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PRINCIPLES OF ENZYME STABILIZATION

V. THE POSSIBILITY OF ENZYME SELFSTABILIZATION UNDER THE ACTION OF POTENTIALLY REVERSIBLE INTRAMOLECULAR CROSS-LINKAGES OF DIFFERENT LENGTH *

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Summary

The denaturing action of guanidine · HCl on modified α-chymotrypsin (EC 3.4.21.1) preparations has been studied. The consecutive treatment of α-chymotrypsin with *N*-acetyl-homocysteine thiolactone, 5,5′-dithio-bis-(2-nitrobenzoic acid) and dithiols of HS-$(CH_2)_n$-SH type, with n ranging from 4 to 10, leads to enzyme stabilization as a result of protein modification. A greater stabilization effect can be achieved by enriching the protein molecule with groups reactive towards dithiols, after first modifying carboxygroups. In this case dithiol with $n = 5$ forms an intramolecular cross-linkage. If an equimolecular mixture of different dithiols is used for enzyme modification, the enzyme gradually 'selects' 1,5-dithiol for the formation of an intramolecular cross-linkage instead of the initial one-point modification. The use of potentially reversible cross-linkages may be generally employed for the preparation of stabilized water-soluble enzymes via the mechanism of selfstabilization.

Introduction

The creation of stabilized enzymes opens vast possibilities of their use in practice [1]. Earlier [2] it was shown that enzyme stability can be increased

* For parts III and IV see Refs. 2 and 18.
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without the use of a carrier by the introduction of intramolecular cross-linkages into the protein molecule. In this case the stability of the enzyme against denaturation increases as a result of its globular structure being made more rigid. The best effect is achieved by introducing into each region of the protein molecule cross-linkages of different lengths corresponding to the distances between the centers to be linked in each region. This approch requires the application of potentially reversible cross-linkages. In our opinion, the reaction of thiol-disulfide interchange [3] gives the greatest opporunity of introducing intramolecular 'brackets' into a protein molecule using bifunctional dimercaptans of different length. Thiol-disulfide interchange has been already used for enzyme mobilization on a carrier [4,5].

If the enzyme molecule contains activated SH-groups, one may expect the formation of bridge linkages during the treatment of the modified enzyme with dithiols of HS-$(CH_2)_n$-SH type of different chain length. Moreover, the linkage formation process is reversible, due to nucleophilic attack of the -S-S- bond formed by the free SH-groups of dithiols. Hence in the case where a mixture of dithiols of different length is used for enzyme modification, the initially formed random one-point modification of the enzyme with a dithiol should be gradually replaced by a two-point cross-linkage formation, which is more stable and advantageous from the thermodynamic point of view: i.e. the enzyme will gradually 'select' the cross-linkage of the optimal length from the variety of available cross-linking agents of different lengths. In other words, selfstabilization of the enzyme will occur.

The present work is the first investigation into the possibility of enzyme self-stabilization. We have studied the process of α-chymotrypsin (EC 3.4.2.1.1) inactivation under the action of guanidine · HCl after the modification of the enzyme in the fashion described above.

Experimental

α-Chymotrypsin must be enriched with SH-groups in order that it may be interacted with dithiols. Several methods of thiolation are known. Thiolation of α-chymotrypsin with methyl-3-mercaptoimidate permits only few SH-groups to be introducted into the protein molecule [4]; moreover, the synthesis of the agent for thiolation is very complicated [6]. We have chosen another thiolation method, using a commercial preparation of *N*-acetyl-homocysteine thiolactone [7,8]. In this case it is possible to introduce up to ten SH-groups into the protein molecule, the enzyme preserving about 90% of its catalytic activity [9].

The activation of SH-groups can be performed by different methods: using β-mercaptoethanol [4,10], iodoacetate [8], 2,2′-diperedyldisulfide [4], etc. In our experiments 5,5′-dithio-bis-(2-nitrobenzoic acid) was used. This reagent activates SH-groups for the reaction on thiol-disulfide interchange [11] and permits the number of SH-groups in the modified enzyme to be spectrophotometrically determined [12].

Materials. Crystalline α-chymotrypsin (active protein 64%, determined by spectrophotometric titration [13]) and its specific substrate *N*-acetyl-L-tyrosine ethyl ester were the product of Koch-Light Laboratories Ltd., (U.K.). 1-Ethyl-

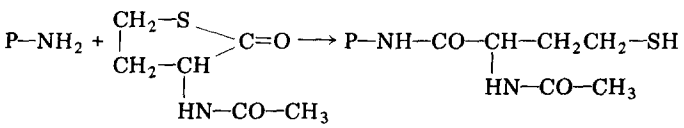
3-(3-dimethylaminopropyl)carbodiimide, *N*-acetyl-homocysteine thiolactone and guanidine · HCl were from Sigma (U.S.A.). Dithiols of HS-$(CH_2)_n$-SH type with $n = 4, 5, 6, 8, 9, 10$ were the products of ICN (U.S.A.). 5,5′-Dithio-bis-(2-nitrobenzoic acid) was the product of Serva, (F.R.G.). All other reagents were analytical grade preparations of Reakhim (U.S.S.R.).

Methods. Activation of the carboxy groups of α-chymotrypsin by carbodiimide. α-Chymotrypsin was treated with carbodiimide as described in [2]. The enzyme concentration in the reaction mixture was 0.5 mM. 43% of the native enzyme activity was retained after this treatment.

Interaction of activated α-chymotrypsin with ethylenediamine. Activated α-chymotrypsin was treated with ethylenediamine [2]; the enzyme concentration in the reaction mixture was 0.25 mM. 17% of the native enzyme activity was retained after this treatment.

Thiolation of native and modified α-chymotrypsin. Thiolation of α-chymotrypsin with *N*-acetyl-homocysteine thiolactone was performed according to [9].

The scheme of the reaction [7] is as follows:

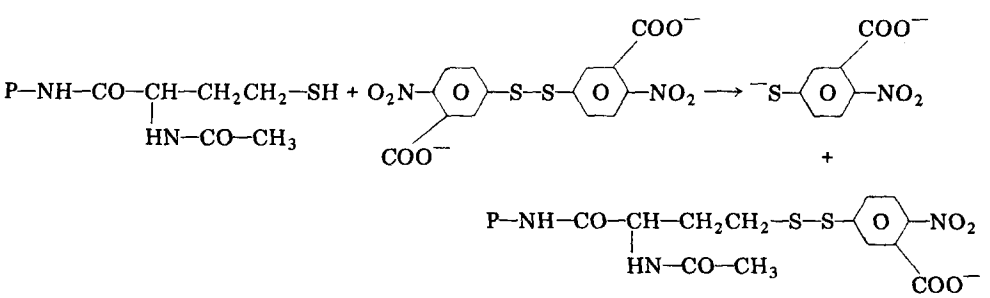


(P = Protein)

The concentration of the native enzyme was 1.3 mM, and of the enzyme modified with carbodiimide and ethylenediamine 0.1 mM.

Activation of SH-groups of modified α-chymotrypsin. Activation of SH-groups of modified α-chymotrypsin with 5,5′-dithio-bis-(2-nitrobenzoic acid) was performed as described in [11].

The scheme of the reaction [5] is:



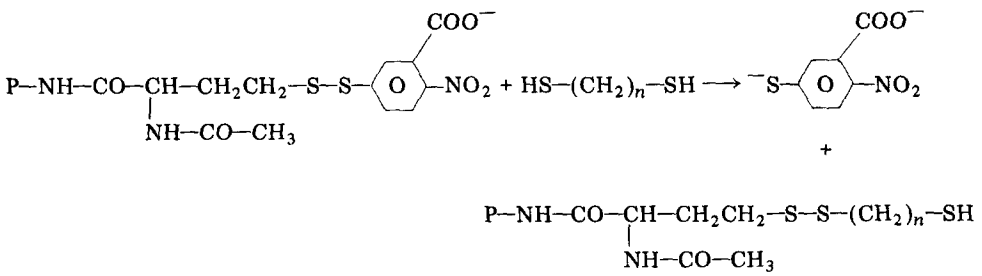
The enzyme concentration in the reaction mixture was 5.0 μM.

Titration of SH-groups of modified α-chymotrypsin. The determination of the number of SH-groups introduced into the α-chymotrypsin molecule as a result of modification was performed spectrophotometrically [12] at $\lambda = 412$ nm, 20°C, pH 8.0, (0.01 M Tris-HCl buffer in 0.3 M KCl and 5 mM EDTA) using a Perkin-Elmer Coleman-55 spectrophotometer (U.S.A.).The concentrations of the enzyme preparations and of 5,5′-dithio-bis-(2-nitrobenzoic acid) in the cuvette were 1 μM and 30 μM, respectively.

Interaction of modified α-chymotrypsin, containing activated SH-groups, with dithiols. The incubation of modified α-chymotrypsin preparations, con-

taining activated SH-groups, with dithiols was performed according to [11], with incubation times from 5 h to 15 days. 1 μM enzyme solution was supplemented with 1 vol.% of Me_2SO solutions of dithiol HS-$(CH_2)_n$-SH type or an equimolar mixture of dithiols, with n = 4, 5, 6, 8, 9, 10. The final dithiol concentration in the reaction mixture was $3 \cdot 10^{-3}$% vol. Before the spectrophotometric titration of SH-groups of the modified enzyme the enzyme preparations were dialyzed against 0.01 M Tris-HCl buffer in 0.3 M KCl and 5 mM EDTA (pH 8.0) in dialysis tubes (Union Carbide Corporation, U.S.A.) for 24 h at 4° C..

The scheme of the reaction [3] is:



Intermolecular cross-link formation under the conditions of the experiment did not occur when enzyme concentrations $1 \cdot 10^{-6}$ M were used. As was shown earlier for the use of diamines as cross-linking agents [2], after 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 indicates that the interaction of the protein with dithiol is a monomolecular process with respect to the former.

Determination of the catalytic activity of the native and modified enzyme. The catalytic activity of the native and modified enzyme was meausred in a TTT-1d pH-stat (Radiometer, Denmark) by the initial rates of enzymatic hydrolysis of 0.01 M *N*-acetyl-L-tyrosine ethyl ester in 0.1 M KCl at pH 7.0, 20° C (cell volume 5 ml).

Study of the denaturing action of guanidine · HCl on the native and modified α-chymotrypsin. The effect of guanidine · HCl on the native and modified α-chymotrypsin catalytic activity was studied in a pH-stat by varying guanidine · HCl concentration in the cell from 0.5 M to 7 M and measuring the catalytic activity of the enzyme preparation as described above. The concentrations of the native and modified α-chymotrypsin in the cell were 0.1 mM and 0.05 mM, respectively.

Results and Discussion

Denaturing effect of guanidine · HCl on α-chymotrypsin treated with N-acetyl-homocysteine thiolactone and dithiols

The treatment of α-chymotrypsin with *N*-acetyl-homocysteine thiolactone leads to the introduction into the protein molecule of an average of 10.3 SH-groups, as determined by spectrophotometric titration. The enzyme retains 91% of its initial catalytic activity, which corresponds to the results of [9] (Table I).

TABLE I

PROPERTIES OF PREPARATIONS OF CHYMOTRYPSIN, TREATED WITH DIFFERENT DITHIOLS OR MERCAPTOETHANOL

For the conditions see Experimental.

Preparation	Number of titrated SH-groups	Relative catalytic activity (%)
Native chymotrypsin		100
Chymotrypsin, treated with *N*-acetyl-homocysteine thiolactone	10.3	91
Premodified chymotrypsin, treated with *N*-acetyl-homocysteine thiolactone	16.4	12
Premodified chymotrypsin, treated with *N*-acetyl-homocysteine thiolactone and then with:		
Mercaptoethanol	0.2	14
1,4-Tetramethylenedithiol	16.2	23
1,5-Pentamethylenedithiol	14.7	53
1,8-Octamethylenedithiol	16.2	18

In aquous solutions of guanidine · HCl, α-chymotrypsin undergoes inactivation (Fig. 1, curve 1). When the enzyme activity decreases to 40% of the initial value, a decrease in guanidine · HCl concentration has no effect.

The same effect of inactivation under the influence of guanidine · HCl was found for modified α-chymotrypsin treated with dithiols. However in this case higher concentrations of guanidine · HCl are required to cause the same degree of inactivation compared to the native enzyme. Hence the modified enzyme possesses a higher stability.

The guanidine · HCl concentration corresponding to 50% of total decrease in the enzyme activity was used as a parameter characterizing the relative stability of individual preparations of the native and modified α-chymotrypsin.

For the dithiols-modified α-chymotrypsin the $[Gdn \cdot HCl]_{half}$ values are

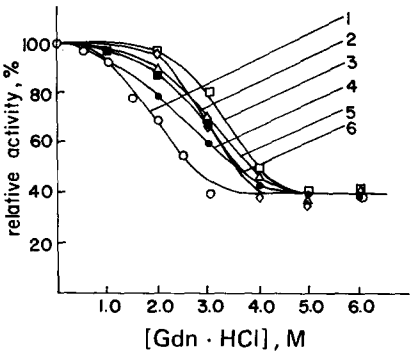


Fig. 1. Decrease in catalytic activity of α-chymotrypsin preparations upon treatment with guanidine · HCl. Conditions: pH 7.0, 20°C, $1 \cdot 10^{-2}$ M *N*-acetyl-L-tyrosine ethyl ester in 0.1 KCL, [Enzyme] = $5 \cdot 10^{-8}$ M. ○———○, native α-chymotrypsin (1); △———△, α-chymotrypsin treated with mercaptoethanol (2). □———□, 1,4-tetramethylenedithiol (3). ●———●, 1,5-pentamethylenedithiol (4). ◇———◇, 1,8-octamethylenedithiol (5). and ■———■, with the mixture of dithiols (6).

2.5—3.4 M, close to that of α-chymotrypsin modified with mercaptoethanol, (2.9 M) which is a one-point modifier (Fig. 1, curve 2). In both cases $[Gdn \cdot HCl]_{half}$ values of the modified enzyme are higher than those of the native enzyme (1.9 M).

One-point enzyme modification occurs even in the presence of bifunctional reagents. This is confirmed not only by the fact of similar α-chymotrypsin stabilization effects when the enzyme is modified by mercaptoethanol and dithiols, but also by the results of spectrophotometric titration of SH-groups in the modified enzyme preparations. 10.0—11.4 SH-groups are found, similar to the value when the enzyme is treated with *N*-acetyl-homocysteine thiolactone [3,10]. In other words, no intramolecular cross-linkage formation took place, otherwise the number of titrated SH-groups should have decreased.

Enzyme stabilization against the denaturing action of urea and guanidine · HCl after similar modification was reported earlier for ribonuclease [14].

In our opinion, the failure of the attempt to introduce intramolecular cross-linkages can be explained by the lack of sufficient reactive centres to be linked in the protein. The enriching of the enzyme molecule with reactive groups should increase the probability of intramolecular cross-linkage formation. For this purpose we performed the enzyme premodification with water-soluble carbodiimide and ethylenediamine, thus increasing the quantity of amino-groups in α-chymotrypsin molecule (as a result of carboxygroup modification [2]). Our preliminary studies have shown that the 'short' modifier ethylene-diamine forms no intermolecular cross-linkages. The increase in the number of aminogroups allows the number of SH-groups in the protein molecule to be increased.

As mentioned above, the idea of the additional introduction of reactive groups into the enzyme molecule was suggested and proved to be successful by Reiner [9], who used reactive SH-groups. The 'two-stage' enrichment of chymotrypsin with SH-groups, suggested by us, gives an opportunity to introduce a greater number of reactive groups into the protein.

Guanidine · HCl denaturing effect on premodified α-chymotrypsin preparations treated with N-acetyl-homocysteine thiolactone and dithiols

The treatment of diamine-premodified α-chymotrypsin with *N*-acetyl-homo-cysteine thiolactone leads to the introduction into the protein of 16.4 SH-groups (average), the enzyme retaining 12% of the initial catalytic activity (Table I).

The enzyme treated with 1,5-dithiol or with a mixture of different dithiols was stabilised by premodification ($[Gdn \cdot HCl]_{half}$ = 4.5 M; Fig. 2, curves 4 and 6). For dithiols with n = 4, 6, 8, 9, 10 the values of $[Gdn \cdot HCl]_{half}$ were the same as in the case of simple modification ($[Gdn \cdot HCl]_{half}$ = 2.5—3.4 M; Fig. 2, curves 2, 3 and 5, where the results for dithiols with n = 4 and 8 are given as typical examples). These data show that the enriching of the enzyme with SH-groups in itself does not cause the effect of stabilization. Together with dithiols, mercaptoethanol was used as a modifying agent; unlike dithiols this does not form any cross-links. Fig. 2 shows that the enzyme treatment with mercaptoethanol, as well as with two dithiols (curves 2, 3 and 5), produces only a slight stabilizing effect, the latter not depending on the length

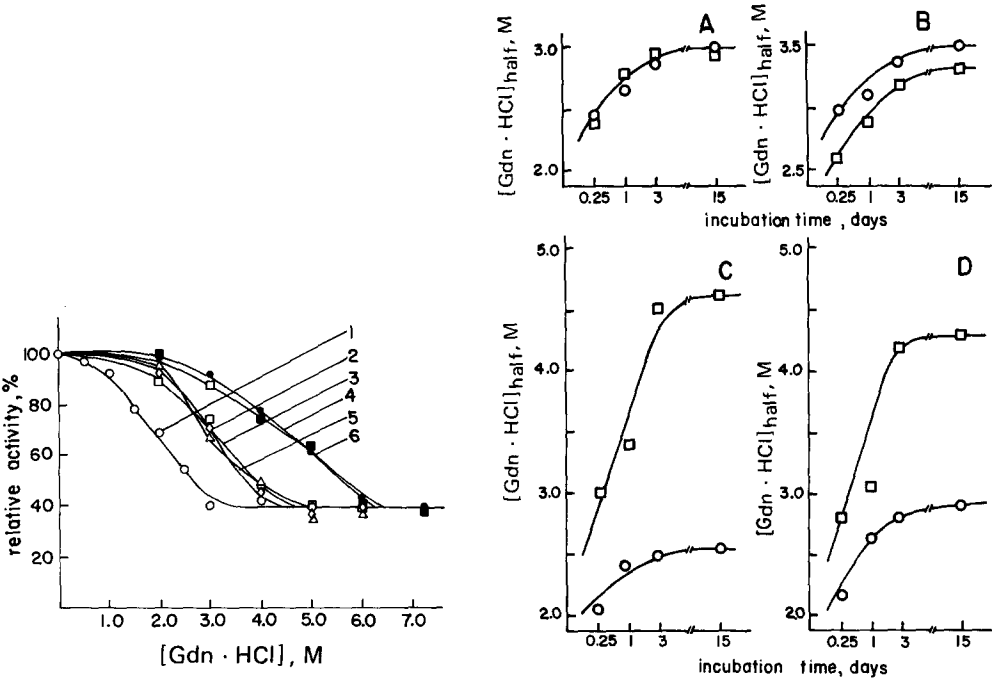


Fig. 2. Decrease in catalytic activity of native an modified α-chymotrypsin upon treatment with guanidine · HCl (experimental conditions and notation as in the legend to Fig. 1).

Fig. 3. Depedence of the parameter $[Gdn \cdot HCl]_{half}$ (see text) on incubation time. α-Chymotrypsin was treated with mercaptoethanol (A), 1,4-tetramethylenedithiol (B), 1,5-pentamethylenedithiol (C), and with the mixture of different dithiols (D). (For experimental conditions see the legend to (Fig. 1). ○, α-chymotrypsin with non-modified carboxy groups; □, α-chymotrypsin with carboxy groups premodified with diamine.

of the dithiol, whereas in the case of 1,5-dithiol, the picture is quite different (curve 4).

The stabilization effect observed in the case of chymotrypsin cross-linking by 1,5-dithiol (curve 4) is not conditioned by a one-point modification, since a thermodynamically more state two-point cross-link is chosen from a mixture of dithiols (curve 6). We conclude that the effects of stabilization with 1,5-dithiol (curve 4) and a mixture of dithiols (curve 6) are similar (compare Fig. 3C and 3D). Otherwise, stabilization by a mixture of dithiols should have had the same effect as stabilization by other dithiols, for the relative amount of 1,5-dithiol in the mixture is small.

Evidently, in the case of 1,5-dithiol intramolecular cross-linking occurs. This assumption is supported by spectrophotometric titration of SH-groups, showing that in preparations with no stabilization effect the number of titrated SH-groups stays unchanged (16.4) after the treatment with dithiols (treatment with mercaptoethanol leads to the disappearance of 16.2 titrated SH-groups, i.e. one-point modification occurs), whereas for preparations with pronounced stabilization by 1,5-dithiol or a mixture of dithiols the number of titrated SH-groups decreases to 14.7 (i.e. two-point linking occurs). The mechanism of the intramolecular cross-link formation in this case is similar to that of the

enzyme stabilization after treatment with diamines of different length [2].

But is it possible to speak in this case of enzyme selfstabilization?

The time dependence of α-chymotrypsin stabilization effects

The effect of enzyme stabilization against denaturing by guanidine · HCl gradually increases with time, showing that the process of -S-S group modification is a slow one (see Fig. 3). For most enzyme preparations the increase in $[Gdn \cdot HCl]_{half}$ values after several days is 0.5—0.7 M (see Fig. 3A and B); for the preparations with intramolecular cross-links these values increase to as high as 1.5—1.6 M, i.e. two to three times higher (Fig. 3C and D). This is of little interest in the case of mofidication by 1,5-dithiol alone. One can presume, that the first step of the process is a fast one-point modification of the enzyme with low $[Gdn \cdot HCl]_{half}$ value, and the second step is a slow transition to the two-point linking in some regions of the protein globule. This phenomenon in itself is far from being selfstabilization.

Quite another situation occurs when an equimolecular mixture of dithiols is used for the modification. The occurrence of the same effect overall as for 1,5-dithiol alone is the only explanation (taking into consideration the fact that different dithiols react identically as one-point modifiers). The first step of the process is the random modification of α-chymotrypsin by different dithiols. Then, step by step, a more advantageous situation is realized, the cross-linking with 1,5-dithiol becomes dominating *.

In other words, as the time passes, the enzyme 'selects' the cross-link of the appropriate length and selfstabilization occurs.

Changes in α-chymotrypsin catalytic activity during modification

The effect of intramolecular thiol-disulfide interchange on the enzyme catalytic activity was previously shown [7,15]. Thiol-disulfide bond redistribution is also known [16,17].

In our experiments the catalytic activity of modified α-chymotrypsin preparations without premodification with diamine was very low (Fig. 4, curve 1). After the enzyme premodification the activity increased and the dependence of the retained catalytic activity on the number of methylene groups [12] in the dithiol $HS\text{-}(CH_2)_n\text{-}SH$ molecule is maximal at $n = 5$. Hence intramolecular cross-linkage formation in case of premodified enzyme and 1,5-dithiol somehow restores the active conformation of the α-chymotrypsin globule showing the reversibility of inactivation during the enzyme premodification, modification and cross-linking.

As a result, the enzyme preparation of maximal stability acquires the maximal preservation of the enzyme activity.

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* This result can also be considered as proof of the fact that the stabilization effect is connected not with one-point modification, but with intramolecular cross-linking. Otherwise, (if there was some specific modifying action of 1,5-dithiol) the effect of stabilization for a mixture of dithiols should have been much smaller, than for pure 1,5-dithiol, due to the lower content of the compound in the mixture.

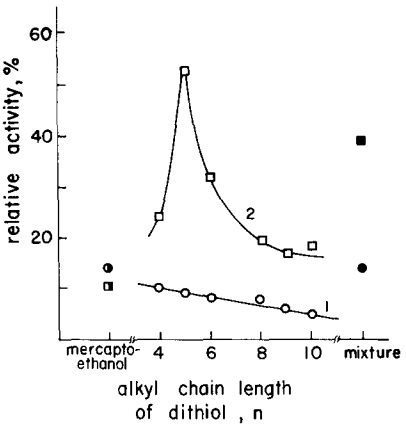

Fig. 4. The dependence of the retention of α-chymotrypsin catalytic activity on the chain length of dithiols. ○ and □, as in the legend to Fig. 3.

Concluding remarks

1. The relatively moderate effects of stabilization in our experiments can only be explained by formation of one intramolecular cross-link in the system under investigation. At the same time the effect in itself supports our new approach. The use of dithiols for reversible modification is most useful for enzymes consisting of several subunits and containing a large quantity of SH-groups. Other potentially reversible cross-linking agents may also be used for enzyme stabilization. The search for such agents is a matter of future.

2. The results obtained in the present study, together with the results of our preliminary studies [2] show that enzyme stabilization by means of intramolecular cross-linking or appropriate modification makes the enzyme more stable against a wide spectrum of denaturing actions: temperature [2], salt action [18] and denaturing agents.

Acknowledgements

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