes. The highest activities were those of the enzymes attached to Akrilex C, after activation of the carboxyl groups with watersoluble carbodiimide. The former enzymes were studied profoundly and the properties of the immobilized enzymes were compared with those of the soluble enzymes.

It was found that the pH-dependence of the immobilized enzymes differed from that of the soluble enzymes. The pH optima of imobilized hexokinase and pyruvate kinase were shifted in the alkaline direction to extents depending on the ionic strength of the medium. Even at an ionic strength of 0.2, the pH shift was about 0.5 pH unit. For 3-phosphoglycerate kinase, the soluble enzyme had a characteristic broad pH optimum range, while the activity of the immobilized form exhibited a relatively sharp pH optimum. No difference was measured between the pH optima of soluble and immobilized lactate dehydrogenase and glucose-6-phosphate isomerase.

It was demonstrated that the apparent temperature optima of the immobilized enzymes were shifted to higher temperatures (hexokinase and lactate dehydrogenase) or the immobilized enzyme showed a broader apparent maximum range (3-phosphoglycerate kinase and pyruvate kinase). The immobilized glucose-6-phosphate isomerase displayed a slightly lower apparent temperatura optimum than the soluble one.

The Michaelis constants of the soluble and immobilized glycolytic enzymes were determined and it was found that the values for some substrates were increased, while for others they were decreased. For 3-phosphoglycerate kinase with both substrates, for pyruvate kinase for ADP, and for hexokinase with ATP the values of $K_{M\ app}$ were decreased relative to those for the soluble enzymes. In all other cases, the apparent Michaelis constants of the immobilized enzymes were higher than those of the soluble enzymes.

The kinetics of the heat-inactivation of the soluble and immobilized enzymes was studied in detail. In most cases the heat-inactivation revealed complex phenomena (hexokinase, immobilized lactate dehydrogenase, glucose-6-phosphate isomerase and 3-phosphoglycerate kinase). The thermal inactivation of soluble lactate dehydrogenase, and immobilized and soluble pyruvate kinase followed first-order kinenetics.

The higher stabilities of the immoblized enzymes compared to those of the soluble enzymes were found in the kinetics of inactivation in the presence of urea. The changes in enzyme activity during the incubation period exhibited results similar to those in the heat-inactivation. Soluble and immobilized pyruvate kinase gave unusual results; both were inactivated according to two apparent first-order

kinetic reactions. It was remarkable that not only the rate constants, but also the amounts of rapidly inactivating molecules, were affected by the urea concentration applied.

For some enzymes (hexokinase and glucose-6-phosphate isomerase), but in particular 3-phosphoglycerate kinase, an activation was observed during the initial phase of heat and urea treatment.

The operation of enzymes bound to Akrilex C was studied in a column reactor and the optimal substrate concentrations were determined. In particular, the pyruvate kinase and lactate dehydrogenase reactors showed good transformations of the substrates, because the conversions of these reactors decreased only slightly when the flow rate was increased from 5 reactor volumes per hour to 50 reactor volumes per hour.

Reactor systems applicable for analytical determinations were constructed. The two immobilized enzyme system (hexokinase and glucose-6-phosphate dehydrogenase) can be used for the determination of glucose and ATP, while the three-enzyme system (the former two plus glucose-6-phosphate isomerase) is applicable for quantitative analyses of fructose.

Immobilized pyruvate kinase and lactate dehydrogenase can be used to perform quantitative measurements of phosphoenolpyruvate and pyruvate in both batch and flow-through cell systems.

Practical importance of the results

First of all, the presented experimental results increase our knowledge about immobilized enzymes and promote further research in this field. The immobilized enzymes having good operational stability afford a possibility for the performance of analytical determinations. The analytical systems mentioned above can be extended to other intermediates, through the use of one further immobilized enzyme.

The flow injection system gives a possibility for relatively simple and accurate measurements. The time used for measurements can be shortened by using several immobilized enzyme columns connected in parallel.

Certain substrates can be produced on a preparative scale with immobilized enzymes, and it is a very significant fact that optically active forms of substrates can be prepared in this way, while the less expensive synthetic procedure gives optically inactive compounds.

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