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Chemical, Physical, and Biological Approaches to the Creation of Stabilised Enzyme Catalysts for Biotechnology

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The problem of the inter-relation between the structure and stability of proteins includes two main questions: firstly, how can we discover the molecular characteristics of the structure of proteins which are responsible for their stability? Secondly, how, knowing the general molecular causes of stability, can we alter the structure of a specific protein in order to increase its stability, i.e. to stabilise it? In the present review an attempt is made to present the current state of both the first (fundamental) and second (applied) aspects of this problem.
The bibliography includes 250 references.

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I. INTRODUCTION

The problem of the inter-relation between the structure and stability of proteins is one of the key problems in modern protein chemistry.[1] Only its solution will permit a close approach to the realisation of the long-standing aim of investigators in the field of protein chemistry: to predict[1,2] the steric structure of the protein from its known aminoacid sequence and to attempt the synthesis[3] of a molecule of a protein type whose property is specified beforehand.

On the other hand, one of the central tasks of chemical (engineering) enzymology is to create stabilised enzyme preparations for various technological applications.[4-11] The pathway leading to the creation of stabilised enzymes was outlined several decades ago when many world laboratories began to elucidate and investigate the molecular mechanisms or at least the molecular causes of the inactivation of protein (see the relevant reviews[12,13]). Only after this "preparatory stage" did it become possible to proceed directly to the development of methods whereby the structure of enzymes could be influenced specifically in order to suppress the inactivation mechanisms established previously. The chemical and physical methods for the stabilisation of enzymes have so far been developed to the greatest extent (see the relevant reviews[14-19]). However, there is no doubt that the biological approach proposed recently (application of methods of genetic engineering)[20-22] merits no less attention.

The present review deals with the entire chemical-physico-biological set of inter-related problems. The data described have been divided into three somewhat separate sections. Firstly, there is a brief discussion of experimental and theoretical approaches used to establish the inter-relation between the stability of proteins and their structure. This is followed by the molecular causes of the stability of proteins themselves. This question has been frequently raised in the literature at least since the studies by Bressler and Talmud.[23] The current level of the problem has been fairly completely dealt with in recent publications, for example, in the monograph of Schultz and Schirmer.[1] We should therefore attempt here mainly to answer the following question: what must be done to the protein (how must its structure be altered) in order to impart to it greater stability. Finally, in the last Section it is shown how this can be achieved at the present time, i.e. the experimental methods for the preparation of highly stable biocatalysts are examined.

II. THEORETICAL AND EXPERIMENTAL APPROACHES TO THE ELUCIDATION OF THE INTER-RELATION BETWEEN THE STRUCTURE AND STABILITY OF PROTEINS

The solution of the problem of the inter-relation between the structure and stability of proteins in the most general form implies the calculation of the free energy of the protein from its steric structure. In principle, one could attempt to calculate separately for this purpose the energy of each of the stabilising (hydrogen bonding, hydrophobic, electrostatic, and dispersion interactions, etc.) and destabilising (in the first place the unfavourable entropy contribution by a strained conformation) factors and to add them up. Unfortunately, this cannot be carried out correctly.[1,24] The reason is that the contribution of each of the above components to the free energy of the protein amounts to tens and hundreds of kcal mol^{-1}.[25,26] This is a lot in view of the fact that proteins as a whole are characterised by a relatively low stability; the experimental free energy of stabilisation (of the native form relative to the denatured form) amounts to between 5 and 15 kcal mol^{-1} for the vast majority of globular proteins.[27]

Another general (experimental) way of solving the problem consists in the following procedure: one selects two proteins of different stability and by comparing their structures one attempts to elucidate how the structural characteristics are responsible for the increased stability of one of them. However, the following question arises: which pair of proteins should be chosen for such a comparison? If we select two arbitrary proteins, then in all probability their stabilities will be found to lie in the statistical average range of free energies: 5–15 kcal mol^{-1}. Consequently the differences in stability will be minute and it will be hardly possible to identify them with any definite structural characteristics, especially against the background of the large structural differences characteristic of functionally different proteins.

The same feature of protein structure (small differences in stability against the background of a great structural variety) lead apparently to a relatively low predictive value of studies of a generalised character.[28-31] In other words, the attempts to analyse the known structure of several tens of proteins in order to find a correlation between their stability and certain general structural parameters (hydrophobic nature,[28-30] volume,[30] or the content of particular aminoacid residues[31]) did not lead to unambiguous conclusions.

Comparison of the stabilities of proteins having fairly close structures proved to be most fruitful. Proteins having the same biological function are characterised by such structural kinship.

A typical experiment designed to elucidate the inter-relation between structure and stability consists of three stages. Firstly, two (or several) functionally related proteins from different sources are selected and are obtained in as pure a form as possible. Next, the following characteristics of the proteins are compared: (1) the aminoacid compositions and primary structures; (2) the steric three-dimensional structures and parameters of their conformational variability; (3) the resistance to the action of reversible denaturing agents and to irreversible investigation; (4) the temperathre dependence of the functional (catalytic) activity.

Finally, an attempt is made to correlate the quantitative stability characteristics obtained (such as the temperature of reversible denaturation, the optimum temperature of enzyme activity, and the equilibrium or kinetic denaturation parameters—ΔG, ΔH, ΔS, etc.) with the structural differences which it has been possible to discover.

The question arises of the origin of functionally related proteins having different stabilities. In the first place, they are found in nature. Firstly, these are related proteins from different mesophilic sources. Secondly, related proteins from thermophilic and mesophilic organisms are compared. Thirdly, proteins from wild strains are compared with proteins altered by mutation. The objects of such research are also obtained artificially. Three methods are used at present for this purpose: "site-directed" mutagenesis, immobilisation, and chemical modification. We shall consider what information can be obtained from the study of the inter-relation between the structure and stability of proteins by the application of each of these approaches.

1. Homologous Proteins of Mesophilic Organisms

Comparison of the structures of enzymes fulfilling identical functions in evolutionary remote organisms has long been used to elucidate the mechanisms of enzyme catalysis.[32] However, this approach is used much more rarely for the study of the inter-relation between the structure and stability of proteins. The point is that the differences between the primary structures of related mesophilic proteins can be extremely large. For example, in two of the most widely different proteins of the family of globins, only 16% of all the aminoacid residues occupy identical positions in the primary structures.[33] On the other hand, the differences between the stabilities of homologous proteins are as a rule very insignificant—not more than 1–3 kcal mol^{-1}. The insuperable problem discussed above therefore arises: how can one interpret the minute changes in stability against the background of large differences in structure?

Nevertheless, comparison of homologous proteins of mesophilic organisms yields some useful information about the inter-relation of their structures and stabilities.[33-36] Firstly, it is sometimes possible to find related proteins which differ only by 1–2 aminoacid residues, which naturally facilitates the problem of the elucidation of their role in the stability of the proteins being compared.[19] Secondly, it is frequently possible to discover "invariant positions" in proteins (the replacement of aminoacid residues in these positions is inadmissible, because it leads to the decomposition of the structure). As a rule, the invariant positions are located either in the inner regions of the globule[33-36] or in the regions corresponding to intersubunit contacts[36] and contacts between neighbouring α-helices.[33] The aminoacid involved in the coordination of haem[34] as well as the fragments of the active centres of the enzyme[36] are also invariant.

2. Comparison of Proteins from Thermophilic and Mesophilic Organisms

The existence of life under extreme conditions has long been known—for the history of the development of the problem, see Brock.[37] It was not until the middle 1960's that it became possible to demonstrate that the stability of microorganisms under extreme conditions is as a rule associated with the increased stability of their constituent biomacromolecules and in the first place proteins.[38]

The search for differences between the structures of related thermophilic and mesophilic proteins has the advantage over the comparison of mesophilic objects that the range of the differences between their stabilities is much wider and hence there is a greater probability of observing the structural differences which are significant for stability. Hitherto ~100 thermophilic proteins have been isolated in a highly pure form and their properties have been investigated. The molecular causes of the increased stability of thermophilic proteins have been discussed in a series of reviews and monographs.[19,37-49]

3. Proteins Modified by Mutation and Proteins from Wild Strains

Under the influence of unfavourable external influences, chemical agents, irradiation, and certain other factors in nature, the phenomenon of the so called mutagenesis takes place.[50] At the level of cells and whole organisms, mutagenesis is expressed by a change in morphology and functions and frequently leads to degeneracy. At the molecular level, nucleotides are replaced, new bases (or even whole sections of the genome) are incorporated or omitted. This results in the synthesis of proteins with a modified structure: with replacement of one (the most frequent case) or several aminoacid residues by different ones; with the polypeptide chain divided into a series of fragments not linked by covaler bonds or, conversely, with covalently cross-linked polypeptide chains.[50]

Comparison of the structures of mutant proteins and proteins from wild strains is actively employed for the elucidation of the mechanisms of enzyme catalysis,[51,52] in particular in order to discover the aminoacid residues significant for catalysis.[53] Using this approach, it has been possible to obtain also information about the role of individual aminoacids and of more complex structural factors in the stability of proteins some examples may be found in the review of Mozhaev and Martinek.[19]

4. Artificially Modified Proteins

There exist at least three ways of obtaining artificially modified proteins: site-directed mutagenesis (using the procedures of genetic engineering), immobilisation, and chemical modification.

(a) The Method of Site-Directed Mutagenesis

In this method manipulation at the level of genes yields analogues of proteins with a known tertiary structure differing by the replacement of only one aminoacid residue in a rigorously specified position.[54]† The latest advances in site-directed mutagenesis[20,55] have made the method available in many laboratories. Thus proteins of "aritificial origin" modified by mutation have come to be used for the study of the role of definite aminoacid residues in catalysis.[56] Enzymes with an improved activity and an altered substrate specificity have been obtained in this way.[57,58] As for mutants of natural origin, proteins with substituted aminoacid residues in specific positions, obtained artificially (by genetic engineering), can yield information about the role of individual structural elements also in the stability of proteins.

(b) Immobilisation of Enzymes

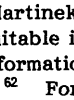
This should be understood in the first place as a method for the significant restriction of the mobility of protein molecules (or their fragments) in space or relative to one another.[59,61] In recent years this method has been used actively in biotechnology,[4-11] in particular for the stabilisation of enzymes.[14-19] However, sometimes one encounters the view that the immobilisation method is effective only for the solution of purely applied problems but does not yield new information for fundamental science. This view is based on the long-standing preconceived notion that immobilisation alters the structure of the enzyme compared with its native structure. However, it has been demonstrated in recent years by physical methods that this is by no means the case: in many instances immobilisation does not change the conformation of the enzyme at all or changes it only insignificantly (see the review of Martinek and Mozhaev[62]). Furthermore, having selected a suitable immobilisation method, one can be certain that the conformations of the native and immobilised enzymes are similar.[62] For this reason, immobilised enzymes have proved to be successful models in the study of a whole series of *in vivo* processes.[62,63] They also yield useful information about the inter-relation of the structure and stability of proteins. Thus the important role of fluctuations in the structure of the protein in its stability has been discovered with the aid of immobilisation.

(c) Chemical Modification of Proteins

This method is used as an approach not only to the stabilisation of enzymes[64-66] but also to the elucidation of the inter-relation between the structure and stability of proteins.[19,66]

The main problem involved in the use of the modification approach is that of obtaining a protein preparation in which only a specific functional group (or a specific series of groups) has been modified. The difficulty of specific modification lies in the fact that the functional groups of proteins exhibit the same type of reactivity: they are all potential nucleophiles and proton donors or acceptors (Table 1). For this reason, the "frontal" modification of proteins by an arbitrary reagent leads to competition between different functional groups of the protein for the reagent and usually ends in failure—non-specific modification. Nevertheless, advances in selective modification have led to the discovery of several reagents for virtually any functional group in proteins which involve only this particular group (for examples, see Table 1). Furthermore, even for reagents exhibiting group specificity, it is frequently possible to select conditions under which aminoacid residues of only one type are modified selectively. For this purpose, one varies either the pH (because as a rule only one of the forms—protonated or deprotonated—is reactive) or one employs reversible modification reactions (Table 2) with the aid of which other groups of the protein competing for the reagent can be subjected to preliminary blocking.

Table 1. The functional groups in proteins which can be subjected to specific modification.[72]

Group	Aminoacid residue	pK_a	Examples of selective reagents*
α-COOH	terminal amino-acid	<1—6.8	carbodiimide + nucleophile
β-COOH	Asp	<1—6.8	ditto
γ-COOH	Glu	<1—6.8	»
	His	6.4—7.5	diethyl pyrocarbonate
—SH	Cys	8.0—9.5	*N*-ethylmaleimide; disulphides
α-NH_2	terminal amino-acid	7.3—>12	dansyl chloride; methyl acetimidate
ε-NH_2	Lys	7.3—>12	ditto
	Tyr	9.4—>12	tetranitromethane, *N*-acetylimidazole
	Arg	11.5—>12	butane-2,3-dione, phenylglyoxal
—OH	Ser, Thr	>12	—
	Trp	>12	*N*-bromosuccinimide
—S—CH_3	Met	—	chloramine T; H_2O_2

*Taken from Lundblad and Noyes.[89]

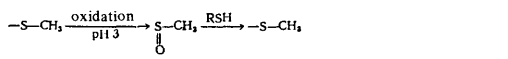
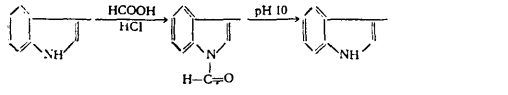
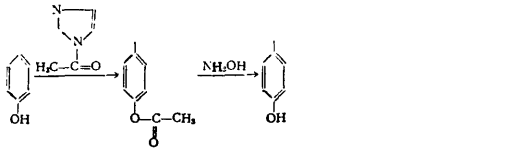
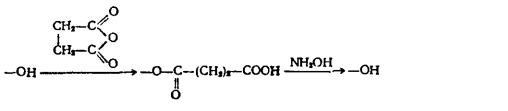
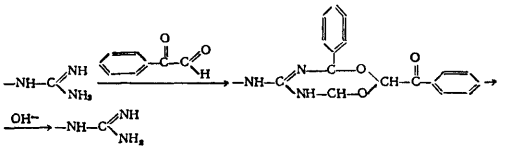
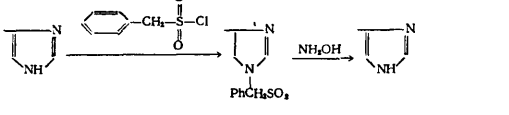
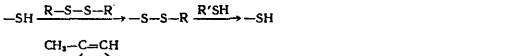
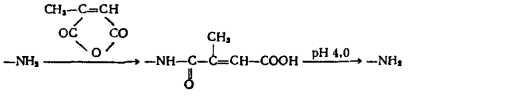
The potential value of the chemical modification method is truly enormous.[67-89] However, its application to the study of the inter-relation between the structure and stability of proteins encounters a series of methodological difficulties, which are frequently fundamental. We shall consider the most important of these difficulties and shall attempt to outline ways whereby they can be overcome.

1. There is a risk that modification will involve the invariant elements of the protein structure and will lead to undesirable conformational changes, up to the denaturation of the protein.[72,84] In order to test this possibility, the chemical modification product must be analysed by a physical method and it must be discovered whether the conformations of the native and modified proteins are different. If such differences exist, it is essential to alter the modifying agent.

†The methodological procedures of site-directed mutagenesis will be examined below.

2. Sometimes it is necessary to modify proteins under fairly severe conditions where undesirable side processes can take place (Table 3). In order to avoid secondary reactions, it is frequently necessary to resort to preliminary protection of the "vulnerable" functional groups (Table 2) and, after the modifications have been carried out, the "protecting groups" must be removed under mild conditions.

Table 2. Reactions leading to the reversible protection of the aminoacid residues in proteins.

Amino-acid	Reaction	Refs.
Met		[90]
Trp		[91]
Tyr		[92]
Ser, Thr		[93]
Arg		[94]
His		[95]
Cys		[96]
Lys		[97]

3. Only in rare cases is it possible to modify the protein in such a way that only a specific aminoacid residue in it is involved. Usually chemical modification results in a set of derivatives of the protein differing both in the number of modified groups and in the ordinal numbers of the residues affected by the modification in the primary structure. This naturally leads to a significant inhomogeneity of the products of the chemical modification of the proteins.[72,84] The difficulty of separating the product is inherent in the fact that the modified proteins frequently differ little in their physical properties (charge, molecular weight, molecular shape, etc.). High performance liquid chromatography proved to be especially useful in this field.[98]

4. Yet another serious problem is how to identify the product of the chemical modification of the protein. Aminoacid analysis is most often used for this purpose. However, the method suffers from the disadvantage that, before the direct determination of the aminoacid composition, the protein is subjected to a treatment under extremely severe conditions (concentrated HCl, temperature above 100 °C) in order to hydrolyse all the peptide bonds. This is naturally accompanied also by the rupture of other chemical bonds (weaker than the peptide bonds) and certain aminoacids undergo chemical decomposition. For this reason, the identification of many aminoacids (Cys, Trp, Met) and of the products of certain types of chemical modification (oxidation, acylation) is either altogether impossible by means of aminoacid analysis or inaccurate results are obtained.[85,86] Fortunately, this difficulty can be overcome[85] by employing certain methodological devices (for example, reactions competing for functional groups or the radioactive tracer method).

Table 3. Undesirable side reactions in the modification of the functional groups of proteins.[84]

Functional group	Conditions	Result
Peptide bond	pH 8—12	hydrolysis
ditto	pH 1—5	N-O acyl shift
SH group	oxidation	S-S bond, S-O acids
S-S bond	reduction	SH groups, new S-S bonds
ditto	pH 8—12	hydrolysis, β-elimination
$S-CH_3$ group of methionine	oxidation	hydroxy-derivative
Amide group	pH 8—12	hydrolysis
O-Glycosyl group	pH 8—12	β-elimination
O-Phosphate group	pH 8—12	β-elimination

Another way of overcoming this difficulty involves the determination of the degree of modification under mild conditions. This is effectively assisted by the reactions involved in the spectrophotometric titration of the residual unmodified functional groups of the proteins with the aid of chromophoric reagents (certain examples are presented in Table 4). The methods for the determination of the primary structure are used to determine the exact site where the modified aminoacid residue is located.[103]

Thus the difficulties arising can be overcome in principle and one can proceed to the search for the inter-relation between the stability and structure of the protein. The experiment must be begun with a comparison of the conformations of the native and modified proteins. The point is that sometimes the increase[104,105] or decrease[106,107] of the stability of proteins as a result of chemical modification is caused precisely by conformational changes. Such data are of limited value, because it is difficult to interpret them and even more difficult to predict them.

One usually has to deal with a chemical modification product which, while conformationally not different from the native protein, nevertheless has a lower or higher stability. In order to elucidate the molecular causes of stabilisation or destabilisation, the following methodological procedures are employed.

The increase of the degree of modification (of the number of modified functional groups). If the stabilising or destabilising effect changes monotonically (smoothly) under these

conditions, the most likely implication is that specific interactions of any kind, for example, the formation of salt bridges or hydrogen bonds, do not arise (or vanish) in the protein on modification. Otherwise, the observed dependence would have been of a discrete type: indeed, it is difficult to imagine that the modification of each successive residue generates or destroys a salt bridge or hydrogen bond. It is more probable that the smooth type of dependence of the stabilising or destabilising effect on the degree of modification reflects a uniform alteration of some integral parameter of the globule (its hydrophobic property or surface charge).

A change in the chain length of the modifying agent for a specified degree of modification. For this purpose, it is convenient to employ homologous series of modifying agents: amines, aldehydes, imidates. Analysis of the type of dependence of the stabilising or destabilising effect on the chain length of the reagent has proved useful for the elucidation of the influence on the stability of proteins of integral parameters such as the surface hydrophobic character or surface charge.[66]

The influence of temperature on the stabilising or destabilising effects can sometimes help to elucidate[66] what type of interactions in the protein makes a decisive contribution to the stabilisation. In particular, if the stabilising effect increases with increase of temperature, then this is important evidence for the enhancement of hydrophobic interactions in the protein.

In conclusion of this Section of the review, we shall draw attention to the possibility of one fundamental error in the interpretation of experimental data. A particular mechanism of inactivation characteristic of the native protein can sometimes be significantly inhibited by chemical modification. For example, the thermal inactivation of bovine serum albumin in solution is sometimes caused mainly by its aggregation.[108] By introducing an excess negative charge on the protein surface (for example by citraconylation), aggregation can be fully prevented. The retardation of the inactivation observed experimentally in this case[108] is caused by a change in the inactivation mechanism and not by the internal stabilisation of the protein (due to its modification). In studies with modified and even with immobilised proteins or proteins modified by mutation, it is always important to make sure that the mechanisms of their inactivation are identical with the mechanisms for the native proteins.

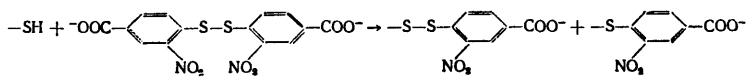
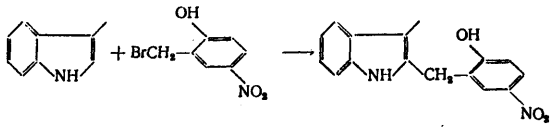
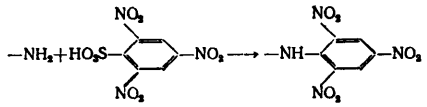
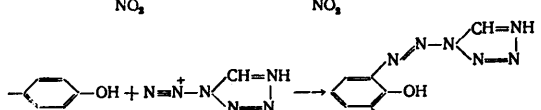
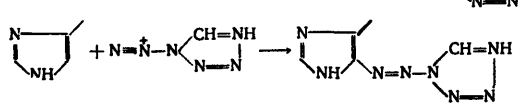
III. MOLECULAR CAUSES OF THE STABILITY OF PROTEINS

The principal molecular causes of the stability of proteins were recently analysed by the present authors.[19] Here we shall consider them only in order to elucidate whether nature has left provision (and what kind of provision) for the artificial increase of the stability of proteins.

1. The Binding of Metal Cations, Substrates, Prosthetic Groups, and Other Low-Molecular-Weight Ligands

The most general explanation of the facts concerning the stabilisation of enzymes in their interaction with ligands was given by Schellman.[109] There exist at least two states of protein—native and denatured, and any compound is in principle capable of binding to either one. The preferential binding to one of the forms of the protein inevitably stabilises it in relation to the other by the amount equivalent to the free energy of formation of the corresponding complex. It is known that the effectors of enzyme activity are bound in the region of the active centre or near it. The formation of the enzyme–ligand complex therefore requires that the protein exist in the native conformation; there is as a rule no binding to the denatured form. This is precisely why specific low-molecular-weight ligands usually stabilise proteins.[109]

Table 4. Spectrophotometric determination of certain functional groups in proteins.

Reaction	λ, nm	Molar absorption	Refs.
	412	13 600	[99]
	410	18 000	[100]
	420	13 000	[101]
	550	13 800	[102]
	480	20 500	[102]

In connection with the preparation of artificially stabilised enzyme preparation by interaction with effectors, we draw attention to the fact that in such interactions proteins frequently undergo conformational changes,[110] i.e. are converted into other, sometimes more stable conformations. An attempt must be made to fix this stable conformation, for example with the aid of immobilisation;[111] a critical analysis of this approach has been carried out in a review.[62]

2. Protein–Protein and Protein–Lipid Interactions

In vivo proteins frequently interact with other protein molecules, lipids, and polysaccharides.[112] The structures then formed can be divided arbitrarily into two fundamentally different types: firstly, complexes with a small number of either protein–protein contacts (for example, subunit enzymes[113] or species comprising proteases associated with protein inhibitors[114]) or protein–lipid contacts (such as the complexes of albumin with aliphatic acids[115]) and secondly systems with a large number of inter-related concerted contacts of different types (protein–protein, protein–lipid, lipid–lipid) of the kind which obtain, for example, in biological membranes.[116] The interaction of the protein with other molecules in both these and other types of structures can significantly increase its stability.

The mechanism of stabilisation on formation of individual protein–protein or protein–lipid contacts has been in principle understood.[114] Together with polar and charged groups, there are "hydrophobic clusters" on the surface of proteins and their contact with water is thermodynamically unfavourable. On formation of the complex, the lipid or protein molecule "sits" on this cluster and thereby prevents its contact with the solvent.[117] The observed free energy of stabilisation amounts to several kcal mol^{-1} per protein–protein contact.[113,114]

Such protein–protein or protein–lipid contacts can also be created artificially: in the presence of lipids[118,119] or high concentrations of proteins (for example, albumin),[120,121] the stability of certain enzymes increases appreciably.

The mechanism of the stabilisation of enzymes when the latter are enclosed in biological membranes appears less unambiguous. Indeed, together with the "shielding effect" discussed above, many other factors should play an important role in this instance. Among them, we shall specify in the first place a change in the rigidity of the protein molecule as a result of its multipoint interaction with other proteins and lipids and we shall consider the influence of this factor on stability.

3. The Rigidity of the Protein Molecule

The rigidity determines the ability of the macromolecule to assume different states in conformational space[122] and is related directly to the stability of the protein. Indeed, both the protein denaturation process (the manifestation of the instability of the protein) and the fluctuations about a certain statistical average stage (the manifestation of the "non-rigidity" or flexibility of the protein) are based on the same molecular mechanisms: the vibrational and rotational movement in the macromolecule. The difference between these processes consists only in the fact that in denaturation a cooperative conformational transition of the entire globule takes place,[26] while local fluctuations in the structure involve only individual sections of the globule without a significant alteration of the overall conformation.[123]

In the present review we shall not attempt to give a rigorous thermodynamic definition of rigidity[122,123] or to relate it to the thermodynamic parameters of the stability of the protein.[124] Undoubtedly many factors which determine the stability of the protein (in the first place hydrophobic interactions) influence also its rigidity.[122,124] Here we shall consider only one question: how a factor such as immobilisation—natural or artificial—affects the rigidity and stability of the protein?

In nature proteins and membranes exist in a naturally immobilised state; this must be understood as having the consequence that, as a result of interaction with other proteins and lipids, their rotational diffusion as well as their vibrational and rotational movement are significantly restricted. The degree of immobilisation of proteins in a membrane depends in many respects on the integrity of the membrane and on the degree of structural formation in the latter. When the latter parameter is altered (for example, in a phase transition within the membrane), the "degree of immobilisation" of proteins changes significantly,[125] which entails changes in the catalytic properties of membrane enzymes.[125,126] However, until recently there have been virtually no data concerning the influence of structure formation in membranes on the stability of membrane enzymes.

A pioneering study on these lines was carried out by Welker.[127] He grew the obligatory thermophilic strain *Bacillus stearothermophilus* at temperatures ranging from 55° to 70 °C and observed that, with increase of the growing temperature, there is a significant increase of the stability of the cell membrane (which he inferred from the decomposition of cell protoplasts at elevated temperatures—Table 5). Simultaneously with the strengthening of the structure of the membrane, there is a significant increase in the thermal stability of membrane enzymes; this has been noted both for peripheral alkaline phosphatase and integral NADH-oxidase (Table 5). An increase of stability is observed only for enzymes forming part of the composition of the membrane. After extraction from the membrane, the stability of alkaline phosphatase first falls sharply and then becomes independent of the temperature at which the given culture of microorganisms has been grown (Table 5). Thus these data confirm the earlier hypothesis[128,129] of the stabilising influence of the membrane structure on proteins.

Table 5. The influence of the growth temperature (T_g) of the culture on the properties of the membranes of the cells of *Bacillus stearothermophilus* and the thermal stabilities of the enzymes.[127]

T_g, °C	Disruption of protoplasts, %				Degree of inactivation, %					
					free A.P.*		membrane A.P.		NADH-oxidase	
	65°	70°	75°	80°	65°	70°	65°	70°	75°	80°
55	0	100	100	100	100	100	87	100	100	100
60	0	80	100	100	100	100	0	92	90	100
65	0	0	70	100	100	100	0	20	75	84
70	0	0	10	100	100	100	0	5	0	10

*A.P. = alkaline phosphatase.

Comparison of the thermal stabilities of enzymes immobilised by natural (in a biomembrane) and artificial (on a polymeric carrier) procedures is extremely instructive. Fig. 1 presents

the temperature dependence of the catalytic activity of the integral membrane enzyme NADH-oxidase.[127] Evidently the stronger the membrane in which the enzyme has been incorporated (i.e. the higher the temperature at which the culture has been grown), the higher the temperature optimum of the catalytic activity. A similar family of experimental curves has been obtained[130] for artificially immobilised trypsin, whose molecules were attached to the carrier by different numbers of bonds (Fig.2). We believe that the identity of the forms of the temperature variations in Figs.1 and 2 is not fortuitous. Apparently it reflects the same trend: the increase of the stability of the enzyme as a result of the strengthening of its structure. The difference consists only in the fact that the strengthening is achieved either as a result of more pronounced structure formation in the natural protein-lipid membrane matrix *in vivo* or via an increase in the number of bonds between the enzyme molecule and the artificial polymer matrix *in vitro*

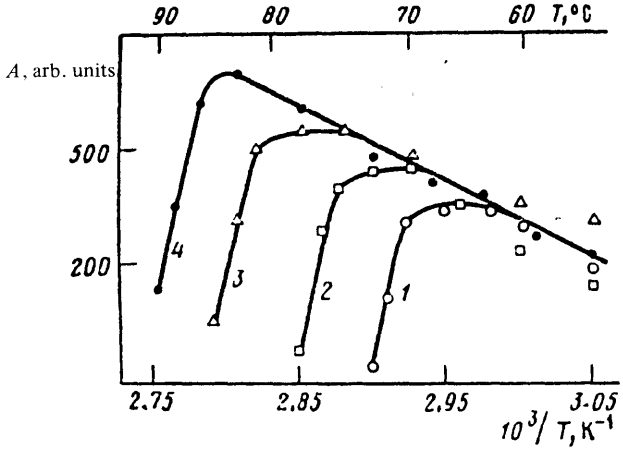


Figure 1. Temperature dependence of the catalytic activity (*A*) of a membrane enzyme (NADH-oxidase from the cells of *Bacillus stearothermophilus* grown at different temperatures): *1*) 55 °C; *2*) 60 °C; *3*) 65 °C; *3*) 65 °C; *4*) 70 °C.[127]

4. Salt Bridges

Among all the stabilising factors based on electrostatic interactions, ion pairs (or, in other words, salt bridges) make the greatest contribution to the stabilisation of protein.

There are relatively few ion pairs in proteins,[131,132] but they make a significant contribution to their stabilisation—up to 5 kcal mol^{-1} per ion pair if the latter is located within the globule and 1–2 kcal mol^{-1} if it is on the surface.[1,113] It is therefore not fortuitous that nature willingly makes use of them and creates thermophilic proteins with increased stability.[19]

We shall quote yet another example. Glyceraldehyde 3-phosphate dehydrogenase, from the moderate thermophile *Bacillus stearothermophilus* has a three-dimensional structure similar to that of the enzyme from rabbit muscle[133] with only a small but significant difference: in the region of subunit contacts, the thermophilic dehydrogenase has a cooperative system of additional salt bridges, which is missing from the mesophilic enzyme. This is in fact the main molecular reason why both the denaturation and the optimum catalytic activity of the thermophilic enzyme are observed at a higher temperature than for the enzyme from rabbit muscle.[133]

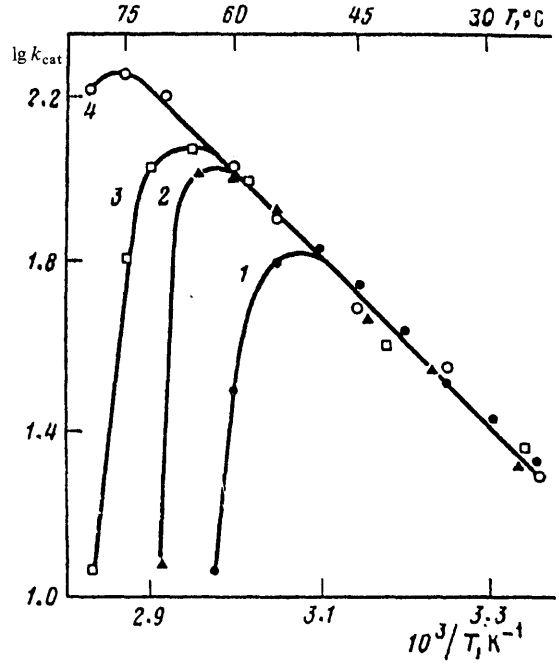


Figure 2. Temperature variation of the catalytic constants k_{cat} for the hydrolysis of the specific substrate catalysed by trypsin: *1*) free enzyme; *2–4*) enzyme immobilised covalently in polyacrylamide gel by 5, 8, and 15 bonds respectively.[130]

An attempt may be made to create in the protein additional ion pairs artificially, for example by site-specific mutagenesis or by chemical modification. However, success of such procedures, i.e. the appearance of a salt bridge, requires detailed knowledge about the steric structure of proteins. The likely usefulness of this approach will probably increase as more new information is obtained about the structure of proteins, in the first place by X-ray diffraction.[134]

5. Hydrogen Bonds

The hydrogen bonds in proteins play an important structural role; they maintain the spatial organisation in the secondary structures (α-helices, β-structures, rotations). It has come to be assumed that the formation of a hydrogen bond in proteins is an energetically favourable process. The energy of the hydrogen bond is frequently calculated as the result of two events: the dissociation of "old" hydrogen bonds between a polar atom (for example, the oxygen of the amide group) and a water molecule and the formation of "new" hydrogen bonds between the polar atoms in the protein molecule and has been estimated as 1–2 kcal mol^{-1}. However, in this treatment no account is taken of the fact that there is a parallel thermodynamically unfavourable process involving the

immersion of the polar atom or group (for example, the amide group) within the hydrophobic nucleus of the globule. Allowance for the energetics of this process leads to the conclusion that[1] the free energy of formation of the hydrogen bond in the protein is close to zero. In other words, the hydrogen bond is the price which the protein has to pay for the characteristic features of its structure. In our view, there is therefore no point in attempting (for example, by methods of protein engineering) to introduce hydrogen bonds into the protein artificially.

6. Disulphide Bridges

The ideas about the stabilisation of proteins with S–S bonds arrived in protein chemistry from polymer chemistry. In the middle 1950's, it was shown that[135,136] the intramolecular cross-linking of macromolecules imparts to them additional rigidity and increases their stability. The nature of this stabilisation is entropic:[135] as a result of the appearance of a cross-link in the macromolecule (a S–S bond in a protein), there is a significant decrease in the entropy of the open-chain form, which entails an increase in the free energy difference between the native and denatured states of the protein. This stabilising contribution in proteins amounts to up to 4–5 kcal mol^{-1} per S–S bond.[137]

Modern methods of protein chemistry lead to extensive possibilities for the introduction of intramolecular cross links into proteins. Firstly, one can use bifunctional modifying agents.[133] The methods developed for the imposition of "molecular yokes" make it possible in the main to cross-link surface regions in the protein. Another approach involves the introduction of a cross link within the protein molecule. Here the method of site-directed mutagenesis can serve as the instrument. For example, it has been possible to introduce S–S bonds into lysozyme T4—a protein from which they are absent—by this method.[139]

7. The Low Content of Aminoacids Susceptible to Oxidative Modification

One of the common mechanisms of the inactivation of proteins involves the oxidation (for example, at an elevated temperature) of aminoacid residues important for the structure.[12,13] In the first place the SH group of cysteine and the indole ring of tryptophan undergo oxidative modification. It is therefore not fortuitous that the highly stable thermophilic proteins contain much smaller amounts of these labile aminoacids (in the first place Cys).[19]

Apparently there exist two pathways to the stabilisation of enzymes unstable with respect to oxidation. The first involves a copy of the procedure which nature has used in thermophilic enzymes: to decrease the number of SH groups. For this purpose, one has to attempt by the method of site-specific mutagenesis to replace the Cys residues, most susceptible to oxidation (provided of course that they are not involved in catalysis), by others less labile, but having similar properties (geometry, hydrophobic properties).[140] The other procedures are methodologically simpler: to create around the enzyme a microenvironment which would shield it from the inactivating factor (oxygen). For example, a charged polyelectrolyte can serve successfully as a shield of this kind: it "coats" the enzyme and significantly hinders the access of oxygen to it. The "salting out" of oxygen by the polyelectrolyte matrix made it possible to increase the stability of labile hydrogenases by a factor of thousands.[141]

8. Compact Packing of Aminoacid Residues

The protein molecule in solution represents a compactly packed structure; the packing density of aminoacid residues in the globule is close to that characteristic of crystals of organic molecules.[1] Nevertheless, there exist "cavities" within the protein which are filled with water molecules (5–15 molecules per protein with a molecular weight of 20 000–30 000). The contact between the polar water molecules and the hydrophobic nucleus of the protein is thermodynamically unfavourable and destabilises the protein.

The stability of the protein can be in principle increased by further compaction, i.e. by the "additional compression" of the protein structure with simultaneous removal of water molecules from the internal region. This type of procedure has been used by nature to create thermophilic enzymes; in their structures, certain internal aminoacid residues have been replaced (compared with the corresponding mesophilic analogues) by more bulky residues.[19] The idea of the compaction of the protein can also serve as the basis for artificial stabilisation if an attempt is made to replace suitable aminoacid residues, for example, by site-directed mutagenesis. It is then necessary to bear in mind that the replacement of aminoacid residues by certain others is impermissible, because it can lead to serious infringements of the protein structure.[142]

9. Hydrophobic Interactions

At the present time ideas about the nature of hydrophobic interactions and their role in the stability of proteins have been arranged to form an elegant system.[143–145] In proteins, about half of the entire volume is occupied by aminoacids with non-polar side groups. Their contact with water is thermodynamically unfavourable, because the introduction of a non-polar fragment into water makes the structure of the latter more ordered. Such structure formation in water diminishes the entropy of the system and hence increases its free energy.# Therefore, in the organisation of the structure of the protein (coiling), the following tendency is manifested: to remove from contact with water the greatest possible number of non-polar fragments in the protein and to hide them in the bulk of the globule.[147] The influence of the unfavourable entropy factor is thereby weakened, hydrophobic interactions are intensified, and the stability of the protein increases.§

As soon as the idea of hydrophobic interactions as a factor stabilising the protein arose, attempts were made to correlate the stability of the protein with its hydrophobic properties. For this purpose, it was natural to attempt the determination

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#Structured clusters of water molecules were previously detected only around very small organic molecules.[143] In the X-ray diffraction analysis of the membrane protein crambin, it has recently been possible to observe a pentagonal cluster formed by water molecules around a non-polar fragment of the protein surface.[146] Thus it was demonstrated for the first time that the protein molecule does not alter qualitatively the character of the interaction of the non-polar fragments with water, i.e. the hydrophobic interactions have the same (entropic) nature for both small molecules and macromolecules.

§ This point of view on the entropic nature of hydrophobic interactions is dominant at the present time but is not the only one—see, for example, Hvidt's review.[148]

of a quantitative criterion of hydrophobic character. Tanford and co-workers suggested[149,150] that the free energy of the transfer of aminoacids from water to the organic phase be used. According to this classification, the more negative the ΔG of the transfer process for the aminoacid, the more hydrophobic it is (Table 6). The creation of a quantitative scale of hydrophobic character stimulated the search for correlations between the stability of the protein and its overall hydrophobic character, calculated as the sum of the hydrophobic character of all its constituent aminoacids. However, unambiguous conclusions could not be obtained from the correlations both for mesophilic proteins[28-30] and on comparing thermophilic and mesophilic proteins.[154,155]

Table 6. Indices of the hydrophobic character of aminoacids.

Amino-acid	ΔG for transfer, kcal mol^{-1}*	f, %**	Hf, arb. units***	Amino-acid	ΔG for transfer, kcal mol^{-1}*	f, %**	Hf, arb. units***
Trp	−3.77	0.27	12.95	His	−0.87	0.17	12.84
Phe	−2.87	0.50	13.43	Thr	−0.07	0.23	11.65
Tyr	−2.67	0.15	12.29	Ser	−0.07	0.22	11.26
Ile	−3.15	0.60	14.77	Asn	−0.09	0.12	11.00
Leu	−2.17	0.45	14.10	Gln	0.00	0.18	11.28
Val	−1.87	0.54	15.07	Asp	−0.66	0.15	10.97
Pro	−2.77	0.18	11.19	Glu	−0.67	0.07	11.19
Ala	−0.87	0.38	12.28	Lys	−1.64	0.03	10.80
Met	−1.67	0.40	14 33	Arg	−0.85	0.01	11.49
Cys	−1.52	0.50	14.93	Gly	−0.10	0.36	12.01

*The free energy of transfer from water to the organic solvent.[151]

**The fraction of the residues hidden within the globule.[152]

***The hydrophobic character of the environment in proteins.[153]

In the first part of the review, we already noted one of the causes of the failure of this approach: unduly small differences between the stabilities of the proteins against the background of large differences in their structures.

Another cause is that, when the aminoacid composition is determined by aminoacid analysis, one finds as a rule the overall contents of Asp + Asn and Glu + Gln, i.e. the acid and the corresponding amide, and one does not take into account the form in which these aminoacids are present. On the other hand, the differences between the hydrophobic properties of the acid and amide forms are large (cf. Asp and Asn, Glu and Gln in Table 6). For example, the highly stable ferredoxin *Clostridium thermosaccharolyticus* has a stability differing from that of the usual protein from *Clostridium tartarivorum*,[156] which has a normal stability, only by the fact that Glu residues are located in positions 31 and 44 and not Gln. With the aid of the usual amino-acid analysis, this difference could not be revealed. The comparison of X-ray diffraction data for the two proteins enabled the authors to resolve this problem.[156]

We may point to yet another source of error which arises in the analysis of correlations between the overall hydrophobic character and the stability of proteins. Sometimes the hydrophobic character of a stable thermophilic protein is compared with that of a statistical-average mesophilic protein and not a less stable mesophilic homologue.[157] This is methodologically absolutely incorrect; for a discussion of this question, see the review of Mozhaev and Martinek.[19]

The question arises whether there is any point (even when all the factors have been taken into account and a comparison made correctly) in seeking correlations between the stability and the overall hydrophobic character of proteins. Such a correlation would apparently be valid only for a certain "idealised" protein (it is sometimes represented by a drop of oil separated from contact with the solvent by a palisade of polar and charged groups[158]). Indeed, in such a hypothetical protein all the non-polar aminoacids would be involved in hydrophobic interactions. Although this simplified picture reproduces correctly the general trend towards the intensification of hydrophobic interactions in proteins, it is not observed in practice. According to X-ray diffraction data, approximately 50% of the area of real proteins is occupied by non-polar aminoacids.[114,145,147,159] They are frequently organised into surface hydrophobic clusters.[145] This has a functional importance because it enables proteins to bind via hydrophobic interactions to other proteins (polyenzyme complexes), lipids (biological membranes), polysaccharides (cell wall), and substrate effectors (enzyme catalysis and its regulation), i.e. ultimately it enables them to function correctly.[160,161] However, in proteins in the free, unbound state (enzymologists have to deal most often precisely with such objects), the disposition of non-polar aminoacids on the surface is "harmful" for stability, because it leads to the loss of free energy as a result of the weakening of hydrophobic interactions.

The non-ideality of protein structure (from the standpoint of the maximum realisation of hydrophobic interactions in them) necessitated the search for new approaches to the definition of a scale of the hydrophobic character of aminoacids. It became evident that the scale[149,150] established on the basis of the extraction model reflects incorrectly many trends in the coiling of the protein. It was therefore suggested that the hydrophobic character of aminoacids be estimated from statistical data for their distribution in the steric structure of proteins.[152,153,162,163]

Chothia's statistical scale[152] reflects the distribution of aminoacids in the bulk of the protein globule: the more frequently is the aminoacid encountered within protein globules and the more rarely on the surface, the more hydrophobic it is thought to be. Another statistical scale, set up by Ponnuswamy and co-workers,[153] is based on the fact that the hydrophobic character is determined by the microenvironment in which the given aminoacid residue is present. In this case the aminoacid which is surrounded by more hydrophobic neighbours in the protein globule is regarded as the more hydrophobic. The hydrophobic character of the microenvironment is then regarded as the sum of such characters[149,150] in the nearest neighbours in the tertiary structure of the aminoacids.

Comparison of these two statistical scales of the hydrophobic character[152,153] shows (Table 6) that they agree fairly well despite the fact that they are based on different principles. Thus in both scales Ile, Val, and Cys are the most hydrophobic and Lys, Arg, Asp, Glu, Asn, and Gln are the least hydrophobic. On the other hand, appreciable discrepancies between the data derived from the statistical scales and the extraction (Tanford) scale are observed. Thus Trp, which is the most hydrophobic on the extraction scale, occupies an intermediate position on the statistical scales and Tyr and Pro, which are closest to it in terms of their hydrophobic properties, should be more properly assigned to hydrophilic aminoacids in terms of the statistical scales.[152,153]

The causes of these discrepancies were first analysed by Chothia,[152,159] who concluded that the introduction of a polar atom into the aminoacid diminishes its hydrophobic character by 1–1.5 kcal mol^{-1}. It is therefore not fortuitous

that the aminoacids which are most hydrophobic on the extraction scale (Trp and Tyr) do not belong to the most hydrophobic aminoacids on the statistical scales (Table 6). Although their large surface area ensures their high hydrophobic character, the presence of a polar atom makes their presence within the protein globule significantly less favourable.

There exist also other causes of the discrepancies between the extraction[149,150] and statistical scales of the hydrophobic property.[152,153] A classification based on the extraction properties has been established solely on the basis of model experiments (transfer of the given substance from the aqueous to the organic phase) and therefore it in no way takes into account the fact that the aminoacids considered in fact enter into the composition of the polypeptide chains. In other words, in real proteins they must adjoin other aminoacids which in principle influence the geometry and energy of their neighbours.[164] Furthermore, the principle of close packing is realised in proteins[1,140,147] and therefore on coiling the most bulky aminoacids (Trp and Tyr) they are forced to remain on the surface of the globule in order not to infringe the compactness of the structure.

Thus there are three main factors which prevent the realisation of hydrophobic interactions in the proteins with the maximum advantage for the stability of the steric structure. Firstly, there is the need to ensure a sufficiently close packing of the aminoacids in the protein. Secondly, the geometry and energy of aminoacids are influenced by the microenvironment, i.e. they are neighbours in the tertiary structure. Thirdly, hydrophobic clusters, which *in vivo* ensure the interaction of the protein with other molecules, should remain on the surface of the protein when it is coiled up. Consequently, there is hardly any point in attempting to find a correlation between the stability of the protein and its overall hydrophobic character. It has been suggested[154] that the content of aliphatic aminoacids be used as a more suitable parameter, because they are indeed more frequently encountered within the protein than on the surface. The so called aliphatic protein index has been defined:[165]

$$A = X_{Ala} + aX_{Val} + b(X_{Ile} + X_{Leu}) .$$

where X_{ala}, X_{Val}, X_{Ile}, and X_{Leu} are the mole fractions of Ala, Val, Ile, and Leu in proteins and a and b are numerical coefficients determined by the size of the aminoacid residues. Fig.3 shows that the aliphatic index of the more stable proteins from thermophilic organisms is indeed higher than that of mesophilic proteins.

The following characteristics of the relation between the hydrophobic interactions in proteins with enhanced stability follow from the data considered. In the most general form, one may say that the increase of stability of proteins *in vivo* (for example of the stability of thermophilic enzymes) occurs not only and not so much at the expense of the increase of the overall hydrophobic character[166] as due to the more "correct" disposition of the non-polar aminoacids in the globule.[167] In other words, the greater the number of non-polar protein fragments are hidden in the interior of the globule and the smaller their number exposed to the solvent, the greater the stability of the protein. This picture has been described quantitatively by Lee and Richards,[147] who introduced the concept of the "surface area accessible (to the solvent)". It has been known that[159] the decrease of the overall free energy of the hydrophobic interactions in the protein ΔG_h depends on the change in the accessible surface area ΔA_s in the following manner:

$$\Delta G_h = \sigma \Delta A_s ,$$

where σ is a proportionality coefficient numerically equal to approximately 25 kcal mol^{-1} A^{-2}. It follows from this equation that the decrease of the surface area of the hydrophobic contact with water should entail the intensification of the internal hydrophobic interactions in the protein and hence should stabilise the latter. This is indeed observed on comparing thermophilic enzymes with the corresponding mesophilic enzymes,[168,169] on comparing the structures of a series of mesophilic homologues,[170] and also from the analysis of the stability of proteins modified by mutation.[161,171]

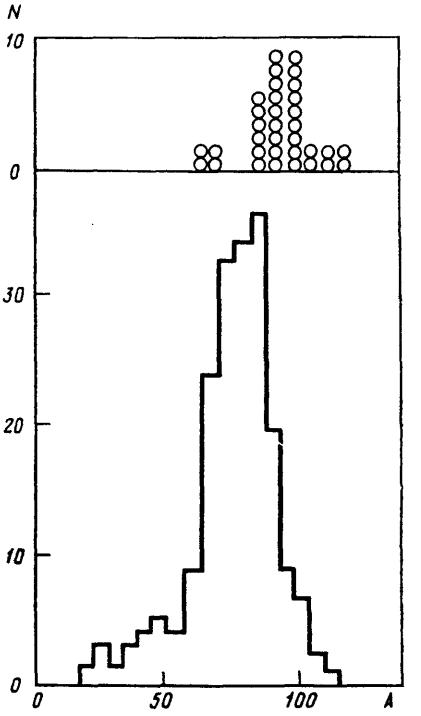


Figure 3. Distribution of the aliphatic index (A) among 34 proteins from thermophilic bacteria (upper section of the Figure) and 208 proteins from mesophilic organisms (lower section);[165] N = number of proteins.

Summarising this Section we may note that the stabilisation of proteins by the intensification of the hydrophobic interactions in them constitutes an extremely promising practical approach.[19,161] One should attempt either to decrease the hydrophobic character of the surface of the protein or to enhance its internal hydrophobic character.

IV. ARE THERMOSTABLE CATALYSTS NEEDED FOR BIOTECHNOLOGY?

The application of thermostable biocatalysts in biotechnolog has a number of advantages.[14–17,172]

The gain in the rates of processes. According to the van't Hoff rule, on raising the temperature by 10 K the rates of chemical, including enzyme-catalysed, reactions increase on average by a factor of 2–3.

The possibility of a shift of the thermodynamic equilibrium. The position of the chemical reaction equilibrium depends on temperature. According to the Le Chatelier principle, by raising the temperature, it is possible to displace the equilibrium towards synthesis for those reactions which take place with absorption of heat (endothermic reactions). Unfortunately, the thermochemistry of enzyme reactions which are of practical value has so far been studied in inadequate detail. As an example of an important reaction whose equilibrium shifts in an unfavourable direction on raising the temperature, we may quote the formation of a peptide bond.[173] Indeed, it has been shown that,[174] only by reducing the temperature for chymotrypsin-catalysed peptide synthesis, is it possible to increase the yield of the final product—the dipeptide.

The increase of the duration of work of the biocatalyst. The biotechnological processes actually introduced into practice involve a high economic cost contributed by the enzymes themselves.[175,176] Evidently the stabilisation of enzymes makes it possible to reduce this cost by increasing the period during which they can be used.

High resistance to other denaturing influences. As a rule, enzymes resistant to the action of high temperatures manifest an increased resistance also to other denaturing influences: concentrated solutions of denaturing agents, extreme pH values, and degradation by proteases. This is true, for example, of natural thermostable enzymes from thermophilic micro-organisms.[177,178] The practical value of this universal stabilisation is high, since the enzymic transformations of many natural compounds and the preparative synthesis of many substances can be effected in the required direction only in organic solvents or in aqueous organic media with a high content of the organic component.[179,180]

Increase of the efficiency of the catalytic process as a result of the increase of the solubility and volatility of the reactants and the decrease of the viscosity of the solutions at elevated temperatures. With increase of temperature, the solubility of the initial reactants increases also as a rule, which makes it possible to work with their more concentrated solutions and, in conformity with the Law of Mass Action, the absolute yield of the final product can be increased. If as a result of heating it has been possible to increase significantly the volatility of one of the final products, then the equilibrium in the synthetic reactions is strongly displaced towards the final product and their yield therefore increases. The decrease of viscosity on heating makes it possible to accelerate and hence to increase the efficiency of the processes in the kinetics of which diffusion plays a significant role.

Sterilisation of the products in the course of the reaction and decrease of the probability of the microbial infection of the reactors. The latter factor must always be reckoned with when biotechnological processes are carried out and its role can be as a rule significantly reduced at elevated temperatures.

The process can be readily arrested by simple cooling.

However, experiments at elevated temperatures also suffer from certain disadvantages. One of these is the increase of the rate of certain side processes, in the first place high-temperature oxidation[13] and racemisation of aminoacids and their derivatives.[181] The economically justified selection of conditions, high-temperature or low-temperature, therefore constitutes a problem which has an independent solution for each specific case.[182]

V. METHODS OF PREPARATION OF STABLE ENZYME CATALYSTS

1. The Search for Enzymes with Increased Thermal Stability in Mesophilic Sources

This approach is the oldest, traditional, and apparently does not require special comment. Its logical development has been the search for enzymes in sources which by their very nature are adapted to existence under high-temperature conditions.

2. Isolation of Enzymes from Thermophilic Sources

A homogeneous preparation of a thermophilic enzyme was first obtained 25 years ago.[183] Since then, the advantages of the use of thermophilic enzymes in biotechnology have been frequently discussed. Apart from the advantages of the thermally stable biocatalysts which were described above and which apply equally to thermophilic enzymes, we shall mention one other. Thermophilic micro-organisms are preferable to mesophilic organisms from the microbiological standpoint. Thus widescale cultivation of thermophilic strains is frequently cheaper than the cultivation of mesophilic strains owing to the decrease of the energy expenditure (there is no need to cool the growing biomass).[172] It is also important that, although thermophilic enzymes are very stable, they have not attained the absolute limit of thermal stability: there exists a certain reserve for additional stabilisation. Thus, having immobilised a thermophilic asparaginase[172] and protease, it has been possible to increase by a factor of 10 the resistance of these enzymes to irreversible thermal inactivation, which was in any case already high without this step.

Nevertheless there is virtually no information in the literature about the use of thermophilic enzymes in biotechnological practice. In our view, this is associated with the fact that, although the maximum catalytic activity of thermophilic enzymes is attained at higher temperatures (by 20–40 K), the activities of the mesophilic and thermophilic enzymes themselves are frequently identical at the temperature optimum.[48,169,185–188] Reports stating that the catalytic activity of a thermophilic enzyme at the temperature optimum is higher than that of the corresponding mesophilic enzyme have been appearing only occasionally.[189]

Not only are the catalytic activities of the thermophilic and mesophilic enzymes equal at the temperature optima but their thermal stabilities are also similar.[169] Thus, when thermophilic and mesophilic enzymes are compared, a correlation is manifested which is the same as that observed previously[190–194] for mesophilic proteins: the higher the catalytic activity, the lower the stability of the protein. In other words, having attained a greater stability, thermophilic enzymes have to sacrifice their catalytic activity partially.[187]

On the other hand, thermophilic enzymes have a property which makes them irreplacable for the purposes of biotechnology: the ability to alter their substrate specificity when the temperature is varied. This property is manifested particularly strikingly in the enzymes (proteases, nucleases, etc.) which act on high-molecular-weight substrates. The reason for this is that the substrates themselves (i.e. proteins and nucleic acids) undergo temperature-dependent conformational changes;[195] other new peptides and nucleotides bonds of the substrate are then exposed to the solvent and become accessible to the action of the enzymes. The appearance of a new specificity[196] is apparently of interest for the vigorously developing genetic

engineering, which requires a set of restrictases with different specificities.[197] One may hope that thermophilic restrictases will become new and highly effective instruments in genetic engineering.

3. Synthesis of Highly Stable Enzymes for the Introduction of a Thermophilic DNA into a Mesophilic Culture

Towards the end of the 1970's, reports appeared[198–200] that the introduction into the cell of a mesophilic organism (for example, *Bacillus subtilis*) of DNA from a related thermophilic organism (for example, *Bacillus caldolyticus*) results in the transformation of the mesophilic culture into the thermophilic culture at an elevated temperature. The transformant then loses its ability to grow at the usual temperature but acquires a new property—the ability to grow at an elevated temperature.[198] The entire biosynthetic apparatus of the transformant then acquires an increased thermal stability[198,199] and the synthesis of highly stable enzymes begins in the cell.[200]

At the beginning of the 1980's, another version of the genetic approach began to be used:[201,202] instead of a whole molecule of thermophilic DNA, only its fragment—the gene coding a specific protein—is introduced into the mesophilic culture. This approach has the undoubted advantage that the required stable enzyme can be obtained with a high yield (actually tens of times greater than in its isolation from a thermophilic culture).[202] Another advantage is that the operations involved in the isolation and purification of the protein are significantly simplified. Indeed, because at the elevated temperature almost all proteins synthesised by the mesophilic culture with the exception of the required high-stable protein are denatured, it is possible to achieve a high degree of purification of the protein at the expense of a small expenditure of time and at a small cost in equipment.

4. The Protein Engineering Method

At the beginning of the 1970's, successful experiments designed to create recombinant DNA were first carried out.[203] It became clear that a new powerful instrument for the wide-scale preparation of natural products and the synthesis of biological molecules with a broad spectrum of properties which do not exist in nature had been placed in the hands of investigators. A new scientific-engineering field was created on this basis—genetic engineering. By genetic engineering, one normally understands the construction *in vitro* of functionally active genetic structures (recombinant DNA), their introduction into cells, and the subsequent biosynthesis by the cells of biological molecules which are not characteristic of them. At the present time the advances in genetic engineering both in our country[204] and abroad[205,206] are generally recognised and the prospects are hopeful.

We shall deal with only one version of the genetic-engineering approach—site-directed mutagenesis[54–58] or "protein engineering".[20] This method makes it possible to obtain modified proteins differing from the prototype proteins by the replacement of, for example, only one aminoacid residue in a specific position in the protein structure. For biotechnology, the method is of interest primarily because it makes it possible to alter specifically the structure of enzymes, their catalytic properties, and their stability.[20] We shall now consider how this is done.

In the first place we shall deal with the primary structure of the initial enzyme[103] and then with its tertiary structure.[134] Then the following stage becomes possible—the selection of the site where the aminoacid residue is to be replaced. This stage does not involve any laborious operations, such as the growing of protein crystals, the determination of the aminoacid sequence, or the interpretation of X-ray diffraction patterns, which have to be performed in the next stage. However, this is in fact the key stage, because it determines the properties of the final product. Next, it is necessary to proceed from the language of protein structure (the aminoacid sequence) to the genetic code. For this purpose, an oligopeptide fragment (4–6 aminoacid residues) in the primary structure of the protein, at the centre of which is located the aminoacid to be replaced, is selected, and an oligodeoxyribonucleotide containing 12–18 bases coding the aminoacid sequence in the chosen oligopeptide, is synthesised.[210] The mutation is programmed in precisely this stage: instead of the nucleotide triplet coding the "native aminoacid", another triplet, coding the new (planned in the preceding stage) aminoacid residue, is introduced into the synthetic oligonucleotide by chemical synthesis. The subsequent operations reduce to genetic-engineering manipulations. Firstly, one finds (or creates artificially) a single-stranded plasmid incorporating the genes coding the given protein. Secondly, using DNA-polymerases and DNA-ligases, one creates enzymically, on the basis of the single-stranded plasmid and the nucleotide synthesised, a heteroduplex double-stranded plasmid. The oligonucleotide then initiates the synthesis of a double-stranded region, fulfilling the role of a seed. Since homoduplex plasmids together with heteroduplex plasmids are synthesised on single-stranded DNA molecules as templates, they are separated using different resistances to heating of homoduplex and heteroduplex plasmids. The heteroduplex double-stranded plasmids are then introduced into a convenient host cell in which they are transformed into two homoduplex plasmids: one contains the gene of the native protein and the other that of the mutant protein. The two genes and their subsequent cloning are carried out at the cellular level. The final result of the multistage process is transformant cells in which the mutant protein, differing from the initial protein by the replacement of one aminoacid residue at a rigorously specified site, is biosynthesised.

The method of site-specific mutagenesis appeared at the beginning of the 1980's and only the first steps have been taken.[20,207] Nevertheless, even the first achievements of the method are striking. We shall consider some of them concerning the synthesis of stabilised proteins.

In the molecule of lysozyme of the T4 phage there are no S–S bonds, but two SH groups are present. One of them, belonging to the Cys-97 residue, is located in the vicinity of the N-terminal region in the tertiary structure. The Ile-3 residue was replaced by cysteine by protein engineering. Since in the tertiary structure of the mutated lysozyme the Cys-3 and Cys-97 residues adjoined one another, the S–S bond was formed in a weakly oxidising medium. The conformations of the native and mutated enzymes were identical but their catalytic properties were different.[139] However, the lysozyme modified by mutation differs from the native protein by a significantly higher thermal stability (Fig.4). The reason for such stabilisation lies in the strengthening of the structure of the protein as a result of the introduction of the S–S bond.[139] Indeed, if the S–S bond in the modified lysozyme is cleaved, then the stability of the protein becomes virtually indistinguishable from that of the initial protein (Fig.4).

Another example of mutation designed to increase stability is provided by the α-subunit of tryptophan synthase. Several forms of the enzyme, differing from one another by the replacement of the aminoacid residue Glu-49 located in

the hydrophobic nucleus of the globule by another aminoacid, were obtained.[208] Such changes do not alter either the conformation or the functional activity of the α-subunit of tryptophan synthase. However, the protein proves to be more stable the more hydrophobic aminoacid has been introduced in position 49. It follows from the correlation illustrated in Fig. 5 that the method of site-specific mutagenesis provides unique possibilities for increasing the internal hydrophobic character (and hence the stability) of the protein by replacing polar aminoacids by more hydrophobic aminoacids. It is then naturally necessary to make specially sure that these replacements do not disrupt the compact packing (conformation) of the protein.

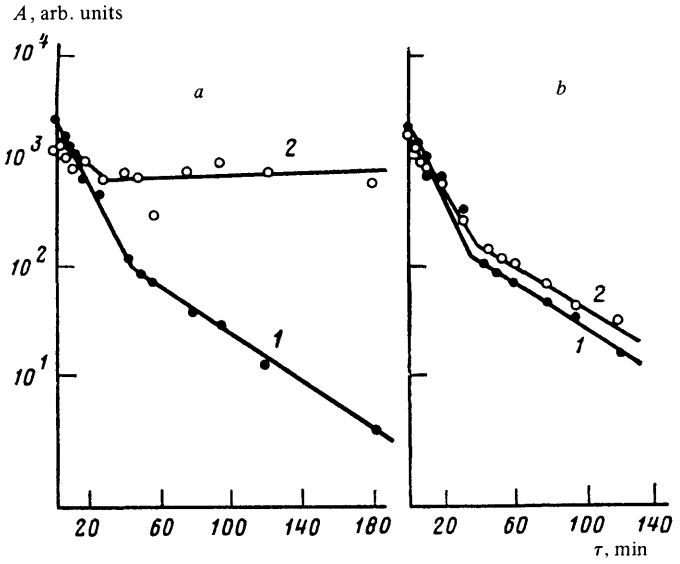


Figure 4. Kinetics of the thermal inactivation of the T4 lysozyme from a wild strain (curves *1*) and the T4 lysozyme with the substitution Ile-3 → Cys and subsequent oxidation with sodium tetrathionate (curves *2*) in the absence (*a*) and presence (*b*) of 10 × 10^{-3} M β-mercaptoethanol;[139] *A* = catalytic activity.

An example of the way that proteins resistant to oxidative inactivation can be obtained by the method of site-specific mutagenesis is provided by the study of Estell et al.[209] The Met-222 residue in subtilisin, which is located in the region of the active centre, readily enters into oxidative reactions, which inactivate the enzyme. It was replaced by the method of protein engineering by other residues, whereupon the activity of the enzyme did not diminish very greatly (within the limits of a factor of 10) but its resistance to inactivation by H_2O_2 increased significantly (Fig. 6). The stabilisation is caused by the fact that, having removed from the active centre the readily oxidisable Met residue (having replaced it by other residues less susceptible to oxidation, namely Ser, Ala, and Leu), it proved possible to eliminate the very cause of oxidative inactivation.[209]

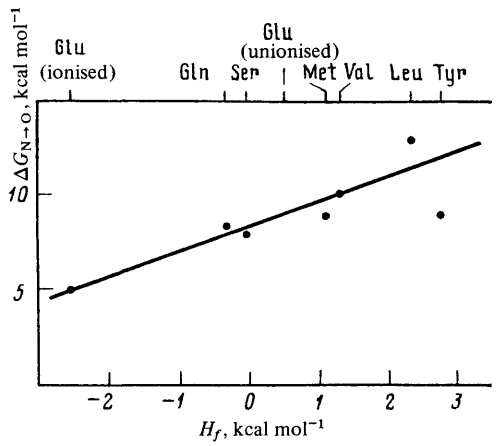


Figure 5. Dependence of the free energy of the stabilisation ($\Delta G_{N \rightarrow D}$) of the native conformation of the α-subunit of tryptophan synthase from *Escherichia coli* on the hydrophobic character (H_f) of the aminoacid residue in position 49;[208] the numerical values of the hydrophobic character of the aminoacids are based on Tanford's data.[150]

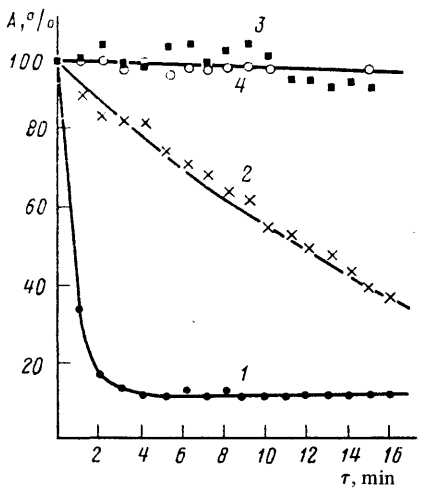


Figure 6. Kinetics of the inactivation (on treatment with 1 M H_2O_2) of subtilisin of a wild type (curve *1*) and mutant forms where position 222 contains the Cys (curve *2*), Ala (curve *3*), or Ser (curve *4*) residues;[209] *A* = catalytic activity.

However, the procedures of protein engineering are not simple. Successful work in this field requires the cooperation of specialists in the structure and chemistry of proteins and nucleic acids, in molecular and cell genetics, and in other branches of biochemistry and molecular biology. The work must be carried out at the highest experimental level using

the latest advances in these fields and requires a large material expenditure. For this reason, traditional methods such as immobilisation and chemical modification, which have long been used to stabilise enzymes, still compete successfully with protein engineering.

5. Immobilisation of Enzymes

A series of special reviews have been devoted to the creation of stabilised enzyme preparations by the immobilisation method.[14-19] Here we shall therefore deal only with the main advances in the immobilisation approach.

As early as 1954, Lumry and Eyring formulated[211] the general ideas concerning the inactivation of enzymes, which still remain valid.[12,13] It was suggested that the inactivation be regarded as a two-stage process:

$$N \rightleftarrows D \rightarrow I$$

where N, D, and I are respectively the native, reversibly denatured, and irreversibly inactivated forms of the enzyme. Any inactivation begins as a rule with a reversible conformational change (the $N \rightleftarrows D$ stage in the scheme), which frequently embraces the entire globule. The reversible uncoiling stage is followed by irreversible processes ($D \rightarrow I$ in the Scheme), such as aggregation,[212] covalent modification of the aminoacid residues,[12,13] and irreversible conformational changes.[213-215]

The general approach to the stabilisation of enzymes based on the immobilisation method consists in a significant retardation or suppression of the primary uncoiling of the protein molecule (the $N \rightleftarrows D$ stage in the Scheme).[216] The multipoint addition of the protein molecule to the surface of a carrier proved to be the most fruitful for this purpose.[217,216] This immobilisation approach made it possible to retard by factors of hundreds and thousands both reversible[130,219] and irreversible[217,218] thermal inactivation of enzymes, their reversible uncoiling under the influence of chemical reagents,[220] and the dissociation of oligomeric fragments into subunits (see the literature references in the review of Arens et al.[216]).

The immobilisation approaches also proved to be extremely useful for the suppression of irreversible secondary processes (the $D \rightarrow I$ stage in the Scheme). Thus, if the inactivation is caused by protein–protein interactions (aggregation or autolysis), then, having immobilised the enzymes, such inactivation can be as a rule fully prevented (see the relevant reviews[14-17,62]). Immobilisation also helps to protect enzymes from inactivation by chemical modification. Such protection is achieved in two ways. Firstly, the enzyme is immobilised within a carrier, which "shields" it from contact with the inactivating molecules. An example of this approach is provided by the protection against oxidative inactivation of hydrogenases incorporated in polyelectrolyte carriers.[118] Secondly, a carrier containing functional groups competing for the inactivating substance or causing its decomposition is selected for immobilisation. For example, if the inactivation takes place under the influence of peroxy- or superoxide radicals, then, in order to achieve protection against them, the enzyme is immobilised jointly with peroxidase or catalase, which catalyse the decomposition of these radicals.[13]

6. Chemical Modification of Enzymes

The first successful studies on the stabilisation of enzymes by their chemical modification were carried out[221] in the middle 1950's. However, the view is still being encountered[65] that it is hardly possible to formulate sufficiently general rules whereby stabilised enzyme preparations can be obtained by chemical modification. Analysis of the numerous studies in this field (see the relevant reviews[18,64-66]) enabled us to reach certain conclusions about the causes of the stabilisation of proteins by their covalent modification.

As a result of the modification, the protein is sometimes converted into a more stable conformation different from the native conformation.[104,105]

Stabilisation takes place on modification of "key" functional groups[64] (they are also referred to as type II groups[66]).

New functional groups, introduced into the protein as a result of chemical modification, are capable to forming additional hydrogen bonds or salt bridges.[222]

Chemical modification by non-polar compounds intensifies the hydrophobic interactions in proteins.[223]

The hydrophilisation of the surface groups of the protein decreases the area of unfavourable contact of the outer hydrophobic residues with water.[19]

The question arises which of the mechanisms enumerated can be used as the basis of general approaches to the stabilisation of enzymes.

1. There is hardly any point in attempting to convert the protein by covalent modification into a more stable conformation different from the native conformation. The point is that the character of the conformational changes introduced by the modification is unpredictable in the vast majority of cases.

2. We shall consider the concept of "key functional groups".[64] The experiment involving chemical modification frequently looks as follows: the stability of the protein does not change with increase of the degree of modification until the attainment of a critical value, whereupon the stability increases sharply.[224] The observed variation is explained as follows:[64,66] for low degrees of modification (in the presence of a small excess of the modifying reagent), mainly the functional groups of the protein which are located on the surface and do not play an important role in the structure and stability are involved. However, in the presence of a large excess of the modifying agent, certain functional groups in deeper layers of the protein globule are modified. The modification of these groups, called "key" groups, improves the balance between the intramolecular interactions in the protein and stabilises the latter.

The application of the idea involving the modification of "key" functional groups in the protein encounters, however, a number of difficulties. The main one is how to discover which functional groups are the "key" groups. This is hardly possible to achieve *a priori*, for example, on the basis of the steric structure of the protein. The only reasonable procedure is to determine them empirically, i.e. by studying the dependence of the stability of the protein on the concentration of the modifying agent (degree of modification). Such studies must be carried out with exceptional care, because there exists a real risk of "overlooking", missing these "key" groups. Indeed, after the modification of the "key" groups, deeper aminoacid residues in the protein are usually involved, which as a rule destabilises it.[64,66] For this reason, if the "key" degree of modification of the protein has been missed in the experiment accidentally, then a stabilisation effect cannot be observed at all. Furthermore, for each specific protein and each new type of functional groups (for example, COOH, NH_2, SH, etc.), the empirical search for "key" groups must be carried out anew.

3. One may attempt to introduce into the protein molecule polar or charged groups by its modification, hoping that they will form new salt bridges with the functional groups already

present in the protein. Unfortunately, it is not easy to apply this approach in practice. For this purpose, it would be necessary to obtain maps of the steric structure of proteins and to search on the latter the uncompensated polar and charged groups which might, in principle, form new hydrogen bonds and enter into electrostatic interactions. Next, it would be necessary to find in the protein a suitable "anchor" group located in the vicinity of the uncompensated polar and charged group. Having selected or synthesised a suitable chemical agent, which would contain, on the one hand, a group reacting specifically with the anchor group and, on the other, a charged or polar fragment located at the specified distance, one could attempt to achieve the specific modification. It is not easy to perform such a delicate experiment.

4. According to some investigators,[223] the stability of proteins can be increased by rendering them hydrophobic. However, the experimental data concerning the influence of hydrophobic modification on the stability of proteins are contradictory. Together with stabilisation effects,[223] cases are known where the stability of proteins rendered hydrophobic is significantly lower than that of the native proteins.[225-227] This apparent contradiction can be explained as follows. If the hydrophobic residue (for example, the CH_3 group) is introduced on the surface of the protein, the latter should be destabilised. Indeed, as a result of the modification, the protein acquires a new thermodynamically unfavourable contact between the hydrophobic CH_3 group and water. However, there are exceptions to this general rule. As mentioned above, together with polar and charged aminoacids, there are on the surface of proteins also hydrophobic aminoacids which are frequently organised into hydrophobic surface clusters.[228,229] If the functional group located near such a cluster has been modified, then the modifying agent of suitable length cuts off the contact between the solvent and the cluster. One can imagine the situation where the interaction of the modifying agent with the cluster does not alter or even diminishes the area of the unfavourable contact between the hydrophobic surface of the protein and water. In any case, however, the modification results in the appearance of an additional contact between two hydrophobic surfaces, which should increase the stability of the protein.[223]

This view is also supported by the observed increase of the stability of certain proteins[230,231] as a result of complex formation with non-polar molecules (benzene and aliphatic acids). The interaction of proteins with these compounds can be represented in the way already described above. However, since this is a relatively weak non-covalent interaction, the protein–ligand complex dissociates readily when the excess of the non-polar substance is removed from the system. The formation of such non-covalent complexes can therefore hardly serve as the basis of a general method for the stabilisation of enzymes.

The modification permitting the introduction of a non-polar molecule into the interior of the hydrophobic core of the protein should be especially effective from the standpoint of stabilisation.[19] The essential feature of the approach permitting the attainment of this situation is that the initial state for modification is not the coiled (native) conformation of the protein but the uncoiled state (statistical coil). This state can be arrived at by treating the protein with a strong denaturant (urea) with the simultaneous cleavage of the S–S bonds[232] (in those proteins where these are present). An attempt may be made to alter the structure of the uncoiled protein in one of the three ways proposed. Firstly, it can be coiled up under "non-native" conditions, i.e. conditions which are not characteristic of the coiling process *in vivo*. There is hope that the protein will coil up into another conformation, which is also catalytically active but more stable. In particular, if the coiling is carried out under conditions favouring the enhancement of the hydrophobic contacts (concentrated solutions of salts, elevated temperature, etc.) then it is possible to obtain a protein conformation in which non-polar aminoacids are more "correctly" packed (the interior of the protein becomes more hydrophobic and the surface more polar). Secondly, it is possible to coil up the proteins in the presence of substances which enter into multipoint non-covalent interactions with them. Here non-polar and biphilic compounds are most promising in our view. The former can be captured in the interior of the hydrophobic core on coiling up the protein and the latter can be "built" into the coiled protein, being in contact via their non-polar fragment with the hydrophobic regions in the protein, while the polar or charged fragments are exposed to the solvent. Thirdly, the uncoiled protein can be modified initially by a chemical agent and then coiled up.

At the present time the approach described above is being actively developed.[233] We shall deal with only one experimental result. Immobilised trypsin was converted into the uncoiled state and then coiled up at different temperatures. The trypsin coiled up at 50 °C and above proved to be[19] more thermally stable than the initial enzyme or enzyme which had been coiled up at the usual (20–35 °C) temperatures. These results agree with data obtained *in vivo* for thermophilic microorganisms.[46,47,234-236] These organisms synthesise two sets of enzymes: stable and labile, and at high temperatures the content of the stable forms is significantly higher. The "high temperature" and "low-temperature" proteins are apparently the iso-forms of the same enzymes, having identical primary structures.[46,47] Consequently, as in model experiments, the protein *in vivo* also coils up into a more or less stable conformation depending on the temperature of the biosynthesis.

5. Finally, we shall consider the approach to the stabilisation of enzymes which we recently proposed[19] and which is being successfully developed at the present time.[237,238] It is based on the hypothesis that contact between the hydrophobic sections of the surface of the protein and water is thermodynamically unfavourable and therefore destabilises the protein. The idea of the approach is that the area of contact between the hydrophobic clusters on the protein surface and water be reduced by chemical modification.

Before applying this approach, it is essential to select a criterion of the hydrophobic character of chemical compounds. Hitherto, in discussing the influence of chemical modification on the stability of proteins, sufficient attention was not paid to this question; (see, for example, the relevant reviews[64-66]). The conclusion whether or not chemical modification of the protein rendered it hydrophobic or hydrophilic is sometimes self-evident and does not require accurate quantitative estimates. As an example of the evident acquisition of hydrophobic properties, one may quote the modification of α-chymotrypsin by aliphatic aldehydes.[224] Indeed, in this case, after the reduction of the Schiff base formed, the aminoacid groups of the enzyme are transformed into $-NH-(CH_2)_n-CH_3$. However, it is then hardly possible to estimate even qualitatively from general considerations how the hydrophilic character of the amino-group changes when it is modified by succinic anhydride [when the NH_2 group is transformed into $-NH-CO_2-(CH_2)_2-COOH$]. Nevertheless, such modification is frequently regarded as imparting hydrophilic properties.

We selected as the quantitative criterion of hydrophobic character the partition coefficient P of the compound considered between water and an immiscible organic phase proposed by Hansch and co-workers.[239,240] According to this

criterion, the more positive the value of P, i.e. the greater the preference for the organic phase by the substance compared with the aqueous phase, the more hydrophobic it is. On the basis of the principle of the additivity of free energies,[241] it is possible to calculate the hydrophobic characters of various functional groups and atoms. For example, the hydrophobic increment per Cl atom (π_{Cl}) can be calculated from the experimental partition coefficient of two compounds: chlorobenzene and benzene:

$$\pi_{Cl} = \log_{C_6H_5Cl} - \log_{C_6H_6} = 2.84 - 2.13 = 0.71 .$$

Using this procedure, we calculated[239,240] the hydrophobic increments for the functional groups and atoms most frequently encountered in biochemistry (more than 150). Some of the values of π are listed in Table 7. The converse problem can be solved by this procedure in its turn—the hydrophobic characters of complex compounds can be estimated.

We suggest the application of the approach described above[239,240] for the estimation of the contribution by the chemical agent (modifying agent) to the change in the hydrophobic character of the protein. Quantitative analysis of this aspect permits a fresh view on certain experimental data concerning the chemical modification of proteins. Thus the oxidation of the Met-192 residue in α-chymotrypsin to the more hydrophilic form $S(O)CH_3$ increases the conformational stability of the protein by 2.1 kcal mol^{-1}. On the other hand, the difference between the free energy increments for the extraction of these groups from water into an organic solvent, namely $2.3RT(\pi_{SOCH_3} - \pi_{SCH_3})$, is very similar, i.e. 2.5 kcal mol^{-1} [240] (p.52). On this basis, one may postulate that the stabilisation of chymotrypsin observed following the oxidation of only one Met residue,[242] is caused precisely by the acquisition of hydrophilic properties by the surface of the hydrophobic cluster in the protein.

Another example of acquisition of hydrophilic properties is provided by the modification of the amino-groups of the protein (for example, by *O*-methylisourea) with their conversion into arginine-like structures. Table 6 shows that Arg is more hydrophilic than Lys. The numerous examples[243–248] of the stabilisation of proteins following the introduction of guanidino-groups into Lys residues can therefore be explained by the decrease of the area of contact between the hydrophobic protein and water.

A major stabilisation effect was achieved by aminating the tyrosine residues in trypsin.[237] The four tyrosine residues in the molecule of this enzyme are distributed on the surface.[249] The appearance of the amino-group in the aromatic ring of tyrosine increases its hydrophilic character by 1.24 kcal mol^{-1}. As a result of such increase in hydrophilic character, the stability in relation to irreversible denaturation of trypsin in which two and more tyrosyl residues have been modified increased by a factor greater than 100.[237]

The stabilisation method designed to render the surface of the hydrophobic clusters in the protein hydrophilic[19] has achieved the greatest successes in the acylation of α-chymotrypsin by the dianhydride of pyromellitic acid.[238] It was shown that the modification involves mainly the protein amino-groups and that three new carboxy-groups are introduced into the enzyme molecule on modification of each group. Thus, the preparation with the maximum degree of modification has been enriched by at least 50 COOH groups.[238] At weakly alkaline pH (thermal inactivation conditions), all the carboxy-groups are deprotonated and a very significant hydrophilic character of the protein surface has therefore been attained (Table 7). The modified enzyme is so much more stable than the native enzyme that it is impossible to select experimentally the temperature at which the kinetics of the inactivation of these two preparations can be compared. In order to estimate nevertheless the difference between their stabilities, it was necessary to extrapolate the experimental data (Fig.7) to an average temperature. For example, at 60 °C the stabilisation effect corresponds to a factor greater than 10^3, becoming still greater with increase of temperature, as can be seen from Fig.7.

Table 7. Parameters of the hydrophobic character of certain chemical groups.[240]

Group	π^{*}_{Ar}	π^{**}_{Alk}	Group	π_{Ar}	π_{Alk}	Group	π_{Ar}	π_{Alk}
H	0	0.23	OH	−0.67	−1.64	COOH	−0.32	−1.11
CH_3	0.56	0.77	SH	0.39	−0.23	COO^-	−4.36	−5.19
C_2H_5	1.02	1.43	OCH_3	−0.02	−1.54	$COCH_3$	−0.55	−1.13
$CH(CH_3)_2$	1.53	1.84	$NHCH_3$	−0.47	−1.38	CO_2CH_3	−0.01	−0.72
C_3H_7	1.55	1.97	CN	−0.57	−1.27	C_2H_3COOH	−0.29	—
C_6H_{11}	2.51	—	NO_2	−0.28	−1.16	$N(CH_3)_2$	0.18	−0.64
C_6H_5	1.96	1.90	NH_2	−1.23	−1.54	$\overset{+}{N}(CH_3)_3$	−5.96	—

*The value of π for a substituent in the aromatic ring.
**The value of π for a substituent in an aliphatic compound.

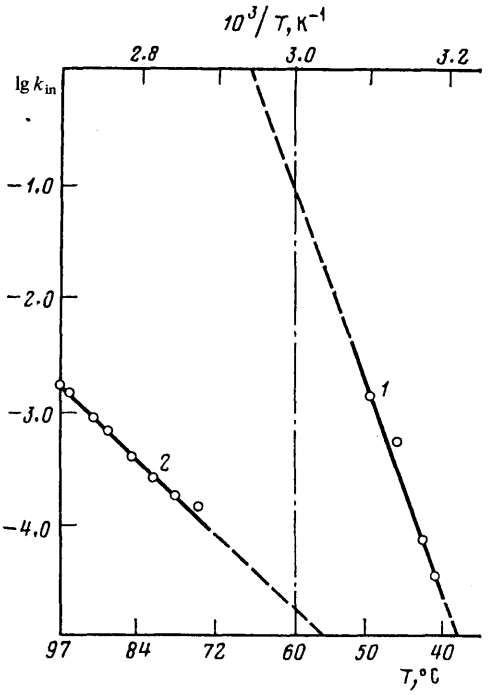


Figure 7. Temperature variation of the rate constant k_{in} for the thermal inactivation of native α-chymotrypsin (line *1*) and α-chymotrypsin modified with pyromellitic dianhydride (line *2*); the degree of modification of the acylated preparation is a maximum.[238]

Thus, by imparting hydrophilic properties to the surface of chymotrypsin (by introducing a large number of carboxylate anions), it is possible to attain[238] enormous stabilisation effects. Previously effects of this order were observed

only on multipoint binding of the enzyme to the surface of a carrier[130,217,218] (see also the relevant reviews[11-19]). Thousandfold (and greater) stabilisation effects[238] are so far the highest achieved using low-molecular-weight modifying agents for proteins. The stability of the chymotrypsin modified by pyromellitic dianhydride is not inferior to that of proteases from extreme thermophiles[250] and is at present the most stable among all the known proteolytic enzymes.

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In the present review we attempted to anser two questions. Firstly, how to discover the structural determinants of the stability of proteins (i.e. the structural features and intra- and inter-molecular interactions which ensure an increased stability of the protein) and, secondly, which approaches and methodological procedures must be used in order to obtain, knowing these structural determinants of the stability of proteins, stabilised enzyme preparations for biotechnology. We do not share the pessimism which is frequently expressed concerning the possibility of recognising the molecular causes of the stability of proteins and of applying them in practice in order to devise stabilisation approaches. On the contrary, the advances achieved hitherto are extremely hopeful. We are confident that new methods and ideas (some of these were discussed or proposed in the present review) will generate an additional stimulus to studies in this field.

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