KINETIC STUDIES OF AN ALLOSTERIC ENZYME UNDER ARTIFICIAL CONFORMATIONAL CONSTRAINTS IMPOSED BY A TIGHT IMMOBILIZATION INTO PROTEIC MEMBRANES

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1. Introduction

It is clear that the catalytic function of enzymes may be affected and controlled by interaction with small molecules, not only directly at or close to the active site, but also indirectly, at distant, secondary allosteric sites [1]. Nearly all the models that have been proposed to rationalize regulatory enzyme behaviour envisage a conformational change of some kind in the protein [2]. The Monod–Wyman–Changeux [3] model assumes spontaneous equilibrium between conformations, with ligand binding having no effect on conformations. Most other treatments have assumed to conformational change to be caused by ligand binding; they may be considered essentially as extensions of Koshland's induced fit hypothesis [4]. In any case the concept of conformational change of regulatory enzyme is not well defined and the study of the effect of artificial conformational constraints on their behaviour is of interest. In accord with Koshland [5], to decide between models it seems reasonable to attempt to 'freeze' some of the conformational states. This 'freezing' can be imposed by a tight chemical immobilization of the regulatory enzymes inside an artificial membrane. The behaviour of enzyme exhibiting Michaelian kinetics when immobilized in an artificial membrane was studied by Goldman et al. [6,7] and by our group [8–10]. The effect of the diffusion limitations on enzyme kinetics is now quite well known [11] and the results obtained during the past ten years make possible a study of immobilized allosteric enzymes [12]. The study of the coupling between regulatory enzyme activity and diffusion processes can throw light on the oscillatory behaviour of metabolic pathway occurring in vivo (Hess and Boiteux [13]). Goldbeter [14] predicted that some allosteric enzymes could induce some 'dissipative structures' at a supra cellular level when coupled with diffusion. The effect was experimentally demonstrated by Hess et al. [15].

For the above reasons the present paper deals with the immobilization of pyruvate kinase, which is indeed an allosteric enzyme [16], in proteic membranes. The membranes produced by a co-crosslinking method are homogeneous in structure and exhibit mechanical properties similar to those of cellophane.

2. Materials and methods

2.1. *Membrane production*

Pyruvate kinase (PK) from rabbit skeletal muscle membranes were produced according to a method described by Thomas et al. [10]. A solution of phosphate buffer 0.02 M, pH 6.8, containing 30 mg ml^{-1} albumin, 2 mg ml^{-1} glutaraldehyde and 1 mg ml^{-1} of PK was spread perfectly flat on a glass plate in order to obtain a membrane of homogeneous thickness. The crosslinking process proceeded at room temperature for 2 h. The plate with the proteic film was then dipped in a distilled water bath. The membrane which easily separated from the glass plate, was rinsed until the rinse water no longer absorbed at 280 nm. No activity and no proteins were observed in the rinse water.

2.2. *Pyruvate kinase activity*

The enzyme is coupled to lactate dehydrogenase.

In solution, measurements were done in a 3 ml quartz cuvette with a 0.2 M Tris–maleate buffer, pH 8, containing 1 mg ml^{-1} ADP, 0.05 mg ml^{-1} $MgCl_2$, 0.233 mg ml^{-1} NADH, 30 I.U. ml^{-1} LDH.

Enzyme membranes (20 cm^2) were tested in a 30 ml of the above solution in a batch reactor. The rate of NADH consumption was recorded spectrophotometrically at 340 nm with a continuous flow quartz cuvette, (DBT Beckman spectrophotometer and Speedex-Ricken Denshi S.P.G. 3 Recorder). The fluid was recirculated from the flow cell to the reactor.

2.3. *Electron microscopy*

Scanning electron microscopy: After drying, the membranes were coated with a carbon layer and observed in a CAMECA microscope at 15 kV.

Transmission electron microscopy: The specimens were post-fixed with 1% osmium tetroxide, dehydrated through ethanol and propylene oxide. Membranes were then embedded into Epon 812. Ultrathin sections were cut with an LKB ultrotome, mounted onto 400 mesh grids and stained with uranyl acetate followed by lead citrate and examined with a JEOL 100 C electron microscope at 80 kV.

3. Experimental results

The active membranes obtained were studied by scanning and transmission electron microscopy (fig.1). The membranes are homogeneous in thickness and the surfaces are regular. A dense structure is observed with no pore and no hole into the protein. The enzymes are tightly immobilized within the 'solid-phase structure'. After immobilization, enzymes are quantitatively stable for weeks.

It was demonstrated by gel chromatography that enzyme molecules are already involved in the proteic macro polymers (mol. wt: 1 million at the beginning of the cross-linking process before the insolubilization step) [17]. The enzyme is chemically linked and not entrapped in the structure. The composition of a dry membrane (w/v) was found of 5% of glutaraldehyde and 95% of protein by nitrogen titration (with a Carlo Erba C.H.N. analyzer). About twenty bonds link each enzyme molecule to the insoluble membrane.

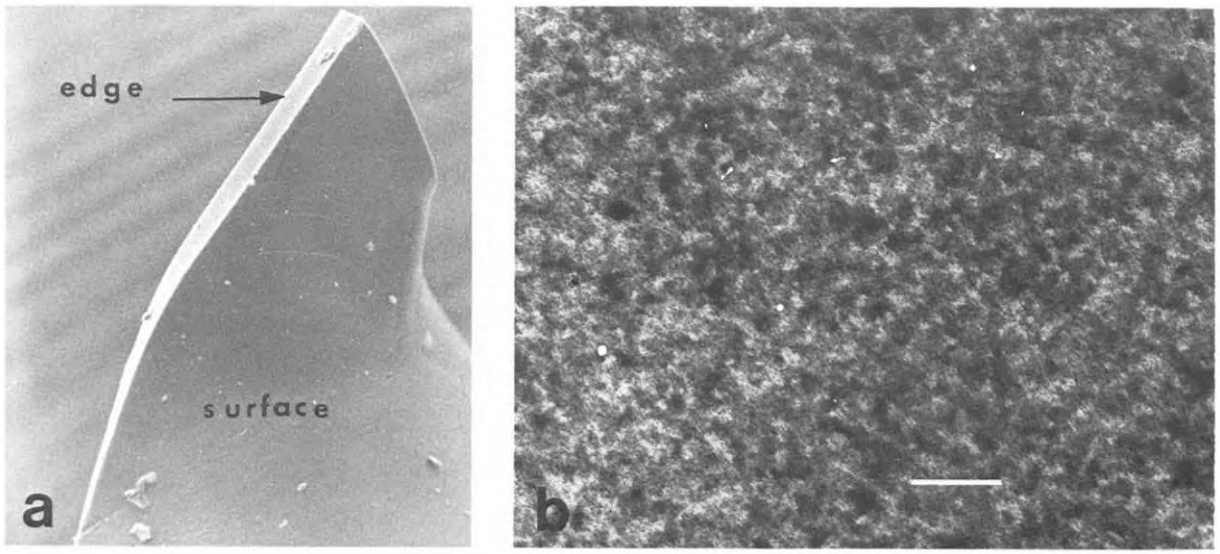

Fig.1. (a) Scanning electron micrograph showing a regular view of a proteic membrane (× 150). Edge and surface are indicated. (b) Transmission electron micrograph of an ultrathin section of a membrane stained with uranyl acetate and lead citrate. Note the presence of a dense structure without holes. The bar indicates 1000 Å.

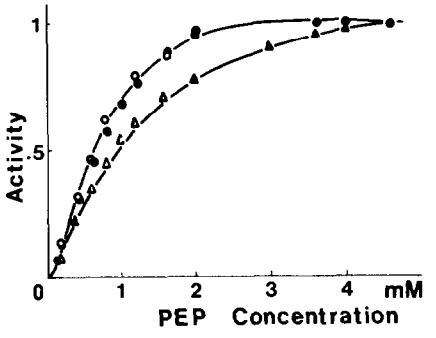

Fig.2. Pyruvate kinase activity in arbitrary units as a function of PEP concentration with the native enzyme in solution (•) and immobilized within membranes exhibiting low (○) and high (△) diffusion limitations. The Mg and K^+ concentrations were 6 mM and 0 respectively. The membrane area and maximum enzyme activity are 20 cm^2 and 0.12 I.U. respectively. The second membrane (△) was 3 times thicker than the first one (0), shown fig.1.

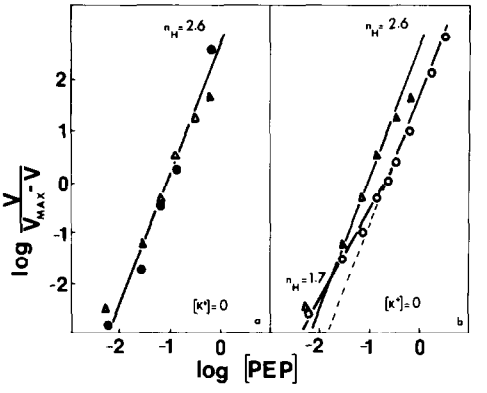

Fig.3. Hill-plot of native (•) and immobilized pyruvate kinase within membranes with low (△) and high (○) diffusion limitations. With low diffusion limitations (fig.2) the native and immobilized enzymes exhibit the same Hill number. A biphasic behaviour is observed (fig.2b) with higher diffusion limitations. [PEP] = mg ml^{-1}, free acid.

The activities of native and immobilized pyruvate kinase were studied as a function of substrate concentration (fig.2). The results obtained with an active membrane exhibiting low diffusion limitations (Thiele modulus smaller than 1) [10] are similar to the behaviour of the free enzyme. With higher diffusion limitations, obtained with a membrane three times thicker, a quite important difference of behaviour is observed. The apparent enzyme substrate affinity is lower, due to the diffusion limitation and was previously discussed by Thomas et al. [10] for Michaelian enzymes.

By using Hill plots (fig.3) the same Hill number is obtained for native enzyme and enzyme membrane with low diffusion limitations. With higher diffusion limitations a biphasic curve is obtained. A modification of the slope is observed for the activity corresponding to one half of the V_{max}. The same Hill number is obtained for concentrations higher than the 'apparent K_m' and a smaller one is observed for lower concentrations.

The effect of an allosteric activator K^+ was tested on both free and immobilized (with low diffusion limitations) pyruvate kinase. The variations of the Hill number as a function of K^+ concentration for both systems are given in fig.4b. The behaviours are similar and a Hill plot for K^+ concentration (13 mM) is shown as an example (fig.4a). It was of interest to study the kinetic properties of an enzyme immobilized in the presence of an activator (K^+, 8×10^{-2} M). After the crosslinking process, K^+ is eluted with a big excess of a 1 M NaCl solution. The activity as a function of substrate concentration was then studied. The results are given as a Hill plot in fig.5. A difference in the Hill numbers obtained with the enzyme immobilized in presence and in absence of K^+ is observed.

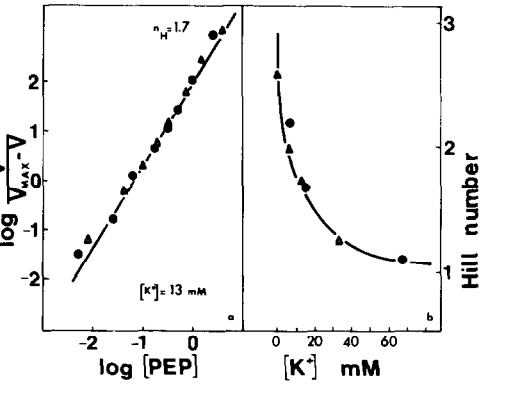

Fig.4. Hill-plot of native (•) and immobilized pyruvate kinase (△) for a K^+ concentration of 13 mM (fig.4a). Hill number as a function of K^+ concentration for native (•) and immobilized enzyme (△) (fig.4b). [PEP] = mg ml^{-1}, free acid.

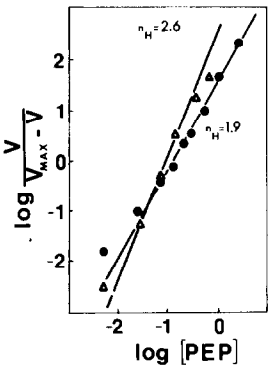

Fig.5. Hill-plot of pyruvate kinase immobilized in the presence (8×10^{-2} M) (•) or absence (△) of K^+ during the crosslinking process. Both measurements were performed in absence of K^+. The K^+ was washed out from the membrane after the immobilization with a Tris-maleate buffer containing 1 M Na^+. [PEP] = mg ml^{-1}, free acid.

4. Discussion

A lot of papers deal with the medical, analytical and industrial applications of immobilized enzymes [18] but little attention has been paid to the basic potentialities of the method. The influence of a structure on enzyme kinetics in terms of diffusion is the only point that has been intensively studied [7,10]. A part of the paper also deals with this aspect: it is shown that diffusion limitations give biphasic Hill plots for an allosteric enzyme. The problem of conformation change of proteins, especially allosteric enzymes, has attracted considerable attention [5] and the introduction of artificial conformational constraints by immobilization seems a new tool of investigation.

From the results obtained with pyruvate kinase the artificial immobilization under low diffusion limitations does not give evidence of modification of cooperativity. The only pointer to it being so is a modification of the Hill number with pyruvate kinase immobilized in the presence of an activator (K^+). Modification of hemoglobin conformation is generally discussed as a general model for allosteric proteins. With hemoglobin there is no doubt that oxygen binding induces changes in quaternary structure of the protein, i.e., a rearrangement of the subunits with respect to each other, as shown by the X-ray data of Muirhead and Perutz [19]. It was demonstrated by Thomas et al. [20] that human hemoglobin immobilized, in the presence of oxygen, under the conditions described here no longer exhibits cooperativity. A Hill number of one and a higher apparent affinity was observed, the protein conformation exhibiting the highest affinity seems to be frozen by immobilization.

From the results presented in the present paper, pyruvate kinase does not exhibit so deep a modification of conformation. Thus, the use of hemoglobin as a model for allosteric enzymes is questionable.

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