INSOLUBILIZATION AND CHARGE EFFECTS ON CROSSLINKED ENZYME POLYMERS

Kinetic studies in solution and in gelified membranes

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1. Introduction

Immobilized enzymes have inspired much interest recently, both because of the theoretical implications they suggest [1] and the possibility of practical application [2]. The various methods used for immobilization have been reviewed [3]. Although generally enzymes have been immobilized in insoluble structures, the binding of enzymes to soluble carriers has been suggested: the grafting of enzymes in solubilized cellulose-containing azide functions [4]; carbodiimide used to immobilize glucose-oxidase on polyvinylic alcohol [5]. Polychymotrypsin has been produced by a crosslinking process [6]. Cyanogen bromide grafted enzymes on enzymatically solubilized polysaccharide [7]. Enzymes have been bound on soluble DEAE-dextran and CM-cellulose [8]. Polyelectrolytes bearing chymotrypsin have been produced in order to study the microenvironment effect [9]. Bienzyme polymers have been produced by a crosslinking method [10–12]. Little attention, however, has been paid to the kinetic properties of these enzyme-containing soluble polymers. In fact, only [9] deals with the problem.

The goal of this paper is to study the kinetic properties of enzymes grafted to polymers. The study was performed with the polymers in solution which were then gelified to form membranes as in [12]. During an ultrafiltration process, due to a concentration polarization layer, a gel of macromolecules is formed along a semipermeable membrane. In the case of a protein macropolymer, the process is irreversible and a membrane is produced.

This method allows the study of the effect of the chemical modification of the enzyme in the first step and of the insolubilization in the second step. During the production of the membrane from soluble polymers no chemical reaction occurs.

The results presented deal with uricase, hexokinase and acetylcholinesterase. Since uric acid is an uncharged substrate, a study of its diffusion in the uricase system is possible. The hexokinase system, on the other hand, employs both neutral (glucose) and negatively-charged (ATP) substrates as well as a positively-charged activator (Mg^{2+}) permitting the determination of charge effects. Likewise, cationic acetylcholine allows the study of diffusion in the acetylcholinesterase system.

2. Materials and methods

2.1. *Enzyme polymer production*

A solution containing 3.7% serum albumin, 2.7 IU/ml urate-oxidase (for example) and 0.4% glutaraldehyde in 0.02 M, pH 6.8, phosphate buffer is mixed and stored for 24 h at 4°C. The soluble polymers obtained are stabilized by adding glycine up to final conc. 1%. The preparation was dialyzed against a 1% glycine solution in a phosphate buffer (pH 6.8, 0.02 M). The existence of the polymers and the presence of associated enzyme activity is checked by gel chromatography on Sepharose 6B. The results obtained with uricase are given in fig.1. Protein elution and activity curves demonstrate that active enzyme is linked to the higher molecular weight polymers.

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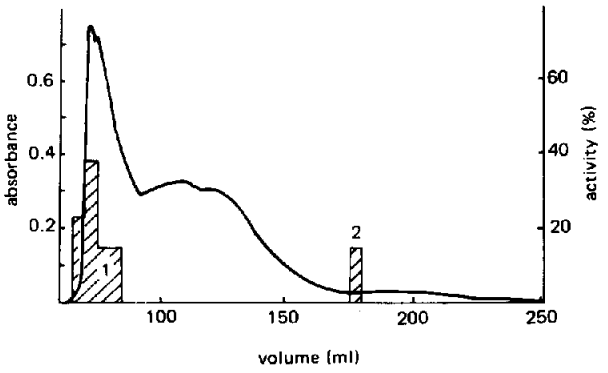

Fig.1. Sepharose 6B gel chromatography of soluble proteic polymers containing active uricase. Tracer quantities of free urate-oxidase were added. Protein elution was followed A_{280}. Enzyme activity was measured in each sample as mentioned in the text. Polymers displaying enzyme activity were eluted in (1) and free enzyme in (2). The activity yield is 30%.

2.2. *Gelified membrane production*

A solution of 2 mg/ml soluble proteic polymers in P_i buffer, pH 6.8, 0.02 M is introduced into a 17 ml ultrafiltration cell (Millipore) with a diameter of 2.5 cm. The diameter of the pores of the cellulose acetate membrane is 0.025 ± 0.003 μm. The thickness of the proteic membrane is about 100 μm. Experiments are performed at 25°C under a pressure of 1 bar. A gel forms during an 8 h period and is then dried for 10 h at 4°C.

2.3. *Enzyme activity measurements*

Free and immobilized uricase (Sigma, *Candida utilis*) activity is tested at pH 9.3 in a 0.05 M glycine–NaOH buffer by observing the A_{293} decrease of uric acid concentration.

Hexokinase activity is tested by its coupling reaction with excess glucose 6-phosphate dehydrogenase, in a solution of 11.1 mM glucose, 5.9 mM ATP, 14.8 mM $MgCl_2$, 1.8 mM NADP in Tris–maleate NaOH buffer 0.2 M, pH 8. The variation of NADPH concentration is measured at A_{340}. Enzyme membranes (10 cm^2) were tested in 30 ml above solution in a batch reactor. The rate of NADPH production was recorded at A_{340} with a continuous-flow quartz cuvette (DBT Beckman spectrophotometer and speedex-Ricken Denshi SPG 3 Recorder). The fluid was recirculated from the flow cell to the reactor.

2.4. *Acetylcholinesterase activity*

Enzyme activity is tested in a 0.05 M Tris–HCl buffer solution, pH 8.5, by adding 0.05 N NaOH with a pH stat (Metrohm). The experiments are performed at 25°C.

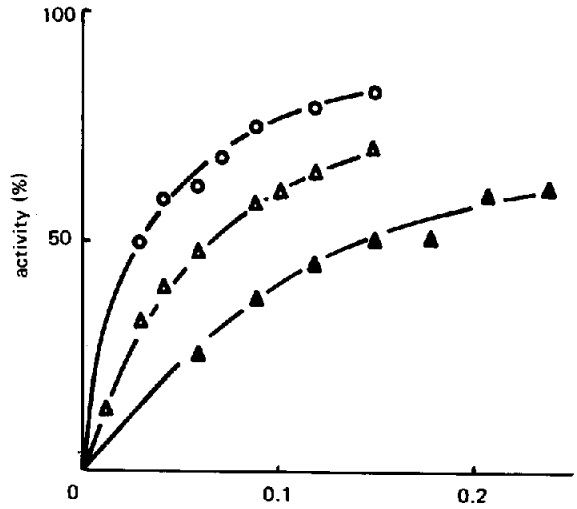

Fig.2a

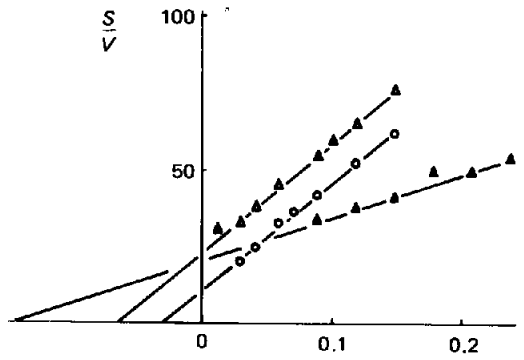

Fig.2b

Fig.2a. Uricase activity free in solution (–○–), bound to soluble proteic polymers (–△–) and to the same polymers gelified as a membrane by ultrafiltration (–▲–) as a function of uric acid concentration. The activity is expressed as a fraction of the maximum enzyme activity in each case. Fig.2b. The ratio of substrate concentration to enzyme reaction velocity as a function of the substrate concentration. Free enzyme (–○–), soluble polymer (–△–), membrane (–▲–).

3. Results

The results obtained with uricase in solution, immobilized on soluble proteic polymers and gelified in a membrane are given fig.2. Activity as a function of substrate concentration (fig.2a) as well as linear transformation (fig.2b) displays a 2-fold K_m increase after grafting on soluble polymers and a 5-fold increase after gelification. At this point there is already some modification of the soluble polymer. The insolubilization effect can be explained by 2 hypotheses: the diffusion limitations and/or a different conformation of the insolubilized enzyme. It is important to note that the kinetic modifications can be fitted by taking into account diffusion and reaction parameters in computer simulations [14].

Hexokinase, with a neutral substrate (glucose), a negatively-charged substrate (ATP) and a positively-charged activator (Mg^{2+}) allows the study of the influence of fixed charges on kinetic behavior. The polymers are produced by polymerisation of albumin molecules. At pH 8 the polymer is a polyelectrolyte, negatively charged. The activities of the free hexokinase, bound hexokinase in a soluble polymer and gelified in a membrane were studied as a function of glucose, ATP and Mg^{2+} concentration. In each case the concentrations of the 2 other molecules were chosen in order to give zero-order kinetics with respect to their concentrations.

Results obtained with glucose are given in fig.3. An increase of 1.3-times the K_m value was observed after binding on the soluble polymer and one of 3.5-times on the gelified membrane.

Results obtained with ATP are given in fig.4. The K_m value was not modified after the binding of the enzyme in the soluble polymer, but an increase of 6.2 times was observed with the gelified membrane. It is possible to try to explain the results by a charge effect.

ATP is strongly negatively charged and an exclusion effect was introduced. Inside the negatively-charged membrane, due to the Donnan effect, the concentration of anions was smaller by far than outside. The concentration of ATP was measured outside and the apparent affinity between the enzyme and ATP was found smaller than in solution.

The results dealing with Mg^{2+} in fig.5, showed the opposite effect. Due to the ion exchange properties of the membrane the local concentration of Mg^{2+} was higher inside the membrane than outside in the bulk solution. The value of the K_a was decreased by a factor 0.38 when the enzyme was insolubilized.

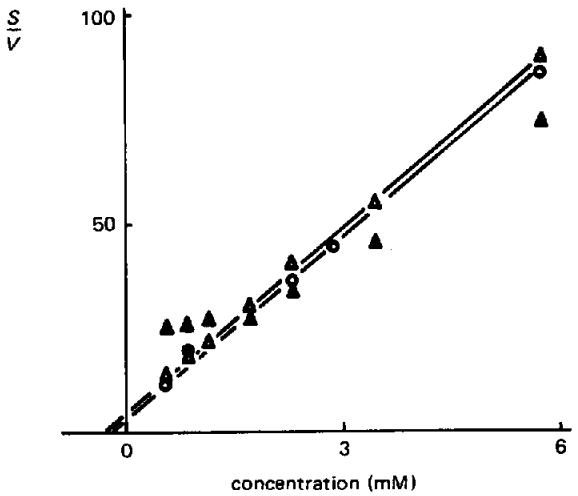

Fig.3. The ratio of glucose concentration to hexokinase reaction velocity as a function of glucose concentration for the enzyme free in solution (–○–), bound to soluble proteic polymers (–△–) and to the same polymers gelified as a membrane by ultrafiltration (–▲–). The S/V ratio is given in arbitrary units. The systems are studied under zero-order kinetic conditions for ATP and Mg^{2+}.

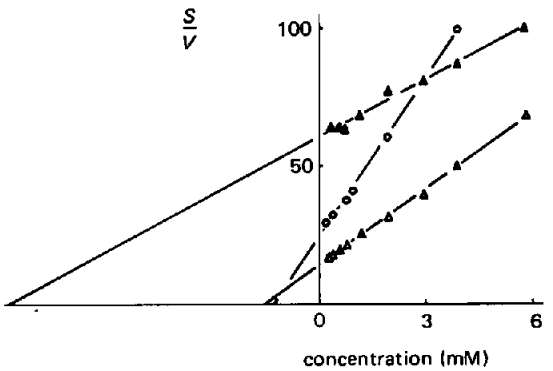

Fig.4. The ratio of ATP concentration to hexokinase reaction velocity as a function of ATP concentration for the enzyme free in solution (–○–), bound to soluble proteic polymers (–△–) and to the same polymers gelified as a membrane by ultrafiltration (–▲–). The S/V ratio is given in arbitrary units. The systems are studied under zero-order kinetic conditions for glucose and Mg^{2+}.

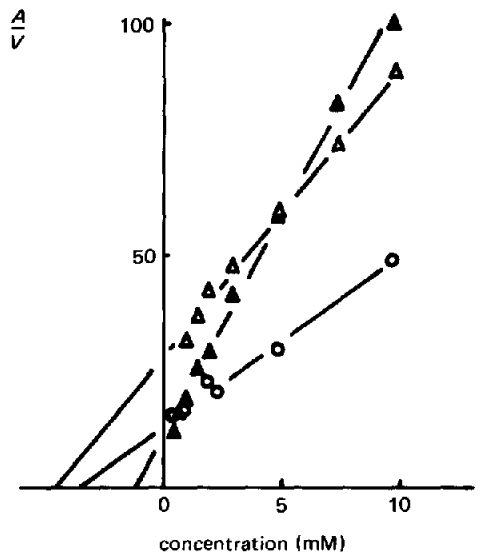

Fig.5. The ratio of Mg^{2+} concentration to hexokinase reaction velocity as a function of Mg^{2+} concentration for the enzyme free in solution (—○—), bound to soluble proteic polymers (—△—) and to the same polymers gelified as a membrane by ultrafiltration (—▲—). The S/V ratio is given in arbitrary units. The systems are studied under zero-order kinetic conditions for glucose and ATP.

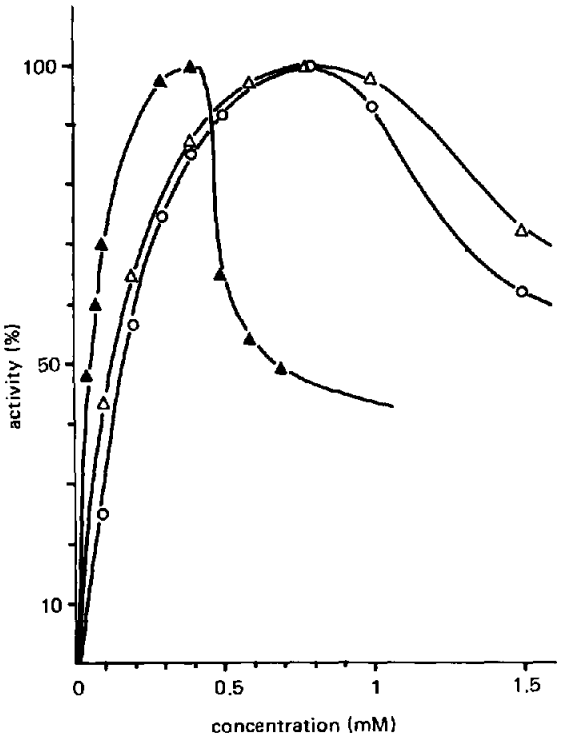

Fig.6. Acetylcholinesterase activity as a function of acetylcholine concentration for the enzyme free in solution (—○—), bound to soluble proteic polymers (—△—) and immobilized in a membrane (—▲—).

The results obtained with acetylcholinesterase are given fig.6. The behavior of the native enzyme and that grafted on soluble polymers are similar. After insolubilization in a membrane, an increase of the apparent enzyme affinity is observed together with a shift of the optimum value of the substrate concentration. Acetylcholinesterase is positively charged and due to fixed charges, the local substrate concentration is higher than outside in the bulk solution.

4. Conclusion

The immobilization of enzymes on artificial support is a good tool for studying the influence of a simple structure on the kinetic behavior of those enzymes [15]. The effect of microenvironment on the enzyme behavior also proves interesting since [16] the study of the effect of local concentrations (i.e., compartmentation) on metabolism provides a better knowledge of the kinetics of multienzyme systems in vivo.

However, with the kinetic information obtained from immobilized enzymes comes a drawback: it is difficult to separate the effects of the chemical modification and the effects of the insolubilization. This is also true for the entrapment methods, since the chemicals introduced in the medium for the polymerization of the carrier are able to modify the protein molecules as well.

In the results presented here, there is a clear separation between the effect of polymerization and the effect of the insolubilized structure. In the formation of gelified membranes from the soluble polymers bearing enzymes, only a mechanical process occurs without further chemical modification.

The results show the possible role of local concentrations of metabolites in the microenvironment of an enzyme. Local concentrations can be modulated not only by the diffusion limitations [17], but also the fixed charged in the insoluble phase.

In the system described here, the support is negatively charged and the local concentration, at the enzyme level, of cations is higher and of anions is lower than outside in the bulk solution.

The insolubilization can also introduce a different conformation of the insolubilized enzyme but in any case diffusion and charge effects are existing when dealing with an insoluble phase. Experimental results can be explained, at least qualitatively, by the charge effects.

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