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General Characteristics of the Changes in the Thermal Stability of Proteins and Enzymes After the Chemical Modification of Their Functional Groups

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The principal structural and physicochemical factors determining the stability of protein macromolecules in solution and the characteristics of the structure of the proteins from thermophilic microorganisms are examined. The mechanism of the changes in the thermal stability of proteins and enzymes after the chemical modification of their functional side groups and the experimental data concerning the influence of chemical modification on the thermal stability of proteins are analysed. The dependence of the stabilisation effect and of the changes in the structure of protein macromolecules on the degree of modification and on the nature of the modified groups and the groups introduced into proteins in the course of modification (their charge and hydrophobic properties) is demonstrated.
The great practical value of the method of chemical modification for the preparation of stabilised forms of biocatalysts is shown in relation to specific examples.
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I. INTRODUCTION

The problem of the stability of globular proteins (and enzymes), i.e. the problem of the determination of the relation between the structure and stability of biomacromolecules, has been and remains one of the most important and complex problems of the modern physical chemistry of biopolymers which have still not been solved. The problem of increasing the stability of enzymes in relation to various denaturing influences is closely related to the first problem as regards practical needs, because, without knowing the fundamental structural factors determining the stability of the protein globule, it is impossible to alter it in a specific manner. The importance and the necessity for the solution of these problems arose as a consequence of the development of modern biotechnology, which involves the carrying out of many enzymic processes under extreme conditions (in particular, at elevated temperatures).

Numerous data show that there is apparently no single general principle governing the formation of a stable protein conformation and hence there is no single universal method for the stabilisation of proteins. The correlations established in recent years between the primary structure and stability of biomacromolecules are valid only for a limited number of proteins within a specific class.[1] Comparison of the primary, secondary, and tertiary structures of enzymes isolated from thermophilic sources and distinguished by a higher stability with their labile analogues reveals a wide variety of structural characteristics of thermophilic proteins and enzymes responsible for their increased stability as well as a few common features. Nevertheless the knowledge of the main structural factors determining the stability of the native conformation of the protein in solution makes it possible to formulate the fundamental principles of its stabilisation.

One of the most widely used methods for the alteration of the properties (in particular, their stability) of proteins and enzymes in solution is the chemical modification of their functional groups not involved in the active centre. The chemical modification is frequently the first stage of the procedure for obtaining stabilised insoluble enzyme preparations. In many cases the change in the stability of the enzyme as a result of covalent immobilisation is caused not only and not so much by its binding (covalent and non-covalent) to the carrier as by the chemical modification of its functional groups. Chemically modified stabilised soluble enzymes have a number of advantages over insoluble enzymes, in particular, they exhibit a high catalytic activity and have a weaker antigenic activity, which is important for medical applications.

The stabilisation of enzymes in nature is achieved as a result of mutations, i.e. the replacement of a small number of some aminoacid residues by "suitable" other aminoacid residues both within and on the surface of the protein globule. Such stabilisation is apparently a consequence of "natural selection" occurring as a result of changes in the conditions of the existence of the given class of microorganisms (for example, following an increase of temperature), whereupon among the multiplicity of isoenzymes modified by mutations, only those which exhibit a sufficiently stable conformation under extreme conditions "survive". On the other hand, the artificial stabilisation of enzymes by chemical modification can be achieved only by modifying the side residues accessible to the modifying agents under the given conditions, whose number in each protein is not quite so large. The set of chemical modification methods suitable for proteins is so far small compared with the scope of natural mutations.

In the reviews[2-5] devoted to the stabilisation of proteins and enzymes in solution which have been published in recent years, the influence of chemical modification on their stability is considered exclusively in phenomenological terms. Some workers assume that it is impossible to predict the effects of the modification.[5] The literature data on this question are not numerous, are incomplete, and are characterised by differences between the criteria used to define the stability of proteins, which hinders their comparison. In the present review the literature and the authors own results concerning the influence of chemical modification on the stability of proteins and enzymes is analysed, which made it possible to formulate certain general rules and the mechanism governing the variation of the stability of biomacromolecules as a result

of the chemical modification of their functional groups. The knowledge of these rules can help investigators to achieve success in the formation of enzyme preparations with a specified stability, having correctly selected the method of modification. From the theoretical standpoint, this is one of the important approaches to the elucidation of the interrelation between the structure and stability of biomacromolecules.

The analysis of studies of the influence of chemical modification on the stability of proteins and enzymes is preceded by the consideration of the main factors which determine the stability of the macromolecules in solution and the possible interrelation between the stability and structural parameters of macromolecules.[6,7]

II. THE STABILITY OF BIOMACROMOLECULES AS A FUNCTION OF THEIR STRUCTURE

1. The Principal Structural and Physicochemical Factors Determining the Stability of Protein Macromolecules in Solution

The native conformation of a protein having the minimum Gibbs free energy G_n under the given physical conditions consists of a three-dimensional structure, determined by the aminoacid sequence and the given microenvironment,[8] which exhibits the flexibility and internal dynamics essential for the manifestation of the functional activity of the protein.[9] Analysis of the three-dimensional structutes of a set of globular proteins have shown that all the aminoacid residues can be divided into three types in terms of their location in the globule: (1) hydrophobic, located preferentially within the molecule (Cys, Ile, Leu, Met, Val, Trp, and Phe); (2) hydrophilic, located preferentially in the surface layer (Asp, Glu, Lys, Asn, Gln, Pro, Arg, Ser, Thr); (3) neutral and ambivalent, indifferent as regards location on the surface or within the inner region of the molecule (Ala, His, Gly, Tyr).[10,11] A tendency towards the preferential cooperation of some aminoacid residues with others is characteristic of proteins,[12,13] which leads to the formation of structural domains in the macromolecule: clusters of hydrophobic or hydrophilic aminoacids.[14,15] Thus a high degree of mutual cooperation has been discovered between the following pairs of residues: Glu–Lys, Met–Arg, Asn–Trp, Gln–Pro.[12]

The transition of the biopolymer, without rupture of covalent bonds, from the native state (N) in which it exhibits its functional activity *in vivo* or *in vitro*, to the denatured (D) state, in which the activity has been lost either reversibly or irreversibly is the denaturation transition.[16,17] The principal feature of a cooperative system is the interaction between individual units whose energy is greater than the energy of thermal movement (kT).[18] The energy of the cooperative interaction is decisive for the operation of a particular kinetic mechanism of the cooperative transition from a disordered to a random state. Three types of interactions are possible in the system: (1) weak; (2) strong; (3) intermediate. In the first case the kinetics of the transition of the system from the ordered to the random state do not differ from those of the rearrangement of an individual unit. In the case of a strong interaction of the units, the system can rearrange as a whole only after an energy sufficient for the disruption of all the bonds of the system has accumulated simultaneously via a fluctuation process. Such transitions (in particular, the fusion of solids) are distinguished by high activation energies. The third, intermediate type of interactions occurs in proteins and the denaturation transition in a protein proceeds via a cooperative mechanism. Nevertheless, proteins resemble solids more closely than liquids and the enthalpy of fusion of the protein structure is a significant factor governing the stability of proteins.[19]

In order to describe the denaturation transition between the N and D states, the "all or nothing" mechanism, put forward *a priori*, is most frequently employed.[20] For the transition to be correctly analysed from the standpoint of the theory of two states and to obtain the true thermodynamic parameters of the transition, the transition must satisfy a number of tests, formulated by Brandts.[20] According to a large number of experimental data, the theory of two states can be used to a first approximation for virtually all globular proteins of small size.[7]

The change in the Gibbs free energy ΔG_{den} on complete and reversible uncoiling of the protein can serve as a measure of the stability of the latter:[6,7]

$$\Delta G_{den} = G_d - G_n,$$

where G_n and G_d are the free energies of the native and denatured forms, ΔG_{den} is the free energy of the stabilisation of the protein, and $a - \Delta G_{den}$ the free energy of its destabilisation. The numerical value of ΔG_{den} for the protein macromolecule is measured as a rule under the conditions involving the disruption of its native structure under the influence of heating, a change of pH, the addition of organic solvents, etc. This quantity is therefore significant only in relation to a specific type of denaturation.[7]

If the denaturation process is reversible, then ΔG_{den} is determined by measuring the constants for the equilibrium between the native and denatured forms of the protein, or by measuring the heat of denaturation with the aid of a scanning microcalorimeter. In the literature, the stability of the proteins is frequently characterised by the average temperature of the transition to the denatured state T_{den} (or T_m), at which 50% of the protein has been denatured and $\Delta G_{den} = 0$, by the temperature of the maximum stability of the protein T_{max} at which ΔG_{den} is a maximum (Fig.1), or by the average concentration of the denaturing agent, etc. In the case of irreversible denaturation (as a consequence of secondary processes) the kinetic stability of proteins is frequently characterised by the rate constants k_{in} for the change in any one property of the protein (the circular dichroism and ultraviolet spectra, fluorescence, and in the case of enzymes also their activity). In the general case the retention of the enzymic activity may not be identical with the stability of the entire protein globule.

The stability of a protein in solution is determined by a number of factors associated with the structural characteristics of the molecule.[20,21] An increase of the conformational entropy (~2–6 e.u. per uncoiled residue) decreases ΔG_{den}. The hydrogen bonds between the peptide groups in the biomolecule (the energy of a single bond is ~1.4 kcal mol^{-1})[17] increase ΔG_{den}.

Depending on the pH of the medium, the long-range electrostatic interactions of the charged groups on the surface of the protein can either stabilise (at pH close to the isoelectric point) or destabilise (at extreme pH values) the native conformation of the macromolecule. The hydrophobic interaction of the non-polar side residues stabilises the native conformation of proteins in aqueous solutions (the ΔG for the system decreases by ~2.8 kcal mol^{-1} when the aliphatic side residue is transferred from water to a non-polar solvent[22]). The intramolecular salt bonds (the energy of a single bond is ~1–3 kcal mol^{-1}), which can be formed either in the form of single electrostatic "fastenings" or within the composition of a spatially limited cluster consisting of several groups of charges of different sign, stabilise the native conformation of the protein. In the clusters a single ionogenic group can be involved in two, three, or four ionic interactions.[23–29]

Specific covalent cross-links in a polypeptide chain (in particular the S–S bonds) stabilise proteins not only as a result of a decrease of the entropy of the uncoiling of the globule[6] but also as a result of the enthalpy contribution (~1.8 kcal mol^{-1} per S–S bond[30]). Prosthetic groups form covalent, ionic, or coordinate bonds with the protein and stabilise the macromolecule as a whole.[31,32] Coordinate bonds involving Ca^{2+}, Mg^{2+} and Zn^{2+} metal ions are very important for the stabilisation of the native conformation of many protein molecules.[33,34]

Thus the total free energy of the denaturation of the protein ΔG_{den} includes the sum of the energies of all the types of interactions indicated above. The stability of the native conformation of a protein is achieved as a result of the displacement of the enthalpy and entropy functions along the temperature scale (Fig.1)[6] and reflects the fine balance between the stabilising and destabilising contributions of interactions of different types. At physiological temperatures, the ΔG_{den} of different globular proteins differ insignificantly and vary from 5 to 15 kcal mol^{-1}.[7] However, even a slight change in the balance between different interactions in the protein can greatly alter its stability, as will be seen below when the experimental data for stabilised proteins and enzymes are considered.

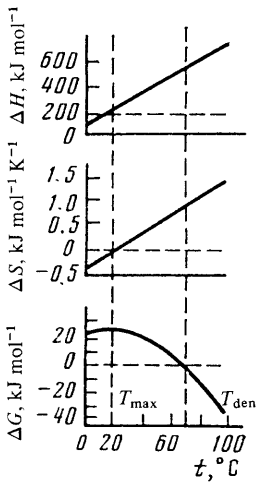


Figure 1. Temperature variation of the enthalpy, entropy, and free energy (ΔG_{den}) of denaturation of cytochrome b_5 (pH 7.4).[6]

Analysis of the principal energy characteristics of the protein structure shows[6] that (1) the protein should be stable under specific external conditions in order to retain its specific structural and functional properties; the energy reserves ensuring the stability of the protein should therefore greatly exceed RT—the minimum necessary value; (2) the protein should not be unduly "rigid", i.e. the structural changes in the course of the functioning of the protein should occur without much energy expenditure and at the same time the protein–water system should be capable of energy exchange in the course of its functioning without its breakdown; (3) since the stability of the protein is not correlated with its molecular weight, the size of the macromolecule should be the smallest possible to ensure its coiling up, transport, and functioning. This principle is realised by the formation of intramolecular domains or a subunit structure.[7] In contrast to the view that the protein structure is "rigid", which was held previously on the basis of X-ray diffraction data, thermodynamic data confirm the existence of fluctuations in the protein structure.[35] The amplitudes of the fluctuations of the atoms about their mean values are greater for less stable proteins and smaller for more stable proteins.[36] In conformity with the proposed mechanism of the dynamic stabilisation of the protein structure,[37] the internal mobility of the clusters of hydrophobic aminoacid residues in the macromolecule is responsible for the overall stability of its native conformation.[38]

The kinetic stability of the protein ($\Delta G^{\neq}_{den}$) is characterised by the inactivation constant k_{in} and the activation parameters determined from the temperature dependence of k_{in}. The kinetic and thermodynamic parameters of the denaturation of proteins vary in parallel as the denaturation conditions are altered,[20,39] which shows that the intramolecular interactions responsible for the thermodynamic stability (ΔG_{den}) determine also the kinetic stability ($\Delta G^{\neq}_{den}$) of proteins.

One of the commonest types of denaturation is thermal denaturation, on which attention has been concentrated in this review. An increase of temperature has different effects on the intramolecular interactions in proteins (Fig.2): (1) the degree of structure formation in water diminishes and up to about 60 °C the strength of the hydrophobic interactions increases; (2) the conformational entropy increases, which makes it possible to attain protein conformations with a higher free energy, i.e. more uncoiled forms of the macromolecule; (3) other intramolecular interactions stabilising the protein (salt, coordination, etc.) are weakened.[40]

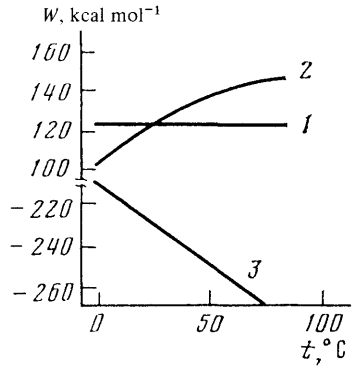


Figure 2. The influence of temperature on the overall contribution W of various factors to ΔG_{den} for native chymotrypsinogen: 1) hydrogen bonds; 2) hydrophobic binding; 3) conformational entropy.[40]

For the majority of globular proteins, the temperature dependence of ΔG_{den} is described by curves (Figs.1 and 3) which pass through a maximum (ΔG^{max}_{den}) at the temperature of the maximum stability of the protein T_{max} and which have upper (T_{den}) and lower temperature limits of the stability of the protein (at $\Delta G_{den} = 0$). T_{max} is usually appreciably lower than physiological temperatures[7] and for many globular proteins is in the range from −3° to +10 °C, which is important for practical purposes and makes it possible to store proteins for a long time at these temperatures.[2] With increase of temperature up to 55 °C, the exposure of the internal aminoacid residues to the solution and the increase of the mobility of the individual sections of the polypeptide chain lead to the reversible local expansion of the native protein macromolecule.[20,41] Above 55 °C, these changes

become irreversible and the protein assumes a fully uncoiled structure.

Water has a destabilising effect on the protein macromolecule. Practical experience has shown that lyophilised proteins keep much longer than in solution. When the water content is more than 0.75 g per gram of protein, the degree of hydration does not affect T_{den} and ΔH_{den} for the protein.[42-44] For a water content below 0.75 g per gram of protein, ΔH_{den} gradually diminishes and T_{den} increases slightly. When the water content is below 0.4 g per gram of protein, T_{den} rises sharply by 30–40 °C. These facts reflect the existence in proteins of various types of bound water. A small number of water molecules are strongly bound to the protein and the system can be regarded as a single whole. The less strongly bound water is distributed over the entire surface of the globule, interacting with its hydrophilic residues. The next layer is transitional in relation to water in bulk.[43] The largest contribution to ΔH_{den} for the protein comes from the ΔH for the disruption of the hydrophobic contacts when the protein is uncoiled, i.e. the ΔH for the ordering of the water molecules around the exposed non-polar groups of the protein. With decrease of the water content, the degree of hydration of the non-polar residues in the denatured state of the protein diminishes, which entails a decrease of ΔH_{den} for the protein.[44] Since the uncoiling of the protein molecules during denaturation is due to the competition between water molecules and protein residues for hydrogen bonds, in the presence of a very low water content the protein molecule is not fully uncoiled.

Although a correlation between the physicochemical properties of proteins and their stability has not been discovered, analysis of data for a large number of proteins suggested the following:[7] (1) in the course of their functioning, proteins forming complexes with other biomacromolecules have as a rule low values of ΔG_{den} (α-lactalbumin, cytochromes *c* and b_5); (2) proteins reacting with low-molecular-weight compounds or with individual sections of other macromolecules have high values of ΔG_{den} (myoglobin, various hydrolytic enzymes).

On the basis of numerous data, a satisfactory correlation has been observed between the stabilities of proteins *in vivo* and *in vitro*.[45-48] This is due to the fact that one of the causes of protein and enzyme inactivation in the cell is the proteolysis of their denatured (uncoiled) conformers, the rate of which is limited by the rate of uncoiling of the native conformation.[47] The turnover rate of the protein *in vivo* is correlated with its T_{den} and with its stability in relation to acid and mechanical denaturation.[46,48] The stability of certain proteins is satisfactorily correlated with the metabolic rate.[49]

However, overall, the thermal stability of the protein is not correlated either with ΔG_{den}, measured under standard conditions, or with T_{den}, i.e. these quantities cannot by themselves serve as objective measures of thermal stability when different proteins are compared.[1,7] For example, cytochrome *c* and lysozyme have the same T_{den} but their maximum values of ΔG_{den} differ approximately by a factor of two. At certain temperatures α-chymotrypsin and lysozyme have almost identical ΔG_{den}, but their T_{den} differ by 20 °C (Fig.3). The thermal stability of the protein is correctly described only by the complete temperature dependence of ΔG_{den} over a fairly wide temperature range. It is particularly important to bear this in mind on comparing the stability of the modified or immobilised enzyme with that of the native enzyme, because the ratio of the ΔG_{den} at a single fixed temperature cannot be extrapolated to other temperatures.

Thus the knowledge of the principal factors responsible for the stability of the native conformation of the protein in solution makes self-evident the general principles by which one should be guided in obtaining protein and enzyme preparations with increased stability over a specific temperature range: (1) the introduction of additional intramolecular bonds (salt and hydrogen bonds) and the enhancement of hydrophobic interactions will increase ΔG_{den} for the protein (one additional bond with an energy of 1–3 kcal mol^{-1} can significantly increase the stability of the protein); (2) a decrease of the conformational mobility of fragments of the polypeptide chain as a consequence of the formation of additional intramolecular (covalent or non-covalent) "cross-links" in the globule (additional S–S bonds, coordinate bonds with metal ions, etc.) will diminish the conformational entropy[6] and hence will increase ΔG_{den}.

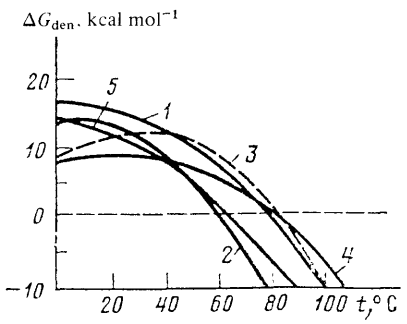


Figure 3. Temperature variation of the free energies ΔG_{den} for globular proteins measured microcalorimetrically: 1) lysozyme; 2) α-chymotrypsin; 3) metmyoglobin; 4) cytochrome c; 5) ribonuclease.[21]

As will be shown below, these principles hold in nature in the formation of proteins and enzymes with increased stability (enzymes from thermophilic microorganisms) and under practical conditions in the preparation of stabilised enzymes by their chemical modification or immobilisation.

2. Relation between the Stability of Biomacromolecules and Their Primary, Secondary, and Tertiary Structures

In order to discover the principal structural factors related quantitatively to the stability of biomacromolecules, a number of attempts have been made in recent years to find a correlation between the stability and the primary, secondary, and tertiary structures of proteins. For this purpose, the aminoacid compositions of proteins with different stabilities were compared and studies were made of the physicochemical causes of the increased stabilities of proteins and enzymes isolated from thermophilic sources and also of the influence of point mutations on the stability of proteins and enzymes.

(a) Correlation between the primary structure and the stability of proteins. An empirical relation between T_{den} and the hydrophobic index X_1 and the average volume of aminoacid residues in the protein X_2 has been discovered for 14 proteins:

$$T_{den} = 226.81 - 0.01270X_1 + 3.847X_2 .$$

It is seen that the main contribution to T_{den} comes from the average volume of the aminoacid residues X_2 and not from their hydrophobic properties. The Glu residues stabilise proteins to the maximum extent, while the Ser residues

destabilise them.[50] However, for a large number of proteins a correlation between T_{den} and X_2 has not been found.[51]

The correlation between the hydrophobic properties and thermal stability of proteins proposed by Bigelow[52] was not subsequently confirmed.[53]

A clear relation has been observed between T_{den} and the ratio S_n/S_d, where S_n and S_d are the surface areas of the protein accessible to the solvent in the native and denatured states respectively: $S_n = 11.116\,M^{2/3}$, while $S_d = 1.44\,M$ (M is the molecular weight of the protein). For 20 of the proteins investigated, T_{den} increases linearly with increase of S_n/S_d, i.e. with decrease of M. The ratio S_n/S_d diminishes with increasing M and there is a decrease of the thermal stability of the protein. For the same ratio S_n/S_d, the quantity T_{den} is greater for subunit proteins than for monomeric proteins. The thermal stability of a long polypeptide chain can be increases in the following way: (1) by the association of the globular subunits to form an oligomer; (2) by coiling up with formation of a multiplicity of globular domains. In such cases S_n/S_d (and T_{den}) increases for an unchanged molecular weight M. The deviations from linearity for certain proteins can be accounted for by the influence on their stability of intramolecular interactions such as those involved in S–S bonds in the binding of metal ions, cofactors, etc.[53]

The microstability of the protein globule differs from its macrostability G_{den} by the fact that the former characterises the local uncoiling of the protein and is determined from the rate of exchange of the protons in the inner part of the macromolecule with water.[54] The microstability, i.e. the "rigidity" of the protein globule, does not characterise the macromolecule as a whole. It depends on the degree of saturation of the protein by non-polar contacts and increases as the hydrophobic nucleus of the macromolecule grows. On the other hand, ΔG_{den} is independent of the hydrophobic properties of the protein globule and is directly related to the number of intramolecular cross-links, which diminish the conformational entropy of ΔS_{conf} of the uncoiling of the polypeptide chain (the binding of M^{n+} ions and cofactors has an analogous influence).[54]

The specific preferential cooperation of various aminoacid residues in proteins with one another, which has been found on the basis of their three-dimensional structure,[12,13] apparently determines the increased or reduced stability of a particular protein with increase of temperature.[55] Comparison of the T_{den} and the aminoacid compositions for a large number of proteins made it possible to establish a relation between the content of each aminoacid residue and T_{den} for the protein. All the aminoacid residues are divided into stabilising (positive correlation with T_{den}) and destabilising (negative correlation with T_{den}). A combination of charged and non-polar helical residues (I), namely Asp, Cys, Lys, GlU, Leu, Arg, Trp, and Tyr has the greatest stabilising effect, while a combination of neutral and short non-polar residues (II), namely Ala, Asn, Gly, Gln, Ser, Thr, Val, and Tyr has a destabilising effect. The overall correlation can be formulated by the equation

$$T_{den} = 64.46202 + 0.89432Y_1 - 0.59065Y_2,$$

where Y_1 and Y_2 are the percentage contents in the protein of the stabilising (I) and destabilising (II) aminoacid residues. Phe, Ile, Met, His, Asn, and Pro do not influence the stability of the protein.[55] The upper limit of the stability of the protein, corresponding to a 100% content of group I residues, is $T_{den} = 164.3$ °C, while the lower limit of the stability, corresponding to a 100% content of group (II) residues, is $T_{den} = 5.4$ °C. However, the proposed correlation between T_{den} and the aminoacid composition of the proteins is valid only for small compact monomeric proteins. Under these conditions, haem or S–S bonds have a lesser influence on the stability of the protein than its aminoacid composition.[55]

A disadvantage of all the correlations discussed above is that, firstly, they are valid only for a limited group of proteins and enzymes, which is as a rule the group for which the given correlation had been derived. Secondly, T_{den} has been used as a general criterion of thermal stability, but, as already mentioned above, it cannot serve as an objective measure of the stability of the protein over a wide temperature range (Fig.3). The determination of a simple thermal stability–temperature correlation is apparently impossible owing to the complex relation between ΔG_{den} for the protein and temperature:[7]

$$\Delta G_{den}(T) = \Delta H_{den}(T_{den} - T)/T_{den} - \int_T^{T_{den}} \Delta C_p \, dT + T \int_T^{T_{den}} \Delta C_p \, d\ln T.$$

In the future, it will probably be necessary to establish a relation between $\Delta G_{den}(T)$, and not T_{den} for proteins, and the structure of the macromolecules. In addition, by virtue of the multiplicity of the structural factors responsible for the stability of the native conformation of the protein, the existence of such a correlation is possible only for a limited number of isotypical globular proteins.

(b) Physicochemical causes of the increased stability of the enzymes derived from thermophilic sources. From the standpoint of the determination of the stability–structure function, comparison of the structures of proteins and enzymes isolated from thermophilic micro-organisms (t-enzymes) and exhibiting an increased thermal stability with their mesophilic analogues (m-enzymes), having structures similar to those of the t-enzymes, is more promising. Such a comparison makes it possible to discover the principal structural characteristics of t-enzymes responsible for their increased thermal stability.

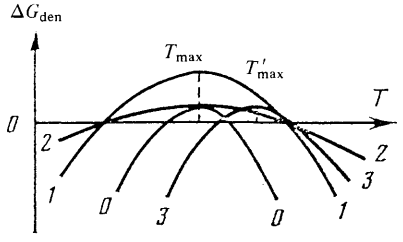


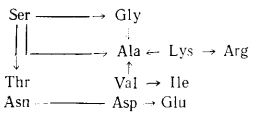
Figure 4. Hypothetical curves for the temperature variation of ΔG_{den} for globular proteins from thermophilic sources: *0*) mesophilic protein; 1) thermophilic protein with a higher value of ΔG_{den} (rise of curve *0*); 2) thermophilic protein with a higher temperature range of stability (the levelling off of curve *0*); 3) thermophilic protein with a displaced stability maximum (shift of curve *0* from T_{max} to T'_{max}).[60]

The thermal stability of microbial enzymes is correlated with the temperatures corresponding to the existence of the micro-organisms.[56] At low culture temperatures (<50 °C) m-enzymes are synthesised, while at high culture temperatures (>50 °C(t-enzymes are synthesised.[57,58] t-Enzymes are also stable in relation to other types of denaturation, namely denaturation by urea, guanidine hydrochloride, and organic solvents and under the influence of pH.[59]

The increased thermal stability of a t-protein compared with an m-protein can result from the operation of the following factors (Fig.4): (1) the increase of the activation energy for the denaturation process; (2) the increase of the free energy of denaturation ΔG_{den}; (3) the equalisation of the free energy of denaturation profiles; (4) a shift of the ΔG_{den} profile.[60] The stability of the majority of t-enzymes increases as a result of the higher position of the curve describing the temperature dependence of ΔG_{den} (from curve *0* to curve *1* in Fig.4). Additional hydrophobic, hydrogen, salt, and S–S linkages, which are present in t-enzymes but not m-enzymes, lead to such alteration of ΔG_{den}. On the other hand, ΔG_{den} for t-enzymes can vary in accordance with curve *2* (Fig.4) as for *t*-phosphoglycerate kinase.[60]

Thermophilic proteins and enzymes have high thermodynamic and kinetic stabilities. Since in terms of the absolute temperature scale, the difference between 35 °C (T_{den} for m-enzymes) and 80 °C (T_{den} for t-enzymes) is relatively small (~15%), even small differences between the activation energies and ΔG_{den} for t- and m-enzymes can lead to appreciable differences between their thermal stabilities for a small difference between their structures. Calculation shows that a decrease of k_{in} by a factor of ten at 70 °C requires an increase of ΔG_{den} by only 1.56 kcal mol^{-1}. In order to increase T_{den} from 35° to 45 °C for a polypeptide chain with $M = 35\,000$, it is necessary to increase the number of hydrogen bonds by only 3%. The differences between ΔG_{den} and $\Delta G^{\neq}_{den}$ for proteins amounting up to 10 kcal mol^{-1} can be obtained following the replacement of one or two aminoacid residues in the molecule.[53]

(c) The fundamental differences between the structures of enzymes from thermophilic and mesophilic sources. The fundamental differences between the aminoacid compositions of t- and m-enzymes have been discovered following a comparison of the aminoacid compositions of a large number of t- and m-enzymes. In relation to ferredoxins, glyceraldehyde-3-phosphate dehydrogenases, and lactate dehydrogenases, the following directions of the preferential exchange of aminoacid residues on transition from m- to t-enzymes have been discovered:[61]



The above changes enhance the hydrophobic properties of the residues and their capacities for helix formation and folding. Under these conditions, the volume of the internal residues increases somewhat and a more compact packing is achieved in the structure of the protein globule. The principal role of the stabilising additional ion pairs in these t-enzymes has been demonstrated on the basis of structural investigations.[62] A higher content of Ala and Gly and charged residues is characteristic of t-lactate dehydrogenase (LDH), while m-LDH contains more Ser, Thr, Gln, Asn, and polar residues. Although the content of hydrophobic residues is the same in the two enzymes, in the t-enzyme they exist only within the globule, while in the m-enzyme they are also present on the surface. The spatial dispositions of the charged and polar residues are also different in t- and m-LDH:[62] in t-LDH the charged residues are located only on the surface of the molecule, while in m-LDH they are also present inside it. Thus the distribution of the aminoacid residues in the t-LDH molecule is thermodynamically more favourable.

Analysis of the aminoacid compositions of 14 t-enzymes and 207 m-enzymes showed that the thermophilic enzymes contain more Gly, Arg, Glx but less Asx and Ser.[63] A positive correlation between the thermal stability and the ratio of Arg and Lys in the protein has also been noted.[64] Arginine is the most hydrophilic aminoacid and its location on the surface is more favourable compared with Lys. The resonance structure of the guanidinium ion enables it to form more than one bond with anionic residues. t-Enzymes are also characterised by a higher content of α-helices and of the β-structure than m-enzymes. The increased Glu content in t-enzymes increases their capacity to bind metal ions. It may be that this is one of the principal causes of the increased stability of t-enzymes.[63]

A positive correlation between the thermal stability and the aliphatic index of the protein A, determined from the formula

$$A = \chi_{Ala} + a\chi_{Val} + b(\chi_{Ile} + \chi_{Leu}),$$

where $a = 2.9 \pm 0.1$, $b = 3.9 \pm 0.1$, and χ represents the content of Ala, Val, Ile, and Leu residues in the protein (in molar percentages), has been established for 34 t-enzymes and 208 m-enzymes. Since the content of the above residues in t-enzymes is much higher than in m-enzymes, each t-enzyme has a greater aliphatic index than its mesophilic analogue.[65]

Comparison of the tertiary structures of t- and m-enzymes revealed two additional salt and hydrogen bonds in t-ferredoxin.[53,66] The increased stability of t-triose phosphate isomerase is due to additional salt and hydrophobic linkages between the subunits.[66] Two additional intersubunit and intrasubunit salt linkages stabilise t-glyceraldehyde-3-phosphate dehydrogenase,[21,67–69] while additional salt linkages, hydrophobic interactions, and bonds with additional Ca^{2+} ions are responsible for the stabilisation of thermolysin.[70–72] The additional salt linkage between Asp (201) [Asn(201) in the m-enzyme] and the His residue increases the activation energy for the thermal denaturation of t-tyrosinase.[73] Among the ten ion pairs in t-LDH, eight are formed by Arg residues, while in different m-LDH only 2, 4, or six ion pairs, containing Lys instead of Arg, have been found.[62] The principal role in the stabilisation of t-LDH has been attributed to additional surface salt linkages formed by Arg residues and also to an enhancement of the intramolecular hydrophobic interactions as a consequence of the decrease of the number of hydrophobic residues on the surface of the globule and of its increase within the globule.[62] The greater thermal stability of other thermophilic enzymes, whose tertiary structures have not been studied in detail, compared with m-enzymes has been explained by the greater number of hydrogen bonds and the stronger intersubunit interaction in the case of enolase,[74,75] the greater number of intramolecular cross-links (S–S bonds) in the case of the iron–sulphur protein[76] and glutamine synthetase,[77] the protective effect of the surface carbohydrates in β-glucosidase,[78] or the change of conformation in the case of dehydrogenase.[79]

Thus the high thermal stability of the enzymes from thermophilic microorganisms is due to the exceptionally small number of additional (compared with m-enzymes) bond both within the macromolecule and on its surface. For the majority of t-proteins, these are mainly salt linkages, which follows from a comparison of the tertiary structures of t- and m-enzymes.

(d) Point mutations and the stability of the protein. The study of the influence of point mutations (which alter only 1–2 aminoacid residues in the polypeptide chain) on the stability of the protein can also yield valuable information about the relation between structure and stability. In contrast to t-enzymes, where the natural selection of the most

stable form from the multiplicity of mutant forms takes place, the artificial mutations are random in their influence on the stability of the protein, which relates them to chemical modification.

The replacement of a single Trp (138) residue by Tyr in the lysozyme of the T4 phage (a polypeptide without S–S bonds) diminishes ΔG_{den} (and T_{den}) over the entire temperature range from 0° to 60 °C by 2–3 kcal mol^{-1} (Fig.5).[80] Here ΔH_{den} diminishes by 20 kcal mol^{-1}. The replacement of a further two Trp residues by Tyr diminishes ΔG_{den} to a still greater extent at all the temperatures studied (Fig.5). Although the overall conformation of the lysozyme hardly changes after the mutations, one of the possible explanations of the marked decrease of ΔH_{den} after the mutation and of its compensation by the simultaneous decrease also of ΔSden might be the local uncoiling of the molecule.[80]

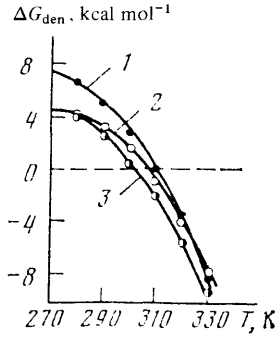


Figure 5. Temperature variation of ΔG_{den} for the native (curve *1*) and mutant (curves *2* and *3*) forms of lysozyme at pH 2.0: 2) Trp (138) replaced by Tyr; 3) Trp (138), (126), and (158) replaced by Tyr.[80]

The single-point replacements of Glu (128) by Lys, of Arg (96) by His, and of Ala (146) by Thr in the T4 phage lysozyme do not alter its tertiary structure but diminish T_{den} by 5°, 14°, and 9° respectively.[81,82] The first mutation increases ΔH_{den}, while the second and third diminish it. The greatest destabilising effect (T_{den} diminishes by 14°) is shown by the replacement of Arg (96) by His. According to the authors, this can be explained by a weakening of the hydrophobic interactions in the molecule and the possible rupture of the α-helix between the aminoacid residues in the 82–90 position.[81]

The replacement of Glu (49) by Met in the α-subunit of tryptophan synthetase from *E.coli* yields a mutant which is twice as stable (at 58 °C) in relation to thermal denaturation and the denaturation by urea.[82] The spatial structure of the enzyme does not then change. According to the authors, the enhancement of the hydrophobic interactions in the mutant following the replacement of the hydrophilic Glu in the hydrophobic nucleus by Met is responsible for the observed stabilisation effect. The nature of the aminoacid residue in the 49-position revealed an interesting relation: the replacement of the charged Glu (49) by the uncharged polar residues Tyr, Ser, and Gln increases the stability of the α-subunit of tryptophan synthetase in relation to guianidine hydrochloride at pH 9.0, while at pH 7.0 and 5.5 it destabilises the enzyme, which is shown most strikingly in the case of Gln.[84] On the other hand, the replacement of Glu (49) by the hydrophobic aminoacid residues Met and Val greatly increases (by 5 kcal mol^{-1}) the stability of tryptophen synthetase relative to guanidine hydrochloride at all pH (particularly at pH 7.0 and 9.0). According to the authors,[83] Glu (49) is in a hydrophobic environment and any diminution of the hydrophilic properties of the residue in the 49-position will therefore naturally induce an increase of the stability of the enzyme. The replacement of Gly (211) in the α-subunit of tryptophan synthetase by Arg or Glu increases somewhat the thermal stability of the enzyme (at 57.8 °C ΔH_{den} increases by 0.1 and 0.6 kcal mole^{-1}, while T_{den} increases by 0.2° and 1.8 °C respectively).[85] On the other hand, ΔH_{den} increases by 17 and 15 kcal mol^{-1} as a result of these mutations. It has been shown that the conformations of the native enzyme and the two mutants are similar. The segment of the chain containing Gly (211) is apparently located on the surface of the molecule and does not participate in any appreciable interactions, so that the replacement of Gly by the more bulky charged Glu and Arg residues induces only small changes in the non-covalent interactions on the surface of the protein.[85]

Studies on point mutations have shown that even the replacement of one aminoacid residue induces an appreciable change in the stability of the protein. Depending on the location of the replaced residue, its immediate environment, and the nature of the substituting residue, the stability either increases or decreases after the mutation as in the case of chemical modification (this will be demonstrated in the next Section). Mutations increasing the polarity within the protein globule (the hydrophobic nucleus) diminish its stability, while those which enhance its hydrophobic properties stabilise it. The mutations of the surface aminoacid residues have only a weak influence on the stability of the macromolecule.

In conclusion it must be emphasised that the stability of the protein is very sensitive not only to changes in its conformation but also to slight variations in the primary structure (as can be seen in relation to point mutations). Therefore a change in the stability of the protein (in particular, its thermal stability) constitutes a sensitive test for the changes in the structure of the protein, which may be below the limits of the resolution of both structural analysis and other physicochemical methods.[7,86]

III. THE MECHANISM OF THE CHANGES IN THE THERMAL STABILITY OF PROTEINS AND ENZYMES AFTER THE CHEMICAL MODIFICATION OF THEIR FUNCTIONAL GROUPS

The chemical modification method is used widely for the identification of the groups in the active centres of enzymes and for the investigation of the topography and conformations of biomacromolecules.[87] In connection with the problem of the stabilisation of biocatalysts, increasing attention has been devoted in recent years to the chemical modification of the functional groups of the protein which do not enter into the active centre.[2–4] Only the surface groups of the protein or those near the surface under the given conditions are as a rule accessible to chemical modification. Depending on the conditions (pH, temperature, etc.), the number of surface groups, i.e. groups accessible to the solvent, can vary.

The reactive groups of the protein can be divided into three components: (1) acid groups (the COOH groups at the C-ends and in the Asp and Glu residues); (2) basic groups (the α-NH_2 groups at the N-ends, the Lys and Arg residues, and the OH groups in the Tyr, Ser, and Thr residues and in the carbohydrates in glycoproteins); (3) neutral groups (the imidazole group in His).[88] The reagents most frequently used for the chemical modification of proteins and enzymes in order to stabilise them or alter their structure are listed in Table 1.

Table 1. The changes in the structure and stability of proteins and enzymes as a result of the chemical modification of their functional groups.

Protein	Modifying agent	Modified residue[a]	Derivatives obtained	Degrees of modification: not influencing structure and thermal stability	Degrees of modification: increasing thermal stability[b]	Degrees of modification: uncoiling and destabilizing the molecule[b]	Refs.
Bovine serum albumin	Succinic anhydride[c]	Lys (59)	$ENH_3^{\pm} \rightarrow ENHCCH_2CH_2CO^{\ominus}$ (O, O)	Up to 60% (up to 30–34 residues)	—	more than 80%	[90—92]
	Methyl acetimidate[c]	»	$ENH_3^{\oplus} \rightarrow ENHC(NH_2^{\oplus})CH_3\ Cl^{\ominus}$	up to 100%	—	—	[92]
	Butyric anhydride	»	$ENH_3^{\oplus} \rightarrow ENHCOCH_2CH_2CH_3$	up to 90%	—	more than 90%	[93]
Human immuno-globin G	Succinic anhydride[c]	Lys[?] (90)	$ENH_3^{\oplus} \rightarrow ENHCCH_2CH_2CO^{\ominus}$ (O, O)	up to 87% (up to 78 residues)	—	more than 87% (expansion of molecule, decrease of β-folds owing to electro-static repulsion)	[90]
	Citraconic anhydride[c]	»	$ENH_3^{\oplus} \rightarrow ENHCCH{=}C(CH_3){-}CO^{\ominus}$ (O, O)	Low degrees of modification	—		[94]
	Potassium iso-cyanate[c]	»	$ENH_3^{\oplus} \rightarrow ENHCNH_2$ (O)	up to 100%	—	—	[94]
	Acetylacetone[c]	Lys (29)	$ENH_3 \rightarrow EN{=}C(CH_3)CH_2CCH_3$ (O)	—	—	from 1 to 6 Lys	[95]
Leucine-binding protein	»	Arg (7)	$ENHC(NH_2^{\oplus})NH_2 \rightarrow E{-}NH{-}C(N{-}C(CH_3){=}CH{-}C(CH_3){=}N)$	up to 3 Arg	—	—	[95]
11S globulin	Acetic anhydride[c]	Lys[e]	$ENH_3^{\oplus} \rightarrow ENHCOCH_3$	up to 21%	—	more than 21%	[96]
Ovalbumin	Succinic anhydride[c]	Lys[e] (19)	$ENH_3^{\oplus} \rightarrow ENHCCH_2CH_2CO^{\ominus}$ (O, O)	Up to 66%	—	More than 66% (uncoiling of protein globule)	[97]
Cytochrome *c*	Tetranitromethane	Tyr (4)	$E{-}C_6H_4{-}OH \rightarrow E{-}C_6H_3(NO_2){-}OH$	—	1 residue (increase of T_{den})	2 residues (extensive uncoiling	[98]
Cytochrome *c*-552	Acetic anhydride[c]	Lys[e] (4)	$E{-}NH_3^{\oplus} \rightarrow ENHCOCH_3$	Up to 100%	—	—	[99]
Horse cyto-chrome *c*	4-Chloro-3,5-dinitrobenzoic acid	Lys (19)	$E{-}NH_3^{\oplus} \rightarrow E{-}NH{-}C_6H_2(NO_2)_2{-}COO^{\ominus}$	2 residues (7 and 60) remote from top and bottom of of haem crevice	2 residues (27 and 72) on frontal surface near exposed portion of haem moiety (formation of new surface salt bonds)	Lys (13) on top of haem crevice [rupture of Lys (13)–Glu (90) salt bond greatly widens haem crevice); lysines 8, 86, and 87 on top of crevice (destabilisation as a consequence of decrease of electrostatic potential in this region)	[29, 100, 101]
	Succinic anhydride	Lys	$ENH_3^{\oplus} \rightarrow ENHCCH_2CH_2CO^{\ominus}$ (O, O)	Up to 95% (up to 18 residues), stability does not change relative to denaturation by urea	—	100%	[102]
	Maleic anhydride[c]	Lys	$E{-}NH_3^{\oplus} \rightarrow E{-}NHCCH{=}CHCO^{\ominus}$ (O, O)	—	—	Modification of all 19 residues	[103]
Myoglobin	Bromoacetate	His (12)	$E{-}C_3N_2H_2{-}NH^{\oplus} \rightarrow E{-}C_3N_2H_2{-}N{-}CH_2{-}CO{-}OCH_3$	Up to 50–58% (6–7 residues)	—	—	[104]
	Iodoacetamide		$E{-}C_3N_2H_2{-}NH^{\oplus} \rightarrow E{-}C_3N_2H_2{-}N{-}CH_2{-}CO{-}NH_2$	Up to 50–58% (6–7 residues)	—	—	[104]
β-Lacto-globulin	Succinic anhydride	Lys[e] (16)	$E{-}NH_3^{\oplus} \rightarrow E{-}NHCCH_2CH_2CO^{\ominus}$ (O, O)	—	1–3 residues (small increase of stability relative to denaturation by urea)	From 4 to 10 residues (lower stability); more than 10 residues (uncoiling of protein also in absence of urea)	[102]
Ribonuclease	Succinic anhydride	Lys[e] (11)	$E{-}NH_3^{\oplus} \rightarrow E{-}NHCCH_2CH_2CO^{\ominus}$ (O, O)	Up to 100% (relative to denaturation by urea)	—	—	[102]

Table 1 (contd.)

Protein	Modifying agent	Modified residue[a]	Derivatives obtained	Degrees of modification: not influencing structure and thermal stability	Degrees of modification: increasing thermal stability[b]	Degrees of modification: uncoiling and destabilizing the molecule[b]	Refs.
	Acetic anhydride	Lys	$E{-}NH_3^{\oplus} \rightarrow E{-}NHCCH_3$ (=O)	—	Low degree of modification: some stabilisation	All 11 residues	[105]
	N-Acetylhomocysteine thiolactone	Lys	$E{-}NH_3^{\oplus} \rightarrow E{-}NHCCHCH_2CH_2SH$ (=O; NHCOCH$_3$)	—	8 residues: increase of stability	—	[106]
Glucoamylase	Acryloyl chloride	Lys	$E{-}NH_3^{\oplus} \rightarrow E{-}NHCCH{=}CH_2$ (=O)	Up to 66 residues	—	More than 66% of residues: stability falls sharply; 100% of residues: stability diminishes by a factor of 2.5 at 65 °C	[107]
Horseradish peroxidase	Acetic and oenanthic anhydrides	Lys (6), OH groups of Tyr(5), Ser (25), Thr (25), and carbohydrate residues	$E{-}NH_3^{\oplus} \rightarrow E{-}NHCOCH_2{\cdot}R$; $E{-}OH \rightarrow E{-}OCOR$	Up to 2 Lys residues, up to 30 surface OH groups	3–4 Lys residues (below 75 °C, k_{in} falls with increase of degree of modification); stabilisation as a result of increase of strength of haem–protein bond	6 Lys residues, more than 30 OH groups (when internal OH groups are also involved)	[34, 108, 109]
	Trinitrobenzenesulphonic acid	Lys (6)	$E{-}NH_3^{\oplus} \rightarrow E{-}NH{-}SO_2{-}C_6H_2(NO_2)_3$	—	3 residues, same effects as in previous case	6 residues: decrease of stability (weakening of haem-protein bond)	[108]
α-Amylase	*p*-Nitrophenyl acetate	Lys	$E{-}NH_3^{\oplus} \rightarrow ENHCOCH_3$	—	—	With increase of degree of modification from 1.5 to 11.5 residues, the protein conformation approaches that of denatured form, k_{in} increases below 67 °C, and $\Delta H^{\neq}$ and $\Delta S^{\neq}$ diminish	[110, 111]
Myosin	β-Naphthoquinone-4-sulphonic acid	Lys			Thermal stability increases with increase of degree of modification from 1 to 15 residues	—	[112]
Lactate dehydrogenase	*O*-Methylisourea	Lys	$E{-}NH_3^{\oplus} \rightarrow E{-}NH{-}C(=NH_2^{\oplus})NH_2$	—	— ↑ 4–5 residues per subunit: sharp increase of stability (formation of stronger salt bonds between COO^- and guanidine groups introduced; from 5 to 15 residues slight increase of stability	—	[113]
Lactate dehydrogenase	Methyl 4-hydroxy-3-nitrobenzimidate	Lys	$E{-}NH_3^{\oplus} \rightarrow E{-}NH{-}C(=NH_2^{\oplus})C_6H_3(OH)(NO_2)$		With increase of degree of modification from 5 to 30 residues, stability increases by a factor of 2–6 at 60 °C		[114]
α-Chymotrypsin	Succinic anhydride	Lys[c] (17)	$E{-}NH_3^{\oplus} \rightarrow E{-}NHCCH_2CH_2CO^{\ominus}$ (=O; =O)	Up to 50% modification; for 100% modification the structure is close to native structure, slight increase of stability	From 60 to 95%: sharp increase of thermal stability (~20 times)	More than 95%: sharp fall of stability (approximately down to that of native protein)	[115, 116]
	Acrolein (with subsequent reduction by $NaBH_4$)	Lys	$E{-}NH_3^{\oplus} \rightarrow E{-}NH_2^{\oplus}{-}CH_2CH{=}CH_2$	Up to 50%: slight increase of stability	From 50 to 95%: sharp increase of thermal stability (~120 times for 95% modification); cause: change in conformation after modification	More than 95%: sharp fall of stability (down to that of native protein)	[116]

[a] The total number of given residues in the protein is indicated in brackets.

[b] The physicochemical causes responsible for the given change in the structure and stability of the protein are indicated in brackets.

[c] The thermal stability was not investigated in these studies.

[d] Alkylation entails the modification, apart from the NH_2 groups of Lys, of the SH groups of Cys and OH groups of Tyr, Ser, and Thr,[117,118] which was not taken into account by the authors of the given investigations.

[e] E = enzyme or protein.

Carboxylic acid anhydrides and chlorides, iminoesters, aldehydes, and chloro- and sulpho-derivatives of tri- or di-nitrobenzenes are employed for the modification of the amino-groups. The Arg residues are modified by various diketones. The nitration or iodination of proteins lead to the modification of the Tyr and Trp residues. The Ser and Thr residues and the OH groups of the carbohydrate residues in glycoproteins are acylated by acid anhydrides and chlorides. Bromoacetate and iodoacetamide modify the His residues. The carboxy-groups in the Leu and Asp residues are modified by means of carbodiimides or the Woodward reagent.[119,120] The above groups of proteins are also used in covalent immobilisation and the alteration of the stability of the biocatalyst can be to a large extent associated with the modification of its primary structure.

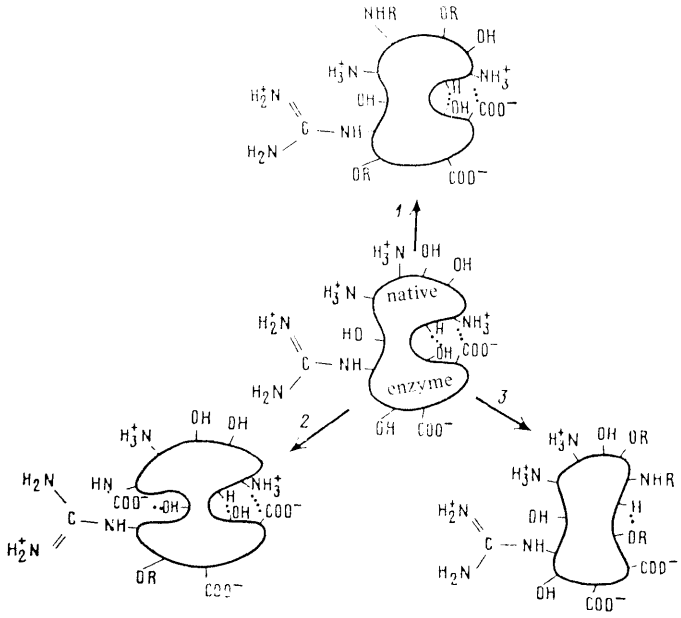


Figure 6. Schematic representation of various types of functional groups of enzymes; types of groups modified: 1) type I; 2) type II; 3) type III.[121]

The same functional groups of proteins and enzymes play different roles in the stabilisation of their native structures depending on their location in the globule and on whether or not they are involved in intramolecular interactions stabilising or destabilising the macromolecule. It is possible to isolate three types of functional groups accessible to modification (Fig.6). *Functional groups of type I* are located on the surface of the globule. These are effectively hydrated polar or charged groups, which do not interact with one another under the given conditions and interact only with the solvent or with ions from the solution. *Functional groups of type II* are located on or near the surface of the protein. In the native enzyme they have an unfavourable environment (charged groups in a hydrophobic environment or in an environment of groups bearing like charges) and destabilise the protein globule. The number of groups of this type in the protein is usually small. *Functional groups of type III* are remote from the surface of the globule and form intramolecular salt or hydrogen bonds stabilising the enzyme. These groups are inaccessible to modification under the usual conditions and are modified only for a loosened conformation of the protein (at an elevated temperature, at extreme pH values, or in the presence of organic solvents).

Chemical modification of functional groups of different types has different effects on the structure and stability of the enzyme. The observed effect also depends on the nature of the residues introduced into the enzyme on modification. Modification of groups of type I in the general case causes little alteration in the conformation and stability of the enzyme regardless of the dependence on the nature of the modifying agent and the degree of modification. If a large number of bulky hydrophobic residues or residues bearing a like charge are introduced into the enzyme at the same time, partial uncoiling of the molecule and its destabilisation can take place. In the same case, when the residues introduced form additional non-covalent bonds (salt and hydrogen bonds) with the neighbouring groups of the protein, the stability of the enzyme can actually increase. A large stabilisation effect is to be expected on modification of groups of type II when the interactions destabilising the native conformation are eliminated. On modification of groups of type II, the degree of modification, the charge, and the hydrophobic properties of the residues introduced actually exert a great influence on the thermal stability. Modification of groups of type III should diminish the thermal stability of the enzyme as a result of the breakdown of the intramolecular bonds stabilising its native conformation. The structure of the enzyme approaches the denatured structure after such modification.

Consequently, depending on which role particular functional groups play in maintaining the native structure of the enzyme, their modification either does not influence the thermal stability (groups of type I) or stabilises (groups of type II) or destabilises (groups of type III) the enzyme. It is to be expected that for each specific enzyme the ratios of the groups of each type are different. A low degree of modification of particular groups of the enzyme is most likely to involve groups of type I, a moderate degree of modification involves groups of types I and II, while exhaustive modification can result also in the modification of groups of type III in the enzyme. After the modification of groups of type III in the protein, the entire system of intramolecular stabilising interactions breaks down and the destabilising effect arising as a consequence of the modification of these groups can therefore overlap the stabilising influence of the modification of groups of type II.

Analysis of the data in Table 1 shows that in the general case there is a possibility of three types of modification of the functional groups of the protein: (1) modification with a change in the charge in the aminoacid residues which alters the electrostatic interactions of the groups on or near the surface of the protein and its isoelectric point and can therefore change the conformation of the globule;[89] (2) modification with introduction of hydrophobic groups into the protein, which can have different effects on the properties of the latter depending on the location and nature of the hydrophobic group introduced, exerting the greatest influence on the conformation, the solubility, and the surface characteristics of the protein; (3) the introduction of hydrophilic groups into the protein molecule, which has little influence on its properties, since the surface of the protein globule is hydrophilic.

The above analysis of the problem of the stability of globular proteins and of the characteristics of the structure of t-proteins permits certain hypotheses concening the possibility of the stabilisation of enzymes as a result of their chemical modification: (1) the stability can increase on formation in the protein after modification of even a small

number of additional non-covalent linkages; (2) modification with introduction of charged groups can exert a great stabilising influence provided that the formation of additional salt bonds is possible; (3) the replacement of the amino-groups of Lys by arginine-like groups following the introduction of amide or guanidine groups should increase the stability of the protein; (4) other conditions being equal, imparting of hydrophobic properties to the protein molecule can hardly appreciably increase its stability. These features have been confirmed in practice, as will be shown below.

The main difficulty in the analysis of literature data is that of comparing the results obtained by different workers on different objects, because almost no systematic and comprehensive investigations of the influence of modification on the structure and stability of proteins are described in the literature. The authors have used different stability criteria, although, as already mentioned, only ΔG_{den} and, in the case of irreversible thermal inactivation, $\Delta G^{\neq}_{den}$, can serve as objective measures of stability. The equilibrium constant K_{den}, related to ΔG_{den}, can be conveniently used as a quantitative characteristic of the thermal denaturation process:

$$\ln K_{den} = -\frac{\Delta G_{den}}{RT},$$

while the rate constant k_{in}, related to $\Delta G^{\neq}_{den}$, can be used for irreversible thermal inactivation: [3]

$$\ln k_{in} = \ln \frac{k_B T}{h} - \frac{\Delta G^{\neq}_{den}}{RT},$$

where k_B is the Boltzmann constant.

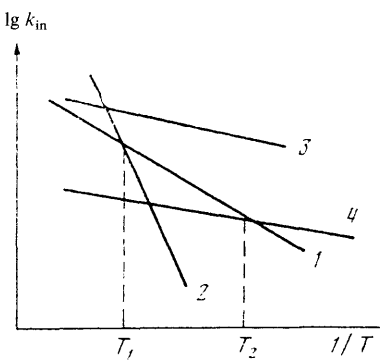


Figure 7. Hypothetical Arrhenius lg k_{in} relations for the native (line *1*) and modified or immobilised (lines *2–4*) enzymes; T_1 and T_2—isokinetic temperatures for the low-temperature (T_1) and high-temperature (T_2) stabilisation. [3]

When the thermal stabilities of the native and modified enzymes are compared, it is essential to compare the temperature variations of $\Delta G_{den}(K_{den})$ or $\Delta G^{\neq}_{den}(k_{in})$. The stabilisation effect θ^t at a given temperature is defined as the ratio of k_{in} for the native enzyme to k_{in} for the modified enzyme, i.e. $\theta^t = k_{in,nat}/k_{in,mod}$. If this ratio is greater than unity, then the modification stabilises the enzyme at a given temperature, while in the case where it is less than unity modification has a destabilising effect. The greater $\Delta G^{\neq}_{den}$, the more stable the enzyme (the smaller the value of k_{in}). The change of $\Delta G^{\neq}_{den}$ after the modification of the enzyme can be caused by the dominant alteration of the enthalpy term ($\Delta H^{\neq}$) or the entropy tern ($\Delta S^{\neq}$) in accordance with the equation

$$\Delta G^{\neq}_{den} = \Delta H^{\neq} - T\Delta S^{\neq}.$$

The quantity $\Delta H^{\neq}$ is determined from the experimental activation energy for the thermal inactivation process E_a: $\Delta H^{\neq} = E_a - RT$. When E_a for the thermal inactivation process is altered following the modification of the enzyme, one can observe two possible cases (Fig.7): [3,86]

(1) An increase of E_a (and $\Delta H^{\neq}$) for the modified enzyme. Depending on the scale in the change of $\Delta S^{\neq}$, the Arrhenius relations for K_{in} for the native and modified enzymes intersect outside or within the limits of the experimental temperature range (Fig.7, curves *1* and *2*). The modified enzyme is more stable than the native enzyme below the isokinetic temperature. The stabilisation effect increases with decrease of temperature. This is the case of the "low-temperature stabilisation" of the enzyme.

(2) A decrease of E_a (and $\Delta H^{\neq}$) after modification (Fig.7, curves *1* and *3*). The stabilisation effect can be observed only above the isokinetic temperature and is greater the higher the temperature at which it is considered. This is the case of the "high-temperature stabilisation". [3]

If $\Delta H^{\neq}$ changes little after modification, then the stabilisation (destabilisation) effect is due solely to the decrease or increase of $\Delta S^{\neq}$. Consequently, the study of the temperature variation of k_{in} makes it possible to discover the conditions under which the modified enzyme is more stable than the native enzyme and the extent to which this happens (and conversely). The knowledge of the temperature variation of k_{in} makes it possible to estimate objectively the enzyme stabilisation (or destabilisation) effects after the enzyme has been modified. The inadequate amount of information derivable from many literature stability data can be explained by the fact that they have been obtained at a single temperature.

1. Analysis of Experimental Data for the Influence of Chemical Modification on the Structure and Thermal Stability of Proteins and Enzymes

Studies of the influence of chemical modification by monofunctional agents are considered in this Section. We shall not deal with the results obtained on modification by bifunctional agents, because the effects of covalent intermolecular or intersubunit binding as well as the effect of the formation of intramolecular cross-links are superimposed on the single-point modification effects, which hinders an unambiguous interpretation of the results.

(a) The influence of the degree of modification on the structure and stability of proteins and enzymes. The modification of ~50% of the side residues frequently does not alter the conformation, the hydrodynamic properties, and the sedimentation constant of the protein (Table 1). The most accessible surface groups of type I are apparently modified in this case. For a number of proteins and enzymes (cytochrome *c*, horseradish peroxidase, and chymotrypsin), local changes in conformation are observed for a specific degree of modification indicating an increase of the number and energy of the intramolecular bonds. [29,108,109,116] At the same time, an increase of the stability of the macromolecules has been noted: the dominant increase of $\Delta H^{\neq}_{den}$ compared with $\Delta S^{\neq}_{den}$. In this case groups of type II were apparently modified. Table 1 shows that proteins and enzymes contain few groups of type II (from one to five). For high degrees of modification, involving internal groups which are masked

in the native conformation and which are involved in the main protein stabilising interactions, the conformation of the macromolecule changes sharply approaching that of the denatured macromolecule. The stability of the protein diminishes as a result of the dominant decrease of $\Delta H^{\neq}_{den}$ compared with that of $\Delta S^{\neq}_{den}$ (Table 1). Consequently, functional groups of type III are modified in this case.

Various acylating agents are used most frequently for modification (Table 1). Many workers assume *a priori* that only the NH_2 groups of Lys are modified and yet the SH groups of Cys and the OH of Tyr, Ser, Thr, and carbohydrate residues are also readily acylated[34,117,118] and a change in the properties of the acylated proteins can be caused by the modification of all the groups indicated and not only the amino-groups. When account is taken of this factor, the literature data might be interpreted somewhat differently. The influence of the degree of modification on their stability has been studied only for a small number of proteins and enzymes (Table 1). Some of these studies must be considered in greater detail.

Interesting results for the influence of the degree of modification on the structure and stability have been obtained for cytochrome *c*—a protein with a known tertiary structure. The stability of cytochrome *c* is determined by the stability of its conformation with a closed structure of the haem crevice[29,101] and is determined by two bonds: the salt bond between Lys (13) and Glu (90) "on top" of the crevice and the hydrogen bond between Lys (79) and the carbonyl residue (47) at the "bottom" of the crevice. The modification of Lys (13) results in a sharp broadening of the crevice and the destabilisation of the protein (k_{in} increases in the temperature range 25–60 °C). The modification of two Lys residues remote from the "bottom" and the "top" of the haem crevice does not influence the conformation and stability of the protein. The two NH_3^+ groups of Lys on the frontal surface of the molecule destabilise the latter as a consequence of electrostatic repulsion and the reversal of their charge therefore stabilises cytochrome *c* as a consequence of the diminution of the repulsion or the formation of new surface salt bonds. The modification of the three Lys residues located "on top" of the crevice in the region of the protein stabilising the Lys (13)–Glu (90) salt bond somewhat diminishes its stability as a consequence of the competition between the negatively charged carboxydinitrophenyl residues introduced and Glu (90) for Lys (13).[29]

With increase of the degree of modification of the amino-groups in bacterial α-amylase by *p*-nitrophenyl acetate to 10.5 groups at 75 °C, the thermal stability improves.[110] The temperature variations of k_{in} for α-amylases with different degrees of modification intersect in one point at 67 °C and have $E_a(\Delta H^{\neq})$ smaller than the native enzyme and to a greater extent the greater the degree of modification.[111] This means that, at temperatures below 67 °C, the thermal stability of the acetylated α-amylase is lower than that of the native enzyme and to a greater extent the higher the degree of modification. Above 67 °C the opposite relation between the thermal stability and the degree of modification is observed.[110] This is an example of the "high-temperature stabilisation". According to the authors,[111] the decrease of $\Delta H^{\neq}(\Delta S^{\neq})$ for the thermal inactivation process from 83 to 46 kcal mol^{-1} on modification of 11.5 Lys residues is due to a change in the conformation of the enzyme to give a partly uncoiled activated state.

We have shown that the "low-temperature stabilisation" of horseradish peroxidase is observed after the modification of Lys residues by trinitrobenzenesulphonic acid (TNBSA) or by oenanthic anhydride.[108] The Arrhenius plots for the $\lg k_{in}$ for the native enzyme and peroxidases with different numbers of modified Lys residues intersect, as for α-amylase, at the isokinetic point (75 °C). The modification of two amino-groups hardly alters $\Delta H^{\neq}$ and $\Delta S^{\neq}$ for the thermal inactivation of the enzyme. For peroxidase with 3–4 modified groups, $\Delta H^{\neq}$ and $\Delta S^{\neq}$ are much greater than for the native enzyme and increase with increasing degree of modification (Fig.8). The stability of peroxidase modified in this way is many times greater than that of the native enzyme at all temperatures below 75 °C and the difference between their stabilities is greater the lower the denaturation temperature. For example, at 50 °C, the modified enzyme is ~100 times more stable than the native enzyme. The modification of all six Lys residues present in peroxidase diminishes $\Delta H^{\neq}$ and $\Delta S^{\neq}$ for thermal inactivation and hence the thermal stability of the enzyme (Fig.8). Thus, we have been able to isolate three types of Lys residues in peroxidase in terms of their accessibility and role in the stabilisation of the globule: two residues on the surface of the enzyme, whose modification does not alter the thermal stability regardless of the nature of the modifying agent (groups of type I); two residues whose modification stabilises the enzyme and the stabilisation effect depends on the nature of the residues introduced (groups of type II); two residues located within the globule whose modification destabilises peroxidase (groups of type III). The increase of thermal stability after the modification of 3–4 Lys residues is correlated with the changes in the circular dichroism spectra of peroxidase indicating an increase of the strength of the haem-protein bond after modification.[108,109] This relation can be understood because the rate-limiting stage in the peroxidase thermal inactivation process is the dissociation of the haem–protein complex with subsequent denaturation and aggregation of the apoprotein.

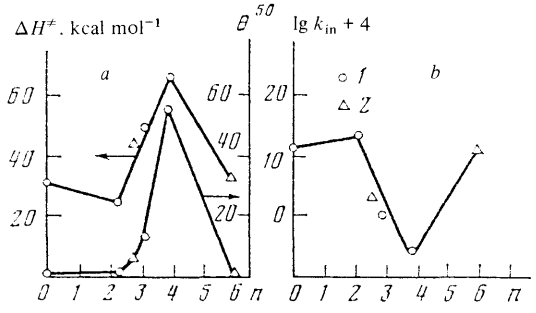


Figure 8. The influence of the number *n* of modified ε-NH_2 Lys groups in peroxidase on $\Delta H^{\neq}$ for irreversible thermal inactivation and on the stabilisation effect ($\theta = k_{in,nat}/k_{in,mod}$) at 50 °C (*a*) and on $\lg k_{in}$ at 50 °C (*b*) (pH 7.0); the peroxidase was modified with oenanthic anhydride (*1*) and TNBS (line *2*).[121]

We have shown[34] that the acetylation of peroxidase entails the modification at 0–22 °C, together with that of the Lys residues, also of the surface OH groups (up to 30 groups) of Tyr, Ser, Thr, and the carbohydrate component of the enzyme, which belong to type I: regardless of its extent, their modification does not influence the thermal stability of the enzyme over the entire experimental temperature range (60–80 °C).[34] With increase of temperature (40 °C), the internal groups are acylated in addition to the three surface NH_2 and OH groups (groups of type I and II): one OH group of Tyr and the OH groups of other residues (groups of

type III). The stability of peroxidase then diminishes by a factor of ~8 at 50 °C compared with the native protein and by a factor of 100 compared with the peroxidase having three acylated amino-groups. The observed destabilisation effect is due exclusively to the modification of the internal OH groups of the enzyme.[34] After the removal of the modification of these groups with the aid of hydroxylamine, the thermal stability of the enzyme again rises sharply (by a factor of 100), i.e. becomes the same as that of the peroxidase with three modified amino-groups (Fig.8). The one internal OH group of Tyr is particularly important for the stability of native peroxidase.

The virtually complete modification of the amino-groups of α-chymotrypsin by succinic anhydride causes little alteration to the structure of the molecule (as can be seen from absorption and circular dichroism spectra) and to its kinetic properties.[115] Following the alkylation of 60% of its amino-group by acrolein and succinylation, the thermal stability of chymotrypsin increases insignificantly. For a degree of modification of 60–95%, the thermal stability of the enzyme increases sharply (by factors of 20 and 100 respectively) and after the complete substitution of the amino-groups it falls to the level for the main enzyme.[116] The authors introduced the concept that the enzyme contains several "key" groups whose modification sharply increases its thermal stability.

Thus the structure and stability of proteins and enzymes depend significantly on the degree of modification of their functional groups. For a large number of other proteins, the dependence of the thermal stability on the degree of modification also passes through a maximum (Table 1): for ribonuclease,[102,105,106] glucoamylase[107] and guanidinated lactate dehydrogenase.[113,114] Groups of types I, II, and III can be detected in virtually every protein.

Local chemical modification. Like point mutations, such modification makes it possible to discover the role of individual aminoacid residues in the stabilisation of the native conformation of the protein. On transamination, the N-terminal amino-group of the main pancreatic inhibitor of trypsin is replaced by the keto-group, which entails the removal of one salt or hydrogen bond. In the presence of 6 M guanidine hydrochloride, T_{den} diminishes after modification from 75° to 68 °C, while at pH 1.0 neither the native nor the modified protein are denatured up to 82 °C. Consequently, the $NH_3^+ \ldots COO^-$ salt bond stabilises the inhibitor at neutral pH but not at acid pH, where the COOH group is protonated. The authors believe that one salt bond with an energy of ~1 kcal mol^{-1} can play an important role in the stabilisation of the native conformation of the inhibitor.[122]

Three other local modifications have also been carried out in the pancreatic inhibitor: (1) one S–S bond was reduced; (2) one Lys–Ala peptide bond was cleaved; (3) the Ala (16) and Arg (17) residues were removed from the active centre. Only local changes were then observed in the conformation near the region of the modifications, while the overall conformations of the modified molecules corresponded to the structure of the native inhibitor. On the other hand, the stability of the globular conformation of the modified protein diminished appreciably and the exchange of the internal amide protons of the protein with the solvent became faster.[123]

After the reduction of the S(14)–S(38) bond [compound (I)], the SH groups formed in the inhibitor were modified by means of charged residues: with iodo acetamide [a neutral group was introduced—compound (II)], with iodoacetic acid [a negatively charged group was introduced—compound (III)]; with ethyleneimine [a positively charged group was introduced—compound (IV)]. The three-dimensional structures of the reduced compound (I) and the three modified proteins proved to be almost identical, but T_{den} was altered: T_{den} = 76 °C for compounds (I) and (II), T_{den} = 79 °C for compound (III), and T_{den} = 70 °C for compound (IV). Thus the maximum stability of the reduced inhibitor was attained when the SH groups were modified with negatively charged groups, because the S–S bond cleaved is located in the positively charged cluster made up of Lys and Arg residues and the groups introduced form with the latter new ion pairs which stabilise the protein. At the same time, the introduction of a positively charged group destabilised the inhibitor for the same reason.[124]

The local chemical modification clearly demonstrates the fact that the modification of the groups involved in protein stabilising interactions diminishes the stability of the protein. An increase of the stability of the protein after modification is observed only on formation of new additional bonds, particularly salt bonds.

(b) The influence of the nature of the residues introduced on modification. The nature of the modifying agent has an appreciable influence on the stability of the macromolecules, particularly in the modification of groups of type II. The possible instances of modification can be divided into several groups: (1) modification with retention of the charge of the modified groups; (2) modification of uncharged residues by uncharged agents; (3) modification involving a change in the charge of the modified groups; (4) modification with change in the hydrophobic properties of the residues introduced.

Modification with retention of the charge of the modified groups. Mainly three modification reactions are used to modify the Lys residues with retention of their positive charge. Amidination with the aid of iminoesters:

$$E{-}NH_3^+ + R'{-}O{-}C(=NH_2^+)R \rightarrow E{-}NH{-}C(=NH_2^+)R \quad (1)$$

Guanidination with *O*-methylisourea:

$$E{-}NH_3^+ + CH_3O{-}C(=NH)NH_2 \rightarrow E{-}NH{-}C(=NH_2^+)NH_2 \quad (2)$$

Modification with aldehydes followed by reduction of the Schiff bases formed:

$$E{-}NH_3^+ + RCHO \rightarrow E{-}N{=}CH{-}R \xrightarrow{NaBH_4} E{-}NH_2^+{-}CH_2{-}R \quad (3)$$

In the first two cases arginine-like groups are introduced into the protein and the p*K* of the Lys residues increases from 10.5 to 12.5 after modification.[125] In the third case the primary amino-group is replaced by a secondary amino-group whose p*K* is somewhat reduced.

We may recall that an increased content of Arg residues compared with Lys residues is characteristic of many t-enzymes (Section II). For example, for less stable proteinases (pig chymotrypsin and trypsin), the Arg : Lys ratios are 4 : 14 and 2 : 14 respectively, while for the more stable enzyme elastase the ratio is 12 : 3.[126] It is therefore logical to expect an increase of the stability of the proteins and enzymes after the guanidination of the Lys residues. Indeed, after the modification of the Lys residues in seven proteins of six different classes with *O*-methylisourea, their stability increases (ΔG for the internal and external tritium exchange increases). This is due to the decrease of the conformational mobility of the local segments of the protein structure.[126]

The guanidination of 15 Lys residues in lactate dehydrogenase does not alter appreciably the protein conformation but greatly increases the thermal stability of the enzyme, which the authors believe to be caused by the formation of the $COO^- \ldots NH_2{=}C(NH_2)E$ salt linkages both within the subunits and between them.[113] A somewhat smaller stabilisation

effect has been observed on acetamidination of the same enzyme.[127] On the other hand, the modification of lactate dehydrogenase with formaldehyde followed by the reduction of the azomethine bonds decreased its thermal stability and even induced partial dissociation of the tetramer.[128] Thus the important role of salt bonds in the stabilisation of the protein has been demonstrated in relation to lactate dehydrogenase: the greater the charge on the modified Lys residues, the stronger the electrostatic interactions stabilising the macromolecule.

The acetamidination of a number of other enzymes (thermophilic glutamine synthetase[129] and peroxidase[34]) also increased their thermal stability and only local changes in the protein conformation were then observed. The increase of the stability of trypsin after the acetamidination of Lys was explained, in particular, by the decrease of its autolysis.[130]

On the other hand, the excess guanidination of proteins by converting their COOH groups into amino-groups with subsequent modification using *O*-methylisourea should apparently lead to their destabilisation as a consequence of the electrostatic repulsion of a large number of positively charged groups, as has been shown for chymotrypsinogen.[131]

Thus the amidination and guanidination of proteins, in which their physicochemical properties are fully retained, can serve as a simple and convenient general method for their stabilisation. The stabilisation mechanism apparently consists in the fact that the arginine-like residues are more hydrophilic than Lys, which reduces the possibility of finding them within the hydrophobic nucleus of the molecule compared with Lys. The arginine-like residues increase the rigidity of the protein globule and, having a higher p*K* than Lys, form stronger salt bonds in the protein.[126]

Less unambiguous results have been obtained in the study of the stability of proteins in which the Lys residues were subjected to the reductive alkylation [reaction (3)].

Thus modification of aminogroups with propionaldehyde or glutaraldehyde decreased the thermal stability of chymotrypsin at 60 °C by a factor of 1.5–3 depending on the pH, while modification with acrolein increased its thermal stability by a factor of 100 under the same conditions.[116,132] Modification with glutaraldehyde decreased the thermal stability of trypsin[133] but increased that of glycogen phosphorylase b [134] and β-glucosidase.[135] Unfortunately, the data for the stability of proteins modified by glutaraldehyde are difficult to interpret owing to the complexity and ambiguity of the processes occurring during modification.[88]

In the general case one should note that the positive charge on the amino-groups of proteins is retained during their alkylation, but hydrophobic residues are introduced whose presence on the surface of the macromolecule is frequently inconvenient. After modification, the capacity of the amino-group for the formation of salt bonds also diminishes. These are apparently in fact the main causes of the decrease of the thermal stability of the majority of proteins and enzymes after reductive alkylation.

Modification of the uncharged residues with uncharged agents. In this case one is dealing primarily with the modification of polar OH groups in the Ser, Thr, and Try residues, as well as the residues of aromatic aminoacids and the carbohydrate groups in glycoproteins. The main reactions used for this purpose were (1) acylation with monocarboxylic acid anhydrides, (2) oxidation of carbohydrate residues, and (3) nitration or iodination of Tyr or Trp residues.

The acylation of up to 30 surface OH groups by monocarboxylic acid anhydrides in horseradish peroxidase does not influence its thermal stability.[34]

Many plant proteins and enzymes contain covalently bound carbohydrate residues which form a polar "coat" round the macromolecules protecting them from proteolysis and stabilising their tertiary structure.[136] The oxidation of carbohydrate residues by sodium periodate or their removal with the aid of enzymes therefore as a rule diminishes the thermal stability of glycoproteins: invertase,[136] cellulase from thermophilic fungi,[77] t-glucosidase from *Mucor miehei* UN-10,[137] and catalase.[138] As was to be expected, the destabilisation effect depends on the degree of modification of the carbohydrate residues. For example, the deglycosylation of 20% of the carbohydrate residues in the catalase of *A.niger* does not influence the stability of the enzyme while after the oxidation of 40% of the carbohydrate residues, $T_{1/2}$† diminishes after 10 min from 75° to 72 °C.[138] The destabilisation effect for glucose oxidase, 50% of the carbohydrate residues of which are oxidised by periodate, is observed only in the presence of denaturing agents: sodium dodecylsulphate or urea. In this case $\Delta H^{\neq}$ for the enzyme diminishes by a factor of 1.3.[139,140] In certain cases, for example, for horseradish peroxidase, the oxidation of carbohydrate residues does not alter the thermal stability of the enzyme.[141]

On the other hand, the covalent addition of polysaccharides to proteins and enzymes as a rule increases their thermal and pH stability as well as their resistance to proteolysis.[2,7] The cause of the universal stabilising action of polyols bound covalently to the protein has become clear in the study of the influence of added sucrose and other polyols on the stability of proteins in solution. It has been established that sugar solutions are a less favourable solvent than water for aromatic and aliphatic aminoacids.[142] Sugars increase the surface tension of water and the degree of structure formation in it near the surface of aminoacid residues of the protein to a greater extent the larger the number of equatorial OH groups in their molecule. This makes the uncoiled (denatured) conformation of the macromolecule thermodynamically less favourable and shifts the equilibrium between the native and denatured protein towards the former, which increases ΔG_{den}.[143] Thus the stabilisation of proteins in sugar solutions or after the covalent binding of sugars is due to the enhancement of the intraglobular hydrophobic interactions. The stabilising action of added glycerol involves a different mechanism: glycerol reduces the surface tension of water and interacts mainly with the polar groups on the surface of the protein molecule.[143] An increase of the glycerol concentration in the protein solution diminishes $\Delta H^{\neq}_{den}$ (and $\Delta S^{\neq}_{den}$) for cytochrome P-450.[144,145]

The nitration of thermolysin with tetranitromethane decreases by a factor of two its activity and thermal stability, while the percentage content of the α-helix remains unaltered. It has been shown that the Tyr and Trp residues are modified in the enzyme, being probably present in the hydrophobic region of the molecule. For this reason, their modification following the introduction of the nitro-group destabilises the enzyme.[146]

Modification with alteration of the charge on the modified groups. The change in the charge (its removal or reversal) of the functional groups of the protein takes pldce on modification of the charged Lys, Arg, Glu, and Asp residues and on acylation of the polar Ser, Tyr, and Thr residues with dicarboxylic acid anhydrides. The modification which alters the sign of the modified group makes it possible to discover the influence of both the overall charge of the molecule and of the local electrostatic interaction on the structure and stability of the protein. The charged and polar residues are located as a rule on the surface of the protein globule and can be involved in local interactions, stabilising or

† $T_{1/2}$ is the temperature at which 50% of the enzyme is inactivated during a specific selected time interval.

destabilising the native conformation of the molecule. Since salt bonds both within the globule and near the surface of the macromolecule play a significant role in its stabilisation, their formation or rupture as a result of modification should be markedly reflected in the stability. The majority of studies of the influence of modification on the stability of proteins and enzymes have been devoted to the acylation of their amino-groups, whose positive charge is removed or reversed. Such modification can also involve the SH and OH groups.[118]

One of the first studies dealt with the stability of acylated trypsin.[147–149] Admittedly, the data obtained are not easy to interpret unambiguously since the blocking of the Lys amino-groups in trypsin diminishes autolysis apart from anything else, which increases the stability of the enzyme.[147] For example, acetyltrypsin is more stable than the native enzyme in an alkaline medium at 25 °C, but is less stable in an acid medium. Its thermal stability is also lower than that of the native enzyme. Modification of trypsin by various monocarboxylic and dicarboxylic anhydrides showed that, for the same degree of modification of the amino-groups at 100 °C and pH 5.5, the monocarboxylic acid derivatives are the least stable. Their stability increases in the sequence acetyltrypsin, propionyltrypsin, butyryltrypsin, diethylacetyltrypsin, phthalyltrypsin $\ll$ β-sulphopropionyltrypsin < itaconyltrypsin < native trypsin < succinyltrypsin < citraconyltrypsin.[148,150] Succinyltrypsin is more stable than the native enzyme also at pH > 5. According to the authors, the electrostatic repulsion of the equally charged COO^- groups introduced is a factor stabilising the enzyme. On the other hand, acylation has little influence on the denaturation of trypsin by urea.[149] Thus, the removal of the charge from the amino-group diminishes the thermal stability of the protein, while its replacement by a negative charge increases somewhat the thermal stability.

The acetylation of alkaline phosphatase increases its thermal stability at 30 °C and at pH from 4.0 to 11.0. The authors believe that the protecting effect of acetylation and alkaline pH is common to many proteins, which is surprising in view of the fact that acetylation greatly increases the overall negative charge of the enzyme molecule, which is in any case high at alkaline pH.[151] Acylation increases the stability of thermolysin,[152] but diminishes the thermal stability of subtilisin and ferredoxin.[153,154]

Invertase from baker's yeast has been modified by citraconic anhydride, acetaminoester, and carbodiimide in the presence of ethylenediamine. The reversal of the sign of the charge on the NH_3^+ and COO^- groups significantly destabilised the enzyme (65 °C), while modification with retention of the positive charge on the amino-groups did not affect the stability. The authors conclude that the native structure of invertase is stabilised by the $NH_3^+ \ldots COO^-$ salt bonds whose rupture on modification of either amino- or carboxy-groups destabilises the enzyme.[155]

For the same degree of modification of the NH_2 groups of α-chymotrypsin, the greatest stabilising effect was shown by the modification of the enzyme with acrolein (the positive charge is retained).[116,153] The weakest stabilisation was observed on acylation with acetic, succinic, and citraconic anhydrides, possibly because, in contrast to the first modification, functional groups other than the NH_2 group were acylated in the enzyme and their modification might have destabilised the latter.

The thermal stability of pepsin increases at 30 °C by a factor of 10–20 (depending on the excess of the nucleophile employed) when its COOH groups are modified with the aid of carbodiimide and methyl glycinate. The stabilisation of the enzyme is due to the decrease of the overall negative charge after modification, because native pepsin is rapidly denatured at pH 7.0 as a consequence of the electrostatic repulsion of the large number of negatively charged COOH groups.[156]

The removal of the charge, as a result of the modification, from the lysine (13) residue, which forms the main salt bond maintaining the closed structure of the haem crevice in cytochrome *c*, fully destabilises the protein. On the other hand, modification involving the removal of the charge from Lys residues destabilising the native protein results in a further closure of the haem crevice.[29]

For each degree of modification of the amino-groups of pig heart lactate dehydrogenase, the neutralisation of the positive charge after their modification with methyl 4-hydroxy-3-nitrobenzimidate (the aminophenoxide form) (Table 1) as well as acetylation greatly destabilise the enzyme. On the other hand, the aminophenol form of the modified amino-groups (the positive charge is retained) increases the stability of the enzyme like its acetamidination and guanidination.[114]

The removal of the positive charge from virtually all the amino-groups of malate dehydrogenase after acetylation or modification with methyl 4-hydroxy-3-nitrobenzimidate diminishes $T_{1/2}$ for the enzyme (after 20 min) by 16–17° and 7 °C respectively. The decrease of stability is apparently caused by the breakdown of intersubunit interactions in which the Lys residues are involved.[157]

The influence of the charge on the amino-group on the thermal stability of horseradish peroxidase has been determined by comparing the stabilities of the enzyme whose four amino-groups have been modified with acetimidate and butyric, succinic, and maleic anhydrides.[34,158] Whilst retaining the positive charge on the amino-group, acetamidination adds the small methyl residue and increases the length of the "foot" at the positive charge by ~1.5 Å. After modification with butyric anhydride, the positive charge of the amino-group disappears, while modification with succinic and maleic anhydrides results in the introduction of a negative charge. All three types of residues introduced have a similar structure. After acetamidination, $\Delta H^{\neq}$ for the thermal inactivation of peroxidase increases and hence the thermal stability at temperatures below 75 °C increases somewhat; it increases by a factor of 2.5 after modification with butyric anhydride and by a factor of 3 after modification with succinic and maleic anhydrides. This can be explained by the decrease of the local and not the total positive charge of the macromolecule. It may be that the two amino-groups of the peroxidase are located in the hydrophobic cavity or in a positively charged cluster of groups and destabilise the native structure of the enzyme. Their modification with removal of the positive charge or the introduction of a negative charge promotes the formation of additional salt bonds in the molecule, which increase the strength of the haem–protein bond, i.e. the thermal stability of the enzyme. Even a slight increase of the length of the "foot" at the positive charge of the amino-group after acetamidination increases somewhat the thermal stability of peroxidase. On the other hand, the appearance of a negative charge on a large number of surface OH groups of peroxidase on succinylation does not affect the thermal stability of the enzyme.[34]

Thus data for the modification of the functional groups involving a change in the charge demonstrate the different roles of the charged aminoacid residues in the stabilisation of the native structure of enzymes. In trypsin, subtilisin, invertase, and lactate and malate dehydrogenases, the amino-groups of the Lys residues are involved in the formation of intramolecular salt bonds or in maintaining the three-dimensional structure of the native enzyme. The removal of the charge from these groups destabilises the enzymes. On the other hand, in a number of enzymes (peroxidase,

cytochrome *c*, chymotrypsin, pepsin, etc., amino- or carboxy-groups destabilising the native protein as a consequence of unfavourable electrostatic interactions have been discovered. Their modification, involving a change in the charge, makes it possible to achieve large stabilisation effects (by factors up to 100 for peroxidase and chymotrypsin).

The influence of the hydrophobic nature of the residues introduced. For the same degree of modification and using modifying agents with similar chemical structures, the influence of the hydrophobic properties of the residues introduced on the stability of proteins and enzymes has been analysed in a number of investigations. In order to elucidate this influence in a "pure" form, it is more correct to compare with one another the properties of members of a series of modified enzymes which differ only by the length of the hydrocarbon residues incorporated in them and not the properties of the native and modified enzymes. Although the number of studies devoted to this question is small, they make it possible to elucidate the overall dependence of the thermal stability of enzymes on the hydrophobic properties of the groups introduced, which is represented by a curve with a maximum (Fig.9). Such relations have been obtained for phosphorylase *b*,[159] α-amylase,[110] horseradish peroxidase,[86, 108] and thermolysin,[152] the accessible amino-groups of which were alkylated or acylated by a series of agents with different hydrophobic properties. In all cases an increase of the thermal stability of the enzymes was observed after their modification [derivatives of the type $E(NHR)_n$ or $E(NHCOR)_n$, where n is constant for each enzyme and R varies].

In phosphorylase *b* ~10% of the Lys residues were modified by aldehydes derived from monocarboxylic acids (from C_2 to C_7) and the azomethine bonds were reduced with $NaBH_4$ (the positive charge on the amino-group is retained under these conditions). Thermal stability at 0° and 50 °C increased with increase of the length of the hydrocarbon residue R in a series of derivatives $E(NHR)_n$ from C_2 to C_5 and then diminished (Fig.9). When the polar glyceraldehyde was used, the stabilising effect was hardly observed.[159]

For bacterial α-amylase, whose amino-groups were modified by the *p*-nitrophenyl esters of monocarboxylic acids (C_1, C_7, and C_{15}), the greatest stabilisation effect was observed after the introduction of the CH_3 group, while for the C_7 and C_{15} derivatives the stability diminished with increase of the length of the hydrocarbon residue.[110] Since data for other derivatives in this series are lacking, it is not known what should be the length of the hydrocarbon residue to give the optimum stabilisation. Possibly it is in the range from C_1 to C_7.

In a series of preparations of peroxidase acylated by monocarboxylic acid anhydrides [the $E(NHCOR)_4$ derivatives, where $R = C_nH_{2n+1}$] the thermal stability $\Delta H^{\neq}_{den}$ increases with increase of the size of the hydrocarbon residue R introduced from C_1 to C_3, while on further increase of R from C_3 to C_6 $\Delta H^{\neq}_{den}$ diminishes (Fig.9). Each succeeding member of the series differs from the preceding one only by the addition of four CH_2 groups. The increment $\Delta(\Delta H^{\neq}_{den})$ per methylene group is 1.5–2 kcal mol^{-1}.[86,108]

The hydrophobic properties of the residues introduced can be varied not only by altering their size but also be replacing the hydrophilic groups in R by hydrophobic groups. For example, the seven accessible amino-groups of thermolysin have been modified by a series of derivatives of normal carboxylic acids, the $[E(NHCOR)_7$ derivatives, where $R = C_{n+8}H_{2n+17}.O_{5-n}]$. The enhancement of the hydrophobic properties in this series was achieved by replacing the ester group by the methylene group. The methylene group enhances the hydrophobic properties of the aminoacid side residue by ~0.7 kcal mol^{-1}, while the ester group diminishes it by ~0.7 kcal mol^{-1}. Thus the replacement of one ester group by the methylene group enhances the hydrophobic properties of the residue by ~1.4 kcal mol^{-1}. The hydrophobic properties of the residues become more pronounced when n is varied from 1 to 5. For n = 4 and 5, insoluble and relatively inactive derivatives are obtained and these were not investigated further. The thermal stability of other derivatives with n = 1–3 varied at 80° and 90 °C as shown in Fig.9, i.e. it passed through a maximum at n = 1.[152]

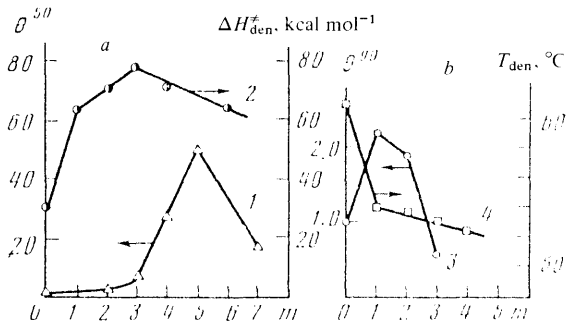


Figure 9. Dependence of the stability of enzymes on the hydrophobic properties of the residues introduced on modification; n is the number of C atoms in the residue for modified derivatives of type $ENHC_nH_{2n+1}$ for phosphorylase *b* (line *1a*),[159] $E(NHCOC_nH_{2n+1})_4$ for horseradish peroxidase (line *2a*),[86,108] $E(NHCOC_{n+8}H_{2n+17}O_{5-n})_7$ for thermolysin (line *3b*)[152] and $E(NHCOC_nH_{2n+1})_{12}$ for ribonuclease (line *4b*);[162] the stabilisation effect ($\theta = k_{in,nat}/k_{in,mod}$) at 50 °C (line *1a*) and 90 °C (line *3b*), the enthalpy of the denaturation process $\Delta H^{\neq}_{den}$ (line *2a*), and the average denaturation temperature T_{den} (line *4b*) have been used as measures of stability.

There is no doubt that the hydrophilic nature of the surface of enzymes is important for their solubility and for the manifestation of their catalytic activity in aqueous solutions. In all the cases considered, the greatest stabilisation of proteins is attained for a definite and comparatively small length of the hydrocarbon residue R: at C_3 for peroxidase and at C_5 for phosphorylase (Fig.9). The interaction of the hydrocarbon residues introduced, having the optimum length for each enzyme, with the nearest hydrophobic groups of the enzyme leads to the greatest stabilisation of the tertiary structure of the protein. On the other hand, when the size of R begins to exceed the size of the hydrophobic cavity in the enzyme molecule, the partial exposure of the R residue to the solution is thermodynamically unfavourable and results in a diminuation of stabilisation effects ($\Delta H^{\neq}_{den}$ decreases) and in certain cases leads to destabilisation. Analogous stabilisation effects for different proteins have been noted when the latter solubilised small amounts of hydrocarbons.[160,161] The dependence of ΔH for thermal denaturation of proteins in the presence of hydrocarbons also passes through a maximum and the increment $\Delta(\Delta H)$ per CH_2 group of the solubilised hydrocarbon is ~2 kcal mol^{-1}.

The dependence of the thermal stability on the hydrophobic properties of the residues introduced passes through a maximum i.e. the introduction of hydrophobic residues of specific dimensions into the protein can stabilise the latter only when the modification does not by itself diminish the stability of the

enzyme (or increases it). In those cases where the modification destabilises the enzyme, an increase in the length of the hydrophobic residues leads to a progressive albeit not very great diminution of the stability of the macromolecule. For example, the thermal stability of ribonuclease modified with different succinimides [the $E(NHCOR)_{11}$ derivatives, where $R = C_nH_{2n+1}$] decreases sharply when the C_1 derivative is used. The increase in the size of the residue R from C_1 to C_4 entails only a slight further decrease of the thermal stability of the enzyme (Fig.9). According to the authors, the destabilisation of the enzyme after modification is similar to the denaturation of ribonuclease by alcohols.[162]

The introduction of very bulky hydrophobic residues decreases sharply the stability of the enzyme in all cases regardless of whether the given modification itself stabilises or destabilises the enzyme. Despite the fact that acetamidination of lactate dehydrogenase stabilises the enzyme,[127] when a very bulky hydrophobic agent is employed, namely methyl *N*-(2,4-dinitrophenyl)-ε-aminocaproimidate, the thermal stability of the modified enzyme becomes smaller than that of the native enzyme. This is due to the marked enhancement of the hydrophobic properties of the enzyme globule and the rupture of the bonds between the Lys residues and other residues, which have a favourable effect on the stability, as a consequence of the introduction of large and bulky substituents despite their positive charge.[163] Thus the introduction of large hydrophobic residues can abolish the stabilising effect of the modification.

The observed change in the thermal stability of enzymes after modification is not always associated with the influence of the hydrophobic properties of the residues introduced. For example, the acylation of α-amylase by various *N*-hydroxysuccinimides and 1-hydroxybenzotriazole increases the thermal stability of the enzyme at 60 °C in the sequence native < α-amylase ($E - NH_2$) ≤ dimethylcarbamoylated α-amylase [$E - NHCON(CH_3)_2$], ethoxyformylated α-amylase ($E - NHCOOC_2H_5$) < formylated α-amylase ($E - NHCHO$) < acetylated α-amylase ($E - NHCOCH_3$) < dimethylpropanoylated α-amylase [$E - NHCOC(CH_3)_3$]. The increase of thermal stability in this sequence is correlated with the weakening of the electron-donating capacity of the amide nitrogen and not with the change in the hydrophobic properties of the residue introduced.[164]

In conclusion one should note that the "pure" contribution of the hydrophobic properties of the residues introduced to the observed stabilisation–destabilisation effect for the modified enzyme is extremely small compared with the effects of the local or overall changes in the charge of the molecule. For example, the acetylation of the four amino-groups of peroxidase increases $\Delta H^{\neq}$ for thermal inactivation by a factor of two (from 30 to 65 kcal mol^{-1}), while the additional contribution to the stability (to the increase of $\Delta H^{\neq}$) by the four CH_2 groups introduced does not exceed 6–8 kcal mol^{-1}.

2. Physicochemical Characteristics of the Change in the Stability of Enzymes after Their Chemical Modification and Immobilisation

The results in the previous Section clearly demonstrate that the chemical modification of the functional groups of enzymes not involved directly in the active centre by monofunctional agents can exert a strong influence on their structure and stability. The studies considered confirm the hypothesis of the existence in proteins and enzymes of three types of functional groups whose modification has different effects on their structure and stability.

The change in the thermal stability of enzymes as a result of their chemical modification is a consequence of local or overall changes in the three-dimensional structure of the protein—in the conformational mobility of individual fragments of the polypeptide chain. Despite the considerable alteration of the stability of the protein, the variations in its structure can frequently be so small that they cannot always be detected by analysing the absorption, fluorescence, or circular dichroism spectra.[127,129,165,166] The stability of proteins is therefore the most sensitive test for any change in their structure.[7,86]

The relation between the changes in the structure and thermal stability after chemical modification and the contributions of different types of groups in the protein have been clearly traced only for several enzymes, for example, for horseradish peroxidase.[34,86,108,109] Modification of groups of type I (up to 30 surface OH groups and two ε-NH_2 Lys groups) does not affect the structure and stability of the peroxidase regardless of the degree of modification. The modification of the other two ε-NH_2 Lys groups (groups of type II) with removal of the positive charge diminishes the conformational mobility of the protein in the haem-binding region, which is reflected in the circular dichroism spectra of the peroxidase. As a consequence of this, there is an appreciable increase of the stability of the enzyme. Finally, the modification of the internal OH and ε-NH_2 groups (groups of type III) reduces the intensity of the haem and aromatic aminoacid bands in the circular dichroism spectra, which indicates a weakening of the heam–protein bond and the approach of the structure of the enzyme to that which obtains after thermal denaturation. The thermal stability of such an enzyme is lower than that of the native enzyme.[108]

Analysis of the literature data shows that, as in the case of the proteins and enzymes from thermophilic sources (Section II), the main cause of the increase of the stability of the modified enzyme is the formation of additional bonds (salt and hydrogen bonds) within and on the surface of the molecule, the enhancement of the hydrophobic interactions in the protein, and also the strengthening of the bonds between the protein and the prosthetic groups, metal ions, etc.

Analysis of the temperature variations of k_{in} for the native and modified (including immobilised) enzymes shows that the stabilisation effect varies with temperature. The term "stabilisation" is significant only for a specific temperature range. "Absolute stabilisation", i.e. the case where the ratio of the constants k_{in} for the native and immobilised enzymes is the same over a fairly long temperature range, is hardly possible. It will be possible only when the $\Delta H^{\neq}_{den}$ for the native and modified enzymes are the same and $\Delta S^{\neq}_{den}$ for the modified enzyme is lower than for the native enzyme by an appreciable amount. However, in all cases known from the literature ΔH_{den} and ΔS_{den} (or $\Delta H^{\neq}_{den}$ and ΔS_{den}) for the enzyme thermal denaturation process vary in parallel after the chemical modification or immobilisation of the enzymes. This is apparently to be explained by the existence of a compensation effect for protein systems in solution, which is most likely to be determined by the properties of water.[167] This effect consists in the fact that, despite the large changes in ΔH_{den} (or $\Delta H^{\neq}_{den}$), the values of ΔG_{den} (or $\Delta G^{\neq}_{den}$) change little in most cases after the chemical modification or immobilisation of enzymes owing to the compensating effect of the entropy term $T\Delta S_{den}$. ΔG_{den} increases either because ΔH_{den} increases more than ΔS_{den} or because ΔS_{den} decreases more than does ΔH_{den}.

Fig.10 presents the experimental compensation relation between $\Delta H^{\neq}_{den}$ and $\Delta S^{\neq}_{den}$ for the thermal inactivation of a series of native and modified enzymes. The relation between $\Delta H^{\neq}_{den}$ and $\Delta S^{\neq}_{den}$ indicates the existing limits of variation of

these parameters as the nature of the modifying agent (or the carrier for immobilised enzymes) is altered or for different degrees of modification. All the points on the compensation relation lying to the left of those corresponding to the native enzymes correspond to preparations of the modified or immobilised enzymes for which $\Delta H^{\neq}_{den}$ and $\Delta S^{\neq}_{den}$ are smaller than for the native enzyme. Such preparations are more stable than the native enzyme above the isokinetic temperature ("high-temperature stabilisation"). All the preparations the points for which lie to the right of the point corresponding to the native enzyme have higher values of $\Delta H^{\neq}_{den}$ and $\Delta S^{\neq}_{den}$ than the native enzyme. These compounds are more stable than the native enzyme below the isokinetic temperature ("low-temperature stabilisation"). If the isokinetic temperatures are similar for different derivatives of the enzyme, then the modified enzymes are more stable below the isokinetic temperature the higher their values of $\Delta H^{\neq}_{den}$.

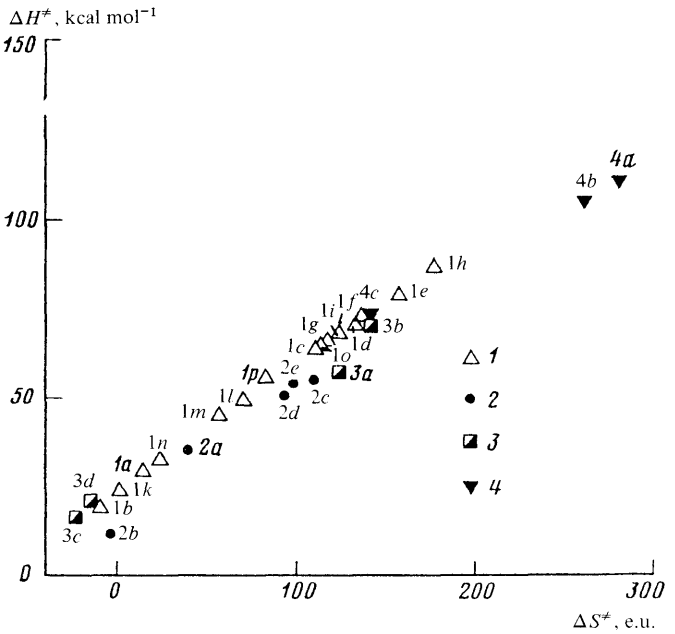


Figure 10. Compensation relation between $\Delta H^{\neq}$ and $\Delta S^{\neq}$ for the thermal inactivation of soluble (native and modified) and immobilised enzymes: 1) horseradish peroxidase: native (*a*), immobilised in polyacrylamide gel (*b*), modified (four NH_2 groups) with acetic (*c*), propionic (*d*), butyric (*e*), valeric (*f*), oenanthic (*g*), succinic (*h*), and maleic (*i*) anhydrides, modified with oenanthic anhydride [*k*) two NH_2 groups; *l*) three NH_2 groups] and trinitrobenzenesulphonic acid [*m*) three NH_2 groups; *n*) six NH_2 groups] and peroxidase–albumin conjugates [*o*) fraction I; *p*) fraction II]; 2) glow-worm luciferase: native (*a*), immobilised on ultragel (*b*), ultradex (*c*), cellophane (*d*), and Sepharose (*e*) and activated with bromocyanogen; 3) α-chymotrypsin: native (*a*), soluble conjugates with dextran (*b*), and sodium alginate (*c*), and immobilised on KM-cellulose (*d*); 4) β-galactosidase: native (*a*), and modified with a dye (*b*) and then sorbed on DEAE-Sephadex (*c*) according to different literature data.[39, 86,108,158,168,169,172,175,178]

Since the native and modified (immobilised) enzymes have different temperature variations of k_{in} (K_{den}), in the general case there is always a temperature range where the native enzyme is more stable and a range where the modified or immobilised enzyme is more stable (Fig.7). As the isokinetic point is approached, the difference between the stabilities of the native and modified enzyme diminishes, while under conditions remote from this point it increases.

Studies involving the comparison of the kinetic and thermodynamic parameters of the thermal inactivation of native and modified proteins and enzymes have shown that in many cases these parameters vary in parallel.[20,39,168] The increase or decrease of $\Delta H^{\neq}_{den}$ and $\Delta S^{\neq}_{den}$ for the thermal inactivation of enzymes after their chemical modification or immobilisation can be accounted for by a change in their thermodynamic stability and hence by a change in the relative contributions of various factors which determine the thermodynamic stability of the protein globule of the enzyme in solution.

Stabilisation of enzymes in solution after the chemical modification of their functional groups. Such stabilisation is caused as a rule by the increase of $\Delta H^{\neq}_{den}$ and $\Delta S^{\neq}_{den}$ for thermal inactivation. This is "low-temperature stabilisation" of enzymes below the isokinetic temperature (Fig.7, curves *1* and *2*), which is as a rule above 50 °C. Chemical modification of enzymes leading to an enhancement of the intramolecular interactions in the globule increases ΔG_{den} for the modified enzyme compared with the native enzyme, whereupon we have $\Delta G_{den,mod} - \Delta G_{den,nat} < 0$. The formation of additional intramolecular bonds on modification increases ΔH_{den}, while the introduction of additional residues increases ΔS_{den} (mainly as a result of an increase in conformational entropy). The greater the amount by which the increase of ΔH_{den} predominates over that of ΔS_{den}, i.e. the greater the number of additional bonds formed following the introduction of a given number of residues, the higher the isokinetic temperature for the native and modified enzymes and the wider the range of experimental temperatures where $\Delta G_{den,mod} < \Delta G_{den,nat}$, i.e. the modified enzyme is more stable than the native enzyme. The activation parameters for the thermal denaturation of the enzyme vary analogously after chemical modification. If chemical modification results in an intensification of the intramolecular interactions in the enzyme globule as a result of the formation of additional bonds, then $\Delta H^{\neq}_{den}$ and $\Delta S^{\neq}_{den}$ for the modified enzyme are greater than for the native enzyme. "Low-temperature stabilisation" obtains in this case (Fig.7, curves *1* and *2*). With increase of inactivation temperature, the intramolecular stabilising interactions are weakened and the conformational entropy $\Delta S^{\neq}_{conf}$ increases in both the native and modified enzymes (see above). As a result of this, the difference $\Delta G^{\neq}_{den,mod} - \Delta G^{\neq}_{den,nat}$ become smaller the higher the inactivation temperature and is zero at the isokinetic temperature. Above the isokinetic temperature, the native enzyme becomes more stable than the modified enzyme.

If modification resulted in the cessation of the enzyme-stabilising interactions (in particular, on modification of groups of type III), then the structure of the enzyme becomes close to that of the denatured enzyme. $\Delta H^{\neq}_{den}$ and $\Delta S^{\neq}_{den}$ diminish after modification (Fig.7, curves *1* and *3*), but, by virtue of the predominance of the decrease of $\Delta H^{\neq}_{den}$ over that of $\Delta S^{\neq}_{den}$, the isokinetic temperature is in this case high and the stability of the modified enzyme is lower than that of the native enzyme virtually over the entire experimental temperature range.

Stabilisation of enzymes after immobilisation. After multipoint interaction with the carrier (covalent and non-covalent), such stabilisation is caused as a rule by the decrease of $\Delta S^{\neq}_{den}$, which predominates over the decrease of $\Delta H^{\neq}_{den}$ for thermal inactivation. This is the "high-temperature stabilisation" of enzymes[39,169–178] (Table 2). The decrease of $\Delta H^{\neq}_{den}$ and $\Delta S^{\neq}_{den}$ (ΔH_{den} and ΔS_{den}) after the immobilisation of

enzymes can be accounted for as follows. When the enzyme interacts with the matrix (the formation of external bonds), the bonds within its molecule are affected, which is accompanied by a decrease of ΔH_{den}. By virtue of the covalent and non-covalent fixation of the polypeptide chain at several points, this is accompanied by the simultaneous decrease of ΔS_{conf}. The greater the decrease of $\Delta S(\Delta S^{\neq})$ (the greater the number of points at which the enzyme is bound to the carrier) compared with ΔH, the lower the isokinetic temperature of the native and immobilised enzymes and the greater the temperature range in which the immobilised enzyme is more stable than the native enzyme and the greater the stabilisation effect ($\Delta G_{den,imm} - \Delta G_{den,nat}$) above the isokinetic temperature (Fig.7, curves *1* and *4*). In multipoint immobilisation the protein is probably fixed in a conformation close to that of the denatured enzyme.[168,176] This may be one of the main reasons for the low activity of enzymes after their multipoint immobilisation. In the absence of external bonds between the enzyme and the carrier (when the enzyme is incorporated in a polymer gel), the thermodynamic parameters of denaturation may not change.[174]

Enzymes functioning in membrane structures in aqueous solutions can have a conformation different from the native conformation. After a successful choice of the carrier, the structure of such protein macromolecules after immobilisation may be closer to the native structure than in solution. It is to be expected that $\Delta H^{\neq}_{den}$ and $\Delta S^{\neq}_{den}$ for such an immobilised enzyme will increase. We observed this type of experimental fact, for example, for the glow-worm luciferase immobilised on polysaccharide carriers.[175] $\Delta H^{\neq}_{den}$ was 35 kcal mol^{-1} for the enzyme in solution and 54 kcal mol^{-1} for the enzyme immobilised on Sepharose.[175] Similar effects are to be expected also for other enzymes functioning in nature in an immobilised state.

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In many cases the modification of enzymes by monofunctional agents appreciably increases their resistance to temperature, extreme pH, and organic solvents. In most cases the stabilisation effect does not exceed a factor of 10–20, but in some cases it reaches a factor of 100 and more.[108,116] In

Table 2. The changes in the thermodynamic parameters of the reversible and irreversible thermal inactivation of enzymes as a result of their modification or immobilisation.

Enzyme	$\Delta H^{\neq}$, kcal mole^{-1}	$\Delta H^{\neq}$, kcal mole^{-1}	ΔS^0, e.u.	$\Delta S^{\neq}$, e.u.	Stabilisation effect θ^a	Refs.
Peroxidase						
native	—	31	—	14	1.0(56)	[86,108]
modified by monocarboxylic acid anhydrides	—	64—87	—	110—178	32—90(56)	[86,108]
incorporated in polyacrylamide gel	—	20	—	—40	0.06(56)	[169]
α-Chymotrypsin[b]						
native	—	109	—	259	1.0	[170]
incorporated in polyacrylamide gel	—	14	—	—36	120(60)[a] T_i 48°C	[170]
incorporated in polymethacrylate gel	—	34	—	21	680(60)[a] T_i 41°C	[170]
α-Chymotrypsin native						
immobilised in soluble form on acrylic acid–maleic acid co-polymer	223	165	692	435	—	[39]
	110	38	346	44	—	[39]
immobolised on poly(maleic acid)	97	61	304	117	—	[39]
Trypsin native						
immobilised on Separon	30	67	93	142	—	[171]
with glucose	10	45	30	62	—	[171]
immobolised on cellulose	—	23	—	—6	—	[171]
β-Galactosidase						
native	—	121	—	282	1.0	[172]
immobilised on DEAE-Sephadex	—	73	—	142	0.14(60)[b] T_i=70°C	[172]
Dextran saccharase[b]						
native	—	90	—	223	1.0	[173]
innobolised on aminated glass	—	25	—	6	7.5(35) T_i=29°C	[173]
Glucoamylase[b]						
native	—	99	—	228	1.0	[174]
immobilised on sulphopropyl-Sephadex	—	64	—	126	0.12(57) T_i=70°C	[174]
Glow-worm luciferase						
immobilised on ultragel	—	35	—	40	1.0	[175]
	—	12	—	—3	0.25(25)	[175]
immobilised on BrCN-Sepharose	—	54	—	100	72(25)	[175]

[a] The temperature at which the stabilisation effect has been measured is indicated in brackets (in °C).

[b] The values of $\Delta H^{\neq}$ and $\Delta S^{\neq}$ as well as the stabilisation effect θ were calculated by ourselves from the temperature variations of $\lg k_{in}$ quoted in different communications.

[c] Values calculated from our own extrapolations.

[d] T_i is the isokinetic temperature.

order to attain the maximum stabilisation effect, account must be taken of those features which have been discovered by the analysis carried out in the present review. The modification of enzymes involving the introduction of carbohydrate residues, amidination, and guanidination as a rule increases the stability of enzymes (at worst it does not diminish it). The greatest influence on stability is shown by the modification involving a change in the charge of the functional groups. In addition, one must avoid the modification of internal groups (type III) of the enzyme by employing for modification the mildest possible conditions (low temperatures and minimum concentrations of organic additives), a high degree of modification, and the introduction into the enzyme of unduly bulky hydrophobic residues.

In the development of methods for stabilisation by chemical modification, it is necessary to obtain data for the thermal stability of enzymes over a wide temperature range as a function of the degree of modification and the nature of the modified groups and the modifying agent. Such analysis makes it possible to optimise not only the conditions in the preparation of the biocatalyst but also its applications. As shown above, chemically modified soluble enzymes are usually more stable at temperatures below 50–60 °C. For experiments at higher temperatures (60–70 °C), it is more desirable to employ enzymes, immobilised on polymer matrices, which are characterised by the high-temperature stabilisation, although immobilisation is frequently accompanied by a decrease of catalytic activity, in particular owing to the approach of the structure of the enzyme to that of the denatured enzyme.

The use of several additive stabilising influences, which makes it possible to achieve the greatest stabilisation effects, is extremely promising.[34]

REFERENCES

1. G.D.Kutuzova, N.N.Ugarova, and I.V.Berezin, Bioorg.Khim., 1982, **8**, 1445.
2. R.D.Schmid, Adv.Biochem.Eng., 1979, **12**, 41.
3. N.N.Ugarova, Symposium, "Itogi Nauki i Tekhniki. Biologicheskaya Khimiya" (Advances in Science and Engineering. Biological Chemistry) (Edited by V.L.Kretovich and I.V.Berezin), Izd.VINITI, Moscow, 1978, Vol.12, p.92.
4. R.Ulbrich and A.Schellenberger, Wiss.Z.-Martin-Luther-Univ., Halle-Wittenberg, Math.-Naturwiss.reihe, 1979, **28**(3), 75.
5. V.P.Torchilin and K.Martinek, Enzyme Microb. Technol., 1979, **1**, 74.
6. P.L.Privalov, Adv.Protein Chem., 1979, **33**, 167.
7. W.Pfeil, Mol.Cell.Biochem., 1981, **40**, 3.
8. G.Nemethy and H.A.Scheraga, Quart.Rev.Biophys., 1977, **10**(3), 240.
9. M.Karplus and J.A.McCammon, CRC Crit.Rev. Biochem., 1981, **9**(4), 293.
10. D.H.Wertz and H.A.Scheraga, Macromolecules, 1978, **11**, 9.
11. H.Meirovitch and H.A.Scheraga, Macromolecules, 1980, **13**, 1406.
12. P.Manavalan and P.K.Ponnuswamy, Arch.Biochem. Biophys., 1977, **184**, 476.
13. P.K.Ponnuswamy, M.Prabhakavan, and P.Manvalan, Biochim.biophys.Acta, 1980, **623**, 301.
14. H.Meirovitch and H.A.Scheraga, Macromolecules, 1981, **14**(2), 340.
15. Sh.I.Wodak and J.Janin, Biochemistry, 1981, **20**, 6544.
16. M.Joly, in "Physical Chemistry of the Denaturation of Proteins" (Translated into Russian), Izd.Mir, Moscow, 1968, p.9.
17. V.P.Kushner, in "Konformatsionnaya Izmenchivost' i Denaturatsiya Biopolimerov" (Conformational Variability and Denaturation of Biopolymers), Izd. Nauka, Leningrad, 1977, p.6.
18. G.I.Likhtenshtein and T.V.Troshkina, Mol.Biol., 1968, **2**, 654.
19. J.Bello, J.Theoret.Biol., 1977, **68**, 139.
20. J.F.Brandts, in "The Structure and Stability of Biological Macromolecules" (Translated into Russian), Izd.Mir, Moscow, 1973, p.174.
21. P.L.Privalov and N.N.Khechinashvili, J.Molec.Biol., 1974, **86**, 665.
22. W.P.Jencks, in "Catalysis in Chemistry and Enzymology" (Translated into Russian), Izd.Mir, Moscow, 1972, p.303.
23. M.F.Perutz, Science, 1978, **201**, 1187.
24. G.I.Likhtenshtein, in "Sostoyanie i Rol' Vody v Biologicheskikh Ob'ektakh" (The State and Role of Water in Biological Objects), Izd.Nauka, Moscow, 1967, p.101.
25. J.-P.Vincent, R.Chicheportiche, and M.Lazdunski, Europ.J.Biochem., 1971, **23**, 401.
26. L.R.Brown, A.De Marco, R.Richarz, and G.Wagner, Europ.J.Biochem., 1978, **88**, 87.
27. M.Levitt and A.Warshel, Nature, 1975, **253**, 694.
28. A.A.Alhrem, M.A.Rodionov, and S.G.Galaktionov, Vestnik Akad.Nauk Beloruss.SSR, Ser.Khim., 1977, 122.
29. N.Osheroff, D.Borden, W.H.Koppenol, and E.Margoliash, J.Biol.Chem., 1980, **255**, 1689.
30. R.E.Johnson, P.Adams, and J.A.Rupley, Biochemistry, 1978, **17**, 1479.
31. M.Ottesen, Methods Biochem.Anal., 1971, **20**, 135.
32. I.V.Berezin, N.N.Ugarova, B.M.Kershengol'ts, and L.Yu.Brovko, Biokhimiya, 1975, **40**, 297.
33. V.V.Filimonov, W.Pfeil, T.N.Tsalkova, and P.L.Privalov, Biophys.Chem., 1978, **8**, 117.
34. G.D.Kutuzova and N.N.Ugarova, Bioorg.Khim., 1981, **7**(1), 75.
35. A.Cooper, Proc.Nat.Acad.Sci.USA, 1976, **73**, 2740.
36. F.R.N.Gurd and M.Rothgeb, Adv.Protein.Chem., 1979, **33**, 73.
37. I.Simon, J.Theoret.Biol., 1981, **90**, 487.
38. K.Wüthrich, G.Wagner, R.Richarz, and W.Braun, Biophys. J., 1980, **32**, 549.
39. L.Ya.Bessmertnaya, L.V.Kozlov, and V.K.Antonov, Biokhimiya, 1977, **42**, 1825.
40. J.F.Brandts, in "Thermobiology", Acad.Press, London–New York, 1967, p.25.
41. G.Lassman, J.Lasch, B.Ebert, W.Damerau, and W.Kudernatsch, Acta Biol.Med.German., 1976, **35**(3–4), 343.
42. Y.Fujita and Y.Noda, Loen Yoshishu–Tanpakushitsu Kozo Toronkai, XXX, 1979, 1. Tokyo Daigaku Rigakubu, Tokyo. Chem.Abs., 1980, **92**(9), 71335y.
43. Y.Fujita and Y.Noda, Int.J.Peptide Protein Res., 1981, **18**, 12.
44. Y.Fujita and Y.Noda, Bull.Chem.Soc.Japan, 1981, **54**, 3233.
45. C.N.Pace, Crit.Rev.Biochem., 1975, **3**(1), 1.
46. C.N.Pace, L.M.Fisher, and J.F.Cupo, Acta Biol. Med.German., 1981, **40**(10–11), 1385.
47. R.M.Daniel, D.A.Cowan, H.W.Morgan, and M.P.Curran, Biochem.J., 1982, **207**, 641.

48. G.McLendon and E.Radany, J.Biol.Chem., 1978, **253**, 6335.
49. G.McLendon, Biochem.Biophys.Res.Communs., 1977, **77**, 959.
50. H.B.Bull and K.Breese, Arch.Biophys.Biochem., 1973, **158**, 681.
51. E.Stellwagen and H.Wilgus, Nature, 1978, **275**, 342.
52. Ch.C.Bigelow, J.Theoret.Biol., 1967, **16**, 187.
53. D.E.Goldsack, Biopolymers, 1970, **9**, 247.
54. P.L.Privalov and T.N.Tsalkova, Nature, 1979, **280**, 693.
55. P.K.Ponnuswamy, R.Muthusamy, and P.Manavalan, Int.J.Biol.Macromol., 1982, **4**, 186.
56. T.I.Bilai, in "Termostabil'nye Fermenty Gribov" (Thermostable Fungal Enzymes), Izd.Naukova Dumka, Kiev, 1979, p.10.
57. H.-U.Habertich and H.Zuber, Arch.Microbiol., 1974, **98**(2), 275.
58. G.A.S.Küttner, M.Bekheit abd el Samei, and G.Sauer, Acta Biol.Med.German., 1979, **38**(10), K19.
59. A.Atkinson, J.Appl.Chem.Biotechnol., 1976, **26**, 577.
60. H.Nojima, A.Ikai, T.Oshima, and H.Noda, J.Mol. Biol., 1977, **116**, 429.
61. P.Argos, M.G.Rossman, U.M.Grau, H.Zuber, G.Frank, and J.D.Tratschin, Biochemistry, 1979, **18**, 5698.
62. H.Zuber, in "Structural and Functional Aspects of Enzyme Catalysis (Edited by H.Eggerer and R.Huber), Springer Verlag, Berlin–Heidelber–New York, 1981, p.114.
63. R.Singleton, C.R.Middaugh, and R.D.MacElroy, Int.J.Peptide Protein Res., 1977, **10**, 39.
64. D.J.Merkler, G.K.Farrington, and F.C.Wedler, Int.J.Peptide Protein Res., 1981, **18**, 430.
65. A.Ikai, J.Biochem., 1980, **88**, 1895.
66. M.F.Perutz and H.Raidt, Nature, 1975, **255**, 256.
67. J.D.Hocking and J.I.Harris, in "Enzymes and Proteins from Thermophilic Microorganisms", Birkhäuser Verlag, Basel–Stuttgart, 1976, p.121.
68. J.D.Hocking and J.I.Harris, Europ.J.Biochem., 1980, **108**, 567.
69. J.E.Walker, A.J.Wonacott, and J.I.Harris, Europ. J.Biochem., 1980, **108**, 581.
70. M.K.Pangburn, P.L.Levy, K.A.Walsh, and H.Neurath, in "Enzymes and Proteins from Thermophilic Microorganisms", Birkhäuser Verlag, Basel–Stuttgart, 1976, p.19.
71. C.Grandi, C.Vita, D.Dalzoppo, and A.Fontana, Int.J.Peptide Protein Res., 1980, **16**, 327.
72. J.T.Barach and D.M.Adams, Biochim.biophys. Acta, 1977, **485**, 417.
73. Ch.Rüegg, D.Ammer, and K.Lerch, J.Biol.Chem., 1982, **257**, 6420.
74. E.Stellwagen and L.D.Barnes, in "Enzymes and Proteins from Thermophilic Microorganisms", Birkhäuser Verlag, Basel–Stuttgart, 1976, p.223.
75. E.Boccu, F.M.Veronese, and A.Fontana, in "Enzymes and Proteins from Thermophilic Microorganisms", Birkhäuser Verlag, Basel–Stuttgart, 1976, p.229.
76. T.Ohnishi, H.Blum, S.Sato, K.Nakazawa, K.Hon-nami, and T.Oshima, J.Biol.Chem., 1980, **255**, 345.
77. F.C.Welder, F.M.Hoffman, R.Kenney, and J.Carfi, in "Enzymes and Proteins from Thermophilic Microorganisms", Birkhäuser Verlag, Basel–Stuttgart, 1976, p.187.
78. Sh.Hayashida and H.Yoshioka, Agr.Biol.Chem., 1980, **44**, 481.
79. T.Oshima, Y.Sakaki, N.Wakayama, K.Watanabe, Z.Ohashi, and S.Nishimura, in "Enzymes and Proteins from Thermophilic Microorganisms", Birkhäuser Verlag, Basel–Stuttgart, 1976, p.273.
80. M.L.Elwell and J.A.Schellman, Biochim.Biophys. Acta, 1977, **494**, 367.
81. M.G.Grütter, R.B.Hawkes, and B.W.Matthews, Nature, 1979, **277**, 667.
82. J.A.Schellman, M.Lindorfer, R.Hawkes, and M.Grütter, Biopolymers, 1981, **20**, 1989.
83. K.Yutani, K.Ogasahara, Y.Sugino, and A.Matsushiro, Nature, 1977, **267**, 274.
84. K.Yutani, K.Ogasahara, A.Kimura, and Y.Sugino, J.Mol.Biol., 1982, **160**, 387.
85. C.R.Matthews, M.M.Crisanti, G.L.Gepner, G.Velecelebi, and J.M.Sturtevant, Biochemistry, 1980, **19**, 1290.
86. N.N.Ugarova, G.D.Rozhkova, and I.V.Berezin, Biokhimiya, 1978, **43**, 1382.
87. A.Wiseman, in "Topics in Enzyme and Fermentation Biotechnology", 1978, Vol.2, p.280.
88. I.V.Berezin, V.K.Antonov, and K.Martinek (Editors), "Immobilizovannye Fermenty" (Immobilised Enzymes), Izd.Moskov.Gos.Univ., Moscow, 1976, Vol.1, p.38.
89. R.E.Feeney, in "Food Proteins. Improvement through Chemical and Enzymatic Modification" (Edited by R.E.Feeney and J.R.Whitaker), Amer.Chem.Soc., Washington, 1977, p.3.
90. A.F.S.A.Habeeb, Arch.Biochem.Biophys., 1967, **121**, 652.
91. A.Jonas and G.Weber, Biochemistry, 1970, **9**, 4729.
92. J.Avruch, J.A.Reynolds, and J.H.Reynolds, Biochemistry, 1969, **8**, 1855.
93. J.Oakes, J.Chem.Soc.Faraday Trans.I, 1976, **72**, 228.
94. Y.Nakagawa, S.Capetillo, and B.Jirgensons, J.Biol. Chem., 1972, **247**, 5703.
95. N.A.Gavrilova, Kh.A.Grinkevich, and V.K.Antonov, Bioorg.Khim., 1979, **5**, 1176.
96. F.Yamauchi, H.Ono, Y.Kamata, and K.Shibasaki, Agr.Biol.Chem., 1979, **43**, 1309.
97. Sh.A.Kidwai, A.A.Ansari, and A.Salanuddin, Biochem.J., 1976, **155**, 171.
98. P.K.Pal, B.Verma, and Y.P.Myer, Biochemistry, 1975, **14**, 4325.
99. I.Aviram, Arch.Biochem.Biophys., 1977, **181**, 199.
100. D.L.Brautigan, Sh.Ferguson-Miller, and E.Margoliash, J.Biol.Chem., 1978, **253**, 130.
101. D.L.Brautigan, Sh.Ferguson-Miller, G.E.Tarr, and E.Margoliash, J.Biol.Chem., 1978, **253**, 140.
102. M.Hollecker and Th.E.Creighton, Biochim.Biophys. Acta, 1982, **701**, 395.
103. A.Schejter, M.Zuckerman, and I.Aviram, J.Biol. Chem., 1979, **254**, 7042.
104. J.F.Clark and F.R.N.Gurd, J.Biol.Chem., 1967, **242**, 3257.
105. D.Puett, J.Biol.Chem., 1973, **248**, 3566.
106. R.Reiner, H.-U.Siebeneick, I.Christensen, and H.Döring, J.Mol.Catal., 1977, 2, 119.
107. V.B.Gerasimas, V.M.Chernoglazov, and A.A.Klesov, Biokhimiya, 1980, **45**, 1086.
108. N.N.Ugarova, G.D.Rozhkova, and I.V.Berezin, Biochim.Biophys.Acta, 1979, **570**, 31.
109. N.N.Ugarova, G.D.Rozhkova, and I.V.Berezin, Biokhimiya, 1978, **43**, 1242.
110. I.Urabe and H.Okada, Proc.IV IFS Ferment.Technol. Today, 1972, 367.
111. I.Urabe, H.Nanjo, and H.Okada, Biochim.Biophys. Acta, 1973, **302**, 73.

112. T.Ito and N.Ando, Agr.Biol.Chem., 1975, **39**, 575.
113. N.Minotani, T.Sekiguchi, J.G.Bautista, and Y.Nosoh, Biochim.Biophys.Acta, 1979, **581**, 334.
114. J.Müller, Biochim.Biophys.Acta, 1981, **669**, 210.
115. D.D.F.Shiao, R.Lumry, and S.Rajender, Europ. J.Biochem., 1972, **29**, 377.
116. V.P.Torchilin, A.V.Maksimenko, V.N.Smirnov, I.V.Berezin, A.M.Klibanov, and K.Martinek, Biochim. Biophys.Acta, 1979, **567**, 1.
117. A.D.Gounaris and G.E.Perlman, J.Biol.Chem., 1967, **242**, 2739.
118. J.F.Riordan and B.L.Vallee, in "Methods in Enzymology" (Edited by S.P.Colowick and N.O.Kaplan), Acad.Press, New York–London, 1972, Vol.25, 494.
119. K.Carraway and D.E.Koshland, in "Methods in Enzymology" (Edited by S.P.Colowick and N.O.Kaplan, Acad.Press, New York–London, 1972, Vol.25, p.616.
120. P.Bodlaender, G.Feinstein, and E.Shaw, Biochemistry, 1969, **8**, 4941.
121. G.D.Kutuzova, Candidate's Thesis in Chemical Sciences, Moscow State University, Moscow, 1981.
122. L.R.Brown, A.De Marco, R.Richarz, G.Wagner, and K.Wüthrich, Europ.J.Biochem., 1978, **88**, 87.
123. G.Wagner, H.Tschesche, and K.Wüthrich, Europ. J.Biochem., 1979, **95**, 239.
124. G.Wagner, A.I.Kalb (Gilboa), and K.Wüthrich, Europ.J.Biochem., 1979, **95**, 249.
125. M.J.Hunter and M.L.Ludwig, "Methods in Enzymology", Acad.Press, New York–London, 1972, Vol.25, p.585.
126. P.Cupo, W.El-Deiry, Ph.L.Whitney, and W.M.Awad, J.Biol.Chem., 1980, **255**, 10 828.
127. P.Tuengler and G.Pfleiderer, Biochim.Biophys.Acta, 1977, **484**, 1.
128. C.S.Tsai, Bioorg.Chem., 1980, **9**, 176.
129. T.Sekiguchi, S.Oshiro,E.M.Goingo, and Y.Nosoh, J.Biochem., 1979, **85**, 75.
130. A.Nureddin and T.Inagami, Biochem.J., 1975, **147**, 71.
131. P.Cupo, W.S.El-Deiry, Ph.L.Whitney, and W.M.Awad, Arch.Biochem.Biophys., 1982, **216**, 600.
132. Å.V.Maksimenko, V.P.Torchilin, A.M.Klibanov, and K.Martinek, Vestnik Moskov.Univ., Ser.Khim., 1978, **19**, 590.
133. N.F.Kazanskaya, O.A.Kost, and I.V.Berezin, Bioorg.Khim., 1975, **1**, 1337.
134. J.H.-Ch.Wang and J.-I.Tu, Biochemistry, 1969, **8**, 4403.
135. J.Woodward, K.S.Whaley, G.S.Sachry, and D.L.Wohlpart, Biotechnol.Bioeng.Symp., 1981, **11**, 619.
136. Y.Tashiro and J.R.Trevithick, Canad.J.Biochem., 1977, **55**, 249.
137. H.Yoshioka and S.Hayashida, Agr.Biol.Chem., 1981, **45**, 571.
138. B.P.Wasserman and H.O.Hultin, Arch.Biochem. Biophys., 1981, **212**, 385.
139. S.Nakamura and S.Hayashi, FEBS Letters, 1974, **41**, 327.
140. S.Nakamura, S.Hayashi, and K.Koga, Biochim. Biophys.Acta, 1976, **445**, 294.
141. I.V.Berezin, N.N.Ugarova, and D.P.Fel'dman, Biokhimiya, 1977, **40**, 926.
142. Hat.Uedaira and His.Uedaira, Bull.Chem.Soc.Japan, 1980, **53**, 2451.
143. T.Arakawa and S.N.Timasheff, Biochemistry, 1982, **21**, 6536.
144. D.I.Metelitza, A.N.Eryomin, and S.A.Usanov, Acta Biol.Med.German., 1982, **41**, 17.
145. A.N.Eremin and D.I.Metelitsa, Biokhimiya, 1982, **47**, 1186.
146. Y.Ohta, S.Tanabe, A.Hachimori, and T.Samejima, J.Biochem., 1974, **76**, 871.
147. J.Sri Ram, L.Terminiello, M.Bier, and F.F.Nord, Arch.Biochem.Biophys., 1954, **52**, 464.
148. L.Terminiello, M.Bier, and F.F.Nord, Arch.Biochem. Biophys., 1958, **73**, 171.
149. J.F.Riordan, M.Bier, and F.F.Nord, Arch.Biochem. Biophys., 1960, **90**, 125.
150. S.A.Barker, C.J.Gray, and A.W.Lomath, J.Appl. Chem.Biotechnol., 1976, **26**, 576.
151. W.Cohen, M.Bier, and F.F.Nord, Arch.Biochem. Biophys., 1957, **67**, 479.
152. I.Urabe, M.Yamamoto, Y.Yamada, and H.Okada, Biochim.Biophys.Acta, 1978, **524**, 435.
153. S.A.Barker, C.J.Gray, and A.W.Lomath, Biochem. Soc.Transactions, 1979, **7**, 5.
154. K.Wada, J.Biochem., 1979, **86**, 1747.
155. J.Woodward and A.Wiseman, Biochim.Biophys.Acta, 1978, **527**, 8.
156. Chin-Yung Ma and S.Nakay, J.Agr.Food Chem., 1980, **28**, 834.
157. J.Mueller and C.Klein, Biochim.Biophys.Acta, 1982, **707**, 133.
158. N.N.Ugarova, L.Yu.Brovko, G.D.Rozhkova, and I.V.Berezin, Biokhimiya, 1977, **42**, 1212.
159. M.A.Shatsky, H.C.Ho, and J.H.-Ch.Wang, Biochim. Biophys.Acta, 1973, **303**, 298.
160. V.N.Izmailova and P.A.Rebinder, in "Strukturoobrazovanie v Belkovykh Sistemakh" (Structure Formation in Protein Systems), Izd.Nauka, Moscow, 1974, p.28.
161. S.Gumpen, P.O.Hegg, and H.Martens, Biochim. Biophys.Acta, 1979, **574**, 189.
162. S.J.Leach and H.Boyd, Int.J.Peptide Protein Res., 1973, **5**, 239.
163. W.Karmeyer and G.Pfleiderer, Biochim.Biophys. Acta, 1977, **481**, 328.
164. J.Hora, Biochim.Biophys.Acta, 1973, **310**, 264.
165. E.A.Alfimova and G.I.Likhtenshtein, in "Molekulyarnaya Biologiya" (Molecular Biology), Izd.VINITI, Moscow, 1976, Vol.8, p.127.
166. N.Citri, in "Advances in Enzymology" (Edited by A.Meister), John Wiley & Sons, London, 1973, Vol.37, 397.
167. R.Lumry and R.Biltonen, in "The Structure and Stability of Biological Macomolecules" (Translated into Russian), Izd.Mir, Moscow, 1973, p.7.
168. L.V.Kozlov, Bioorg.Khim., 1980, **6**, 1243.
169. N.N.Ugarova, B.M.Kershengol'ds, I.D.Artamonov, and I.V.Berezin, Biokhimiya, 1976, **41**, 1662.
170. K.Martinek, A.M.Klibanov, V.S.Goldmacher, and I.V.Berezin, Biochim.Biophys.Acta, 1977, **485**, 1.
171. J.Turkova, L.V.Kozlov, L.Ya.Bessmertnaya, L.V.Kudryavtseva, V.M.Krasil'nikova, and V.K.Antonov, Bioorg.Khim., 1980, **6**, 108.
172. V.S.Baeva, L.V.Kozlov, and V.K.Antonov, Biokhimiya, 1977, **42**, 706.
173. H.Kaboli and P.J.Reilly, in "Proceedings of III Joint US/USSR [Conference on?] Enzyme Engineering, Tallin, 1978" (Edited by Bungay), NSF, Washington, p.102.
174. Sn.Moriyama, A.Noda, K.Nakanishi, R.Matsuno, and T.Kamikubo, Agr.Biol.Chem., 1980, **44**, 2047.

175. N.N.Ugarova, L.Yu.Brovko, and N.V.Kost, Enzyme Microb.Technol., 1982, **4**, 224.
176. E.Brynda and M.Bleha, Biopolymers, 1977, **16**, 1945.
177. Yu.Yu.Kulis, Biokhimiya, 1979, **44**, 417.
178. O.B.Kinstler and L.V.Kozlov, Biokhimiya, 1977, **42**, 1674.

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