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THE PRINCIPLES OF ENZYME STABILIZATION *

IV. MODIFICATION OF 'KEY' FUNCTIONAL GROUPS IN THE TERTIARY STRUCTURE OF PROTEINS

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Summary

The dependence of α-chymotrypsin thermostability and catalytic activity on the degree of its amino groups modification has been studied. Modification was carried out by both alkylation (using acrolein with further reduction of Schiff bases by sodium borohydride) and acylation (with siccinic or acetic anhydrides). It has been determined that modification of the majority of titrated amino groups (approximately 80%) only has a slight effect on the first-order rate-constant characterizing the monomolecular process of enzyme thermoinactivation (50°C, pH 8). Thermostability sharply increases (by 120 times) only for a degree of modification higher than 80%, but, nevertheless, the complete substitution of all the titrated amino groups again leads to enzyme destabilization. The conclusion has been drawn that there is only one or two amino groups out out approximately fifteen titrated ones, the modification of which plays a key role in the lateration by the enzyme of its thermostability.

The degree of the stabilization effect has been studied relative to both the nature and concentration of the salt added Na_2SO_4, NaCl, KCl, CCl_3COOK, $(CH_3)_4NBr$. Ultraviolet absorption (280 nm) of chymotrypsin has also been elucidated with respect to the degree of alkylation of its NH_2-groups. The data obtained allowed the conclusion to be drawn that enzyme modification leads to a decrease in the non-electrostatic (hydrophobic) interactions on the

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surface layer of the globule. As a result, a protein conformation more stable in respect to denaturation (unfolding), is formed.

Introduction

Only the first steps in the formation of the physicochemical principles of enzyme stabilization have been made [1—4]. The chemical modification of the enzyme using low molecular weight ligands seems to be the simplest method of allowing its stability to be changed. The method of chemical modification was successfully used when studying the interrelation between the structure and the function of the proteins [5]. It is of great importance that even the lowest degree of protein modification very often leads to a considerable alteration in their properties, see for example ref. 6. But, in any case it is difficult to predict how the chemical modification of the enzyme will alter its thermostability. This is connected with the extremely complex molecular structure of the biocatalysts (which is often still unknown for some enzymes). But, nevertheless, the results of the present investigation and an analysis of the literary data may serve as the basis for the general principle: when modified, the functional groups of the same type in the protein globule do, as a rule, exhibit high heterogenity, i.e. there are several groups, the modification of which has a great effect on the biocatalyst properties studied as well as the functional groups, the modification of which has a slight effect (or none at all) on the enzyme thermostability. Let us call such functional groups 'key' groups.

By way of example, we have studied the thermostability of α-chymotrypsin with respect to the degree of modification of its NH_2-groups (by their alkylation and acylation), see preliminary communication [7].

Experimental

Materials

Crystalline α-chymotrypsin, *N*-acetyl-L-tyrosine ethyl ester, sodium borohydride and succinic anhydride were commercial preparations of Koch-Light Laboratories Ltd (U.K.). Acrolein was obtained from BDH (U.K.), 1-ethyl-3-(3-dimethylaminopropyl)-carbodimide was purchased from Sigma (U.S.A.) and 1-aminopropanol-3 and acetic anhydride were the products of Reakhim (U.S.S.R.). Inorganic salts and components of buffer solutions were analytical grade preparations.

Alkylation of α-chymotrypsin by acrolein

Modification was carried out in accordance with two methods: (A) for constant incubation time varying the acrolein concentration or (B) at constant modificator concentration varying incubation time.

(A) 1 ml of $5 \cdot 10^{-4}$ M aqueous chymotrypsin solution and 0.1 ml of freshly prepared aqueous acrolein were added to 8.9 ml of 0.02 M borate (pH 8.5), so that the final concentration of the modifier in the reaction mixture was (M) $4.5 \cdot 10^{-1}$, $1.8 \cdot 10^{-1}$, $1.4 \cdot 10^{-1}$, $4.5 \cdot 10^{-2}$, $1.4 \cdot 10^{-2}$, $4.5 \cdot 10^{-4}$ or $4.5 \cdot 10^{-5}$, respectively. The mixture was stirred for 2 h at 5°C.

(B) 1 ml of $5 \cdot 10^{-4}$ M aqueous chymotrypsin solution and 0.1 ml of freshly prepared aqueous acrolein were added to 8.9 ml of 0.02 M borate buffer (pH 8.5), so that the final concentration of the modifier in the incubation mixture was $4.5 \cdot 10^{-3}$ M or 4.5 M respectively, the incubation time (at 5°C) varied from 30 min to 27 h.

The product of protein condensation with acrolein, obtained in accordance with method (A) or (B), was reduced according to ref. 8. For this purpose 20 mg of sodium borohydride were added to the solution of modified α-chymotrypsin and the mixture was stirred in an ice bath (5°C) for 1 h. Then the mixture was acidified (0.1 M HCl) to pH 3.0 to remove the excess sodium borohydride. Then the pH value was restored with 0.1 M KOH to pH 6—7 and the mixture was stored in a refrigerator.

Succinilation of α-chymotrypsin

The enzyme modification by succinic anhydride was performed according to the method given in ref. 9. 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 for 3 h in an ice bath. To obtain enzyme preparations with a lower substitution degree, 150, 30, 15 or 1.5 mg of acylating agent were used. Modification was carried out at a constant pH maintained with the help of 0.1 M KOH. Then the solution was passed through a column (2.5 × 60 cm) with Sephadex G-50 (Pharmacia, Sweden) in 0.01 M KCl at an elution rate of 1.5 ml/min.

Modification of the succinylated α-chymotrypsin by acrolein

Partly succinylated α-chymotrypsin preparations were modified additionally (modification of NH_2-groups remained free) by acrolein in line with the following method: 1 ml of $5 \cdot 10^{-5}$ M enzyme solution was introduced into 8.9 ml of 0.02 M borate buffer (pH 8.5) and 0.1 ml of 2.5% aqueous acrolein solution was added. The mixture was stirred for 45 min at 5°C, then the reduction by sodium borohydride was carried out as shown above. The value of catalytic activity retained by the enzyme is 15% to that of unmodified α-chymotrypsin.

Acetylation of α-chymotrypsin

The enzyme acylation by acetic anhydride was carried out according to method [9] slightly changed by us. The procedure was the following: 50 mg of α-chymotrypsin were solved in 40 ml of half saturated sodium acetate solution (the final enzyme concentration is $5 \cdot 10^{-5}$ M) and while mixing, 1.9 ml of freshly distilled acetic anhydride were added dropwise for 1 h at 4°C and pH 7.8 (using 0.1 M KOH for retaining constant pH value). Then the modification product was dialysed against distilled water in dialysis tubes (Union Carbide Corporation, U.S.A.) for 24 h at 5°C. The value of catalytic activity retained was approximately 5% of the initial value.

Determination of the modification degree of protein NH_2-groups

As a result of the modification of protein by carboxylic anhydrides [5] or aldehydes [10], first of all, the substitution of the NH_2-groups occurs. The degree of substitution can be controlled by titrating free NH_2-groups by trini-

trobenzensulfonic acid (TNBS) [11]. For this purpose 2 ml of 0.1 M borax solution (pH 9.2), 1 ml of $5 \cdot 10^{-6}$ M solution of modified α-chymotrypsin and 0.06 ml of 1 M TNBS aqueous solution were placed in a cuvette. Having mixed it for 30 min at room temperature we made a record (420 nm) of the kinetic curve on a Perkin-Elmer-402 spectrophotometer (the same substances, except the enzyme preparation, were placed in a reference cuvette). The degree of substitution of α-chymotrypsin NH_2-groups was determined in relative units: absorbance, elucidated for non-modified enzyme, corresponds to 100% of free amino groups, which can interact with TNBS. In experimental conditions the absorbance at 100% was taken as $A_{420} = 0.95$. The absorbance of α-chymotrypsin treated with $4.5 \cdot 10^{-2}$ M of acrolein is 0.12, which corresponds to about 13% of the free amino-groups.

Measurements of enzyme activity

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

Thermoinactivation experiments

The enzyme ($5 \cdot 10^{-8}$—$5 \cdot 10^{-6}$ M) in $5 \cdot 10^{-3}$ M Tris-HCl (pH 8.0) and 0.1 M KCl (other values of the salt concentration or its nature will be considered separately) were incubated at 50°C. Aliquots were taken at certain intervals and the residual catalytic activity was determined as described above. Preliminary it was specially shown that thermoinactivation is a monomolecular process and does not involve either autolysis or protein aggregation. Two facts support this suggestion. The first is that the nature of the 'relative enzyme activity versus time' profiles does not depend on the concentration of the native ($5 \cdot 10^{-8}$—10^{-6} M) and modified ($5 \cdot 10^{-8}$—$5 \cdot 10^{-6}$ M) α-chymotrypsin. Secondly, the values of the thermoinactivation rate constants observed in the above mentioned concentration interval are practically identical in the free enzyme and in the enzyme covalently bound with Sephadex G-50 [29] via 1 or 2 bonds.

The apparent first-order rate-constant measured at 70% degree of conversion, see refs 1—3, was taken for the parameter characterizing enzyme stability.

Results and Discussion

The dependence of the enzyme thermostability on the degree of NH_2-groups modification

Fig. 1 indicates that both the alkylation and acylation (succinylation) of the NH_2-group first involved in modification, slightly influence α-chymotrypsin thermostability. Modification of less reactive (evidently, resistant to modification, i.e. located inside the globule) functional groups of protein plays a decisive role. So, enzyme thermostability at the final stage of its modification (when less than 20% of the NH_2-groups are left non-substituted) increases sharply (10—100 fold), but then the sharp decrease in thermostability is observed as a result of almost complete disappearance of the free (titrated) amino groups.

Thus the change in α-chymotrypsin thermostability under modification of its NH_2-groups mainly depends on the degree of their substitution. The following facts prove it:

(i) The enzyme is not 'sensitive' to the nature of modifier. It follows from this fact that approximately the same effect on thermostabilization is observed at the same degree of protein substitution for both alkylation or succinylation (see Fig. 1) and acetylation. In the latter case, we observe more than an 80-fold increase in the enzyme thermostability, when about 10% of the amino groups in its molecule are still free.

(ii) Moreover, the thermostability of α-chymotrypsin does not depend on whether the given degree of modification (corresponding, for example, to points III, III′ and III″ on Fig. 1) is gained by using one modifier (succinic anhydride, point III) or NH_2-groups modification was carried out by succinic anhydride only in part (prior to the degree of modification corresponding to points I or II) and then the enzyme was treated by acrolein. Proceeding from Fig. 1, all the enzyme preparations of the same modification degree, gained by various methods, possess approximately equal thermostability.

(iii) Finally, the thermostability effect is independent of the kinetics of the modification reaction. The example of α-chymotrypsin alkylation has proved it: either we perform short time enzyme incubation in the solution of acrolein high concentration (see Method A in Experimental) or long time enzyme incu-

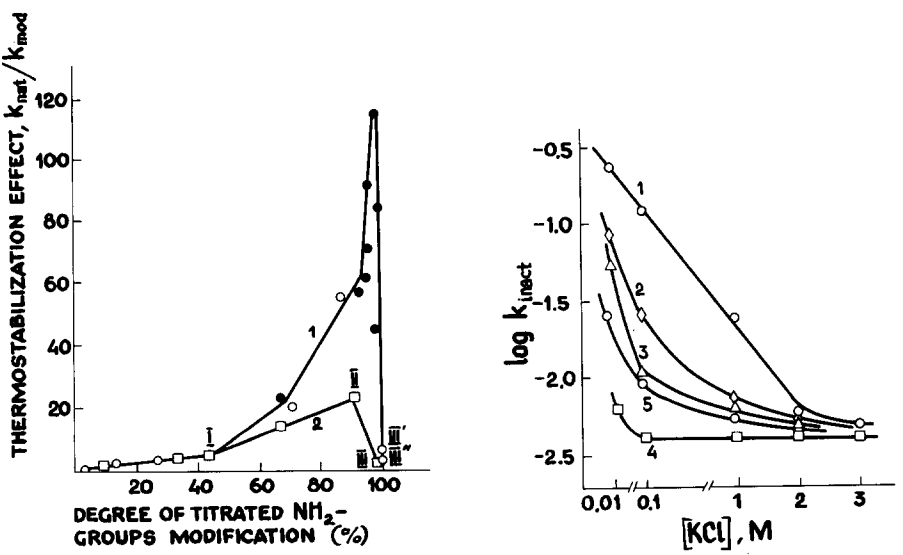

Fig. 1. The dependence of α-chymotrypsin thermostabilization (as a ratio of first-order rate constants for thermoinactivation of native, k_{nat}, and modified, k_{mod}, enzyme) on degree of substitution of amino groups by alkylation (curve 1) and succinylation (curve 2). Points I, II, III, III′ and III″ see in the text. Modification by acrolein (curve 1) was carried out according to method (A) (○———○) or (B) at acrolein concentration of $4.5 \cdot 10^{-3}$ M (●———●). Thermoinactivation conditions: 50°C, pH 8.0 ($5 \cdot 10^{-3}$ Tris-HCl); 0.01 M KCl, enzyme concentration $5 \cdot 10^{-7}$ M.

Fig. 2. The dependence on the ionic strength of the solution, observed for the first-order rate-constant (min^{-1}), characterizing thermoinactivation of native α-chymotrypsin (curve 1) and alkylated enzyme (according to method A) with various substitution degree of titrated NH_2-groups: curve 2 66%, curve 3 71%, curve 4 87%, curve 5 100%. For thermoinactivation conditions see Fig. 1.

bation at the low concentration of the modificator (Method B) the value of the stabilization effect in both cases is modified only by the degree of substitution of NH_2-groups gained (compare white and black points on curve 1, Fig. 1).

Effect of ionic strength on the thermostability of the native and modified α-chymotrypsin

Native (non-modified) α-chymotrypsin becomes more stable with the increase in the ionic strength of the solution (concentration of KCl), i.e. the rate constant of thermoinactivation is decreased (curve 1 in Fig. 2), see ref. 12 also. But the dependence of k_{inact} on the ionic strength becomes less sensitive to the alterations in the salt concentration, while the surface layer of the chymotrypsin globule is being alkylated (i.e. as the enzyme becomes more stable as a result of modification, cf. curves 1—5, Fig. 2) and, finally, in the case of a more stable enzyme preparation (according to the data in Fig. 1) k_{inact} does not depend on the ionic strength (curve 4, Fig. 2). In other words, the differing effects on the protein globule (precisely, its chemical modification or the increase in the ionic strength of the solution) result in a higher thermostability of the enzyme and eventually lead to the same value of the rate constant of thermoinactivation (Fig. 2).

It is known [13—15] that at least two conformational states are typical of the α-chymotrypsin molecule, their equilibrium depending on the ionic strength of the solution. Thus, on the basis of the data given in Fig. 2, we may suppose that the increase in the ionic strength of the solution as well as the chemical modification of the enzyme shifts the equlibrium of the same process, to be more precise, the equilibrium between two conformational states of the protein molecule differing in their thermostability. In other words, it is believed that either the influence on the enzyme results in the structural change in the protein globule of the same type, providing its higher conformational stability (thermostability).

Conformational transition in the α-chymotrypsin molecule induced by the increase in ionic strength

It has previously been determined that α-chymotrypsin conformers, dependent on salt concentration, not only differ in catalytic activity [13] or the binding capacity of the active center [14], but in a number of spectrum and other physical properties of the protein globule [15]. In our work data were obtained which allowed us to judge of the nature of this conformational transition:

(i) The modification of 15 from 17 (see Experimental) of the carboxy groups of α-chymotrypsin by 1-aminopropanol-3 (with the formation of amide groups, $-C(O)NH(CH_2)_3OH$) though changes the enzymes thermostability slightly (value of k_{inact}), but has no effect on the thermostability dependence on the ionic strength, see Fig. 3. As this modification should considerably decrease the globule negative charge it may be concluded that the electrostatic effects involved the protein carboxyl groups do not play a significant part in the conformational transition induced by salt.

(ii) Succinylation of all the titrated NH_2-groups of α-chymotrypsin has no effect on its thermostability. Moreover, the completely succinylated enzyme is

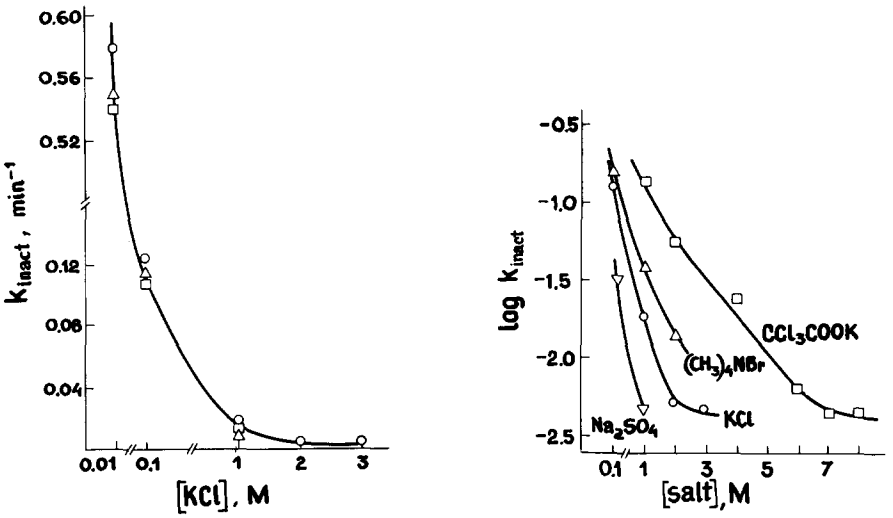

Fig. 3. The dependence on the ionic strength of the solution, observed for the first-order rate-constant, characterizing α-chymotrypsin thermoinactivation: native enzyme (○———○), completely succinylated (△———△) and the enzyme with carboxy groups substituted by amidation with the help of 1-amino-propanol-3 (□———□). For thermoinactivation conditions see Fig. 1.

Fig. 4. The dependence of the first-order rate-constant (min^{-1}), characterizing thermoinactivation of native α-chymotrypsin, on salt concentration. For the conditions of thermoinactivation see Fig. 1.

characterized approximately by the same thermostability dependence on the ionic strength as the native (non-modified) enzyme (cf. the data on Fig. 3). As succinylation considerably decreases the globule's positive charge, it may be thought that the electrostatic effects involved in the protein amino groups do not play an important part in the alteration of α-chymotrypsin thermostability induced by salt.

The results obtained (Fig. 3) agree with the assumption now accepted [16], that the salt interaction with the charged groups does, as a rule, play an insignificant role in denaturation (conformational-dependent) processes in the proteins at neutral pH values. It can be explained by the fact that the charged groups located on the surface of the protein globule interact with the solvent both in the 'folded' and 'unfolded' states of the polypeptide chain.

Hence, the salt-induced change in the activity coefficients of the globule's non-charged sites, and, above all, in either the hydrocarbon side groups of the amino acid residues or the fragments of the polypeptide chain itself is the driving force of conformational transition under investigation. Let us examine this question in detail.

(iii) Fig. 4 indicates that the α-chymotrypsin thermostability is highly sensitive to the nature of the salt added. Precisely, Na_2SO_4 has the greatest stabilizing effect, the NaCl and KCl effect is weaker, while that of CCl_3COOK is the weakest. The observed ratio of effectiveness of the salts is similar to that of many denaturation processes in proteins [16,17]. The results, represented in Fig. 4, allow us to think that the salts added change (according to Hofmeister's lyothropic series) the activity coefficients of the enzyme molecule's non-charged fragments. Benzyl radicals of phenylalanine residues situated in α-chy-

motrypsin on the globule surface [18] could appear, for example, as non-charged fragments. But this disagrees with the nature (intensity) of the salt effect observed in the case of $(CH_3)_4NBr$ (Fig. 4). Proceeding from the salt effect on activity coefficient of benzene [19] this salt should produce a minimum 'salting out' effect or could even lead to 'salting in'. Thus, if the observed salt effects (Fig. 4) are supposed to be explained by alteration of the activity coefficients of aromatic protein fragments, then $(CH_3)_4NBr$ should have a weaker stabilizing effect compared to CCl_3COOK (or could lead even to enzyme destabilization). But Fig. 4 indicates that $(CH_3)_4NBr$ has the same effect on α-chymotrypsin as KCl does.

This result agrees with the data [20,21] on the effect of concentrated salt solution on activity coefficients of acetyltetraglycine ethyl ester Ac-Gly_4-OEt). As Ac-Gly_4-OEt can be viewed [16] as fragment model of the polypeptide chain then it should be concluded that the phenomenon we observed (enzyme stabilization in concentrated salt solutions, Fig. 4) is based on salting out of the polypeptide chain itself. In other words, it may be supposed that, as a result of changes in the activity coefficient of the amide fragments, a protein globule tends to gain (by conformational changes) another degree of contact with the solvent. Loss of contact with the aqueous solution by some polypeptide chain fragments may result in strengthening the hydrophobic interactions in the globule's surface layer. Such a 'salted out' conformation (possibly, the conformation shrinks) of protein naturally would be hardly revealed unfolded while heating, and hence the higher thermostability will be observed.

Mechanism of α-chymotrypsin thermostabilization observed as a result of its chemical modification

Enzyme modification leads both to its thermostabilization and to changes in its spectral properties (280 nm) as Fig. 5 indicates. It is important to stress that the change in the absorption of enzyme solution occurs at a high substitution degree of protein NH_2-groups when the increase in enzyme thermostability is observed (compare Figs. 1 and 5).

It is a fact that the spectral effects in the region of 280 nm are explained by the change in the micro-environment of the aromatic chromophores in the protein molecule [22,23]. Principally, we can explain the effect observed (Fig. 5) by the fact that the modifier molecule in the globule alters the micro-environment of the surface layer. But it is highly unlikely for several reasons. The first, considerable change in the enzyme absorption results from alkylation of only 2—3 out of 15 titrated amino groups [1,3]. The second, even more important, spectral effect, observed as a result of modification, alters the sign as the degree of NH_2-groups substitution is increased: at first, the decrease occurs and then the sharp increase in absorption at 280 nm takes place. It is based, evidently, on dramatic conformational changes in the protein molecule.

What are the driving forces of such conformational transition? As it was shown above, the increase in α-chymotrypsin thermostability, observed as a result of its NH_2-groups modification, only slightly depends on the nature of the modifier. On being alkylated by acrolein, protein amino groups evidently retain a positive charge $E\text{-}NH_3^+ \rightarrow E\text{-}NH_2^+\text{-}CH(OH)CH{=}CH_2$, while as a result of acetylating ($ENH_3^+ \rightarrow E\text{-}NH\text{-}C(O)CH_3$) or succinylating ($E\text{-}NH_3^+ \rightarrow E\text{-}NH\text{-}C(O)$-

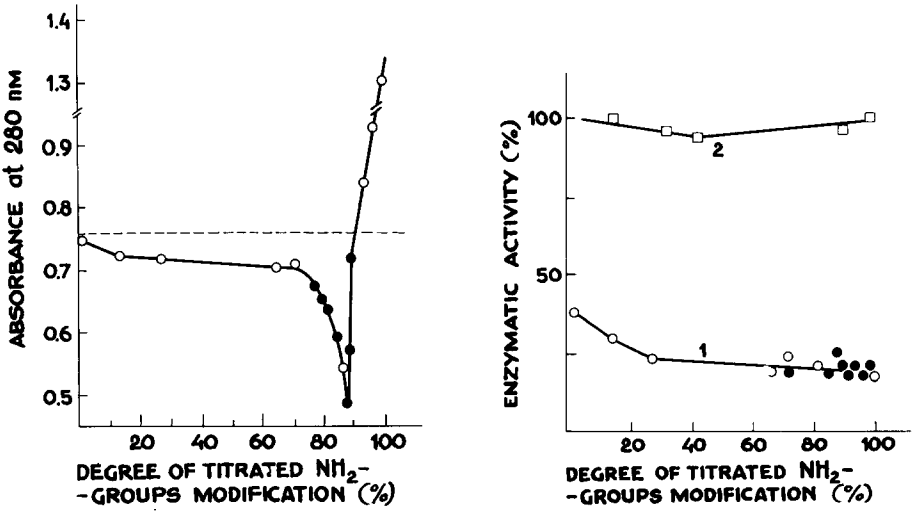

Fig. 5. The dependence of α-chymotrypsin ($1.7 \cdot 10^{-5}$ M) absorption (280 nm) on the degree of NH_2-groups substitution by alkylation according to method A (○———○) or B at acrolein concentration of $4.5 \cdot 10^{-3}$ M (●———●). Conditions: 25°C, pH 6.9 ($5 \cdot 10^{-3}$ M Tris-HCl). The absorption of the non-modified enzyme solution is marked by the dotted line.

Fig. 6. The alteration of α-chymotrypsin catalytic activity as a result of its NH_2-groups modification by alkylation according to method A (○———○) and B (●———●) (curve 1) and succinylation (curve 2).

$(CH_2)_2COO^-$) the positive charge disappears and in the latter case it is substituted for a negative charge. It appears as the grounds for the conclusion that the change in thermostability, resulted from the enzyme modification, does not depend on the alteration (decrease) of positive charge of globule. Hence, electrostatic effects hardly play an important role in the mechanism of thermostabilization. This agrees with the accepted view [16] that charged groups, as a rule, play an insignificant role in the process of protein denaturation at neutral pH values (see the discussion of this problem above).

We may think that the formation of a more thermostable enzyme state takes place owing to the increase in hydrophobic interactions when involving modifier residues in the globule. Such an explanation was given earlier, when investigating the influence of modification on the thermostability of glycogen phosphorylase [24] and albumin [25]. This mechanism appears to be possible for one more reason: free energy of hydrophobic interactions on the surface layer of the globule plays an important part in the conformation stability of proteins [26].

The effect of modification on the catalytic activity of the enzyme

Despite the dramatic effects of thermostabilization the modification has hardly any effect on the catalytic activity of α-chymotrypsin (Fig. 6). The small (approximately two-fold) decrease in the enzyme activity, observed under alkylation (curve 1), is explained by the influence of the reducing agent (sodium borohydride) on the enzyme (even for non-modified enzyme). Hence, chemical modification even indirectly does not influence the active center. This means that α-chymotrypsin becomes more stable on 'strengthening' (by modification) of above all, peripheral sites of protein globule.

Concluding remarks

(i) There are two important factors in using stabilized enzyme preparations in practice. The first, the effect of thermostabilization, observed as a result of the modification (namely the increase of enzyme 'life time' by dozens of times, see Fig. 1), is sufficiently large to solve the applied tasks. The second, enzyme catalytic activity changes only slightly, see Fig. 6.

(ii) The 'key' functional groups of various enzymes, the modification of which entails thermostabilization, may differ in the degree of sensitivity to the modifier. So, glycogen phosphorylase is stabilized at a low degree of stabilization [24], while ribonuclease thermostability is changed only at the highest degree of substitution [27] after 'loosing' the protein structure by the preliminary modification possibly had taken place [28], and thus, the key groups became sensitive to the modifier.

All these data show that the modification method can be of great importance in obtaining stabilized enzyme preparations to study the dependence of the parameter under examination (thermostability and so on) on a substitution in detail for each enzyme. Moreover, the results of the present study indicate that the thermostabilization effect is only observed in rather limited interval values of a substitution degree of protein groups of a given type. So, it may appear difficult to determine the optimal ratio of the free and modified functional groups of the enzyme (particularly, if there are dozens in a protein molecule).

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