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REACTIVATION OF ENZYMES IRREVERSIBLY DENATURED AT ELEVATED TEMPERATURE

TRYPSIN AND α-CHYMOTRYPSIN COVALENTLY IMMOBILIZED ON SEPHAROSE 4B AND IN POLYACRYLAMIDE GEL

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Summary

Trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.2) covalently immobilized on Sepharose or in polyacrylamide gel has been irreversibly denatured at 70—90°C and then reactivated in an almost 100% yield. Thermoinactivated enzyme is first made to unfold under the action of urea with S-S bonds being simultaneously reduced and then made to refold (under the optimal conditions for the thiol-disulfide exchange) into its native conformation. It is demonstrated that the 'irreversible monomolecular thermoinactivation-reactivation' cycle can be repeated many times.

The contribution of various mechanisms to thermoinactivation of the enzymes is discussed. Based on the data obtained, the irreversible thermoinactivation of enzymes under investigation should be ascribed only to changes in their secondary and tertiary structures; the primary structure is not likely to be affected.

Introduction

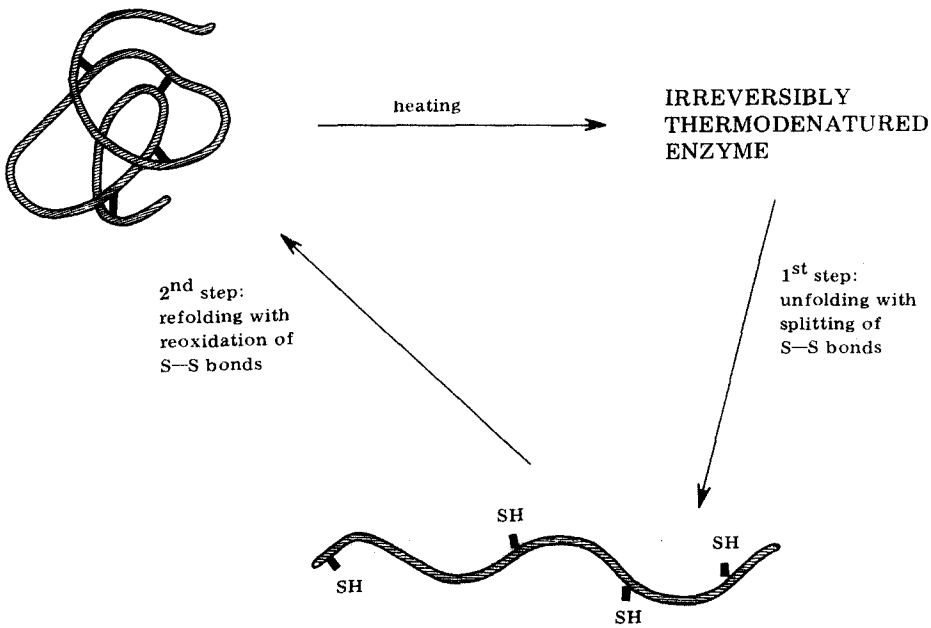
Application of biocatalysis in chemical technology, analytical chemistry and organic synthesis, medicine and other applied areas is hindered by the well known instability of enzymes that readily denature in vitro (see review of Ref. 1). Hence, the necessity to elaborate the physico-chemical principles of enzyme stabilization against denaturing actions (see reviews of Refs. 1, 2). On the other hand, enzymes could be used several or many times if irreversibly

denatured proteins could be reactivated. This was the aim of the present investigation.

Also, the study of renaturation of enzymes should help elucidate mechanisms of the reverse process, i.e. denaturation. These mechanisms are still obscure (see literature to Ref. 3, 4), although intensive studies in this area have been carried out for decades now (see reviews of Refs. 5—9). It is not clear to which factors the irreversible character of denaturation should be attributed. It is commonly believed that denaturation of enzymes becomes irreversible owing to the secondary processes, such as aggregation, chemical modification of the functional groups (for example, their oxidation or 'poisoning' by metal cations), autolysis or breakage of the peptide bonds under the action of admixtures of other enzymes, splitting of the 'native' disulfide bonds (for example, as a result of intra- or intermolecular thiol-disulfide exchange or their alkaline or catalytic splitting) [5,6]. Very often this proves to be the case. For example, about 40 years ago Anson, while studying hemoglobin and some other proteins showed that thermoinactivation results in aggregation of macromolecules until a precipitate is formed. Anson was also the first to reactivate irreversibly denatured proteins by dissolving the precipitate in concentrated urea and by varying the pH values (see Refs. to 5). A similar procedure was successfully used with thermodenatured β-lactamase: as a result of incubation of the precipitate in 5 M guanidinium chloride or 8 M urea and subsequent removal of the denaturants, the enzyme was observed to renature [10]. Another example is inactivation of urease which occurs on 'poisoning' of its SH-groups by heavy metal cations. The enzyme can be renatured by an excess of I^- [11]. The mechanism of the reactivation is actually the binding of metal ions with iodide; as a result, the Me-S bond in the inactivated protein is split.

However, the reasons for the irreversible character of denaturation can be basically different. It is known that the conformational changes that accompany irreversible denaturation can be rather significant [6]. This was demonstrated, for example, by physico-chemical methods for dihydrofolate reductase (inactivated at 45°C) [12] and ATPase (inactivated at 46°C) [13]. Therefore, the molecular mechanism of irreversible denaturation is possibly the following: at high temperatures the 'correct' noncovalent interactions in the enzyme, that maintain the native structure of protein existing at room temperature, are disrupted and thermodynamically more favourable 'non-native' bonds are formed; when temperature is lowered these 'incorrect' noncovalent interactions, although thermodynamically unstable, can be retained for purely kinetic reasons, as the molecular mobility of the polypeptide chains should decrease. Hence, with the lowering of the temperature the protein may remain denatured. In this case the protein exists in a kinetically maintained (metastable) conformation from which it cannot spontaneously renature under low temperature.

This monomolecular physical mechanism of irreversible denaturation is supported by the fact [3,4,14] that the thermoinactivated enzyme (trypsin covalently immobilized on Sepharose) could be reactivated in the following way: in the first stage the simultaneous action of 8 M urea and dithiothreitol on the enzyme seemed to induce destruction of all (including the 'non-native') noncovalent interactions and S-S bonds (Scheme I). The second stage of the



Scheme I. Scheme of an 'irreversible thermodenaturation-reactivation' cycle. Regeneration includes the unfolding of an irreversibly thermodenatured enzyme with splitting of S-S bonds, and the subsequent folding of the protein into a native conformation as a result of reoxidation of S-S bonds in the presence of catalysts of thiol-disulfide exchange.

reactivation procedure is visualized according to Anfinsen and Scheraga [15]. It has been shown by them (see review of Ref. 15), that the protein unfolded from the native state into random coil, can refold under favourable conditions into the native conformation. Moreover, immobilized (Refs. 16—21, also Mozhaev, V.V. and Martinek, K., unpublished data) forms of some proteins are capable of successful oxidative refolding after being completely unfolded and reduced. In contrast to researchers in the host of early works we applied the 'unfold-refold' approach to an irreversibly thermodenatured protein [3,4,14] (see reviews of Refs. 22, 23), also.

Previously we showed [3,4,14] that the irreversibly thermodenatured protein can be reactivated, if it is first unfolded (by urea and dithiothreitol) and then refolded (under the optimal conditions for thiol-disulfide exchange). The present work contains a detailed description of this experiment (performed with thermoinactivated trypsin immobilized on Sepharose 4B). Moreover, we studied the following questions: firstly, whether this approach [3,4,14] is applicable to another thermodenatured enzyme (α-chymotrypsin) immobilized on a different support (polyacrylamide gel); secondly, whether the 'thermoinactivation-regeneration of catalytic activity' cycle can be repeated several times.

Experimental

Materials

The following enzymes were used in the present work: bovine trypsin (EC 3.4.21.4) produced by the Olaine plant (U.S.S.R.) and bovine α-chymotrypsin (EC 3.4.21.2) manufactured by Koch-Light Laboratories.

For the enzymatic activity assay the following specific substrates were used: *N*-α-benzoyl-L-arginine ethyl ester for trypsin and *N*-acetyl-L-tyrosine ethyl ester for α-chymotrypsin, both manufactured by Reanal (Hungary).

The covalent immobilization of α-chymotrypsin in polyacrylamide gel was carried out using acryloyl chloride and acrylamide manufactured by Koch-Light Laboratories, and *N*,*N*′-methylenebisacrylamide, *N*,*N*,*N*′,*N*′-tetramethylenediamine and ammonium persulfate manufactured by Reanal (Hungary).

Sepharose 4B activated by cyanogen bromide (Pharmacia) was used as a support for trypsin immobilization.

For regeneration of thermoinactivated enzymes we used urea produced by Reakhim (U.S.S.R.), EDTA by Chemapol (Czechoslovakia), dithiothreitol by Sigma, reduced (Serva) and oxidized (Reanal) forms of glutathione.

The buffer solution components, salts, acids and KOH were all products of Reakhim (U.S.S.R.).

Methods

Immobilization of trypsin on Sepharose 4B. Immobilization was carried out as previously described [17]. To this end, 30 mg trypsin were incubated in a 15 ml suspension of 5 g of the support (CNBr-activated Sepharose) in borate buffer (0.1 M H_3BO_3/0.05 M $CaCl_2$, pH 8.5) in the presence of 25 mg of benzamidine at 4°C with gentle stirring for 24 h. The support with the immobilized enzyme was washed several times to remove adsorbed trypsin with solutions of 1.0 M KCl; 0.2 M $NaHCO_3$ (pH 10.5); 0.001 M HCl; 0.1 M Tris-HCl (pH 8.0); 8 M urea and finally with water. The resulting preparation contained approx. 1 mg of catalytically active trypsin per g of the support. The amount of the immobilized enzyme was measured by active site titration, for used by Sinha and Light see methods [17]. Furthermore, as judged from the specific enzymatic activity assay, the k_{cat} value did not significantly change as a result of immobilization [17].

α-Chymotrypsin covalently entrapped in polyacrylamide gel was obtained as previously described [24]. As determined by active site titration, 25% of α-chymotrypsin molecules retain their catalytic activity upon immobilization. Specific enzymatic activity assay shows that the k_{cat} value of the immobilized preparation is practically the same as that of native α-chymotrypsin.

Enzymatic activity assay of trypsin and α-chymotrypsin immobilized on/in the support. Initial rates of the enzymatic hydrolysis of *N*-α-benzoyl-L-arginine ethyl ester or *N*-acetyl-L-tyrosine ethyl ester were measured with a Radiometer TTT-1c pH-stat (Denmark). Gel granules with immobilized enzyme were separated from the solution on a glass filter and dried until a constant weight was achieved. A weighed amount of immobilized enzyme (0.01—0.1 g) was added to the cuvette of the pH-stat containing 5 ml of $5 \cdot 10^{-3}$ M substrate solution in 0.1 M KCl, and the activity was assayed at 25°C, pH 8.0. We have demonstrated (in terms of criteria formulated previously, see Refs. in Ref. 24) that for the

conditions used by us the diffusion factors do not affect the rate of the enzymatic reaction.

Study of the kinetics of thermoinactivation of immobilized enzymes. 0.2—1.0 g enzyme preparation were incubated in 5—10 ml 0.005 M Tris-HCl buffer (pH 8.5) with stirring in a stoppered thermostatted cell (70—95°C). At certain time intervals, aliquots were taken, cooled to room temperature, and the residual catalytic activity of the immobilized enzyme was measured.

The observed thermoinactivation is irreversible in terms of the accepted views as after cooling of the enzyme preparation to room temperature the residual catalytic activity remains at a low level for several days. Moreover, the apparent value of the residual catalytic activity of the thermodenatured enzyme does not depend on the mode of cooling: whether it was carried out rapidly (within several minutes) or slowly (that is, for hours by reducing the temperature every time by 5°C).

Regeneration of irreverisbly denatured enzymatic preparations involved two consecutive operations:

(a) The unfolding of the immobilized enzyme with simultaneous cleavage of its S-S bonds. A weighed amount of the thermoinactivated immobilized enzyme (0.1—0.5 g) was incubated in 5 ml of a 10 M solution of urea (pH 3.0) for 30 min. Then rapidly (within several seconds) the pH was adjusted to 8.5 and 0.5 ml of 0.1 M Tris-HCl buffer (pH 8.5) containing 10 mg EDTA and 20 mg dithiothreitol were added to the suspension. The suspension was stirred for at least 3 h with nitrogen flushing. As a result, the enzyme preparation totally lost its catalytic activity.

(b) Reoxidation of S-S bonds in the presence of the thiol-disulfide exchange catalysts. The immobilized reductively-unfolded enzyme was separated on a glass filter from the solution of denaturants and thoroughly washed with a $1 \cdot 10^{-3}$ M solution of HCl and water to remove dithiothreitol. The enzyme preparation was placed in 5 ml of 0.1 M Tris-HCl buffer (pH 8) containing 2.4 mg oxidized glutathione, 12 mg reduced glutathione and 0.05 M $CaCl_2$ and was left to stand in air with stirring.

Study of the stability of immobilized trypsin in urea. 5 ml of a substrate solution was added to the pH-stat ($1 \cdot 10^{-2}$ M of *N*-benzoyl-L-arginine ethyl ester/0.1 M KCl, pH 8.0) and 0.01—0.03 g immobilized trypsin. Then a weighed amount of urea was added. After 3—5 min the catalytic activity was measured. Then a weighed amount of urea was added again to increase its concentration in the cuvette. After 3—5 min the catalytic activity was measured again. In this manner the denaturant concentration in the cuvette was gradually raised (after each rise of urea concentration the catalytic activity was measured), until a saturated solution of urea (about 11 M at room temperature) was obtained. Then the suspension in the cuvette was gradually diluted with a substrate solution (after each decrease of urea concentration the catalytic activity was measured) until a very diluted solution of urea was obtained.

Results and Discussion

Regeneration of the catalytic activity of thermoinactivated trypsin covalently immobilized on Sepharose 4B

The enzyme was first inactivated at high temperature (70, 80 or 90°C) with

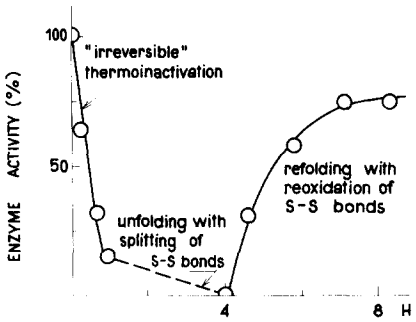


Fig. 1. Regeneration of the catalytic activity of trypsin (immobilized on Sepharose 4B), after irreversible monomolecular thermoinactivation (70°C, pH 8), as a resul of reduction of S-S bonds in the presence of urea (broken line) followed by their reoxidation.

15% (or less) of the initial catalytic activity being retained (Fig. 1). The thermoinactivation observed can be considered irreversible in terms of the accepted views (see Methods).

Reactivation of the enzyme preparation was carried out according to the Scheme I. Thermodenaturated enzyme was unfolded in 10 M urea and the S-S bonds were exhaustively reduced. We followed the experimental conditions developed by Sinha and Light [17] who demonstrated that all the six S-S bonds of trypsin immobilized on Sepharose 4B result in 12 SH-groups. As a result, the enzyme completely lost its catalytic activity (Fig. 1, broken line). Finally, after removal of the denaturants the S-S bonds were reoxidized again following the recommendations in [17] for the enzyme unfolded from the native state (we should bear in mind that in our case the enzyme was unfolded after irreversible thermodenaturation).

Fig. 1 shows that we can (by more than 70%) regenerate the catalytic activity of irreversibly thermoinactivated enzyme. Sometimes, less than 100% of the catalytic activity was obtained, due to the fact that part of the enzyme immobilized on Sepharose was washed off the support on heating as a result of the hydrolysis of the uretane bond [25]. This was quantitatively (spectrophotometrically) shown by us in a previous study [26].

Regeneration of the catalytic activity of thermoinactivated α-chymotrypsin covalently immobilized in polyacrylamide gel

The α-chymotrypsin molecule consists of three polypeptide chains. As a result of unfolding and cleavage of its five S-S bonds, two interchain disulfide bridges should also split. In this case, it should be expected that the three polypeptide chains should separate, and the protein may fail to renature. It was shown by Anfinsen and coworkers [27] that this is just the way the enzyme behaves in solution. However, the situation is different with the immobilized enzyme when the enzyme was made to bind with the complementary surface of the support in a multipoint fashion. Then each of the chains may be attached to a support with at least one bond; hence regeneration may be a successful as with a single chain protein (such as trypsin, see above).

To avoid the limitation of the cyanogen bromide method of attachment of

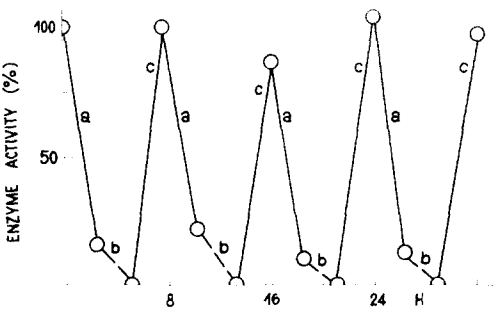


Fig. 2. Regeneration of the catalytic activity of α-chymotrypsin (immobilized in polyacrylamide gel), after irreversible thermoinactivation (95°C, pH 8, curve a) as a result of reduction of S-S bonds in the presence of urea (curve b), followed by their reoxidation (curve c).

the enzyme to Sepharose (where the protein is washed off the support, see above), we used covalent entrapment of α-chymotrypsin in polyacrylamide gel. In this case attachment of the enzyme is multipoint with more than a dozen of the protein NH_2 groups being involved [24].

The resultant preparation of immobilized enzyme possesses (due to the multipoint interaction with the complementary surface of the gel support) a high thermostability [24]. Therefore, thermoinactivation was performed at 95°C and, as a result, the catalytic activity dropped to 15% of the initial value. The loss of the catalytic activity is irreversible (see Methods).

On reduction of the S-S bonds in the presence of 10 M urea, less than 1% of the enzymatic activity is retained. It may well be that the extent of protein unfolding in this case is different from random coil. Therefore, to distinguish this state from the situation in solution, we name it 'immobilized random coil' [20].

As seen in Fig. 2, the subsequent reoxidation of the S-S bonds by oxygen in the presence of the thiol-disulfide exchange catalysts resulted in almost 10% regeneration of the catalytic activity.

We conducted four consecutive 'thermoinactivation-reactivation' cycles (Fig. 2). This shows the possibility of manifold regeneration of irreversibly denatured enzymes.

Properties of regenerated enzymes

An important question is whether the protein after regeneration acquires its native conformation or a different conformation which is also catalytically active. Unfortunately, it is not easy to investigate the structure of immobilized protein by direct physico-chemical methods (fluorescence, absorption, and so forth) [28]. But the value of the catalytic activity and resistance of the enzyme against denaturants (urea) or heating should be considered as indirect characteristics of protein conformation (especially in the region of its active centre). The regenerated enzymes possess the following properties:

(i) from the data in Fig. 3 it is obvious that the stability of immobilized trypsin to reversible denaturation with urea does not change after a 'thermoinactivation-regeneration' cycle;

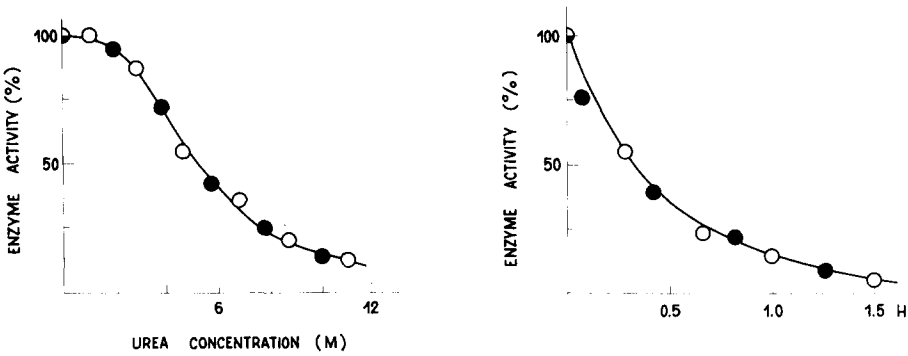


Fig. 3. Alteration in the catalytic activity of trypsin immobilized on Sepharose 4B in the presence of various concentrations of urea (pH 8.0). Preparations subjected to a 'thermoinactivation-regeneration' cycle (●———●) and not subjected to this preliminary procedure (○———○) were studied.

Fig. 4. Alteration in the catalytic activity of trypsin immobilized on Sepharose 4B as a result of irreversible thermoinactivation (80°C, pH 8). Preparations subjected to a 'thermoinactivation-regeneration' cycle (●———●) and not subjected to this preliminary procedure (○———○) were studied.

(ii) the kinetics of thermoinactivation of regenerated α-chymotrypsin (Fig. 2) and trypsin (Fig. 4) are the same as that of the initial (non-denatured) enzymes;

(iii) the catalytic activity of the regenerated enzyme is almost the same as that of the initial preparation (see above).

Thus we propose that the regenerated enzyme has the same conformation as prior to the irreversible thermoinactivation.

Conclusion

The mechanism of irreversible denaturation of proteins

It should be emphasized that our study involved immobilized enzymes. Therefore, the observed thermoinactivation should not be ascribed to intermolecular processes, such as aggregation, autolysis, degradation induced by admixtures of other enzymes, and so forth. It is obvious from the present work that monomolecular thermoinactivation of the enzymes studied (α-chymotrypsin and trypsin) does not involve (up to a temperature of about 100°C) processes which could have changed the primary structure of the protein (hydrolysis of peptide bonds or destruction, e.g. oxidation of the functional groups, and so forth). Otherwise, we would have failed to carry out complete reactivation of irreversibly denatured enzymes under the action of urea (affecting only the secondary and tertiary structures) and compounds (dithiothreitol and thiol-disulfide exchange catalysts) affecting S-S bridges only. These data confirm our view developed in preliminary communications [3,4,14] that sometimes irreversible denaturation of enzymes may have a purely physical monomolecular mechanism; in other words, we believe that irreversibility is not necessarily associated with chemical changes of the protein.

Why reactivation should involve the breakage (and subsequent restoration) of S-S bonds

We failed to regenerate thermodenatured enzymes under the action of urea

only (without breakage of S-S bonds). So the proposed physical mechanism of monomolecular irreversible denaturation could be disputed; i.e. it could be stated that the reason for denaturation irreversibility is chemical and consists of intramolecular disulfide exchange.

However, this mechanism does not seem plausible to us for the following reasons. First, it is known that the monomolecular inactivation of trypsin and α-chymotrypsin occurs even at neutral pH values under very moderate temperatures (40°C) (see Ref. 29 for example). For these conditions the rate of intramolecular disulfide-disulfide exchange should be negligibly low [30].

Secondly, it should be emphasized that in the presence of concentrated solutions of urea we managed to completely protect trypsin and α-chymotrypsin against irreversible thermoinactivation even at 100°C [31]. It is known that in concentrated solutions of urea these enzymes exist in an unfolded state and hence, have mobile polypeptide chains [6]; this could facilitate the S-S exchange. The absence of thermoinactivation in these conditions [31] could be regarded as indication that the denaturation irreversibility is not associated with intramolecular disulfide-disulfide exchange.

Thirdly, our attempts to regenerate the thermoinactivated enzymes that, at the stage of unfolding were confined to only dithiothreitol (without urea), were unsuccesful.

Therefore, we believe that the reactivation method should involve the splitting of S-S bonds because, firstly, the unfolding of the protein in concentrated solutions of urea does not affect the whole molecule; it is known that in the vicinity of S-S bonds there are regions of ordered structure which disappear only after additional splitting of S-S bonds. This is shown for example, for pepsinogen [32], see also literature to [3,4,14]. In other words, the breakage of S-S bonds should facilitate degradation of non-covalent (including non-native) interactions in thermodenaturated protein and can thus facilitate its conversion to a random coil. Secondly, the splitting of S-S bonds in thermodenatured protein should be of primary importance for the second step of the reactivation process: it is possible that in this case the kinetic (steric) hindrances for the refolding of the thermodenatured protein into a native conformation are eliminated.

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