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THE PRINCIPLES OF ENZYME STABILIZATION

II. INCREASE IN THE THERMOSTABILITY OF ENZYMES AS A RESULT OF MULTIPOINT NONCOVALENT INTERACTION WITH A POLYMERIC SUPPORT

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Summary

The catalytic activity, thermostability (resistance to monomolecular thermoinactivation) and molecular mobility of chymotrypsin and trypsin mechanically entrapped into polymethacrylate and polyacrylamide gels have been studied. It has been established that the thermostability of the enzymes does not depend on the concentration of electroneutral polyacrylamide gel over the range of 0—50 w/w%. However, in polymethacrylate gel of concentration higher than 30 w/w%, when a high catalytic activity is retained, the thermostability of chymotrypsin dramatically increases: in 50 w/w% gel the first-order rate constant for thermoinactivation of the enzyme at 60°C is 10^{-5} that in water.

Based on these data and also on experimentally obtained results on transitional and rotational diffusion of both native and modified enzymes, the following mechanism of enzyme stabilization is formulated and proved. In principle, the protein molecule of an enzyme may form with the three-dimensional lattice of polyelectrolyte gel multiple noncovalent linkages (via electrostatic or hydrogen bonds); as a result, the structure of the enzyme becomes more rigid and its thermostability should increase. However, since these bonds are relatively weak, in diluted gels they can hardly be realized, as the "quenching" of the transitional movement of the enzyme molecules, accompanying complex formation would have required a heavy entropy loss. At the same time, in concentrated gels, this unfavourable entropy contribution is absent as the polymer's lattice provides significant steric hindrances for the transitional diffusion, so that the molecules almost stop moving. That is why weak linkages between the protein globule and the support can be realized here. That the complex formation does take place is indicated by the fact the rotational diffusion of the protein molecules is almost completely frozen. When there is no specific protein-

support interaction (in polyacrylamide gel), no deceleration of the rotational movement of the protein molecules occurs and no noticeable increase in the thermostability of the enzymes is observed.

It is possible that the mechanism discovered by us functions in vivo and is responsible for the stability (and, which is important, for stability regulation) of the proteins incorporated in biomembranes. On the other hand, the results obtained by us may enrich enzyme engineering, as they allow the general strategy of production of stabilized enzymes to be outlined.

Introduction

We demonstrated previously [1] that the thermostability of enzymes may be drastically increased if they are attached to a complementary surface of a relatively rigid support in a multipoint fashion. This finding allows one to attain unfailingly stabilization of enzymes by their being chemically immobilized. It is known [2], however, that in addition to chemical immobilization methods, immobilization by physical methods is often used now (for example, mechanical entrapment into gels or adsorbtion on supports). Hence the question: is it possible to apply the principles of multipoint protein-support interaction for stabilizing enzymes by physical immobilization methods? In other words, is it possible to increase considerably the thermostability of enzymes if their multipoint interaction with the support is not covalent but relatively weak, i.e. provided by electrostatic, hydrogen, hydrophobic, etc., bonds?

This problem has another very important aspect. In vivo the overwhelming majority of enzymes function not in a free state, but incorporated into biological membranes or adsorbed on their surfaces [3,4]. In this case the enzyme is associated with other components of the membrane by multipoint noncovalent linkages (hydrophobic, electrostatic, hydrogen etc.) [5]. Therefore it is very important to elucidate whether there are general mechanisms of enzyme stabilization involving such linkages and if so, to simulate these mechanisms.

In the present work, we have carried out a detailed study of the thermostability, catalytic activity and molecular mobility of model enzymes, chymotrypsin and trypsin, mechanically entrapped in polymethacrylate gels of different concentrations. In this gel, the protein molecule of the enzyme may, in principle, form weak linkages (for example, electrostatic and hydrogen) with the polyelectrolyte support. This is why enzymes mechanically entrapped in polymethacrylate gel may be considered as a realistic model of biomembrane systems.

Experimental

Materials

Bovine α-chymotrypsin was a commercial preparation of Koch-Light (3 times crystallised); the concentration of the active enzyme determined by spectrophotometric titration [6] was 64%. Bovine trypsin was obtained from the Leningrad Kirov meat-packing plant; the concentration of the active enzyme determined by spectrophotometric titration [7] was 56%. The substrates, *N*-acetyl-L-tyrosine ethyl ester and *N*-α-benzoyl-L-arginine ethyl ester, were com-

mercial preparations of Koch-Light and Reanal, respectively. The active centers titrants, *N-trans*-cinnamoylimidazole and *p*-nitrophenyl-*p'*-guanidinebenzoate, were obtained from Serva. Picryl sulfonic acid, a titrant of $-NH_2$ groups of proteins, was a product of Sigma. Dansyl chloride (1-dimethylaminonaphthalene-5-sulfonylchloride) was a commercial preparation of Fluka. Succinic anhydride was a product of Koch-Light and *p*-aminobenzylcellulose of Serva.

Sodium methacrylate was prepared from methacrylic acid as described in [8]. To remove fluorescing contaminations (for example, hydroquinone) from methacrylic acid (Koch-Light), it was distilled in vacuum over reduced copper. Acrylamide (Koch-Light) and *N,N'*-methylene bisacrylamide (Reanal) were purified by recrystallization from chloroform and acetone, respectively [9]. The initiators of polymerization, ammonium persulfate and *N,N,N',N'*-tetramethylethylenediamine, were commercial preparations of Reanal.

Sephadexes G-75 (Fine) and G-50 (Fine) were obtained from Pharmacia. The rest of the reagents used in this work, i.e. inorganic salts, buffer solutions components, acids, KOH, were analytical grade preparations.

Methods

Measurements of the catalytic activity of the enzymes. The activity of the enzymes was determined potentiometrically in a Radiometer TTT 1c pH-stat, by the initial rate of catalytic hydrolysis of the specific substrates, *N*-acetyl-L-tyrosine ethyl ester for chymotrypsin and *N*-α-benzoyl-L-arginine ethyl ester for trypsin. An aqueous solution or gel ground into powder (weight less than 0.1 g and particle size not more than 0.1 mm) containing the enzyme, were added into the cuvette of the pH-stat to 5 ml of the $5 \cdot 10^{-3}$ M substrate solution in 0.1 M KCl at pH 8.0 and 25° C. The acid liberated as a result of enzymatic hydrolysis was titrated by 10^{-2} M KOH.

We have purposely demonstrated (in terms of the criteria formulated previously [10]) that in the conditions used by us, the diffusion rate of the reagents was sufficiently high not to affect the rate of enzymic hydrolysis; to this end, we studied the dependence of the rate of the reaction catalyzed by the immobilized enzyme on the stirring rate, temperature, substrate concentration and the activity of the immobilized enzyme and the size of gel particles. It appeared that we followed the true value of catalytic activity of the enzymes entrapped into the gel (without diffusion restrictions).

Incorporation of the fluorescent (dansyl) label in chymotrypsin molecule. 150 mg of chymotrypsin were dissolved in 9 ml of 0.1 M phosphate buffer, pH 7.5, then 0.9 ml of acetone containing 4 mg/ml of 1-dimethylaminonaphthalene-5-sulfonylchloride (dansyl chloride) were added. The mixture was incubated for 15 h in the dark. The stained protein was separated from other products by gel filtration in a 3 × 45 cm column packed with Sephadex G-75 equilibrated with 0.01 M NaCl. The protein in the central region of the main peak (approx. 75 mg) was collected, dialyzed against distilled water and lyophilized. It was shown spectrophotometrically [11] that, in these conditions approx. 1.3 dansyl label is bound to a molecule of protein.

Succinylation of the enzymes. Modification of chymotrypsin (and dansyl chymotrypsin) by succinic anhydride was carried out as described in [12]. It was shown by spectrophotometric titration of protein amino groups by picryl

sulfonic acid [13], that, as a result of succinylation, chymotrypsin loses approx. 85% out of the 14—15 titrated NH_2 groups. The enzyme thereby retains all its catalytic activity; moreover, the Michaelis-Menten equation parameters determined by us for the reaction of succinylated chymotrypsin with *N*-acetyl-L-tyrosine ethyl ester are very similar to those given in [12].

Entrapment of the enzymes into polymethacrylate gel. Chymotrypsin, succinylated chymotrypsin and trypsin were entrapped into polymethacrylate gels of different concentrations according to the procedure described in [8]. The enzyme was introduced into a solution of sodium methacrylate of a given concentration (up to 50% w/w, which is the solubility limit of the monomer), and *N,N'*-methylene bisacrylamide (their weight ratio was always 35 : 1), then *N,N,N',N'*-tetramethylethylenediamine, a polymerization initiator, was added (0.15 w/w%). The mixture was cooled and pH was adjusted to 8.0 with the help of a concentrated solution of KOH. Then, after 0.3 w/w% of the other initiator, $(NH_4)_2S_2O_8$, were added, the solution was rapidly stirred, placed into thin test-tubes (or into spectrofluorometric cells for fluorescent measurements) and then into an ice bath. The process of gel formation lasted 6 h.

The concentration of the enzymes in the starting solution of the monomer (prior to polymerization) did not exceed $5 \cdot 10^{-5}$ M. It was found out by titration [8] that polymerization does not lead to loss of chymotrypsin active centres. However, the relative catalytic activity of both chymotrypsin and trypsin entrapped into polymethacrylate gel proved to be 30—40% of that in the native state; the observed decrease in the activity is, probably, caused by change in the enzyme's microenvironment due to immobilization (rather than by diffusion restrictions, see above). The measurements were carried out both with swollen gel and with the particles of gel placed in the solution of the monomer that had the same concentration as in the studied gel; in the latter case the swelling of the gel was not so intensive.

The pH of the gel was assumed to be that of the solution of the monomer before polymerization. As we showed with the help of microelectrodes, these two values differ by not more than 0.2 pH units.

Entrapment of chymotrypsin into polyacrylamide gel. This was carried out by two methods. (a) To make fluorescent measurements concentrated solutions of acrylamide and *N,N'*-methylene bisacrylamide were passed through a column packed with ion exchange resine "Elgalite" to remove fluorescing admixtures. Then to the solutions of the monomer and the cross-linking agent (the w/w ratio of which was always 19 : 1) the enzyme (1 mg/ml) was added and one of the initiators, *N,N,N',N'*-tetramethylethylenediamine (0.08% w/w); the mixture was cooled by water and ice and pH was adjusted to 8.0 by a concentrated solution of KOH. Finally, 0.15 w/w% of the second initiator, $(NH_4)_2S_2O_8$, were added, the mixture was rapidly stirred and poured into spectrofluorimetric cells placed in an ice bath. Polymerization was allowed to take place for 4 h. The relative catalytic activity of chymotrypsin entrapped into polyacrylamide gel was almost equal to that of the native enzyme.

(b) Polyacrylamide gel prepared by method (a) contains a certain amount (up to several per cent w/w) of the non-polymerized monomer and, possibly, of oligomers which are not incorporated into the three-dimensional covalent lattice of the gel. These compounds per se stabilize the enzyme against thermo-

inactivation [14]. To avoid this, for thermoinactivation experiments the gel was prepared without the enzyme and washed until all the low-molecular-weight admixtures were removed. To this end, the gel (35% w/w) was ground into powder (particle size not more than 0.1 mm) and was incubated, with intensive stirring, 10 times for 1 h at room temperature with a 10-fold excess (w/w) of distilled water. Then the enzyme was mechanically entrapped into the washed gel. For this purpose the gel particles were incubated for 24 h at 4°C and pH 8.0 ($2 \cdot 10^{-2}$ M Tris · HCl + 0.1 M KCl) in $5 \cdot 10^{-6}$ M chymotrypsin solution, then they were filtered and washed with distilled water. The partition coefficient of the protein in the gel-buffer system is about 0.1. As a result, we obtained gel with $5 \cdot 10^{-7}$ M enzyme, the concentration of the polymer being 22% w/w (after swelling). To obtain a preparation with a higher content of polyacrylamide, the gel particles were frozen in liquid nitrogen and lyophilized until the required weight was reached. As a result of all these operations, the relative catalytic activity of the entrapped enzyme decreased by not more than 1.5—2 times.

Study of enzyme thermoinactivation. To study the thermoinactivation of native or succinylated chymotrypsin, an approx. 10^{-7} M solution of the enzyme with pH 7.8 (0.02 M Tris · HCl + 0.1 M KCl) was incubated at a given temperature for a certain time, then it was rapidly cooled and the relative enzymatic activity was measured at 25°C.

To thermoinactivate the enzymes entrapped in the gel, the thin test-tubes with the enzyme-containing gel were heated in a water bath to a certain temperature, incubated for a certain time; then the test-tubes were taken out, rapidly cooled with ice-cold water, the gel was extracted from the test-tubes, ground into powder and placed into the cuvette of the pH-stat and the relative enzymatic activity was determined at 25°C (for experimental details, see ref [1]).

We purposely demonstrated that the studied process is a monomolecular thermoinactivation that does not involve autolysis or aggregation. This is evidenced by the experimental fact that the catalytic activity vs. incubation time profiles do not depend on the initial concentration of the enzymes over the concentration range tested, i.e. $2 \cdot 10^{-8}$—$2 \cdot 10^{-7}$ M at 55°C in the solution and $2 \cdot 10^{-6}$—$5 \cdot 10^{-5}$ M at 60°C in the gel. In spite of this, the thermoinactivation pattern cannot be described in terms of the first-order reaction kinetics (Fig. 1). That is why, as previously [1,8], we determined experimentally only the effective first-order rate constant with respect to a certain degree of the conversion in the process (most often, 50—75%) of enzyme inactivation.

Study of the transitional diffusion of the enzymes in the polymethacrylate gel. To estimate the upper limit of the polymethacrylate gel concentration at which there occurs a deceleration of the transitional diffusion of the enzyme molecules, an approach was used based on the results of [15—17] where a study was made of bimolecular protein-protein interaction in polyacrylamide gel (to be more exact, trypsin activation of chymotrypsinogen [15] and autolysis of trypsin [16]). It was found that the rate of these reactions does not depend much on the gel concentration in the range of 0—30 w/w% but at higher concentrations the rate sharply decreases. This seems to be due to the fact that at a high concentration of the gel, the rate of the protein-protein interaction

goes from the kinetic region to that of diffusion. The rate constant k_{app}, characterizing the interaction of two uncharged particles is described [10,18] (with an accuracy to a steric factor) by the following equation:

$$k_{app} = \frac{k}{1 + \frac{k}{4\pi D\delta}} \quad (1)$$

where k is the true (not diffusion-dependent) rate constant of the reaction, D is the sum of the diffusion coefficients of the reagent's molecules, δ is the equilibrium distance of the interaction. For most of chemical reactions occurring in water, the following relation is usually true:

$$k/4\pi D\delta << 1$$

and, consequently, $k_{app} \approx k$; this means that the diffusion does not affect the process in the least. If the properties of the medium are changed, and the coefficient of diffusion becomes so low that $k/4\pi D\delta >> 1$ then

$$k_{app} = 4\pi D\delta \ . \quad (2)$$

Hence the apparent reaction rate constant will decrease proportionally to the decrease in the diffusion coefficients of the reagents. This is the situation that seems to be realized in concentrated polyacrylamide gels, where, as the monomer concentration increases the protein-protein interaction should be retarded due to the small size of the pores, and the reaction should in the long run be diffusion controlled [17]. In this case its rate is described by Eqn. 2.

In the present work, we have studied autolysis (bimolecular self-cleavage) of trypsin in polymethacrylate gel. Trypsin was chosen because, having physicochemical properties similar to those of chymotrypsin [19], it undergoes autolysis much more readily and is more convenient from the point of view of methodology.

In aqueous solutions at pH 8.0 (0.02 Tris · HCl + 0.1 M KCl), 45°C and an initial concentration of trypsin of $2 \cdot 10^{-5}$ M, the process of its autolytic inactivation strictly obeys the second order kinetics up to the degree of conversion of 90%. The measurement procedure was the same as in the case of monomolecular thermoinactivation of the enzyme (see above). The second order rate constant (found: 250 $M^{-1} \cdot s^{-1}$) does not change even if the initial concentration of the enzyme is changed 5-fold. This means that at such (high) concentrations of the enzyme we are dealing with an autolytic process, and not with monomolecular thermoinactivation. In polymethacrylate gels of low concentration (up to 25% w/w), autolysis of trypsin (under the same conditions as in solution) also obeys rather well the second order kinetics.

Study of the rotational diffusion of protein molecules in polymethacrylate and polyacrylamide gels. For studying the rotational diffusion of proteins in gel, we used the method of fluorescence polarization [20—23]. The gist of the method is the following: if a fluorescing molecule in solution is excited by polarized light, in a certain period of time characterized by a value of τ (an average lifetime of the molecule in the excited state) this molecule may emit a photon. However, the emitted light will be partially depolarized, as the fluores-

cing molecule, which takes part in the Brownian rotation, will change its orientation within this time. That is why, measuring the value of fluorescence polarization, one may judge about the rate of rotational diffusion of the molecule in solution. As the fluorescence of chymotrypsin molecules in the solution is weak, we used the method [22] that implies introduction into a protein molecule of a fluorescing dansyl label (the method of dansylation see above).

Rotational relaxation time (ρ_h) of the enzyme molecule was calculated from Eqn. 3 derived in ref. [24] for the case of thermally activated rotation of the label which is firmly fastened to an ellipsoid approximating the studied macromolecule:

$$\frac{1}{P}-\frac{1}{3}=\left(\frac{1}{P_0}-\frac{1}{3}\right)\left(1+\frac{3\tau}{\rho_h}\right). \tag{3}$$

The values of τ and P (fluorescence polarization) were measured for every concentration of the gel as described in [11] (with excitation by linearly polarized light at 365 nm). To estimate the value of P_0 (the highest possible polarization in the absence of Brownian movement), we took into consideration that, according to Einstein's equation for molecular rotation, the value of ρ_h is directly proportional to the η/T ratio (η is the viscosity of the solution, T the absolute temperature) [21,24] and, consequently Eqn. 3 may be presented as:

$$\frac{1}{P}-\frac{1}{3}=\left(\frac{1}{P_0}-\frac{1}{3}\right)\left(1+\beta\frac{T}{\eta}\right) \tag{4}$$

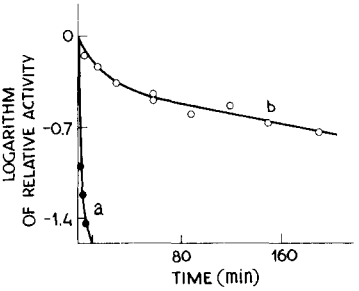
where β is the constant. The value of $1/P_0 = 3.17$ was established in the experiments carried out in aqueous solutions, from the ordinate intercept that is cut off by the asymptote of the linear part of the $1/P$ vs. T/η curve. The viscosity of the solutions was varied by addition of sucrose (from 0 to 40% w/w) [11] at a permanent temperature (25°C). The rest of the measurements were carried out at 5°C.

Study of rotational diffusion of dansylchymotrypsin was carried out at pH 7.0 (and not at pH 8.0 as in the inactivation measurements). This is due to the fact that with protein concentrations necessary for fluorescent measurements (approx. 1 mg/ml) at pH 8 (unlike the case with pH 7.0) the protein may undergo an aggregation. At the same time, we have purposely demonstrated that the dependences of $1/P$ on the gel concentration at the two pH values are qualitatively the same. No aggregation is observed for succinylated dansylchymotrypsin.

Results and Discussion

Thermostability of chymotrypsin in polymethacrylate gels of various concentrations

Fig. 1 shows kinetic curves characterizing the loss of chymotrypsin catalytic activity in aqueous solution (curve a) and in 44% w/w polymethacrylate gel (curve b). It was ascertained that, in the conditions used by us, there takes place monomolecular thermoinactivation, and not autolysis or aggregation (see Methods and refs. 1 and 8). It is obvious from the figure that the thermostability of the enzyme in the gel is by far higher than in the aqueous solution.



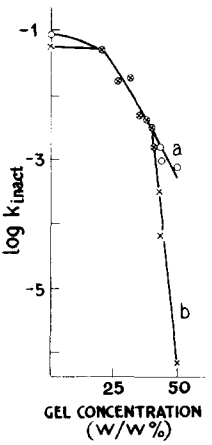


Fig. 1. Kinetic curves of monomolecular thermoinactivation of chymotrypsin: (a) in an aqueous solution in the absence of a monomer; (b) in 44% w/w polymethacrylate gel. Conditions: 60°C, pH 8.0 (in solution: 0.02 M Tris · HCl + 0.1 M KCl, in gel: bufferless medium), initial concentration of the enzyme in solution: 10^{-7} M, in gel: $5 \cdot 10^{-5}$ M; for other conditions see Methods.

Fig. 2. Dependence of the logarithm of the effective value of first-order rate constant (k_{inact}, s^{-1}) characterizing thermoinactivation of chymotrypsin, on the concentration of polymethacrylate gel at degrees of conversion (%): a, 0; b, 75. The points at 0 and 20% w/w gel correspond to thermoinactivation in solution, without a monomer, and in the presence of a soluble polymer, respectively (see ref. 1) at a chymotrypsin concentration of approx. 10^{-7} M when there is no autolysis. The points at gel concentrations 0 and 50% w/w were obtained by extrapolating the temperature dependence of k_{inact} observed at lower or, respectively, higher temperatures (see Fig. 3). Conditions: 60°C, pH 8.0, initial concentration of the enzyme in the gel $5 \cdot 10^{-5}$ M; for other conditions see Methods.

Fig. 2. shows the dependence of the logarithm of the effective rate constant of thermoinactivation of chymotrypsin (at different degrees of conversion) on the concentration * of polymethacrylate gel in the range of 0—50% (the solubility limit of the monomer in water). One can see that at a gel concentration of 0—30% w/w, the rate of thermoinactivation (at 60° C) of chymotrypsin is almost constant, but drops sharply as the concentration of the gel is raised. In 50 w/w% gel the value of the stabilization effect is as high as 10^5 times. Moreover, extrapolating linearly the log k_{inact} vs. $1/T$ plot (see Fig. 3, curve c), one may obtain the average lifetime of the immobilized enzyme as being, at room temperature, hundreds of millions of years (which, by the way, is commensurable with the age of the Earth!)

The stabilization effect observed depends reversibly on the concentration of the gel. For example, if the enzyme is entrapped into concentrated (44% w/w) gel, which is then made to swell (up to a concentration of about 30% w/w), almost no stabilization can be observed.

The possible mechanism of enzyme stabilization in concentrated polymethacrylate gel

Stabilization of the enzyme cannot be explained by the fact that, on its being entrapped into the gel, the protein becomes covalently attached to the

* In all the cases, by concentration of the gel is meant the concentration of the monomer in the polymerization mixture.

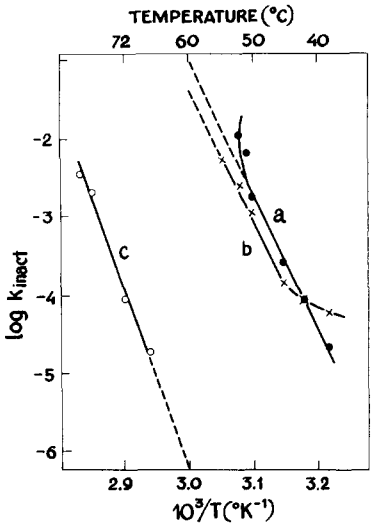


Fig. 3. Temperature dependence for the effective value of first-order rate constant (k_{inact}, s^{-1}) characterizing thermoinactivation of chymotrypsin. a, b: in aqueous solution in the absence of a monomer at different degrees of conversion: 0% (curve a), 75% (curve b); c: in 50% w/w polymethacrylate gel at 75% of conversion. For experimental conditions see legends to Figs. 1 and 2.

polymeric support; we have demonstrated that practically all chymotrypsin entrapped into polymethacrylate gel may be washed out into the aqueous solution [8]. Neither can the reason for the enzyme being stabilized in concentrated gel be the nonpolymerized monomer [14], as we have demonstrated experimentally [8] that its residual concentration in the gel (2% of that prior to polymerization) can only weekly affect the rate of chymotrypsin inactivation. A possible increase in the ionic strength as the concentration of polymethacrylate gel increases cannot account either for the fact of stabilization, because it is known [8,25] that ionic strength affects the thermostability of chymotrypsin only insignificantly.

The phenomenon of a sharp increase in the thermostability of chymotrypsin on its being mechanically entrapped into concentrated polymethacrylate gel (Fig. 2) should evidently be ascribed to the interaction between the enzyme molecules and the polymeric support. The following mechanism seems to be the most plausible. In principle, a protein molecule may form with carboxy groups of polymethacrylic acid electrostatic and hydrogen bonds. These bonds, however, are rather weak and are not realized in solutions of a polymer or diluted gels, where formation of such complexes entails great entropy losses on "quenching" of the rotational and transitional movements of the enzyme molecules. In concentrated gels, where the transitional (and, possibly, rotational) movement must, due to steric hindrances introduced by the three-dimensional lattice of the polymer, be to a great extent decelerated (regardless of the complex formation), the situation should be different. Hence, in concentrated gel, formation of a protein-support complex should be thermodynamically much more favourable, as there are no (or much less) free energy losses due to the rotational and transitional entropy of the enzyme globule on its being sorbed on the polymeric support. It is not excluded that in concentrated gel the mobil-

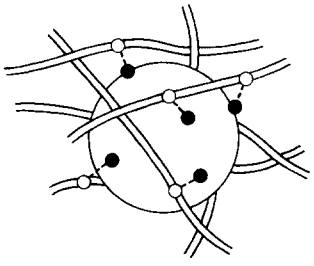
ity of the polymer chains is somewhat limited, and, compared to diluted gels (or a polymer solution), the entropy expenditure of free energy necessary for the support to be 'frozen' (when its complex with the enzyme is formed) are much lower. Finally, with concentrated gel, the interaction of the enzyme with the support should be expected to be multipoint at least for steric reasons, as the polymer chains adher to the enzyme globule from all the sides (Fig. 4). And, as we have demonstrated [1], such multipoint interaction may lead to a sharp increase in the thermostability of the enzyme.

Experimental verification of the suggested mechanism of enzyme stabilization

We believe that the postulated [26] mechanism is, from the thermodynamic point of view, a general physico-chemical mechanism of stabilization of enzymes mechanically entrapped into supports. That is why its experimental verification is of such importance. If the suggested mechanism of stabilization is correct, the enzyme support system should possess the following properties:

(1) The transitional diffusion of enzyme molecules, being more or less free in diluted gels, is considerably hindered in concentrated gels (up to a complete stop of the protein molecules). Furthermore, the model implies that the cessation of the transitional movement of the enzyme's molecules is not associated with the enzyme-support complex formation, but is a result of the fact that in concentrated gels the pores are smaller. Consequently, the threshold concentration of the gel, at which the diffusion of the protein molecules is inhibited, should be lower than that at which a sharp increase in the thermostability of the enzyme is observed (over 30% w/w for the chymotrypsin-polymethacrylate gel system; see Fig. 2).

(2) On the other hand, if the transitional movement of the protein molecules is a priori inhibited (i.e. inhibited independently of increase in the density of the support) a stabilization of the enzyme against inactivation could be observed at sufficiently low (<30% w/w) concentrations of the gel.



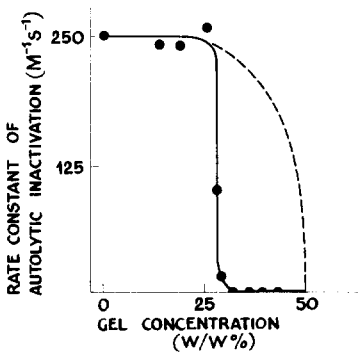


Fig. 4. Schematic representation of multipoint noncovalent interaction of a protein globule of an enzyme with a three-dimensional lattice of a polymeric gel.

Fig. 5. Dependence on the concentration of polymethacrylate gel for the second-order rate constant characterizing autolysis of trypsin. Conditions: 45°C, pH 8.0 (in solution: 0.02 M Tris · HCl + 0.1 M KCl; in gel: bufferless medium), initial concentration of trypsin $2 \cdot 10^{-5}$ M; for other conditions see Methods. Broken line shows decrease in the relative rate constant of monomolecular thermoinactivation as the concentration of gel increases (with the data of Fig. 2).

(3) The protein-support interaction preventing the enzyme from being inactivated is multipoint. If this is the case, then, as the thermostability of the enzyme increases, the rotational mobility of the protein molecules should be decelerated (up to a complete stop of them).

(4) The enzyme-support interaction is specific being evidently due to the formation of electrostatic and hydrogen bonds between the carboxy groups of polymethacrylic acid and the functional groups of the protein (most likely, amino groups). Consequently, if the enzyme is modified (to prevent its being adsorbed on the support) the effect of stabilization (as well as inhibition of the rotational movement of the enzyme molecules) is not to take place at high concentrations of the gel.

As will be shown below, these phenomena do take place experimentally.

(i) Inhibition of the transitional diffusion of trypsin in polymethacrylate gels. To estimate the rate of the transitional diffusion of the enzyme molecules in polymethacrylate gels, we have studied the bimolecular reaction of trypsin autolysis (see Methods). Fig. 5 shows how the rate constant of autolytic inactivation of trypsin changes with increase in the concentration of polymethacrylate gel. One can see that at the concentrations of the gel up to 25% w/w, autolysis of trypsin proceeds at approximately the same rate as in water, but drastically (almost to zero) drops as the density of the support increases further. The inhibiting effect entailed by an increase in the gel concentration, is completely reversible, as on swelling of the 30% w/w gel the rate of bimolecular autolysis of trypsin greatly (almost up to the level in the aqueous solution) increases.

This phenomenon seems to be due to the fact that, as the density of the support increases (hence the pore size decreases), autolysis of trypsin becomes diffusion-controlled (see Methods). If this is the case, one can estimate to what extent the diffusion coefficient of the protein molecules decreased. According to Eqn. 1, in the point where the reaction leaves the kinetic region to become diffusion controlled (25% w/w gel) we have $k_{app} \approx 4\pi D\delta$. The value of k_{app}, determined by us without the gel is equal to $250\ M^{-1} \cdot s^{-1} \equiv 4 \cdot 10^{-19}\ cm^{-3} \cdot s^{-1}$ (see Fig. 5). Consequently, $D \lesssim 10^{-13}\ cm^2 \cdot s^{-1}$, if one assumes $\delta = 5$—50 Å [27]. At the same time, in water the diffusion coefficient of trypsin is approx. $10^{-6}\ cm^2 \cdot s^{-1}$ [28]. Thus, when concentrated (>25% w/w) polymethacrylate gels are used instead of water, the coefficient of transitional diffusion of trypsin drops by more than 7 orders of magnitude, that is the protein almost stops moving.

Finally, let us cite a result that agrees with the idea that the inhibition of the transitional diffusion of the enzyme molecules proceeds independently of the enzyme-support complex formation, i.e. independently of the observed effect of stabilization of enzyme activity. Fig. 5 shows in arbitrary units (broken line) a drop in the relative rate of monomolecular thermoinactivation of chymotrypsin as the concentration of the gel increases (with the data of Fig. 2). One can see that the inhibition of the bimolecular autolysis (solid curve) occurs earlier (at lower gel concentrations) than does the increase in the thermostability of the enzyme (broken line).

(ii) Thermostability of chymotrypsin attached to p-aminobenzyl cellulose in polymethacrylate gel. The transitional movement of enzyme molecules may be

retarded in a different fashion (independently of increase in the density of the three-dimensional lattice of the gel) for example, by attaching the enzyme to the particles of a water-insoluble support. Such an immobilized enzyme, even if entrapped in a relatively diluted polymethacrylate gel, should have a higher stability in terms of our model.

We have studied the thermostability (at 60° C) of chymotrypsin attached to the particles of *p*-aminobenzylcellulose (size of granules 0.01 to 0.1 mm) by azo-coupling reaction with the tyrosine groups of the enzyme [29]. Native chymotrypsin in 30% w/w polymethacrylate gel is almost as labile as in water (see Fig. 2.); whereas *p*-aminobenzylcellulose-attached enzyme, even in such diluted gel, has a several dozens of times lower effective rate constant of thermoinactivation (in comparison with the process where chymotrypsin immobilized on *p*-aminobenzylcellulose is incubated in water).

Thus, this experiment proves the thermodynamic concept put forward by us for explaining the fact of stabilization of the enzyme mechanically entrapped in polyelectrolyte supports.

(iii) Rotational diffusion of chymotrypsin in polymethacrylate gel. Using the method of fluorescence polarization (see Methods), we studied the rotational diffusion of dansylchymotrypsin in polymethacrylate gels of different concentrations. Fig. 6 shows the dependence of the apparent relaxation time (ρ_h) on the gel concentration. One can see that up to the gel concentration of 33% w/w, the rotational freedom of chymotrypsin remains almost constant and the same as in water (and the experimental value of $\rho_h \approx 5 \cdot 10^{-8}$s agrees with the value calculated from the Einstein's formula [21,24] if a molecule of chymotrypsin is approximated as a sphere with a diameter of approx. 40 Å [19]). However, if the gel concentration is raised further, the rotation of the enzyme is inhibited in an abrupt manner (the relatively small residual level of rotation at the gel concentration above 40% w/w seems to be due to the rotation of the fluorescent label which does not depend on the rotation of the protein globule).

A sharp inhibition of the rotation of the enzyme molecule observed in concentrated polymethacrylate gels (Fig. 6) cannot be accounted for by an in-

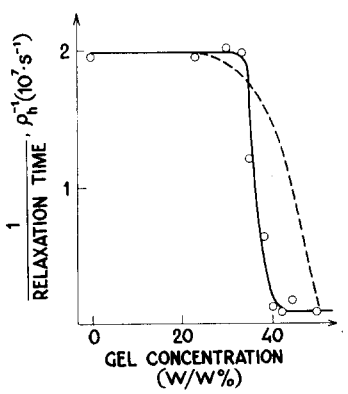


Fig. 6. Rotational molecular mobility (characterized by apparent relaxation time, ρ_h) for dansylchymotrypsin mechanically entrapped into polymethacrylate gel. Conditions: 5°C, pH 7, concentration of the protein 1 mg/ml; for other conditions see Methods. Broken line shows (in arbitrary units) change in the rate constant of monomolecular thermoinactivation of chymotrypsin (with the data of Fig. 2).

crease in the microviscosity of the support as, firstly, in this case the dependence of ρ_h on the gel concentration would have been smooth and not steep as in Fig. 6; secondly, as was demonstrated experimentally [30,31], increase in the concentration of polymers in an aqueous solution only weekly retards the rotational diffusion of proteins.

This effect (Fig. 6) may be explained in terms of the mechanism of enzyme stabilization suggested by us (see above). In diluted polymethacrylate gels, where the transitional diffusion of the protein is relatively free, its interaction with the polymeric support (at the expense of weak, electrostatic and hydrogen, bonds) is hardly ever realized. This means that the rotation of the protein molecules is not inhibited. The situation is different in concentrated polymethacrylate gels, where, as the transitional diffusion of the protein molecules is retarded, multipoint interaction of the protein with the support should (for entropy reasons) come into force. This multipoint protein-support interaction will inevitably cause deceleration and eventually a complete stop of the rotation of the protein molecules.

Fig. 6 shows, for comparison's sake (in arbitrary units), how the rate constant of monomolecular thermoinactivation of the entrapped enzyme decreases as the concentration of the gel increases (with the data of Fig. 2). One can see that retardation of the rotation of the protein globules (solid line) occurs at lower concentrations of the gel than does the stabilization of the enzymatic activity. This seems to be associated with the fact that for the complete inhibition of the rotation of the protein globule to occur, a three-point interaction of it with the support would be sufficient. Such a three-point complex may well be realized in gels of moderate concentrations. At the same time, if the gel concentration is raised further, the probability that the protein will interact with other polymer chains of the support, which surround the molecule of the enzyme, increases (see Fig. 4). This means that the number of the protein-support interaction points will increase and, together with it, will symbatically increase the thermostability of the entrapped enzyme.

(iv) Rotational mobility and thermostability of succinylchymotrypsin in polymethacrylate gel and chymotrypsin in polyacrylamide gel. One could think that the multipoint enzyme-support interaction is due to the fact that in a concentrated gel the chains of the polymer mechanically compress the molecule of the enzyme, thereby preventing it from unfolding [32—35]. This is not so. The enzyme-support interaction has a specific character and seems to be due to electrostatic and hydrogen bonds being formed between the carboxy groups of polymethacrylic acid and, primarily, the amino groups of the protein. To verify this suggestion, we studied two systems in which there is no such specific interaction. The first is polymethacrylate gel with entrapped chymotrypsin which is devoid of the sorbtion ability with respect to the negatively charged support; to attain this, the amino groups of the enzyme were modified (by more than 80%) by acylation with succinic anhydride. The second system is non-modified chymotrypsin, entrapped, however, into polyacrylamide gel which is inert with respect to the proteins [9,36].

It turned out that in the former and in the latter systems, where specific protein-support interaction is absent, both the rotational molecular mobility of the enzyme (characterized by apparent relaxation times ρ_h; see Fig. 7,A) and its

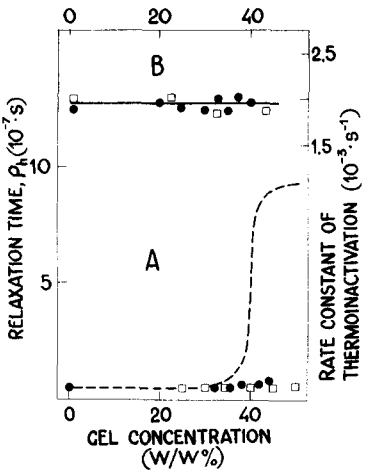


Fig. 7. Rotational molecular mobility (characterized by apparent relaxation time, (ρ_h) (A) and thermostability (characterized by the rate constant of thermoinactivation) (B) for succinylated chymotrypsin in polymethacrylate gel (●———●) and chymotrypsin in polyacrylamide gel (□———□). Broken line shows change in the apparent value of ρ_h for chymotrypsin mechanically entrapped into polymethacrylate gel (with the data of Fig. 6). Conditions: thermoinactivation was carried out at the degree of conversion of not more than 50% at 51°C, pH 8, initial concentration of the enzyme in the gel $5 \cdot 10^{-7}$ M, study of rotational diffusion: 5°C, pH 8, concentration of the protein in gel approx. 1 mg/ml; for other conditions see Methods.

thermostability (characterized by thermoinactivation rate constants; see Fig. 7, B) do not depend on the concentration of the gel up to 45 to 50% w/w *. This allows the conclusion to be made that the effects observed in the chymotrypsin-polymethacrylate gel system (i.e. increase in the thermostability of the enzyme, see Fig. 2, and the loss of rotational molecular mobility, see Fig. 6) are really due to the specific interaction between the carboxy groups of the support and the amino groups of the protein.

To sum up, all the experimental data analysed in (i)—(iv) support the suggested mechanism of stabilization of the enzymes mechanically entrapped into polymeric gel capable of specific interaction with the protein.

Concluding remarks

(a) The results obtained by us (see also ref. 1) allow the following recommendation to be made to enzyme engineers: to obtain highly stable enzyme preparations, one should rigidify the molecule of the enzyme by attaching it with

* This result is also important for an unambiguous interpretation of the results given in (iii). Change in the fluorescence polarization with increase in the gel concentration (Fig. 6) could be explained by the fact that in polymethacrylate gels it is only rotation of the dansyl label that is inhibited (for example, at the expense of electrostatic interaction of its positively charged tertiary amino group with the carboxylate groups of the support), whereas the protein itself does not interact with the support. If this were the case the fluorescence polarization of succinylated dansylchymotrypsin should have changed on its being entrapped in polymethacrylate gel. This, however, does not agree with the experimental results (see Fig. 7,B). Consequently, the alteration in the rotational behavior observed for the nonmodified enzyme (Fig. 6) is solely due to the interaction of the polyelectrolytic support with the protein.

multiple bonds (covalent or noncovalent) to a complentary surface of a relatively rigid support. The statement holds for the cases, and there are a great many such cases [37], when an important step of inactivation of the enzyme is the unfolding of its protein globule.

(b) In terms of the mechanism of enzyme stabilization suggested by us some of the reported data become explainable. Previously [38,39] studies were made on thermostability of acetylcholinesterase, glucose oxidase and peroxidase mechanically entrapped into hydrophobic silicon-organic gel, silastic. The thermostability of the first enzyme entrapped into such gel increased many-fold, whereas the thermostability of the latter two enzymes remained unchanged on immobilization. No explanation was offered for this phenomenon. We believe that all the three enzymes could, in principle, become stabilized against unfolding, and hence thermoinactivation, having formed multipoint hydrophobic contacts with the support. However, the enzyme-support interaction may, as we have shown, be thermodynamically effective only if the molecular mobility of the protein molecules mechanically entrapped into gel is retarded as early as in the ground state. In silastic of the given concentration, inhibition of the transitional diffusion is likely to occur to the large molecules of acetylcholinesterase and not to relatively small molecules of glucose oxidase and peroxidase.

(c) The postulated mechanism of stabilization of enzymes mechanically entrapped into concentrated polymeric supports (forced cessation of the transitional movement of the protein molecule followed by a multipoint interaction of the protein with a relatively rigid support) possibly functions in vivo in membrane enzyme systems, where, firstly, protein molecules possess a rather low mobility (rotational and transitional movements are inhibited) [40], and, secondly, enzyme molecules may interact with phospholipids and structural proteins of the membrane phase forming multiple relatively weak linkages, hydrophobic, electrostatic, hydrogen etc. [5].

(d) It is known that membrane enzymes change their thermostability when organisms adapt themselves to environmental temperature alteration. The molecular mechanism of such regulation is possibly [41] that a fraction of unsaturated lipids present in membranes may produce an effect on the viscosity of the membrane phase and hence on the molecular mobility of the enzyme molecules. Both molecular mobility and thermostability of enzymes mechanically entrapped into a support are, as is demonstrated in the present study, interrelated.

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