Biochimica et Biophysica Acta, 522 (1978) 277—283

BBA 68361

THE PRINCIPLES OF ENZYME STABILIZATION

III *. THE EFFECT OF THE LENGTH OF INTRA-MOLECULAR CROSS-LINKAGES ON THERMOSTABILITY OF ENZYMES

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(Received May 9th, 1977)
(Revised manuscript received October 4th, 1977)

Summary

The effect of intramolecular cross-linkages of different length on the thermostability of α-chymotrypsin has been studied. To this end, the carboxy groups of the enzyme were first activated by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; then the activated protein was treated by diamines of $NH_2(CH_2)_n NH_2$ type with n ranging from 0 to 12. The dependence of the rate constant of monomolecular thermoinactivation of the enzyme on the length of the cross-linking agent gives a minimum corresponding to tetramethylenediamine. The number of cross-linkages may be increased by enriching the protein molecule with carboxy groups; for this purpose α-chymotrypsin was succinylated. For succinylated α-chymotrypsin, the dependence of the rate constant of monomolecular thermoinactivation of the enzyme on the length of the cross-linking agent has a minimum for a shorter bifunctional reagent, ethylenediamine. In addition, the maximum stabilizing effect (compared to the native enzyme) increases (from 3- to 21-fold) if instead of α-chymotrypsin modified with tetramethylenediamine, succinylated α-chymotrypsin modified with ethylenediamine is used. This approach may be generally employed for preparation of stabilized water-soluble enzymes.

Introduction

It was previously [1,2] established that enzymes can be stabilized against denaturing action by rigidifying their globular structure. It is known that the

* For parts I and II, see refs. 32, 33.

initial and essential step of denaturation (inactivation) is, as a rule unfolding (i.e. an important conformational alteration) of protein globules [3—5]. With the protein globule structure having been rigidified one may expect that its unfolding (denaturation) will be hindered. This idea was realized by attaching enzymes to a complementary surface of a water-insoluble support [1,2]. However, in medicine or new technological processes involving water-insoluble substrates, such as cellulose, starch or collagen, there has arisen a need for stabilized water-soluble enzyme preparations [6,7]. Solution of this problem will evidently require approaches that do not involve the use of water-insoluble support; all these approaches will, however, have one common principle, i.e. the more rigid the molecule of an enzyme, the more stable it is.

In nature this rigidity (and, as a consequence stabilization) is attained by intramolecular linkages [8]. They may be [9] both covalent, e.g. S-S bonds [10,11] and weaker, such as salt bridges [12] or Ca^{2+} ions incorporated into a protein molecule [13] etc.

One may introduce "man-made" cross-linkages ("brackets") into protein molecules by treating them with bifunctional reagents [14]. Such reagents are glutardialdehyde, diimidoesters, diisocyanates etc. Analysis of the literature [15—23] shows that in most cases the stabilization (against thermoinactivation) effects are not very great; sometimes they even seem to be due to a chemical modification of the enzyme rather than to the presence of cross-linkages [15—17], as was pointed out by Zaborsky [18]. In other cases the effects of stabilization proved to be more pronounced [21—23] and higher stability correlated with higher rigidity of the protein molecule [22].

In all probability, success or failure to increase the stability of the enzyme by treating it with a bifunctional agent largely depends on the length of the latter and the distance between the centres to be linked on the protein globule. It is obvious that every given protein requires an intramolecular cross-linking agent of an optimal size. Research with the aim of elucidating the size of a bifunctional agents may furnish, in addition to what they are meant for, interesting information on protein structure. In other words, study of the dependence of enzyme thermostability on the length of the cross-linking agent will allow one to judge about the topography of the surface layer or a protein globule in solution. These data may contribute to the knowledge of crystalline enzymes obtained by X-ray analysis.

Studies of the above kind have hardly ever been carried out, mainly due to the fact that series of bifunctional cross-linking agents have not been commercially available; their chemical synthesis is very labour-consuming. We suggest using bifunctional reagents that cannot be applied directly to intramolecular cross-linking of enzyme unless the latter is premodified in a certain fashion. For this purpose it is tempting to try a series of aliphatic diamines: they are commercially available (with the length of the hydrocarbon chain of 0—12 methylene groups) and relatively inexpensive; these two factors are of primary importance for practical use. Diamines may be used for cross-linking carboxy groups of enzymes (of which there are many in proteins), if they are preliminary activated with carbodiimide. As a result, between the amino group of the modifier (diamine) and a carboxy group of a protein there forms an amide linkage [24].

In the present work, the enzyme used was well-known α-chymotrypsin. It

was first activated by 1-ethyl-3(3-dimethylaminopropyl)carbodiimide and then treated with diamine of various length (from hydrazine to dodecamethylenediamine); the alteration in the thermostability of the biocatalyst was thereby followed.

Experimental

Materials

Crystalline α-chymotrypsin (with a concentration of the active protein of 64% determined by spectrophotometric titration as described in [25]) and its specific substrate *N*-acetyl-L-tyrosine ethyl ester were products of Koch-Light Laboratories Ltd. (Great Britain). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide was manufactured by Sigma (U.S.A.). All the amines were commercial preparations, i.e. hydrazine, ethylenediamine, pentamethylenediamine, 1-aminopropanol-3 were products of "Reakhim" (U.S.S.R.), tetramethylenediamine of Ferak (West Berlin), hexamethylenediamine and dodecamethylenediamine of Koch-Light Laboratories Ltd. (Great Britain). All other reagents (inorganic salts, buffer solution components etc.) were analytical grade preparations.

Succinylation of α-chymotrypsin

α-Chymotrypsin was acylated with succinic anhydride following the recommendations of Goldstein [26]. 300 mg of α-chymotrypsin were dissolved in 30 ml of 0.2 M phosphate buffer (pH 7.7); then 300 mg of succinic anhydride were introduced in small portions in cold (4°C) with pH being maintained constant. In these conditions over 80% of 14—15 titrated amino groups of the enzyme become acylated [2]. After that the solution was passed through a 2.5 × 60 cm column packed with Sephadex G-50 (Pharmacia, Sweden) in 0.01 M KCl, the elution rate being 1.5 ml/min. The resulting preparation of succinylated α-chymotrypsin possesses both the catalytic activity and thermostability of the native enzyme (in the conditions employed by us; see below).

Activation of the carboxy groups of α-chymotrypsin by carbodiimide

α-Chymotrypsin was treated with carbodiimide as described in [27,28], with a slight modification. 63 ml of 10^{-6} M solution of α-chymotrypsin (or succinylated α-chymotrypsin) were supplemented with 7 ml of 10^{-2} M aqueous solution of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and incubated in a bufferless medium pH 4.5 (pH-stat) for 1 h at 20°C. In these conditions 15 out of 17 exposed carboxy groups of chymotrypsin are modified [29].

As a result of treatment with carbodiimide, the relative catalytic activity of α-chymotrypsin drops 3-fold.

Interaction of activated chymotrypsin with diamines or 1-aminopropanol-3

The reaction was carried out as described, for example, in [30], slightly modified to suit our purposes. 10 ml of the solution obtained after treatment of α-chymotrypsin or succinylated α-chymotrypsin by carbodiimide were supplemented with 4 ml of 0.02 M phosphate buffer (pH 8.2) and 1 ml of amine solution in the following concentrations: 0.01 M for hexamethylenediamine or dodecamethylenediamine, 0.1 M ethylenediamine, tetramethylenediamine and

pentamethylenediamine or 10 v/v% of hydrazine (or 1-aminopropanol-3). The incubation was carried out at 20°C and pH 8.2 for 1 h.

In these conditions the thermostability (see Results and Discussion) and the relative catalytic activity (10—30% of the native enzyme level) reach the limiting values (i.e. do not change during 3 h of incubation as was the case for the reaction with dodecamethylenediamine). This enzyme preparation was dialyzed against distilled water in dialysis tubes (manufactured by Union Carbide Corporation, U.S.A.) for 24 h at 4°C. Such purification of the modified enzyme from the nonreacted diamines is important because, as we have found, the thermostability of chymotrypsin added to a solution of diamine increases. The reason for this is that noncovalent protein · ligand complex seems to arise. This is shown by the fact that after the 24-h dialysis the thermostability of unmodified chymotrypsin returns to the level of the native enzyme.

Determination of the catalytic activity of the native and modified enzyme

The catalytic activity of the native and modified enzyme was measured in a TTT-1d pH-stat (Radiometer, Denmark) by the initial rates of enzymatic hydrolysis of 0.01 M solution of *N*-acetyl-L-tyrosine ethyl ester in 0.1 M KCl at pH 7.0, 20°C (cell volume 10 ml).

Thermoinactivation experiments

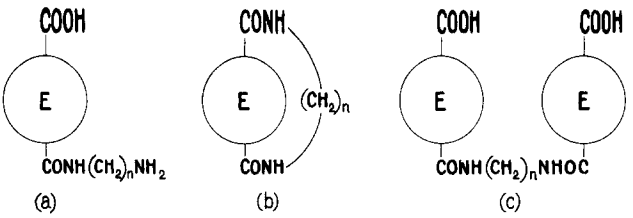
10 ml of α-chymotrypsin or a corresponding derivative (10^{-6} M) were supplemented with 5 ml of 0.02 M phosphate buffer (pH 7.0) and incubated at 50°C; 1 ml aliquots were taken at certain time intervals, and the residual catalytic activity was determined in the fashion described above.

The process of thermoinactivation does not obey the first-order kinetics, see Fig. 1, 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. Nevertheless, its kinetics does not depend on the starting concentration of the enzyme ($5 \cdot 10^{-8}$—$5 \cdot 10^{-7}$ M for native α-chymotrypsin and $7 \cdot 10^{-7}$—$1.5 \cdot 10^{-5}$ M for the tetramethylenediamine-modified enzyme), see, for example, Fig. 1. This means that the observed inactivation of the enzyme is a monomolecular process involving no aggregation or autolysis of the protein. As a parameter characterizing the relative thermostability of individual preparations of modified α-chymotrypsin (compared to the native enzyme) use was made, as in [31—33], of the apparent first-order rate constant measured with a certain amount of the catalytic activity having been lost by the enzyme (at a certain degree of conversion of the thermoinactivation process, i.e. 70%, for details see [32] and Fig. 3 therein).

Results and Discussion

Intramolecular nature of cross-linking in α-chymotrypsin molecules

The preparations of α-chymotrypsin activated by carbodiimide and then treated with diamine apparently contain both modified carboxy groups (when only one of the two amino groups of the modifier is covalently bound with the protein, see Scheme 1a), and the cross-linkages (when both amino groups of diamine have interacted with the protein, see Scheme 1b). It is not excluded that



Scheme 1.

treatment of carboddimide-activated chymotrypsin by diamines may result in the protein molecules being linked in the intermolecular fashion, i.e. in the formation of the oligomers of the enzyme (Scheme 1c). To prevent this, we used minimal concentrations of activated chymotrypsin ($<10^{-6}$ M) for modification with diamine. In addition, it was demonstrated for each diamine that with a ten-fold increase in this concentration the enzyme preparations have the same thermostability and relative catalytic activity as those obtained at a lower concentration of the protein. This is indicative of the fact that in our conditions the interaction of the protein with diamine is a monomolecular process with respect to the former.

Increase in the thermostability of intramolecular linked α-chymotrypsin

The thermostability of diamines-treated α-chymotrypsin markedly increases (for example, curves a and c in Fig. 1). The effect of stabilization observed cannot be accounted for just by chemical modification of the enzyme (Scheme a), as the modification of activated α-chymotrypsin by monoamine, 1-aminopropanol-3, hardly affects its thermostability, if only slightly reduces it, see Fig. 1. It should be concluded therefore that increase in the thermostability of the enzyme modified by diamines is due to the intramolecular "brackets".

Fig. 2 shows the dependence on the number of methylene groups (n) in the molecule of the modifier, $NH_2(CH_2)_nNH_2$ for the apparent first order rate constant for thermoinactivation of α-chymotrypsin cross-linked by various diami-

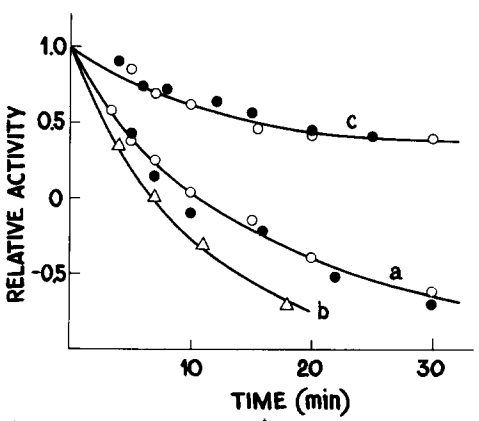
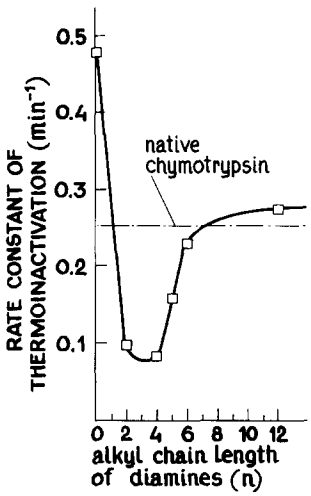


Fig. 1. Kinetic curve of thermoinactivation of α-chymotrypsin and its derivatives in semilogarithmic coordinates: a, native enzyme; b, activated α-chymotrypsin modified by 1-aminopropanol-3; c, activated α-chymotrypsin modified by tetramethylenediamine. Conditions: 50°C. pH 7 (0.02 M phosphate). The initial concentration of the enzyme preparations: (a) Filled circles $1 \cdot 10^{-6}$ M, empty circles $1 \cdot 10^{-7}$ M; (b) $7 \cdot 10^{-7}$ M; (c) filled circles $7 \cdot 10^{-7}$ M, empty circles $1 \cdot 10^{-5}$ M.



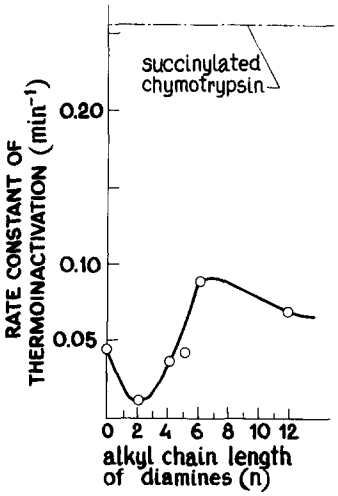


Fig. 2. First-order rate constant for monomolecular thermoinactivation (degree of conversion 70%) of α-chymotrypsin cross-linked intramolecularly by diamines, depending on the number of methylene groups in a molecule of the modifier, $NH_2(CH_2)_nNH_2$. For conditions see Fig. 1. Broken line shows the level of thermostability of succinylated α-chymotrypsin.

Fig. 3. First-order rate constant of monomolecular thermoinactivation (degree of conversion 70%) of succinylated α-chymotrypsin cross-linked intramolecularly by diamines, depending on the number of methylene groups in a molecule of the modifier, $NH_2(CH_2)_nNH_2$. For conditions see Fig. 1. Broken line shows the level of thermostability of succinylated α-chymotrypsin.

nes. The curve has a minimum at $n = 4$, although at $n = 2$, 5 and 6 stabilization of the enzyme also occurs.

That the treatment by tetramethylenediamine results in the greatest stabilization of chymotrypsin seems to be due to the fact that this very agent has the length fitting to the distance between the carboxy groups of the protein molecule; this facilitates the formation of a greater than in the case of other diamines number of intramolecular cross-linkages.

Increase in the thermostability of intramolecularly cross-linked succinyl-α-chymotrypsin

It may be believed that the more cross-linkages there are in a protein molecule, the more stable it must be against unfolding and hence against inactivation. It is possible that the number of the cross-linkages is determined by the quantity (and mutual orientation) of the functional groups on the surface of the protein (carboxy groups in our case) which may in principle interact with a cross-linking agent. It is clear that this quantity (and the possible number of cross-linkages) may be increased by premodifying the protein in a certain way. This is what we did, modified α-chymotrypsin by succinic anhydride. As a result, ε-amino grpoups transformed into carboxy groups [26]. Then succinylated α-chymotrypsin was treated by diamines of different length using the same procedure as with the native enzyme. Fig. 3 shows the dependence obtained thereby. One can see that the maximum stabilization effect increase from 3-fold (for nonsuccinylated enzyme, Fig. 2) to 21-fold; secondly, the maximum stabilizing effect is produced not by tetramethylenediamine (see Fig. 2), but by a shorter bifunctional reagent, ethylenediamine. The latter fact

should be interpreted as indicating that the surface of succinylated α-chymotrypsin globules is more densely "populated" with carboxy groups than that of the native α-chymotrypsin. Thus, the premodification of the enzyme makes possible regulation of the stabilization effect both with respect to the degree and the optimal length of the cross-linkage.

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