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ON THE MECHANISM OF IRREVERSIBLE THERMOINACTIVATION OF ENZYMES AND POSSIBILITIES FOR REACTIVATION OF "IRREVERSIBLY" INACTIVATED ENZYMES

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SUMMARY

A possible mechanism for so-called irreversible thermoinactivation of enzymes is suggested. We postulated that in many cases "irreversibly thermoinactivated enzyme" is an incorrectly folded polypeptide chain which, during a reasonable experimental interval, cannot transform into a correctly folded, thermodynamically stable and catalytically active conformation because of high kinetic barriers (intrinsic steric hindrances). Proceeding from this model, reactivation of "irreversibly" thermoinactivated trypsin (covalently attached to CNBr--activated Sepharose to prevent intermolecular processes such as aggregation or autolysis) was carried out. Thermoinactivated trypsin was first transformed to random coil by reduction of its disulphide bonds in 8M urea solution and then renaturated into a catalytically active form by reoxidation of disulphide bonds in the presence of dithiothreitol and in the absence of the denaturant.

INTRODUCTION

Mechanisms of reversible inactivation (denaturation) of enzymes have been investigated in detail and in many cases are well understood (1-3). At the same time, mechanisms of so-called "irreversible" (experimentally irreversible) inactivation (in particular, thermoinactivation) of enzymes have been studied to a lesser extent and many remain unknown (2,4). Usually this is due to the inability to apply thermodynamic treatment to the process of irreversible inactivation, which is often complicated by accessory reactions. However, from the viewpoint of applied enzymology, irreversible inactivation of enzymes is especially important, because this process could result in a decrease of catalytic effectiveness of enzymatic systems.

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Irreversible thermoinactivation of enzymes is often attributed to intermolecular processes such as aggregation and precipitation or to autolysis in the case of proteases (2,4-6). However, as a result of developments in the field of immobilized enzymes (7,8), particularly studies of inactivation and stabilization of immobilized enzymes (9), it became evident that although these mechanisms could play a role in irreversible inactivation of enzymes, they could not be the primary cause. It is clear that intermolecular processes are very restricted or even impossible for enzymes covalently attached to solid supports, but the rates of thermoinactivation of these immobilized enzymes in many cases are comparable to those for soluble enzymes (8,10). Therefore, general mechanisms of irreversible thermoinactivation of enzymes remain unclear, and attempts to stabilize enzymes against such inactivation (or to reactivate them) are in most cases empirical (11).

On the basis of analysis of mechanisms of reversible denaturation of proteins (1-3), it appears reasonable to suggest a possible mechanism of irreversible thermoinactivation of enzymes. With an increase in temperature, protein molecules partially unfold and then may refold into new structures, different from the native enzyme conformations to form kinetically or thermodynamically stable structures which are enzymatically inactive. Even after cooling, these incorrect structures remain because a high kinetic barrier prevent spontaneous refolding to the native structures. Thus "irreversibly thermoinactivated enzyme" may be defined as an incorrectly folded enzyme (whose structure is supported by the same interactions (1) - hydrophobic, electrostatic, hydrogen bonded, Van der Vaals's - as the native conformation) which during a reasonable experimental interval cannot transform into correctly folded, catalytically active conformation because of intrinsic steric hindrances.

This model is supported by results of our work (13), which showed that reversibly inactivating (unfolding) reagents, such as urea or acrylamide protected both soluble and immobilized enzymes against irreversible thermoinactivation. In terms of the present concept this is attributed to the ability of reversible denaturants to prevent incorrectly folded structures, which result from either a heating or a heating/cooling cycle, by reducing the non-covalent interactions which provide the kinetic barrier to formation of the thermodynamically most stable structures.

From works of Anfinsen and coworkers (12) as well as numerous subsequent authors (6,14,15), it is known that the native structure of many proteins can be reversibly unfolded (to produce a conformation close to a random coil) by urea if further chemical reduction of their disulphide bonds is performed. The unfolded polypeptide chains can be refolded into native structures by reoxidation of thiol groups following removal of denaturant. It is evident, that the

structures of "irreversible" thermoinactivated enzymes are supported by the same forces as native structures. Therefore, they should also be transformed into an unfolded state close to a random coil structure by reduction of disulphide bonds in urea solution. From this unfolded conformation, according to Anfinsen, the native structure should be produced. As a result of unfolding and refolding (with reduction and reoxidation of disulphide bonds), irreversibly thermoinactivated enzyme can be reactivated. In the present work the reactivation of "irreversibly" thermally inactivated enzyme was demonstrated.

MATERIALS AND METHODS

Bovine trypsin (crystallized) was obtained from Reanal (Hungary). The content of the enzyme active centres determined by spectrophotometric titration (16) was 67%. Dithiothreitol, CNBr-activated Sepharose, N-benzoyl-L-arginine ethyl ester were products of Sigma. 2-Mercaptoethanol was obtained from Eastman and p-nitrophenyl-p'-guanidinobenzoate from Merck. All other chemicals used were of the highest available purity.

Immobilization of trypsin was carried out according to published methods (17,18). The coupling mixture contained 0.1% (w/v) protein and a 30% (v/v) suspension of CNBr-activated Sepharose 4B in 0.1M borate buffer at pH 8.5 containing 0.05M $CaCl_2$. The reaction mixture was gently stirred for 15 hours at 4°. Excess protein was removed by repeatedly washing with buffers, as described in (17). The suspension was stored at 4° in 1mM HCl.

Enzymatic activity of Sepharose-trypsin was measured potentiometrically, (19), using a Radiometer type TTT 1c titrator. Approximately 0.05-0.30ml of a suspension of immobilized trypsin were added to 5ml of substrate solution (0.01M N-benzoyl-L-arginine ethyl ester, 0.1M KCl) at pH 8.0 and 25°. Catalytic activity of Sepharose-trypsin was about 400 nmol of hydrolized N-benzoyl-L-arginine ethyl ester /s per ml of gel.

The procedure "unfolding-refolding" (reduction-reoxidation) of Sepharose--trypsin was carried out according to published methods (18). Reduction of disulphide bonds of a 30% (v/v) suspension of immobilized trypsin was accomplished with 0.1M mercaptoethanol in 8M urea, 1mg/ml EDTA and 0.1M Tris·HCl, pH 8.5. The suspension was maintained under anaerobic conditions for 5 hours at 25° with slow stirring. Then pH was lowered to 3.0 with 1M HCl and the gel was washed on a glass filter with 1mM HCl. All solutions used in the procedure were degassed with argon immediately before use. Reoxidation was carried out with a 5 to 10% (v/v) suspension of reduced Sepharose-trypsin at 37° in 0.1M Tris·HCI (pH 8.5), containing 1mM dithiothreitol and 0.05M $CaCl_2$. During reoxidation the mixture was gently agitated. At the desired time for the removal of samples, the suspension was adjusted to pH 3 with 1M HCI, and the gel was thoroughly washed with 1mM HCI.

Thermoinactivation of immobilized trypsin was carried out as previously described (9,13). The suspension of Sepharose-trypsin was incubated at 60° in 0.1M Tris·HCI (pH 8.0) and aliquots were withdrawn for enzyme assay.

RESULTS AND DISCUSSION

The well-studied enzyme, bovine trypsin, was chosen as a model for our work. To prevent possible intermolecular processes (autolysis, aggregation) all

TABLE 1 Effect of protein unfolding and refolding (reduction and reoxidation) on the enzymatic activity (towards N-benzoyl-L-arginine ethyl ester) of catalytically active and thermoinactivated Sepharose-trypsin. For experimental conditions see Materials and Methods.

Type of Sepharose-trypsin	Activity#, nmoles/sec/ /ml of gel	Activity after reduction of disulphide bonds in 8M urea, nmoles/sec/ml of gel	Activity# after reoxidation of disulphide bonds, nmoles/sec/ml of gel
Catalytically active enzyme	408±6	0	318±19
Reoxidized catalytically active enzyme	318±19	0	307±23
Partially thermoinactivated enzyme	16±6	0	314±20
Reactivated thermoinactivated enzyme	314±20	0	309±11
Thermoinactivated reoxidized enzyme	11±8	0	321±14

Mean values from three independent experiments.

experiments were made with the enzyme covalently attached to CNBr-activated Sepharose. Sinha and Light (18) recently showed that this immobilized and catalytically active trypsin can be unfolded and refolded (with reduction and reoxidation of disulphide bonds) with recovery of its activity.

As a result of cleavage of disulphide bonds of trypsin (attached to CNBr-activated Sepharose) with mercaptoethanol in 8M urea, totally inactive enzyme was produced. When the filtered and washed, immobilized enzyme was resuspended in buffer solution, containing 1mM dithithreitol, and stirred under air at 37^{o} for 4-5 hours, 70-80% of initial enzymatic activity was recovered (Table 1). Further incubation of the mixture under these conditions did not increase the yield of Sepharose-trypsin activity. (The fact that activity here as well as in

TABLE 2 Effect of incubation of thermoinactivated Sepharose-trypsin in different solutions on its enzymatic activity (towards N-benzoyl-L-arginine ethyl ester).

Residual activity of thermoinactivated immobilized enzyme was 19 nmoles/ /sec/ml of gel.

Solution	Incubation time, hours	Incubation temperature, °C	Activity after incubation, nmoles/sec/ml of gel
0.1M Tris·HCl (pH 8.5)	10	20	21
0.1M Tris·HCl (pH 8.5)	10	37	15
8M urea in 0.1M Tris·HCl (pH 8.5)	4	37	28
8M urea in 0.1M Tris·HCl (pH 8.5)	8	37	26
4M guanidine·HCl in 0.1M Tris·HCl (pH 8.5)	4	37	5

(18) returns to less than the 100% level is evidently due to presence of forms containing proteolytic cleavages in the polypeptide chains in the commercial trypsin preparation.) When an additional cycle of "unfolding-refolding" with the reoxidized enzyme was carried out, nearly 100% recovery of its catalytic activity was achieved (Table 1).

After incubation of a suspension of immobilized trypsin in buffer (pH 8) at 60° for several minutes, the enzyme was inactivated. The time of inactivation was chosen to obtain 95-97% inactivation of the immobilized enzyme. This thermoinactivation is "irreversible": only slight reactivation of the enzyme could be achieved even after incubation of the suspension of inactivated trypsin either in buffer or in urea solutions at room or elevated temperatures during several hours (Table 2).

"Irreversibly" thermoinactivated trypsin was then treated by the same procedure of "unfolding-refolding" as the non-inactivated enzyme. As a result of this procedure, the heat denatured enzyme activity sharply increased up to

70-80% of the initial activity (Table 1). A repeated cycle of thermoinactivation-unfolding(reduction)-refolding(reoxidation) produced complete recovery of reactivated trypsin activity (Table 1). When we thermoinactivated and then reactivated preliminarily refolded enzyme we also achieved nearly 100% recovery of its enzymatic activity (Table 1).

Therefore, using the described procedure we have shown that it is possible to reactivate "irreversibly" inactivated trypsin. This method may be quite general and analogous reactivation can probably be achieved for many other enzymes (providing there has been no proteolytic cleavage of peptide chains).

We think that this approach can be very useful in enzyme engineering for reactivation and repeated use of inactivated enzymes in catalytic systems.

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