DENATURING AGENTS (UREA, ACRYLAMIDE) PROTECT ENZYMES AGAINST IRREVERSIBLE THERMOINACTIVATION: A STUDY WITH NATIVE AND IMMOBILIZED α-CHYMOTRYPSIN AND TRYPSIN

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1. Introduction

It is common knowledge that, when heated or treated with denaturing agents of urea type, enzyme solutions lose their catalytic activity. Countless investigations both experimental and theoretical have been devoted to these inactivation processes. Nevertheless, the mechanisms of inactivation are in many respects still obscure [1–5].

It has been for the first time demonstrated in this work that classical denaturing agents (of the type of urea and other amides) which are known to reversibly inactivate enzymes, are sharply increasing thereby their stability with respect to irreversible first-order thermoinactivation. For instance, heating α-chymotrypsin solution at 100°C brings about an instantaneous loss of its catalytic activity; see curve a in fig.1. The situation is different in 9.6 M acrylamide where first-order inactivation process is actually eliminated; see curve b in fig.1 (catalytic activity was measured on subsequent cooling the solution in the absence of the denaturant; see Methods). A more detailed quantitative study of this phenomenon was carried out by us at 57°C for α-chymotrypsin and trypsin, both in the native and immobilized state (attached to *p*-aminobenzyl cellulose).

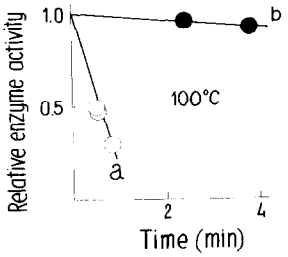


Fig.1. Protection of α-chymotrypsin against irreversible thermoinactivation with acrylamide. Curve a - in the absence of acrylamide, curve b - in 9.6 M acrylamide solution.
Conditions: 100°C, pH 5.0, 0.01 M acetate buffer, 0.1 M KCl.

2. Experimental

2.1. *Materials*

Crystalline bovine α-chymotrypsin (E.C. 3.4.4.5), urea and acrylamide were the products of Olaine chemical reagents plant (USSR). Bovine trypsin (E.C. 3.4.4.4) was obtained from the Leningrad meat packing plant (USSR). *N*-acetyl-L-tyrosine ethyl ester and *N*-tosyl-L-arginine methyl ester were also commercial preparations (Reanal, Hungary). Trans-cinnamoylimidazole and *p*-nitrophenyl-*p'*-guanidine benzoate were synthesized according to [6] and [7], respectively. *p*-Aminobenzyl cellulose was a commercially available sample (Serva).

2.2. *Methods*

The normality of α-chymotrypsin and trypsin solutions was estimated by spectrophotometric titration according to [6] and [7], respectively.

Relative catalytic activity of enzyme solution was determined by the initial rate of steady-state hydrolysis of specific substrate (i.e., *N*-acetyl-L-tyrosine ethyl ester and *N*-tosyl-L-arginine methyl ester, respectively). Measurements were performed in a TTT-1c pH-stat Radiometer (at $[S]_o \approx 10^{-2}$ M in 0.1 M KCl).

Irreversible inactivation of enzymes (see, e.g., fig.2, A-B and 3, A-B) was studied in a special thermostated

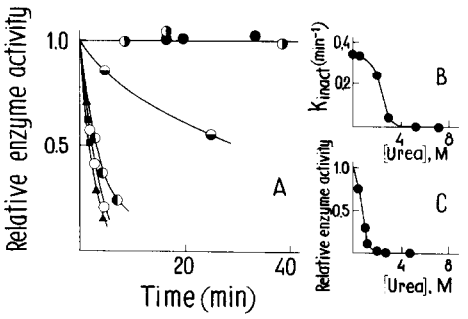


Fig.2. Thermoinactivation of α-chymotrypsin in urea solutions. Conditions: 57°C, pH 5.0, 0.01 M acetate buffer, 0.1 M KCl. (A) Kinetics of α-chymotrypsin irreversible thermoinactivation in the presence of various urea concentrations (M): ● - 7, ◑ - 5, ◒ - 3, ◐ - 2, ○ - 0.5, ■ and ▲ - in the absence of urea. Initial enzyme concentration: ■ 7×10^{-8} M, the rest of the curves, 2×10^{-7} M. (B) Dependence of first-order rate constant for α-chymotrypsin irreversible thermoinactivation on urea concentration (k_{inact} values are estimated from initial regions of the curves drawn in fig. 2,A). (C) Dependence of α-chymotrypsin relative catalytic activity on urea concentration. Enzyme concentration is 2×10^{-7} M.

cell at 57°C and pH 5.0 (α-chymotrypsin) and pH 8.3 (trypsin). To measure thermoinactivation rate, samples were taken which were then cooled to 20°C, diluted (to reduce the concentration of the denaturant to <0.5 M; the enzyme reactivation is over within 5 min)

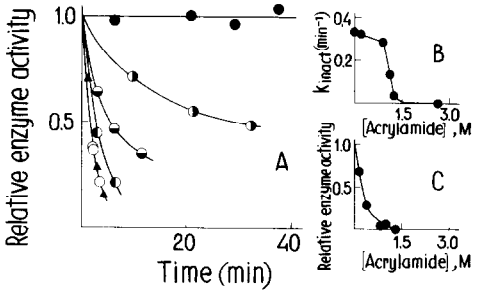


Fig.3. α-Chymotrypsin thermoinactivation in acrylamide solution. For experimental conditions see legend to fig.2. (A) Kinetics of α-chymotrypsin irreversible thermoinactivation in the presence of various acrylamide concentrations (M): ● - 2.8; ◑ - 1.4; ◒ - 1.1; ◐ - 0.92; ○ - 0.24; ▲ - in the absence of acrylamide. Initial concentration is 2×10^{-7} M. (B) Dependence of first-order rate constant for α-chymotrypsin irreversible thermoinactivation on acrylamide concentration (k_{inact} values are estimated from initial regions of the curves drawn in fig. 3,A), (C) Dependence of α-chymotrypsin relative catalytic activity on acrylamide concentration. Enzyme concentration is 2×10^{-7} M.

and added into a pH-stat cuvette for relative catalytic activity to be measured.

Reversible inactivation of α-chymotrypsin under the action of urea (fig.2, C) or acrylamide (fig.3, C) was studied in the pH-stat cuvette at 57°C and pH 5.0 in the presence of substrate. To this end, relative catalytic activity of α-chymotrypsin was determined from the initial region of the kinetic curve (see above) when thermoinactivation is sufficiently insignificant to be neglected. Equilibrium of reversible denaturation is achieved whithin 2 min.

α-Chymotrypsin and trypsin were covalently bound to *p*-aminobenzyl cellulose by azocoupling [8].

Relative catalytic activity of the suspension of immobilized enzyme preparations was determined by the same method as in the case of native catalysts.

3. Results and discussion

The data on α-chymotrypsin irreversible thermoinactivation (57°C) in the presence of various urea concentrations are presented in fig.2, A. The fact that increase in the urea concentration inhibits thermoinactivation is readily observable.

It should be emphasized that, with low enzyme concentrations used (2×10^{-7} M and less) neither autolysis (as follows from the value of second-order rate constant of this process [9]) nor other polymolecular interactions, i.g. aggregation, are involved in the inactivation process observed by us. This is evidenced by that 'relative activity-time' profiles for different initial concentrations of the enzyme (fig.2, A) have the same pattern.

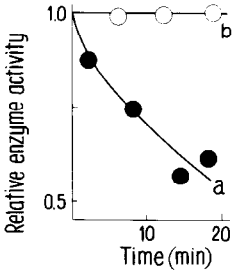
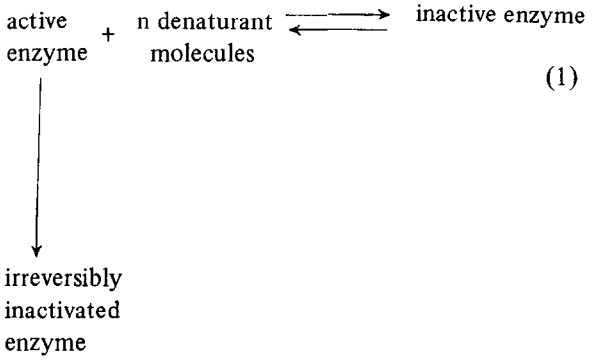


Fig.4. Protection of α-chymotrypsin covalently linked to *p*-aminobenzyl cellulose against irreversible thermoinactivation with urea. Curve a - in the absence of urea, curve b - in 8 M urea solution. For conditions, see legend to fig.2.

Fig.2B shows the plot of first-order rate constant values for α-chymotrypsin irreversible thermoinactivation against the concentration of urea. It is obvious that with urea concentration increasing from 0 to 4 M, the thermoinactivation rate goes down to zero.

On the other hand, in the presence of urea which protects α-chymotrypsin against irreversible thermoinactivation (fig.2B), it undergoes reversible loss of catalytic activity (fig.2C). On a whole, the results obtained may be presented as the following scheme:



(1)

where the coefficient is $n \approx 7$.

The other denaturant, acrylamide, behaves similarly with respect to α-chymotrypsin. It may be drawn from the data presented in fig.3 in terms of equation (1) that $n \approx 8$.

The effect of α-chymotrypsin stabilization by denaturing agents against thermoinactivation is specific not only for this enzyme. The other enzyme, trypsin (for which in the absence of denaturants at 57°C and pH 8.3, $k_{inact} = 0.19\ min^{-1}$) is also protected against irreversible thermoinactivation both with urea ($n \approx 3$) and acrylamide ($n \approx 7$).

To make absolutely sure that no autolysis is involved in the irreversible inactivation observed by us, we showed that denaturing agents are also operative with immobilized enzymes (fig.4). It is apparent that in this case any polymolecular processes of inactivation (like autolysis or protein aggregation) are non-existent.

As to an intimate mechanism that could explain the effect of enzyme protection against irreversible thermoinactivation by inactivating them reversibly with denaturants, we cannot unfortunately offer any. Suggestions are all we may resort to.

It is known that at high temperatures and in denaturant solutions α-chymotrypsin and trypsin molecules unfold [10–12]. In the case of thermoinactivation, a high-rate stage of reversible unfolding is followed by irreversible (kinetically or thermodynamically) inactivation of the enzyme consisting, probably, of chemical reactions (oxidation, formation of 'wrong' disulphide linkages, etc.) [12]. Why do not enzymes unfolded by urea undergo further irreversible inactivation? Reversible inactivation by denaturants is a process which may occur via various mechanisms: rupture of intraprotein hydrogen bonds [13], weakening of interprotein hydrophobic interactions [14], changes due to lower activity coefficients of peptide groups [15] etc. Perhaps, reversible inactivation by denaturants leads to spatial 'estrangement' of the chemical groups, the interaction between which predetermines irreversible denaturation.

Denaturants' protective effect appears to be significant first of all for understanding general mechanisms of enzyme denaturation (inactivation). Second, this phenomenon may be laid as a basis for creating catalytically active enzyme preparations (in immobilized state, possibly) of high thermostability [16]. And, finally, denaturants' protective capacity may be utilized as a method for conservation of enzyme preparations.

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