Biochimica et Biophysica Acta, 485 (1977) 1—12

BBA 68259

THE PRINCIPLES OF ENZYME STABILIZATION

I. INCREASE IN THERMOSTABILITY OF ENZYMES COVALENTLY BOUND TO A COMPLEMENTARY SURFACE OF A POLYMER SUPPORT IN A MULTIPOINT FASHION

KAREL MARTINEK, A.M. KLIBANOV, V.S. GOLDMACHER and I.V. BEREZIN
Lomonosov State University of Moscow, Bldg. "A", Moscow 117234 (U.S.S.R.)
(Received March 7th, 1977)

Summary

The general principle of enzyme stabilization has been formulated: inactivation of the enzyme due to unfolding of its molecule under a certain denaturing action may be sharply retarded provided the protein globule is rigidified by being attached to a complementary surface of a relatively rigid support in a multipoint fashion.

A method has been elaborated allowing a support with a surface geometry strictly congruent to that of the enzyme globule to be prepared and ensuring multipoint covalent binding to be effected. To this end, the enzyme molecule is modified in many points by a monomer analogue and the resulting enzyme preparation is copolymerized with the monomers. As a result, the enzyme proves to be attached with multiple linkages in the three-dimensional lattice of polymeric gel.

The method of enzyme stabilization has been subjected to experimental verification. Model enzymes, chymotrypsin and trypsin, were first acylated by acryloyl chloride or coupled with acrolein then copolymerized with the monomers, sodium methacrylate or acrylamide. The thermostability of the immobilized enzymes obtained as a result is by several orders of magnitude higher (10^3—10^8 times above the 60—102°C temperature range) than that of native enzymes, with high catalytic activity being retained. This technique allows preparation of both highly stable water-insoluble enzymes (on formation of gel cross-linked with *N,N'*-methylene bisacrylamide) and soluble stabilized enzymes (if polymerization is performed without other than the enzyme cross-linking agents).

Introduction

Elucidation of the principles of enzyme stabilization is one of the most important goals of enzymology. This problem, if solved, will allow preparation of

highly stable enzymes that will find application in medicine, chemical technology, for analytical purposes etc. [1,2]. From the point of view of the theory, the attempts at stabilizing enzymes may be regarded as a new approach to studying the mechanisms of their denaturation; see, for example, [3].

In the recent years, enzymes are stabilized by being immobilized on a support (as a result of covalent binding or adsorption); such works are reviewed very comprehensively in [1,2,4—6]. However, in certain cases, stability of enzymes to inactivating actions increases on their being immobilized, in other cases decreases, sometimes it remains unaltered. All in all, unfortunately, higher stability enzymes are an exception, rather than a rule [1,6].

In the cases when stability of enzymes increases as a result of their immobilization, the following two explanations are offered [1,4—6]: alteration in the conformation of the enzyme on interaction with the support and alteration in the microenvironment of the enzyme molecule when it leaves water to go to the micromedium surrounding the support. Unfortunately we do not know now how the stability of the enzyme is associated with its conformation and its microenvironment, the more so that the relationship may be specific for each enzyme. Therefore, these considerations can hardly be laid as a foundation for the elaboration of the general principles of enzyme stabilization. Moreover, it was recently demonstrated by direct physical methods that, on binding between proteins and supports, the protein macromolecules hardly undergo any conformational changes [7—12].

Another handicap in elaboration of enzyme stabilization principles is that the general mechanisms of their inactivation have not been unraveled [13] in spite of the fact that research in this area has been carried out for dozens of years now. It may well be that a unique, general mechanism of protein denaturation does not exist at all [14].

It is beyond doubt, however, that inactivation of the enzymes under the action of, for example, heating or denaturing agents, involves considerable conformational changes in the protein molecules, that is unfolding [13—15] (Fig. 1, a and b). This, in our opinion, may serve as a starting point in working out the principles of enzyme stabilization. In fact, if unfolding is recognized as being an indispensable step of enzyme inactivation, then the more rigidly fixed the protein globule of the enzyme on a support, the more difficult it is to unfold and, as a consequence, the harder it is to inactivate its catalytic centre. By the way, in nature variation of rigidity of proteins is a common environmental adaptation feature of organisms [16].

Let us imagine now that we have linked a molecule of an enzyme to a rigid support by several strong chemical bonds (Fig. 1, c). It will obviously make the structure of the protein molecule much more rigid, and hence unfolding and inactivation (for example, on heating) of such immobilized enzyme will be much more difficult to accomplish than that of the starting, native, enzyme (transition a → b in Fig. 1). The model shown in Fig. 1, although schematic, seems to be true to reality. For example, when a study was made of the conformational mobility of immobilized proteins (with the use of a number of physical methods), it was found that, as a result of the protein-support binding, the former acquires a more rigid molecular structure (becomes 'frozen') [7,8,10]. This conclusion is supported by abundant indirect data (obtained by both

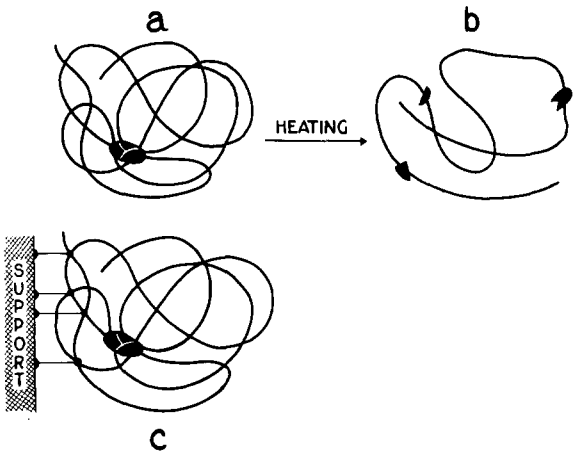


Fig. 1. Schematic representation of the unfolding (and, as a consequence, inactivation) of a native molecule of enzyme (a) on its being heated (transition a → b). For the enzyme attached by many linkages to a rigid support (c) the unfolding becomes considerably hindered. Filled region: the active centre on the enzyme.

physical methods and enzymatic activity measurements) [8,10,17–19]. Indeed, conformational transitions in support-bound proteins are hindered. Gabel, in his pioneering study [20] showed that the more linkages there are between trypsin and Sephadex, the more stable is the protein against inactivation (i.e. unfolding [21]) by urea.

So, all the above considerations give grounds for believing that the general principle of enzyme stabilization consists in multipoint binding of the catalyst molecule to the support. This binding is to make the conformation of the molecule more rigid (without necessarily altering it), hence more stable against unfolding and, ultimately, inactivation. This principle is, however, difficult to realize methodologically for steric reasons, as both the surface of the support and that of the protein have configurations of their own that are by no means congruent. Besides, even when a multipoint binding is realized, it is a very small part of enzyme molecule surface that is bound to the support (see Fig. 1,c); hence one should not expect that the whole of its molecule will become rigid. This may be the reason why many attempts at stabilizing enzymes failed, see for example [7,22,23]. The problem then is actually that of providing a support with a surface absolutely complementary to that of the enzyme molecule. Only in this case the multipoint interaction conducive to stabilization of the enzyme will take place.

The gist of our approach to this problem (see preliminary communications [24,25]) is the following (Fig. 2). The enzyme is modified by an analogue of a monomer and the resulting preparation is copolymerized with the monomer proper (sometimes also with a cross-linking agent). As a result, one has an enzyme chemically attached to the three-dimensional lattice of a polymeric gel, the points of the enzyme-support binding being the premodification sites of the enzyme molecules. It is obvious that the underlying principle of the method presupposes that the microsurface of the gel around the chemically entrapped enzyme molecule should be complementary to the surface of the latter.

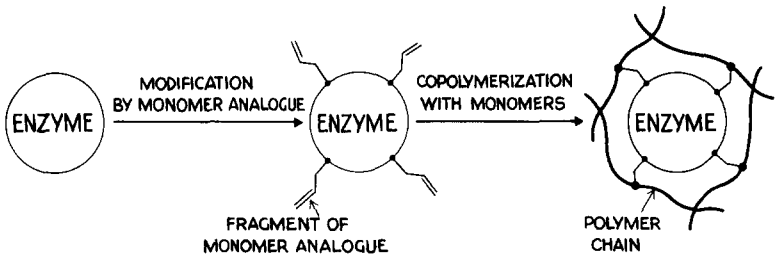


Fig. 2. Schematic representation of the novel method of enzyme immobilization: copolymerization of the enzyme modified by a monomer analogue with the monomer proper.

As analogues of the monomer, one may use, for example, amino groups modifiers (because in proteins there are many such groups, say ϵ-NH_2 groups of the lysine residues; this will ensure a multipoint binding of the monomer analogue with the protein): for example, acryloyl chloride and methacryloyl chloride that acylate NH_2 groups, or acrolein that alkylates NH_2 groups.

As monomers, use may be made of gel-forming substances, such as acrylamide, sodium methacrylate or ethylene glycol methacrylate; *N,N'*-methylene bisacrylamide may be employed as a cross-linking agent. All these agents are commercially available and inexpensive.

We have studied the thermostability of well-known proteolytic enzymes, chymotrypsin and trypsin, chemically entrapped in different polymeric gels by the above method.

Experimental

Materials

Bovine α-chymotrypsin used was a commercial preparation of Koch-Light (3 times recrystallised); the concentration of the active centres determined by spectrophotometeric titration [26] was 64%. Bovine trypsin was supplied by the Leningrad Kirov meat-packing plant; the concentration of the active centres determined by spectrophotometric titration [27] was 56%. The determined values of k_{cat} and $K_{ma,app}$ for chymotrypsin- and trypsin-catalyzed reactions of the hydrolysis of specific substrates (*N*-acetyl-L-tyrosine and *N*-α-benzoyl-L-arginine ethyl esters, respectively) were almost the same as in the literature [29].

Acrolein and acryloyl chloride were distilled preparations of BDH and Reachim, respectively. A commercial acrylamide was a product of Koch-Light. Sodium methacrylate was synthesized from methacrylic acid (Koch-Light) as described in [28]. *N,N'*-methylene bisacrylamide, *N,N,N',N'*-tetramethylethylenediamine, ammonium persulfate, riboflavin were commercial preparations of Reanal.

The substrates, *N*-acetyl-L-tyrosine ethyl ester and *N*-α-benzoyl-L-arginine ethyl ester were commercially available samples (Koch-Light and Reanal, respectively). Titrants for the enzymes' active centres, *N-trans*-cinnamoyl imidazole and *p*-nitrophenyl-*p'*-guanidinebenzoate were commercial reagents of Serva. Picryl sulfonic acid, a titrant for NH_2 groups of proteins, was supplied by Sigma.

Benzamidine hydrochloride was a product of Merck. Inorganic salts and buffer solutions components were analytical grade preparations.

Methods

Determination of the catalytic activity of the enzymes. The enzyme activity was assayed potentiometrically with the help of a Radiometer TTT 1c pH-stat. The specific substrate for chymotrypsin was *N*-acetyl-L-tyrosine ethyl ester and that for trypsin *N*-α-benzoyl-L-arginine ethyl ester [29].

An aqueous solution or powdered gel (nor more than 0.1 g; particle size under 0.1 mm) with the enzyme were added into the cuvette of the pH-stat containing 5 ml of $5 \cdot 10^{-3}$ M solution of the substrate at pH 8.0 and 25°C (unless otherwise stated). To ensure the necessary ionic strength, 3 M KCl was used (so high an ionic strength is necessary for the electrostatic interactions in the polymethacrylate gel to be eliminated). The acid liberated as a result of the enzymatic hydrolysis was titrated with 10^{-2} M KOH.

We have purposely demonstrated (in terms of the criteria formulated previously [30]) that in the conditions used by us the diffusion factors do not affect the rate of the enzymatic reaction.

Modification of the enzymes by the monomer analogue. If proteins are acylated by chloranhydrides of carboxylic acids [31] or reductive alkylated by aldehydes [32], it is mostly NH_2-groups that are affected. A molecule of bovine chymotrypsin contains 17 NH_2-groups [29], out of which 14—15 can be titrated.

(a) Chymotrypsin was modified by acryloyl chloride in the following way: to $3 \cdot 10^{-5}$ M solution of the enzyme at pH 8.0 (0.2 M KH_2PO_4) and 0°C a 1000-fold molar excess acryloyl chloride was added in several portions during 5 min of intensive stirring (within this time almost all of the chloranhydride is hydrolysed); pH is maintained at 8.0 by adding concentrated KOH. As a result of such modification, chymotrypsin at first become completely inactivated (apparently, due to its active centre having been acylated by chloranhydride [33]) and then at pH 8 and 25°C became reactivated to the level amounting to 80—90% of that of the native enzyme, the half-time of conversion being approx. 20 min. Reactivation seems to result from deacylation of the functional group(s) localized in the enzyme active centre, see ref. 33.

It was demonstrated (by titrating the amino groups of the enzyme with picryl sulfonic acid [34]) that acryloyl chloride in these conditions acylates all the titrated protein NH_2-groups.

To obtain the preparations of the enzyme containing a lower number of acryloyl residues per molecule, the reaction was carried out with a lower amount of acryloyl chloride. The degree of modification in every case was determined by the same method of titration of free amino groups [34].

(b) Chymotrypsin was modified by acrolein in the following way: to $5 \cdot 10^{-4}$ M enzyme solution in 10^{-2} M borate buffer (pH 8.5) an acrolein solution was added (to a concentration of 0.25 v/v %). The mixture was incubated for 2 h at 4°C. It was demonstrated (by titrating the amino groups of the enzyme with picryl sulfonic acid [34]) that acrolein in these conditions modifies all the titrated protein NH_2-groups. The catalytic activity of chymotrypsin does not thereby decrease more than 2-fold.

(c) Trypsin was modified by acrolein in the following way: $5 \cdot 10^{-4}$ M solution of the enzyme in 10^{-2} M borate buffer (pH 8.5) was incubated with 1% v/v acrolein in the presence of 10^{-2} M $CaCl_2$ and $2 \cdot 10^{-2}$ M benzamidine (to prevent autolysis of the enzyme) and 30% w/w acrylamide (to prevent precipitation of the enzyme modification product) for 3 h at 4°C. The activity of the enzyme does not thereby decrease more than 2-fold.

Copolymerization of the modified enzymes with the monomers (chemical entrapment into gel). To prepare the gels, the recommendation given in ref. 35 were followed.

(a) Entrapment of acryloyl chymotrypsin in polymethacrylate gel was done in the following way: to 10^{-5} M solution of acryloyl enzyme in 0.1 M phosphate buffer there were added 25% w/w sodium methacrylate (the monomer) with 0.25% w/w *N,N'*-methylene bisacrylamide (the cross-linking agent) and 0.24% w/w ammonium persulphate and 0.15% w/w *N,N,N',N'*-tetramethylethylenediamine (the initiating agents); pH was adjusted to 8.0. The mixture was polymerized at 0°C for 24 h in thin test-tubes (to prevent local overheating). The resulting gel was taken out of the test-tubes, ground into powder, washed with $5 \cdot 10^{-3}$ M borate buffer (pH 9.5). It was purposely demonstrated that the enzyme cannot be washed out of the gel (for the methodological details of these experiments see ref. 28). The activity of the thus immobilized enzyme is 30% of that of the native enzyme.

The same method was used to copolymerize sodium methacrylate with the preparations of the given enzyme acryloylated only partially (and hence containing free amino groups). The number of linkages with which the protein globule of chymotrypsin is covalently bound to the polymethacrylate support seems to be mainly determined by the number of amino groups modified (at the first step of immobilization, see Fig. 2) by the acryloyl residue. In principle, the number of acryloyl residues per enzyme molecule may be greater as it is not only the amino group that have been modified by chloranhydrides, but, it seems, other functional groups of the protein [31]. On the other hand, it is not certain that every acryloyl residue bound to the enzyme will be attached, as a result of copolymerization, to the polymethacrylic chains of the gel. However, when all is said and done, one may say that between the true value and the experimentally determined value there undoubtedly exists a simple relationship, that is the greater "the number of acryloylated amino groups in a protein", the greater is "the number of linkages between the enzyme globule and the support".

(b) Acryloyl chymotrypsin was entrapped in polyacrylamide gel in the following way: to $3 \cdot 10^{-5}$ M solution of acryloyl enzyme in 0.2 M phosphate buffer were added 30% w/w of acrylamide (the monomer), 1.5% w/w of *N,N'*-methylene bisacrylamide (the cross-linking agent) and 30 mg/l riboflavin (the initiating agent). The mixture was placed in narrow test-tubes at 0°C and illuminated with a 300-W tungsten lamp for 1 h. The gel prepared in this way was taken out of the test-tubes, ground into powder and washed with water. It was purposely demonstrated that the enzyme cannot be washed out of such gel. The activity of the enzyme immobilized in such a way amounts to 25% of that of the native enzyme.

(c) The method of chemical entrapment of chymotrypsin (or trypsin) modi-

fied by acrolein into polyacrylamide gel was similar to that used for immobilization of acryloyl chymotrypsin (see above). The suspension of the resulting gel was treated with sodium borohydride (1 mg/ml) in distilled water for 1 h for the double bonds of the Schiff's bases to be reduced [32]. Then the gel was washed. The immobilized chymotrypsin and trypsin could not be washed out of such gel; their activity was not less than 50% of that of the native enzymes.

A study of thermoinactivation of the native, modified and immobilized enzymes. Thermoinactivation of native chymotrypsin was studied in the following way: 10^{-7} M solution of the enzyme at pH 8.0 ($5 \cdot 10^{-3}$ M of Tris · HCl + 3 M KCl) was incubated at a given temperature for a certain time, then quickly cooled and the enzymatic activity relative to *N*-acetyl-L-tyrosine ethyl ester was determined at 25°C. We have ascertained that the process studied is a monomolecular thermoinactivation and does not involve autolysis or aggregation: this is evidenced by the experimental fact that the thermoinactivation curves of chymotrypsin do not depend on the initial concentrations of the enzyme over the range of $5 \cdot 10^{-8}$—$5 \cdot 10^{-7}$ M (Fig. 3). A similar phenomenon is observed for the preparations of modified chymotrypsin and trypsin (at the concentrations of the latter of $<10^{-7}$ M and in the presence of 10^{-2} M $CaCl_2$).

To thermoinactivate the immobilized enzymes, gel suspension in $5 \cdot 10^{-3}$ M Tris · HCl buffer (+3 M KCl) at pH 8.0 was prepared. The suspension was placed into thin test-tubes which were incubated at a higher temperature for a certain time, then instantaneously cooled to 20°C (as a result of addition to the excessive volume of thermostatically temperature controlled substrate solution) and the relative enzymatic activity was immediately determined (for details of the experiment see [3,28]). We made it a point to prove that the catalytic activity of the immobilized enzyme does not depend on the time elapsed between cooling the sample and measurement (from 5 min to 1 h). It goes without saying that no autolysis or aggregation can occur in the case of immobilized enzymes [1]. Thus, both with the native and immobilized enzymes, the monomolecular thermoinactivation of the enzymes was studied.

It is seen in Fig. 3 that the process of thermoinactivation in spite of being

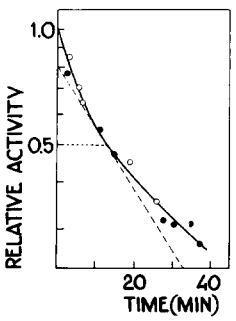


Fig. 3. Kinetic curve of thermoinactivation of chymotrypsin in semilogarithmic coordinates. The initial concentration of the enzyme: filled circles $5 \cdot 10^{-7}$ M; empty circles $1 \cdot 10^{-7}$ M. Broken line: slope of the kinetic curve at the point of half-inactivation; the slope was used to calculate the effective value of the first-order rate constant as a relative characteristic of the thermostability of the enzyme (see Methods). Conditions: 55°C, pH 8.0 ($5 \cdot 10^{-3}$ M Tris · HCl + 3 M KCl), for other conditions see Methods.

clearly monomolecular, cannot be described in terms of first-order kinetics which may be due, among other things, to a multistep character of the inactivation process or to the presence in the enzyme of several forms inactivating at different rates). That is why, when choosing a parameter to characterize the relative rate of thermoinactivation, we decided on, as previously [28], the effective first-order rate-constant with the degree of conversion being 50% (see Fig. 3).

Results and Discussion

In Fig. 4 there are thermoinactivation curves of native chymotrypsin and acryloyl chymotrypsin chemically entrapped in polymethacrylate gel at 60°C. It is obvious that the thermostability of the immobilized enzyme exceeds by far that of the native one: the latter almost totally inactivates within several minutes, whereas the former retains 70% of its activity even after a 3-h incubation.

Dependence of the stabilization effect on the number of linkages between an immobilized enzyme and a support

To get a more comprehensive, quantitative, idea of the effect of enzyme stabilization, we tried to find out how the rate of thermoinactivation depends on the number of linkages between an immobilized enzyme and a support. The experimentally determined (see Methods) number of amino groups of protein acryloylated at the first step of immobilization (see Fig. 2) were assumed as being those by which the protein globule of chymotrypsin is covalently bound with polymethacrylic chains of the gel.

The thermostability of immobilized chymotrypsin is so much higher than that of the native enzyme that they are impossible to compare experimentally, that is at the same temperature. Therefore we compared the values of the ther-

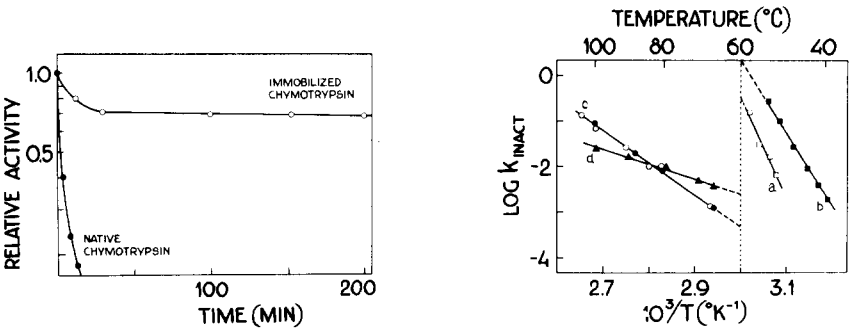


Fig. 4. Thermoinactivation of native chymotrypsin and acryloyl chymotrypsin chemically entrapped in polymethacrylate gel. Conditions: 60°C, concentration of the native enzyme $1 \cdot 10^{-7}$ M, pH 8.0 ($5 \cdot 10^{-3}$ M Tris · HCl + 3 M KCl), for other conditions see Methods.

Fig. 5. Temperature dependence of the first-order rate constant of monomolecular thermoinactivation of chymotrypsin (k_{inact}, min^{-1}). a, native enzyme; b, chymotrypsin modified by acryloyl chloride; c, acryloyl chymotrypsin chemically entrapped in polymethacrylate gel (filled circles: water-insoluble enzyme; empty circles: water-soluble enzyme (see text)); d, acryloyl chymotrypsin chemically entrapped in polyacrylamide gel. For conditions see Methods and the caption to Fig. 4, also.

moinactivation rate constants at 60°C determined by extrapolating the Arrhenius plots, as is examplified by maximally modified preparations of the enzyme in Fig. 5 (broken line).

It was found (Fig. 6) that as the number of acryloylated amino groups of the immobilized enzyme increases (this means that the number of covalent linkages between the protein globule and the polymethacrylate support increases) chymotrypsin becomes more and more thermostable. As a result, the effective rate constant of thermoinactivation (at 60°C) drops by more than 1000 times. All the further experiments were carried out with a preparation of the enzyme that displays a maximum thermostability being attached to the support with the greatest number of linkages.

Mechanism of stabilization

The effect of stabilization cannot be explained by alteration in the thermostability of the enzyme resulting from its having been chemically modified by acryloyl chloride, because, as we showed, acryloyl chymotrypsin is even less stable than the native enzyme (see Fig. 5, cf. lines a and b) regardless of the degree of modification.

Neither can stabilization be attributed to the effect of the gel microenvironment on the enzyme, because, as demonstrated by us previously [36], the thermostability of chymotrypsin physically entrapped in polymethacrylate gel of such concentration (25% w/w before swelling) is almost the same as that of native chymotrypsin.

The nature of the gel is actually no decisive factor for stabilization of the enzyme. For example, the thermostability does increase if acryloylated chymotrypsin is chemically entrapped in another, polyacrylamide, gel (30% w/w before swelling). This is shown in Fig. 5, cf. lines a and c.

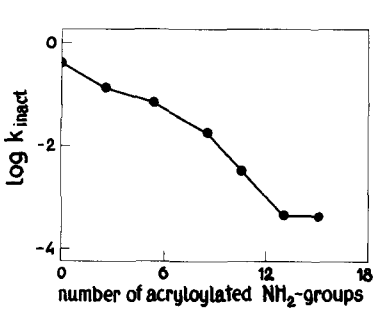
The effect of stabilization (of the type shown in Fig. 4) also took place with a different method of enzyme premodification, i.e. alkylation by acrolein followed by entrapment into polyacrylamide gel *.

Finally, it should be pointed out that the results hardly depend on the nature of the enzyme used: they are similar for chymotrypsin and trypsin. Consequently, for the stabilization observed the critical factor is not the method of premodification or the nature of the gel, but the chemical entrapment of the enzyme molecule in the three-dimensional structure of the support. The mechanism of stabilization is that the unfolding of the globule on heating of the protein is hindered as the latter is fixed (Fig. 1). Thereby, the native (catalytically active) conformation of the active centre may only be retained if the enzyme-support interaction is multipoint, as is shown in Fig. 6, to prevent denaturation (unfolding) of a protein on heating, a sufficiently great number of linkages between the enzyme globule and the polymeric chains of the gel is to be realized.

Temperature dependence of enzyme inactivation

The effect of stabilization greatly depends on temperature. The data are

* Moreover, it has recently been shown in our laboratory [48] that thermostable enzyme preparation may be obtained by attaching chymotrypsin to a support not only via protein amino groups (as is the case in the present study), but also via carboxy groups.



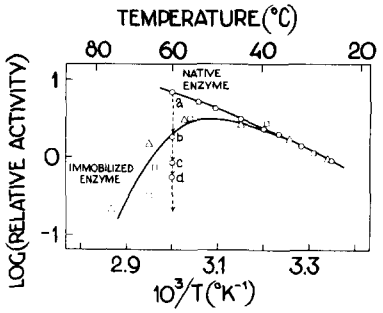


Fig. 6. The dependence of the effective first-order rate constant (k_{inact}, min^{-1}) for monomolecular thermoinactivation of acryloylated chymotrypsin chemically entrapped in polymethacrylate gel on the degree of modification of the enzyme at the first step of immobilization (see Fig. 2). For the conditions see the caption to Fig. 4 and Methods.

Fig. 7. Temperature dependence of the relative enzymatic activity of native chymotrypsin and its immobilized forms (the value at 25°C for every form is assumed as being a unity). Circles: native enzyme; broken line corresponds to decrease in the activity of the enzyme during its measurement at 60°C; point a corresponds to the initial moment of time, points b, c and d to the activity of the enzyme in 10, 20 and 30 min, respectively after the first measurement. The immobilized enzyme (acryloyl chymotrypsin chemically entrapped into polymethacrylate gel). Triangles: water-insoluble form; squares: water-soluble form. For conditions see Methods.

presented in Fig. 5 as Arrhenius plots. As seen, the effective values * of the activation energy of thermoinactivation of acryloylated chymotrypsin entrapped in polymethacrylate and polyacrylamide gels (35 and 15 kcal/mol, respectively) are much lower than that of the native enzyme (110 kcal/mol). This means that, as the temperature rises, the effect of stabilization of the immobilized enzyme will increase compared to the native enzyme. For example, extrapolation of line a in Fig. 5 shows that at 102°C the entrapped enzyme is 10^8 times as stable as the native one. The difference is all the more striking if comparison is made with free acryloyl chymotrypsin (line b).

Temperature dependence of enzymatic activity

As seen in Fig. 7, chymotrypsin chemically entrapped in polymethacrylate gel in the temperature range of 25—75°C possesses a high catalytic activity that hardly ever changes during the time of the measurement (30 min). The deviation (decrease) at higher temperatures in the catalytic activity from the straight line relationship that is characteristic of lower temperatures, is due to fully reversible inactivation of the immobilized enzyme.

At the same time, native chymotrypsin as early as at 60°C rapidly, and what is more important, irreversibly inactivates during the activity measurements (points a—d in Fig. 7). Thus, our immobilization method has allowed to obtain

* It follows from Fig. 5 that below 82°C chymotrypsin entrapped in polyacrylamide gel is more stable than that entrapped in polymethacrylate gel, and above 82°C the situation is the opposite. This is due to the difference in the effective values of activation energy for thermoinactivation in different gels. This may in principle be caused by two factors: (1) it is possible that the apparent value of the activation energy is indicative of the energy barrier of flexibility of the polymeric chain of the support; (2) it is known [14,15,37] that even far from the thermodenaturation point, proteins in the solution noticeably change their conformation as the temperature rises. Hence, in the case of an immobilized enzyme, different supports may produce a different effect on these predenaturation conformational changes and, ultimately, on the mechanism of thermoinactivation.

a highly stable preparation of the enzyme possessing a high catalytic activity at an elevated temperature.

Properties of water-soluble stabilized enzymes

We have also obtained a preparation of water-soluble chymotrypsin bound to a support, polymethacrylic acid. This was done by copolymerizing acryloyl chymotrypsin with sodium methacrylate in the absence of a cross-linking agent (other conditions were the same; see Methods). The resulting non-cross-linked gel-like polymer completely dissolves in an excess of water. It was found that the thermostability of such an enzyme solution within the whole temperature range studied closely resembles that of acryloyl chymotrypsin chemically entrapped in the gel (Fig. 5). The temperature dependences of the catalytic activity of the water-soluble and water-insoluble enzyme preparations also proved to be similar (Fig. 7).

These results testify to the fact that the high stability of the enzymes immobilized in the gel is determined by the microstructure of polymethacrylate chains localized in the immediate proximity of the chemically entrapped enzyme, rather than by the macrostructure of the entire gel block. In other words, a polymeric structure in the vicinity of the entrapped enzyme, being congruent to the surface of the enzyme molecule (i.e. predetermined by the number of the linkages with the protein) remains intact even in solution.

This result is important because soluble-stabilized enzymes have not yet been prepared, and not for lack of trying [1,2]. Several approaches have actually been used in this area [2]; two of them are worth mentioning here, i.e. those where the enzyme molecule was attached (as in our case) to a water-soluble support: (1) polymeric chains "grafted" to an enzyme molecule were made to grow by polymerization [38]; and (2) an enzyme molecule was attached to another polymeric (protein as well) molecule or particle. Both approaches have so far failed to give satisfactory results: the increase in the thermostability is very small, if any [39—42]. More pronounced effects obtained with proteolytic enzymes are clearly due to a trivial mechanism, elimination of autolysis [43—47]. It follows from our experiments that the enzyme molecule can be reinforced with the help of the novel method described above: for making a molecule of an enzyme thermally stable it is sufficient to surround it with a gel-like microstructure of a soluble polymer interacting with it in a multipoint fashion.

Acknowledgment

It is our pleasant duty to thank Dr. Z.A. Streltsova for taking interest in our work and helping us out with some equipment.

References

1 Zaborsky, O.R. (1973) Immobilized Enzymes, CRC Press, Cleveland, Ohio
2 (1976) Immobilized Enzymes (Berezin, I.V., Antonov, V.K. and Martinek, K., eds.), Vol. 2, Lomonosov State University Press, Moscow
3 Martinek, K., Goldmacher, V.S., Klibanov, A.M. and Berezin, I.V. (1975) FEBS Lett. 51, 152—155
4 Goldman, R., Goldstein, L. and Katchalski, E. (1971) in Biochemical Aspects of Reactions on Solid Supports (Stark, G.E., ed.), pp. 1—78, Academic Press, New York
5 Melrose, G.J.H. (1971) Rev. Pure Appl. Chem. 21, 83—119

6 Vieth, W.R. and Venkatasubramanian, K. (1974) Chem. Technol. 4, 309—320
7 Gabel, D., Steinberg, I.Z. and Katchalski, E. (1971) Biochemistry 10, 4661—4669
8 Giacometti, G.M., Colosimo, A., Stefanini, S., Brunori, M. and Antonini, E. (1972) Biochim. Biophys. Acta 285, 320—325
9 Berliner, L.J., Miller, S.T., Uy, R. and Royer, G.P. (1973) Biochim. Biophys. Acta 315, 195—199
10 Moore, T.A. and Greenwood, C. (1975) Biochem. J. 149, 169—177
11 Lasch, J. (1975) Acta Biol. Med. Germ. 34, 549—554
12 Barel, A.O. and Prieeles, J.-P. (1975) Eur. J. Biochem. 50, 463—473
13 Joly, M. (1965) A Physico-Chemical Approach to the Denaturation of Proteins, Academic Press, New York
14 Blumenfeld, L.A. (1974) Problems of Biological Physics, chapter 5, Izd. Nauka, Moscow
15 Tanford, C. (1968) Adv. Protein Chem. 23, 121—182; (1970) Adv. Protein Chem. 24, 1—95
16 Alexandrov, V.Ya. (1975) Cells, Macromolecules and Temperature, Izd. Nauka, Leningrad; (1977) Springer-Verlag, in the press
17 Glassmeyer, C.K. and Ogle, J. (1971) Biochemistry 10, 786—792
18 Royer, G.P. and Uy, R. (1973) J. Biol. Chem. 248, 2627—2629
19 Klibanov, A.M., Samokhin, G.P., Martinek, K. and Berezin, I.V. (1976) Biochim. Biophys. Acta 438, 1—12
20 Gabel, D. (1973) Eur. J. Biochem. 33, 348—356
21 Delaage, M. and Lazdunski, M. (1968) Eur. J. Biochem. 4, 378—384
22 Gabel, D. and Kasche, V. (1972) Biochem. Biophys. Res. Commun. 48, 1011—1018
23 Mosbach, K. and Gestrelius, S. (1974) FEBS Lett. 42, 200—204
24 Martinek, K., Klibanov, A.M. and Berezin, I.V. (1975) U.S.S.R.—U.S.A. Seminar on Immobilized Enzymes, Moscow (to be published in the Symposium Volume)
25 Martinek, K., Goldmacher, V.S., Klibanov, A.M., Torchilin, V.P., Smirnov, V.N., Chazov, E.I. and Berezin, I.V. (1976) Dokl. Akad. Nauk S.S.S.R. (Russ.), 228, 1468—1471
26 Schonbaum, G.R., Zerner, B. and Bender, M.L. (1961) J. Biol. Chem. 236, 2930—2935
27 Chase, T., Jr. and Show, E. (1967) Biochem. Biophys. Res. Commun. 29, 508—514
28 Tchernysheva, A.V., Martinek, K., Klibanov, A.M., Mevkh, A.T. and Berezin, I.V. (1975) Izv. Akad. Nauk S.S.S.R., Ser. Khim. No. 8, 1764—1768
29 Mosolov, V.V. (1971) Proteolytic Enzymes (Russ.), Izd. Nauka. Moscow
30 Berezin, I.V., Klibanov, A.M. and Martinek, K. (1975) Uspekhi Khimii (Russ. Chem. Rev.) 44, 17—47
31 Schröder, E. and Lübke, K. (1965) The Peptides, Vol. 1, Acad. Press, New York
32 Feeney, R.E., Blankenhorn, G. and Dixon, H.B.F. (1975) Adv. Protein Chem. 29, 135—203
33 Dixon, G.H. and Neurath, H. (1957) J. Biol. Chem. 225, 1049—1059
34 Fields, R. (1971) Biochem. J. 124, 581—590
35 Maurer, H.R. (1968) Disk—electrophorese. Theorie and Praxis der diskontinuierlichen Polyacrylamidgel-Electrophorese. Chapt. 2, Walter de Gruyter and Co., Berlin
36 Martinek, K., Klibanov, A.M., Tchernysheva, A.V. and Berezin, I.V. (1975) Dokl. AN S.S.S.R. (Russ.) 223, 233—236
37 Lumry, R. and Biltonen, R. (1969) in Structure and Stability of Biological Macromolecules (Timasheff, S.N. and Fasman, G.D., eds.), pp. 65—212, Marcel Dekker, Inc., New York
38 Sela, M. and Arnon, R. (1972) Methods Enzymol. 25, 553—558
39 Hixson, H.F. (1973) Biotechnol. Bioeng. 15, 1011—1016
40 Foster, R.L. and Thomas, A.R. (1973) Biochim. Biophys. Acta 321, 409—414
41 Wykes, J.R., Dunnill, P. and Lilly, M.D. (1971) Biochim. Biophys. Acta 250, 533—529
42 Lasch, J., Bessmertnaya, L., Kozlov, L.V. and Antonov, V.K. (1976) Eur. J. Biochem. 63, 591—598
43 Goldstein, L. (1972) Biochemistry 11, 4072—4084
44 von Specht, B.-U., Seinfeld, H. and Brendel, W. (1973) Hoppe-Seyler's Z. Physiol. Chem. 354, 1659—1660
45 Kol'tzova, S.V., Glikina, M.V., Illarionova, N.G. and Samsonov, G.V. (1971) Molek. Biol. (Russ.) 5, 225—231
46 Torchilin, V.P., Bobkova, A.S., Smirnov, V.N. and Chazov, E.I. (1976) Bioorg. Khim. (Russ.) 2, 116—124
47 Marshall, J.J. and Rabinowitz, M.L. (1976) J. Biol. Chem. 251, 1081—1087
48 Goldmacher, V.S., Martinek, K., Klibanov, A.M., Torchilin, V.P., Smirnov, V.N. and Berezin, I.V. (1977) Bull. Moscow Univ. (Vestnik MGU), in the press