Biochimica et Biophysica Acta, 364 (1974) 193–199

BBA 67311

THE MECHANOCHEMISTRY OF IMMOBILIZED ENZYMES. HOW TO STEER A CHEMICAL PROCESS AT THE MOLECULAR LEVEL BY A MECHANICAL DEVICE

ILYA V. BEREZIN, ALEXANDER M. KLIBANOV and KAREL MARTINEK

Laboratory of Bioorganic Chemistry, Building "A", Lomonosov State University, Moscow 117234 (U.S.S.R.)

(Received April 22nd, 1974)

SUMMARY

1. A kinetic study of the tryptic activation of chymotrypsinogen in polyacrylamide gel has been performed. It was found that an increase in the gel concentration up to 32% (w/w) produces only a weak effect on the rate of protein–protein interaction. However, if the gel concentration is further raised by only 1–2% (w/w), the reaction appears to be arrested. This is due to the fact that, as the concentration of the gel increases, the protein–protein interaction begins to be controlled by diffusion.

2. Mechanical compression of the gel results in the diffusion-controlled reaction being accelerated by more than 20 times. After subsequent decompression of the gel the reaction rate returns to the initial level. In terms of the suggested structure of the gel, it is inferred that mechanical deformation allows the reaction to be reversibly switched from a diffusion to a kinetic regime.

3. The phenomenon discovered by us simulates regulatory mechanisms which may function in the enzymic systems localized in gel-like membranes in vivo.

4. The system studied, in which the "generation" of an enzymic activity occurs as the result of mechanical deformation of the polymeric support, may be used as a chemical amplifier of weak mechanical "signals".

INTRODUCTION

There are several reasons why immobilized enzyme catalysis is developing so rapidly. Firstly, it is due to the new possibilities which the use of the immobilized enzymes have opened up for chemical technology and medicine. Secondly, the enzymes incorporated into a polymer or gel film may be regarded as being a model of real membrane enzymic systems [1]. In this connection it was interesting to find out whether the rate of protein–protein interaction in the gel support can be regulated by mechanical compression. This question, if solved, may have both theoretical and practical significance.

We have studied the kinetics of activation of chymotrypsinogen by trypsin in polyacrylamide gel. The enzymic activation of zymogen [2] was made the object of investigation because this kind of protein–protein interaction has much in common

with the reaction which occurs in the membrane in vivo and which plays an important role in nerve excitation transmission [3]. The choice of polyacrylamide gel for a support [4] is primarily due to its being chemically inert. The three-dimensional lattice of this gel does not interact with low molecular substances (electrolytes and non-electrolytes) [5], or with proteins [6].

EXPERIMENTAL

Preparation of gel. The gel was prepared by riboflavin (10 mg/l) initiated photochemical polymerization of the mixture of acrylamide and *N,N'*-methylenebisacrylamide (95:5, w/w) in the presence of all the components of the enzymic process (also in the presence of α-chymotrypsin titrant; see Determination of the rate of the process studied). Polymerization was carried out by cooling with ice-cold water. The time of illumination, 20 min, should be amply sufficient to ensure that total polymerization occurs.

Determination of the rate of chymotrypsinogen activation by trypsin. The quantity of the α-chymotrypsin formed was determined by titration with *p*-nitrophenyltrimethylacetate ($1.5 \cdot 10^{-4}$ M; 2% acetonitrile (v/v)) [14]. The gel was prepared directly in the optical cuvette of a "Hitachi-356" double-wave spectrophotometer (l = 1 cm). The rate of formation of *p*-nitrophenol was determined at 400 nm, subtracting the absorbance at 550 nm.

Experimental procedure for compression. A 1 cm thick parallelepiped cut out of a piece of gel was placed between two parallel framed glass plates, the distance between which could be changed. This system was then placed in the spectrophotometer cuvette. 5 min after the beginning of the reaction the gel was compressed and in another 5 min the load was removed. The decrease in the length of the optical pathway due to gel compression is taken into consideration.

Process 1, "initiation" of catalytic activity on mechanical compression.

Trypsinogen + a catalytic amount of trypsin incorporated in the gel. The system is not active due to diffusion hindrances	Mechanical compression of the gel ——→	System without diffusion hindrances	→ Trypsin

Process 2, amplification of a weak mechanical action. The accumulation of Product P should be taken as a measure of amplification.

$$\text{Substrate} \xrightarrow{\text{Trypsin}} \text{Product (P)}$$

Scheme 1.

RESULTS

The three major results we have obtained are: (i) Fig. 1 shows a plot of the rate of formation of α-chymotrypsin (on the activation of chymotrypsinogen by tryp-

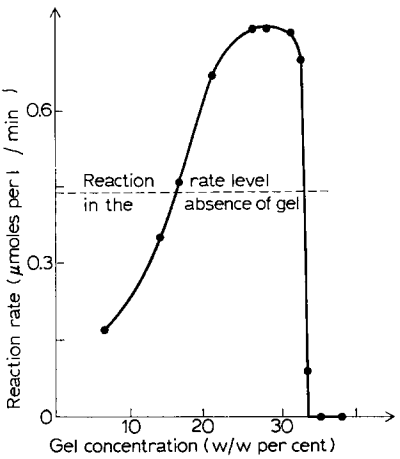

Fig. 1. Dependence of the rate of tryptic activation of chymotrypsinogen on the concentration of polyacrylamide gel. Conditions: 20 °C, pH 7.9 (0.01 M Tris–HCl buffer), $2 \cdot 10^{-7}$ M trypsin and $1 \cdot 10^{-4}$ M chymotrypsinogen. On the ordinate, the rate minus the background (about 3% of the optimal rate of the process). The background is evidently due to trypsin reacting with the α-chymotrypsin titrant.

sin) versus the concentration of polyacrylamide gel. As is seen in the figure, the rate of the process increases as the concentration of the gel increases up to 25%. The phenomenon seems to be due to the fact that the monomer, as well as the movable polymer chains, produce an inhibiting effect on the process. For example, under the conditions specified in the caption to Fig. 1, the rate of activation of the zymogen in the absence of acrylamide is 0.45 μmoles/min, whereas in the medium of the 20% acrylamide monomer (which corresponds to the optimal concentration of the gel) the rate is 0.02 μmoles/min.

(ii) Of great interest is another observation, i.e. that the rate of the protein–protein interaction sharply decreases at gel concentrations exceeding 32%. For example, if the concentration of the gel is changed by only 2%, the rate of activation of zymogen goes down to zero. This result cannot be accounted for by any kind of protein denaturation, since, as is seen in Fig. 2, the concentration of polyacrylamide gel from 20–45% produces only a slight effect on the reaction rate of both trypsin and α-chymotrypsin with *p*-nitrophenylacetate, their low molecular substrate. It is most feasible to interpret the data obtained (the right hand part of the curve in Fig. 1) in terms of the concept of protein diffusion in polyacrylamide gel [7]. It may be supposed that at gel concentrations exceeding 32% the protein–protein interaction is a diffusion-controlled process. On the other hand, it is understandable that a reaction involving a low molecular substrate (Fig. 2) may proceed without diffusion hindrances, the fact following from the general theory describing the effect of diffusion on the rate of a chemical reaction [8].

(iii) We have found that the rate of activation of the zymogen may be significantly increased by mechanical compression of a sufficiently dense matrix obtained at a gel concentration exceeding 32% (deformation of a gel of a lower concentration does not in the least alter the rate of the enzymic reaction). It is shown in Fig. 3 how the rate of the enzymic process studied in a 33.5% polyacrylamide gel depends on the degree of compression of the gel plate. This effect, as shown in Fig. 4, is almost

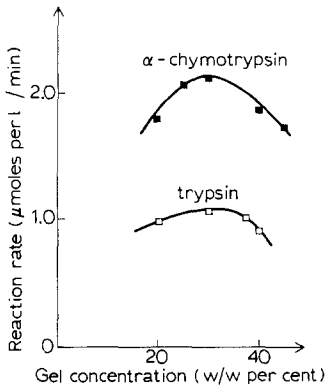

Fig. 2. The dependence of the reaction rate of α-chymotrypsin and trypsin with *p*-nitrophenylacetate, upon the concentration of polyacrylamide gel. Conditions, 20 °C, pH 7.0, buffer 0.01 M Tris–HCl, $2 \cdot 10^{-5}$ M α-chymotrypsin, $2 \cdot 10^{-5}$ M trypsin, $3 \cdot 10^{-4}$ M substrate and 2% acetonitrile (v/v).

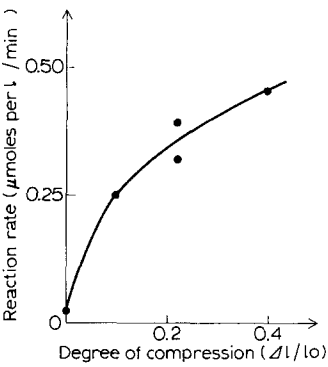

Fig. 3. The dependence of the rate of reaction between trypsin and chymotrypsinogen on the value of the relative degree of compression of 33.5% polyacrylamide gel. For the conditions see the captions to Fig. 1.

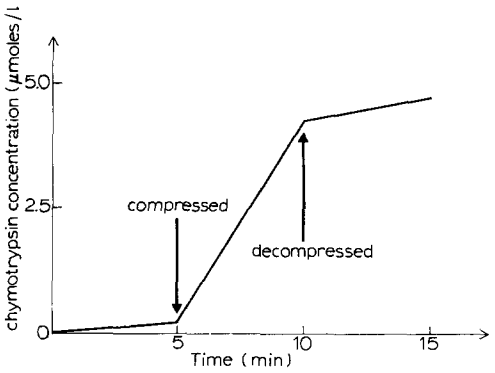

Fig. 4. The product concentration vs time dependence for the reaction of trypsin with chymotrypsinogen in 33.5% polyacrylamide gel. Conditions: a piece of gel was placed in the spectrophotometer cuvette. 5 min after the beginning of the reaction the gel was compressed by 35% and in another 5 min the load was removed. For the other conditions see the caption to Fig. 1.

completely reversible. For example, 5 min after the beginning of the reaction the gel was compressed ($\Delta l/l_0 \approx 0.4$), which resulted in the rate being increased almost 20-fold. In another 5 min the load was removed; thereby, the rate of the enzymic process almost instantaneously returned to the initial level. At low degrees of deformation the "compression–decompression" procedure may be repeated many times, the reversibility of the "increase–decrease" effect of the reaction rate being maintained all the time.

DISCUSSION

The results obtained by us may be explained in terms of the following model of protein diffusion in gel. Let us suggest, after Tombs [7], that at sufficiently high concentrations of gel (when the size of pores is close to that of the diffusing protein), the diffusion coefficient depends on the quantity of the pores whose size exceeds a critical value rather than on the average size of the pores (in the first approximation the critical value may be taken as an effective diameter of a protein globule). Let us imagine that in the initial state (prior to compression) the protein molecule is incorporated into a certain elementary unit of polymeric gel (Cube a in Fig. 5), which, since the size of the facets is too small, does not allow the diffusion of the protein

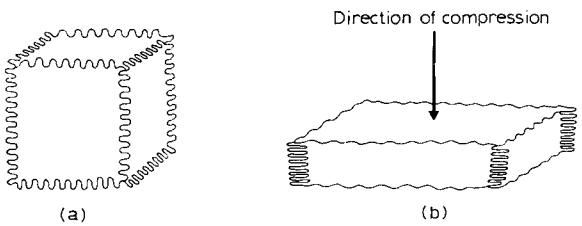

Fig. 5. A scheme of the shape of an elementary unit of polyacrylamide gel prior to compression (a) and after compression (b).

globule. If one exerts some pressure, normally to the upper facet, one obtains a paralellelepiped shown in Fig. 5b. The volumes of these two figures are bound to be equal, as gel, like water, is practically incompressible. This means that the decrease in the side facets of the unit due to compression should entail an increase in the upper and lower facets. The latter seems to have a very great significance for the rate of diffusion, as the compression of the gel may bring about an increase in the number of pores whose size exceeds the critical value. Hence the conclusion, that, in terms of this model, compression of the gel may basically facilitate diffusion, thereby increasing the rate of the protein–protein interaction, which has been observed experimentally. This phenomenon opens up a lot of possibilities in the mechanochemistry of immobilized enzymes, as the use of other kinds of gel (or polymer) deformation, e.g. stretching, shearing, bending and twisting may also be possible.

For the practical purposes, the above system may be used for amplifying weak mechanical effects. This is due to the fact that, in a system subjected to a weak mechanical effect, the level of catalytic activity increases (Figs 3 and 4). The measure of amplification of a weak primary "mechanical" signal will be the quantity of the sub-

strate processed by the generated enzyme. This is very well exemplified by an autocatalytic process shown in the scheme.

Let us estimate the specific energy expenditure for the generation of one molecule of the catalyst. It is obvious that the quantity of the enzyme "generated" when the gel was being compressed depends not so much on the energy consumed by compression but also on the initial concentration of the immobilized enzyme and the volume of the gel. Let us make a calculation for the conditions employed in the experiment when the rate of activation of chymotrypsinogen was measured in a gel cube of a 1 cm edge, which contained 10^{-4} M of trypsin. In 35% gel the reaction does not occur, see Fig. 1. For the cube to be relatively compressed by about 20%, it should be subjected to a pressure of about 1 atm = 10 newton/cm^2, which corresponds to an energy expenditure of 10 newton $\cdot$ 0.2 cm = $2 \cdot 10^{-2}$ J = $5 \cdot 10^{-3}$ cal. As a result of such compression, the rate of the reaction goes up to about one-third of the maximum possible value (which corresponds, apparently, to the total absence of diffusion hindrances in the gel, see Fig. 1). Therefore, one may tentatively assume that about one-third of the immobilized enzyme molecules have been liberated as a result of compression, i.e. $0.33 \cdot 10^{-7}$ mole/ml $\cdot 6 \cdot 10^{23} = 2 \cdot 10^{16}$ molecules. Thus the specific energy expenditure for initiating enzymic activity is $5 \cdot 10^{-3}$ cal/$2 \cdot 10^{16}$ molecules = $2.5 \cdot 10^{-19}$ cal/molecule of catalyst. It is evident that the level of catalytic activity of the immobilized enzyme may be regulated mechanically by means of a moderate energy expenditure. (We have previously suggested a model in which the catalytic (enzymic) activity may be initiated by light [9–12]. In this model, which is based on the mechanism of the primary act of visual reception after Wald [13], we have $h\nu/0.1 = 10^{-18}$ cal/molecule of the "generated" catalyst, the quantum yield being 0.1 at 313 nm wavelength.) Moreover, it should be taken into account that in the case of an ideally elastic gel, no energy is evidently spent as deformation is totally reversible. In a descriptive fashion, initiation of the catalytic activity by exerting mechanical action may be compared to the energy-independent opening of a door in a reservoir where enzyme molecules are stored.

It should also be pointed out that the regulation of the rate of the protein–protein interaction (affected in the mechanochemical model described above) may actually function in vivo, for example in some enzymic processes occurring in the membranes with ordered gel-like structures. One may expect when various mechanical deformations of the membrane occur (the mechanism of hearing?), its structure may undergo alterations ranging from a free diffusion state to the one with diffusion hindrances (and vice versa). This may affect, as has been demonstrated in the present communication, the catalytic properties of the enzymes immobilized in the membrane.

REFERENCES

1 Goldman, R., Goldstein, L. and Katchalski, E. (1971) Biochemical Aspects of Reactions on Solid Supports (Stark, G. R., ed.), pp. 1–78, New York
2 Kunitz, M. and Northrop, J. H. (1935) J. Gen. Physiol. 18, 433–441
3 Segal, H. L. (1973) Science 180, 25–32
4 Bernfeld, P. and Wan, J. (1963) Science 142, 678–679
5 White, M. L. and Dorion, G. H. (1961) J. Polymer Sci. 55, 731–740
6 Marinis, S. and Ott, H. (1965) Protides Biol. Fluids Proc. (Peeters, H., ed.), pp. 420–421, Elsevier, Amsterdam

7 Tombs, M. P. (1965) Anal. Biochem. 13, 121–132
8 Noyes, R. M. (1961) In Progress in Reaction Kinetics (Porter, G., ed.), Vol. 1, pp. 129–160, London
9 Berezin, I. V., Varfolomeyev, S. D. and Martinek, K. (1970) Dokl. Acad. Nauk S.S.S.R. 193, 932–935
10 Martinek, K., Varfolomeyev, S. D. and Berezin, I. V. (1971) Eur. J. Biochem. 19, 242–249
11 Varfolomeyev, S. D., Klibanov, A. M., Martinek, K. and Berezin, I. V. (1971) FEBS Lett. 15, 118–120
12 Berezin, I. V., Kazanskaya, N. F. and Aisina, R. B. (1972) Dokl. Acad. Nauk S.S.S.R. 207, 1383–1386
13 Wald, G. (1968) Nature 219, 800–807
14 Bender, M. L., Begue-Canton, M. L., Bleakeley, R. L., Brubacher, L. J., Feder, J., Gunter, C. R., Kezdy, F. J., Killheffer, J. V., Marshall, T. H., Miller, C. G., Roeske, R. W. and Stoops, J. K. (1966) J. Am. Chem. Soc. 88, 5890–5913