STRUCTURE-STABILITY RELATIONSHIP IN PROTEINS: FUNDAMENTAL TASKS AND STRATEGY FOR THE DEVELOPMENT OF STABILIZED ENZYME CATALYSTS FOR BIOTECHNOLOGY

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I. INTRODUCTION

The relationship between protein structure and stability is one of the key problems of modern chemistry[1,2] whose solution should contribute to a better understanding of another important problem, namely, how the self-orgnization of protein molecules proceeds in vivo. Only the solution of these two problems will enable us to approach the materialization of an old dream: to predict the spatial structure of a protein from its known amino acid sequence (primary structure)[1,3] and to synthesize by chemical methods[4] protein molecules with the desired properties.

On the other hand, one of the primary tasks of enzyme engineering is to produce stabilized enzyme preparations to be used in fine organic synthesis and analysis, in medicine, and in various areas of biotechnology.[5-11] The way to the development of stabilized enzymes was paved many years ago by researchers studying molecular mechanisms or at least the molecular basis of protein inactivation (for review, see References 12 and 13). This initial stage provided us with possibilities for changing artificially the structure of enzymes in order to suppress their inactivation. The *chemical* and *physical* methods of enzyme stabilization belong today to the most successful ones (for review, see References 14 to 19). However, there is no doubt that the *biological* approaches recently proposed (such as application of genetic engineering)[20-22] are also very promising.

The present review, which covers the chemical, physical, and biological aspects of protein stability and stabilization, is divided into several parts for reasons of convenience. In the first part, the experimental and theoretical approaches to studies on the relationships between protein structure and stability are discussed briefly. The molecular basis of protein stability is treated in the second part. This problem has been discussed consistently in the literature

ever since the pioneering work of Bresler and Talmud[23] appeared in 1944. In addition, the modern aspects of the problem have also been reviewed exhaustively, e.g., in the monograph by Schulz and Schirmer.[1] Therefore, we have made an attempt to answer only the principal question: how should a protein be treated, i.e., how should its structure be changed in order to become more stable? In the third part of the review, the experimental methods of obtaining highly stable enzyme preparations are described.

II. THEORETICAL AND EXPERIMENTAL LINES OF APPROACH TO THE ELUCIDATION OF THE RELATIONSHIP BETWEEN PROTEIN STRUCTURE AND STABILITY

To solve the problem of the structure-stability relationship in the most general sense means to calculate the free energy of a protein from its known three-dimensional structure. Hence, the energy of all the factors, both stabilizing (hydrogen bonds, hydrophobic and electrostatic interactions, etc.) and destabilizing (above all, the thermodynamically unfavorable entropic contributions to the standard free energy of the protein caused by its strained conformation), should be calculated and summed up. Unfortunately, this cannot be done exactly[1,24] since the contribution of each of the factors mentioned to the free energy of the protein represents dozens to hundreds of kilocalories per mole.[24-26] Therefore, the standard deviations of these values (even when the most accurate calculation methods are used) are dozens of kilocalories per mole, i.e., too high, particularly in view of the fact that proteins possess marginal stability only; namely, the experimental values of free energy of protein stability (of the native conformation vs. a denatured one) determined in this manner for most of globular proteins are only 5 to 15 kcal/mol.[27]

However, the structure-stability relationship in proteins can be studied experimentally. A common experimental approach to this problem is the following: proteins of different stability are chosen and their structures (determined by X-ray studies and/or other physicochemical methods) are compared in order to find out the structural features responsible for the higher stability of one of them. The question arises, however, concerning which proteins should be chosen for such a comparison. The problem is that two proteins arbitrarily taken for comparison will most probably differ only marginally in stability. It will be hardly possible, therefore, to correlate the difference found between these two proteins with any of their *particular* structural features (especially if one takes into account the large differences in the structures of these proteins not functionally related).[27]

The same peculiar characteristics of proteins, i.e., a negligible difference in stability and a great structural variety, also make difficult the prediction of protein stability from the knowledge of their *general* structural features.[28-31] In other words, all the attempts to analyze the structures of many different proteins in order to find a relation between their stability and some of their general structural parameters (e.g., hydrophobicity,[28-30] volume or content of a particular amino acid residue[30,31]) have resulted in ambiguous conclusions.

The comparison of the stability of proteins having similar structures has yielded better results. Ideally, the tertiary structures of the proteins compared should be almost identical and the difference in their stabilities should result only from minor differences in their amino acid sequences. Such a structural relationship is typical of proteins from different sources but with the same biological function.

A typical experiment aimed at the elucidation of the relationship between protein structure and stability involves three principal steps.

1. The isolation and purification of two (or more) functionally related proteins from different sources

2. The analysis of the following parameters characterizing the structure and the stability of these proteins: amino acid composition and primary structure; spatial (three-dimensional) structure and conformational mobility; resistance to reversible and irreversible denaturation; and temperature dependence of functional (enzymatic) activity
3. The search for relationships between the quantitative characteristics of stability (e.g., temperature of reversible denaturation, optimal temperature of enzyme activity, and thermodynamic and/or kinetic parameters of denaturation) and the structural differences found

Where could a pair of functionally related proteins differing in stability be found? First of all, they could be found in nature, taking, e.g., the two functionally related proteins compared from mesophilic sources or taking one protein from a mesophilic source and the other one from a thermophilic microorganism. Moreover, a protein from a wild strain can be compared with its counterpart from a mutant strain. Finally, a native protein can be compared with a protein prepared in the laboratory by site-directed mutagenesis, immobilization, or chemical modification. Let us consider each of the lines of approach in detail.

A. Homologous Proteins from Mesophilic Microorganisms

The comparison of the structures of enzymes having a similar function in different organisms has been used frequently to study the mechanism of enzyme catalysis;[32] seldomly, however, has it been used to elucidate the structure-stability relationship in proteins. This is due to the fact that the differences in the stability of homologous proteins are rather insignificant (<1 to 3 kcal/mol). On the other hand, there are usually marked differences in the amino acid composition of functionally related mesophilic proteins. In some related proteins of the globin family, for example, only 16% of the amino acid residues occupy the same positions in the primary structures. Hence, in comparing homologous mesophilic proteins, we are usually faced with the problem discussed previously, namely, how to interpret small changes in stability in terms of major differences in structure.

Nevertheless, the comparison of homologous proteins from mesophilic microorganisms has yielded valuable information on the structure-stability relationship, especially on globins, cytochromes, dehydrogenases, ferredoxins, and lysozymes.[33-36]

If two proteins can be found which only differ in one to two amino acid residues, then the elucidation of the role that these residues play in their stability is much easier. Such examples have already been considered.[19]

Specifically, we can reveal invariant sites in proteins, i.e., sites where the substitution of amino acid residues is not permitted in any case since it would result in the destruction of the unique spatial structure and hence loss of activity. As a rule, the invariant sites are located inside the globule,[33-36] e.g., in the interface of two α-helixes[33] or of subunits of oligomeric proteins.[36] The amino acids involved in the binding of the heme and of other cofactors[34] or constituting the active sites of enzymes[36] are also invariant. The investigation of the invariant sites is of special value for experiments with amino acid substitutions, e.g., in protein engineering[20] (see Section IV.I).

B. Comparison of Proteins from Thermophilic and Mesophilic Microorganisms

It has been known since ancient times that life can exist under extreme conditions. Back in the 1st century B.C., Pliny the Elder in his *Natural History* mentioned organisms living in hot springs.[37] A detailed study on thermophilic microorganisms adapted to life at elevated temperatures (up to 100°C) was undertaken in the beginning of the 20th century. However, it was not until the mid-1960s that it was unambiguously demonstrated (e.g., see Reference 38) that the stability of microorganisms under extreme environmental conditions is well correlated with the enhanced stability of the cell constituents (biomacromolecules) of pro-

teins. As a rule, isolated thermophilic proteins, when not in contact with the other components of the cell, are very stable (even though some opposite observations have also been made, see Section III.B).

The investigation of differences between the structures of thermophilic and the corresponding mesophilic proteins is more advantageous than the comparison of many mesophilic proteins since the differences in the stability of these structures are much larger (up to 5 to 10 kcal/mol). Therefore, the probability that structural features which are of key importance for the stability of proteins will be discovered is higher. About 100 thermophilic proteins have been isolated and their properties have been studied in detail. Moreover, the molecular basis of the higher stability of thermophilic proteins has been discussed in numerous special reviews and monographs.[19,37-49]

C. Proteins from Mutant and Wild Strains

Mutagenesis occurs in nature as a result of unfavorable external factors, such as chemical reagents and irradiation.[50] At the level of cells or whole organisms, mutagenesis manifests itself by morphological and functional changes which very often cause the death of the organisms. At the molecular level, either replacement of some nucleotides in DNA and incorporation of new ones or elimination of some bases (or even of large areas of the genome) take place. As a result, proteins with altered primary structures are synthesized where either one (the most common case) or several amino acids are replaced by others or polypeptide chains are covalently cross-linked; vice versa, some amino acid residues may be deleted and the proteins then consist of covalently unbound polypeptide chains.

Despite the experimental difficulties encountered with mutants,[51] the comparison of structures of proteins from mutant and wild strains is widely used in studies on the mechanism of enzyme catalysis,[51,52] particularly in the search for amino acids involved in the catalytic process.[53] Such studies also provide information on the role of individual amino acid residues in maintaining the stability of the protein; some examples have been reviewed in our earlier study[19] (see Sections IV.D., E., and I).

D. Proteins Prepared in the Laboratory

There exist at least three experimental methods for changing the structure of proteins: site-directed mutagenesis (based on genetic engineering), immobilization, and covalent modification.

1. Site-Directed Mutagenesis

This method provides a unique possibility for obtaining protein homologs differing from the starting protein in a single amino acid localized in a preselected site of the polypeptide chain. The experimental aspects of site-specific mutagenesis[54,55] are considered later. Recent advances in site-specific mutagenesis have made this method more available.[20,21]

Artificially prepared mutant proteins (muteins) are now used in studies on the role of the individual amino acid residues in catalysis.[21,56] Moreover, the first attempts have been made to increase the activity of enzymes and to change their specificity.[57,58]

2. The Immobilization of Enzymes

This method is usually considered one which restricts the mobility of protein molecules or their fragments in space (or relative to each other).[59-61] Recently, this method has received wide application in biotechnology,[5-11] particularly for the stabilization of enzymes (for a review, see References 14 to 19). The opinion that immobilization can be used only for problems of *applied* research and that it is not likely to provide useful information for *fundamental* research is based on the old prejudice that immobilization profoundly changes the structure of the native enzyme. It has recently been shown by physical methods, however, that, as a rule, immobilization does not cause any significant changes in the conformation

of the enzymes (for a review, see Reference 62). Moreover, the choice of the proper immobilization technique will practically guarantee the similar conformations of native and immobilized enzymes (cf. Chapter 2 in Reference 62). Immobilized enzymes are suitable models, therefore, for studies on various processes in vivo[62,63] and yield useful information on the relationship between protein structure and stability as well as on the important role of structural fluctuations of proteins on their stability.[14-17]

3. The Chemical Modification of Proteins

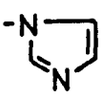
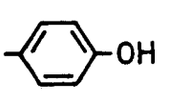
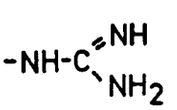
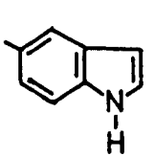
This method is used not only for the stabilization of enzymes (for a review, see References 64 to 66), but also in studies on the relationship between protein structure and stability. The main problem here is to obtain a protein with only *one* particular functional group (or a few well-defined groups) modified. The functional groups of proteins possess the same reactivity: they are all potential nucleophiles and proton donors (or acceptors) (see Table 1). Therefore, efforts to modify a certain functional group of the protein by a chemical reagent usually are only unsuccessful because of the competition of different functional groups for the same reagent, which leads to nonspecific modification.

Nevertheless, because of the great advance in studies on the chemical modification of proteins,[67-89] a large number of specific reagents with a high selectivity are available today for any biochemical laboratory (see Table 1). Moreover, even for the group-specific reagents, conditions can be chosen under which amino acid residues of only one type are selectively modified. This can be achieved, e.g., by varying the pH and thus making only one form of the functional group (either protonated or deprotonated) reactive, or by reversible blocking of the functional groups (for some examples, see Table 2).

So, the potentiality of chemical modification of proteins is tremendous. However, there are several principal difficulties which make the application of modification techniques in studies on structure-stability relationship of proteins complicated. Let us consider some of the main difficulties and the ways in which they can be overcome:

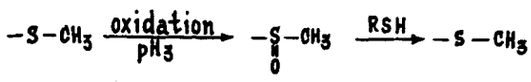
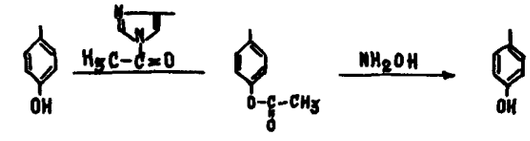
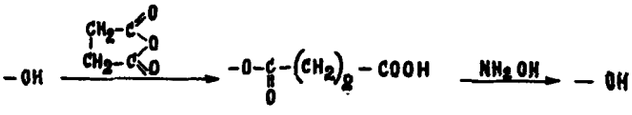
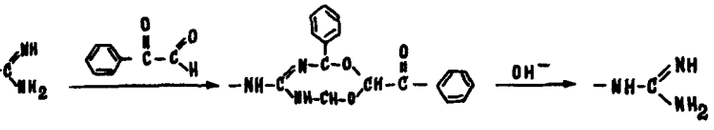
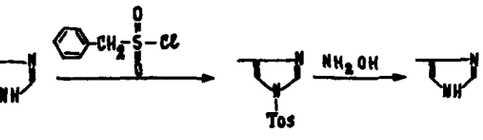
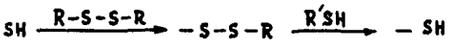
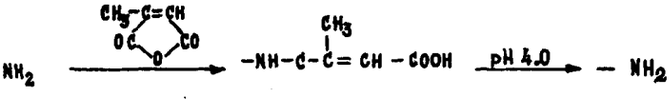
1. Modification can lead to unfavorable conformational changes and even denaturation.[72,84] Hence, any work on a modified protein should start its analysis by physical (spectral) methods to determine whether the conformations of the native and modified protein are identical or different.
2. Sometimes the modification procedure is carried out under rather hard conditions (at high or low pH, in the presence of oxidants and reductants, etc.) and undesired side reactions (such as scission of peptidic and other bonds, oxidation, and reduction of some functional groups) can parallel the modification (see Table 3). Modification can also affect the functional groups of the active sites of enzymes. To avoid such side reactions, the "vulnerable" functional groups should be protected before the modification (see Table 2) and the "protecting reagent" should be removed under mild conditions after the reaction.
3. As a rule, the chemical modification of proteins results in several derivatives that differ in the number of the functional groups modified. Moreover, protein molecules that contain the same number of modified groups can differ in the positions of the amino acid residues modified. This leads to a considerable heterogeneity of the products.[72,84] The separation of the individual forms of proteins modified to similar degrees is usually complicated because of the similarity of their physical properties, e.g., charge and molecular weight. High performance liquid chromatography (HPLC) techniques may be used to advantage in such cases.[98]
4. The other serious problem represents the identification of the products of chemical modification. The method used most widely for this purpose is amino acid analysis which, however, has a serious drawback: before analysis, the protein must be hydro-

Table 1
FUNCTIONAL GROUPS OF PROTEINS WHICH ARE SELECTIVELY MODIFIED

Group	Amino acid	pK_a	Examples of selective modification reagents[89]
α–COOH	C-terminal amino acid		
β–COOH	Asp	1—6.8	Carbodiimide + *nucleophile*
γ–COOH	Glu		
	His	6.4—7.5	*Diethylpyrocarbonate*
–SH	Cys	8.0—9.5	N-ethylmaleimide disulfides
α–NH_2	N-terminal amino acid	7.3—12	Dansyl chloride, methyl acetimidate
ε-NH_2	Lys		
	Tyr	9.4—312	Tetranitromethane, N-acetylimidazole
	Arg	11.5—12	2,3-Butanedione, phenylglyoxal
–OH	Ser, Thr	12	
	Trp	12	N-bromosuccinimide
S–CH_3	Met		Chloramine-T, H_2O_2

lyzed under rough conditions (concentrated HCl, temperature above 100°C) to cleave all peptide bonds. Under such conditions, all the less stable chemical bonds undergo cleavage as well and some amino acids are destroyed. The identification of a number of amino acids (cysteine, tryptophan, methionine) and of products of some chemical reactions (oxidation, acylation, etc.) is thus either impossible or ambiguous.[85,86] Fortunately, this difficulty can be overcome, most commonly by using competitive reactions of functional groups (see Table 2) and the method of radioactive labels.[85] Another way to cope with the same difficulty is to determine the degree of modification under mild, nondenaturing conditions (i.e., at room temperature, physiological pH, etc.). This can be accomplished to advantage by spectrophotometric determination of unmodified functional groups using chromogenic labels (see examples in Table 4). Then the position of the modified amino acid residue can be determined by conventional methods used for the analysis of the primary structure of proteins.[103]

Table 2
REVERSIBLE BLOCKING OF AMINO ACID RESIDUES IN PROTEINS

Amino acid	Reaction scheme	Ref.
Met		90
Trp		91
Tyr		92
Ser and Thr		93
Arg		94
His		95
Cys		96
Lys		97

Studies on the structure-stability relationships in proteins are generally started by a comparison of the native and the modified protein. Sometimes the conformational changes may cause the protein stability to increase[104,105] or decrease.[106,107] Such findings are usually of little value for the structure-stability studies since the conformational changes are difficult to interpret and even more difficult to predict. More often we are dealing with modification products that have the same conformation as the native protein, but show a different stability. The effect of the following factors is analyzed most commonly to cast light on the molecular reasons of protein (de)stabilization.

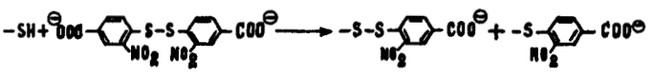
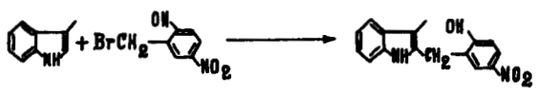
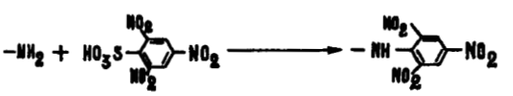
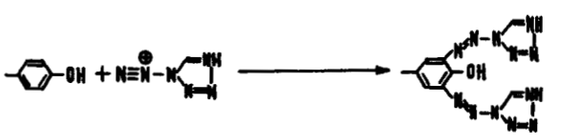
a. Increase of the Modification Degree

If the (de)stabilizing effect is changing monotonously with the modification degree, then

Table 3
UNFAVORABLE SIDE REACTIONS ON MODIFICATION OF FUNCTIONAL GROUPS IN PROTEINS[84]

Group	Conditions	Result
Peptide bond	Alkaline pH	Hydrolysis
	Acid pH	N → O acyl shift
SH group	Oxidation	S–S bond, S–O acids
S–S bond	Reduction	SH groups, new S–S bonds
	Alkaline pH	Hydrolysis
		β-Elimination
S–CH_3 of methionine	Oxidation	Oxy-derivatives
Amide group	Alkaline pH	Hydrolysis
O-glycosyl group	Alkaline pH	β-Elimination
O-phosphate group	Alkaline pH	β-Elimination

Table 4
SPECTROPHOTOMETRIC DETERMINATION OF FUNCTIONAL GROUPS IN PROTEINS

Reaction scheme	Wave length (nm)	Molar absorbance	Ref.
	412	13 600	99
	410	18 000	100
	420	13 000	101
	550	13 800	102
	480	20 500	102

specific interactions (salt bridges, hydrogen or disulfide bonds, etc.) may hardly occur (or vanish) as a result of chemical modification. In other words, the continuous profile of the dependence of the (de)stabilizing effect on the modification degree appears to reflect a rather gradual change of some integral parameter (e.g., hydrophobicity or surface charge) of the protein modified.

b. Change of the Modifier Size

The dependence of the (de)stabilizing effect on the size of the modifying reagent sometimes may be used to investigate the role that some integral parameters of the protein globule, e.g., surface hydrophobicity or charge, play in maintaining the stability of proteins. For this purpose, different homologous series of reagents have been employed (e.g., amines, aldehydes, and imidates).[66]

c. Temperature Dependence of (De)stabilizing Effect

The analysis of this effect sometimes enables us to determine which interaction type makes the largest contribution to the (de)stabilization of the protein.[66] In particular, if the (de)stabilizing effect increases with temperature, the hydrophobic interactions in the protein are very likely to become stronger as a result of the modification.

E. Summary

Concluding our comments on chemical modification, we should be aware of the fact that it may sometimes largely suppress only one of the inactivation mechanisms typical of the native protein. For instance, thermal inactivation of native albumin in solution is mainly the result of its aggregation.[108] Aggregation of a protein can be suppressed by establishing additional negative charges in the protein, e.g., by citraconylation. The observed[108] slowdown in inactivation is then caused by a change of its inactivation mechanism and not by internal stabilization of the protein resulting from its modification. When working with modified, immobilized, or mutant proteins, we should therefore identify first the mechanisms causing their inactivation and the differences from the mechanism observed with the native protein.

III. MOLECULAR REASONS FOR PROTEIN STABILITY

The principal molecular features responsible for protein stability have been recently reviewed by us.[19] If we consider them here briefly, it is only to find out whether nature still has some reserves for artificial stabilization of proteins.

A. Binding of Metal Ions, Substrates, Cofactors, and Other Low Molecular Weight Ligands

Metal ions dramatically increase protein stability due to their binding to the labile fragments of the polypeptide chain, in particular to the bends. An increase in protein stability is observed when enzymes interact with substrates, cofactors, and other low molecular weight ligands as well; some examples of such stabilization have been discussed before.[19]

The most general explanation of this phenomenon has been presented by Schellman.[109] Proteins can exist in at least two states — in the native state and in the denatured state — and any ligand can bind to both these protein forms. The preferential binding of the effector molecule to one of the forms results in its stabilization, e.g., of the native form compared with the denatured one. Quantitatively, this stabilization can be expressed by the value of free energy of the formation of the corresponding protein-ligand complex. The binding of substrates, cofactors, and other effectors of enzyme activity occurs as a rule in either the active site or its vicinity. Hence, the enzyme should be in the native (catalytically active) state for the complex to form. Specific low molecular weight ligands therefore usually stabilize proteins.[109]

There exists still another mechanism by which substrates and other specific ligands stabilize enzymes. The interaction of proteins with effectors very often results in conformational changes of the former;[110] e.g., enzymes adopt more stable conformations. This mechanism may be utilized to obtain artificially stabilized enzyme preparations for biotechnology: the substrate-induced stable conformation of the enzyme can be fixed, e.g., by cross-linking or immobilization.[111] A critical analysis of this line of approach can be found in Reference 62.

B. Protein-Protein and Protein-Lipid Interactions

In vivo proteins very often interact with lipids or polysaccharides.[112] The resulting complexes fall into two groups: (1) complexes with a limited number of either protein-protein solitary contacts (as are intersubunit contacts in oligomeric enzymes,[113] or contacts in proteinase-proteinase inhibitor complexes[114]) or protein-lipid solitary contacts (as in the complex

of serum albumin with fatty acids[115]); (2) systems with a large number of interrelated and concerted contacts of different types (protein-protein, protein-lipid, lipid-lipid), e.g., in biological membranes.[116] The interaction with other molecules in both cases can significantly increase the stability of the proteins.

The stabilization mechanism that is due to protein-protein or protein-lipid solitary contacts is fairly clear.[114] Hydrophobic clusters are localized on the surface of protein molecules side by side with polar and charged groups. The contact of these clusters with water is thermodynamically unfavorable. When a protein forms a complex, the molecules of lipids or proteins settle down to these clusters and thus block their contacts with the solvent.[117] The observed value of the free energy of stabilization is usually several kilocalories per mole *per* protein-protein or protein-lipid contact.[113,114]

Such solitary contacts can be created artificially as well: in the presence of lipids[118-120] or high concentration of proteins (e.g., albumin),[121,122] the stability of a number of enzymes dramatically increases.

The mechanism of enzyme stabilization which is due to the incorporation of enzymes into biological membranes is more complex. In addition to the shielding of the hydrophobic regions on the surface of the protein from the thermodynamically unfavorable contacts with water, many other factors must be considered, e.g., the microenvironment of the protein and the change of the rigidity of the protein molecule resulting from its multipoint interaction with other proteins and lipids.

The rigidity of the protein molecule is usually determined thermodynamically as the opposite of the flexibility (or mobility) of the protein macromolecule. Flexibility characterizes the ability of the macromolecule to undergo conformational changes[123] and is therefore directly related to protein stability. As a matter of fact, the two processes (protein denaturation and fluctuations near an equilibrium state) are governed by the same molecular mechanism, namely, oscillatory and rotatory motions of the macromolecule and/or its fragments. The difference between the two processes is that during denaturation a cooperative conformational transition of the whole globule occurs,[26] whereas local fluctuations may affect only some regions of the globule and need not necessarily change the conformation of the whole molecule.[124]

It is not our aim to present here an unambiguous thermodynamic definition of protein rigidity, which has been discussed elsewhere,[123,124] or to correlate[125] rigidity with thermodynamic parameters of protein stability. Here we wish to consider only one question: how can immobilization, both natural and artificial, influence the rigidity and stability of proteins?

Membrane proteins function in vivo in a naturally immobilized state. As a result of interaction with other proteins and lipids, the translational diffusion of proteins in biomembranes is significantly retarded. The degree of the immobilization of the protein in the membrane, i.e., the extent to which its motion is limited, depends greatly on the phase state of the membrane. The "melting" of the membrane at the point of phase transition results in the destruction of the microcrystalline structure of the membrane. Hence, the translational diffusion of proteins along the membrane accelerates, and oscillatory and rotatory motions in proteins become faster. In other words, the structure of the protein molecule becomes more flexible and mobile.[126] Dramatic changes of different properties of the protein therefore may be expected to occur after the melting point of the membrane has been achieved. There are numerous examples showing that the catalytic activity of membrane enzymes is very sensitive to phase transitions in biological membranes.[126,127] However, little is known about how deeply the phase state of the membrane can affect protein stability.

Pioneer studies in this field were carried out by Welker.[128] He cultivated an obligatory thermophilic strain of *Bacillus stearothermophilus* at 55 to 70°C and found that the higher the temperature of cultivation, the more rigid the cell membrane (as judged by the destruction of protoplasts at elevated temperatures) (Table 5). From his investigation of the lipid and

Table 5
EFFECT OF CULTIVATION TEMPERATURE ON PROPERTIES OF *BACILLUS STEAROTHERMOPHILUS* CELL MEMBRANE AND THERMOSTABILITY OF ENZYMES[128]

Cultivation temperature (°C)	Protein-to-lipid ratio	Inactivation degree (%)									
		Destruction of protoplasts				Alkaline phosphatase				NADH-oxidase	
						Solubilized		In membrane			
		65°C	70°C	75°C	80°C	65°C	70°C	65°C	70°C	75°C	80°C
55	3.65	0	100	100	100	100	100	87	100	100	100
60	4.27	0	80	100	100	100	100	0	92	90	100
65	5.22	0	0	70	100	100	100	0	20	75	84
70	5.85	0	0	10	100	100	100	0	5	0	10

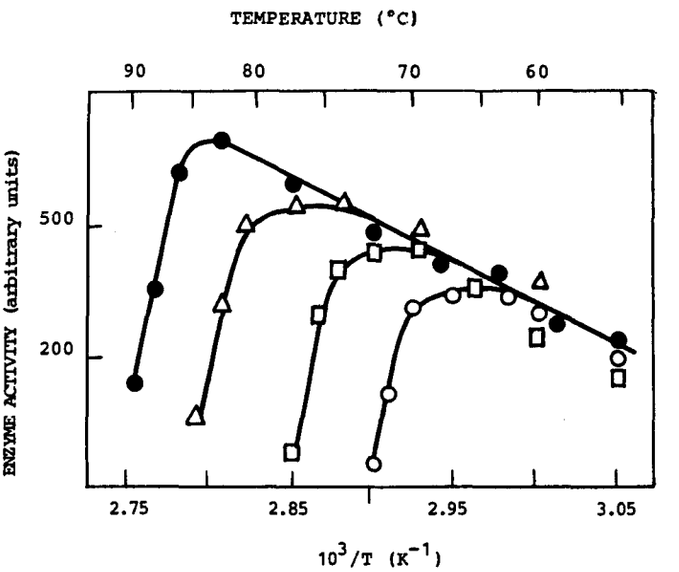


FIGURE 1. Temperature dependence of enzyme activity of a membrane enzyme, NADH-oxidase, from *Bacillus stearothermophilus* cultivated at 55°C, (-○-), 60°C (-□-), 65°C (-△-), and 70°C (-●-).[128]

protein composition of membranes, Welker[128] concluded that the observed increase in the temperature at which the membrane was destroyed can be correlated well with both the increase in the content of saturated and branched lipids and the increase in the protein-to-lipid ratio: the more rigid the membrane structure, the more thermostable the membrane proteins. This was observed with both peripheral (alkaline phosphatase) and integral (NADH-oxidase) enzymes (Table 5). Thermostability increased only with enzymes incorporated into the membrane. When alkaline phosphatase was isolated from the membranes, its stability dramatically decreased and became insensitive to the temperature of cultivation of the microorganism (Table 5). Thus, Welker unambiguously proved the hypothesis previously voiced[129,130] of the stabilizing effect of the membrane structure on proteins.

A comparison of the thermostability of enzymes immobilized naturally (in biomembranes) and artificially (on a polymeric support) is very instructive. Figure 1 shows the temperature dependence of catalytic activity of an integral membrane enzyme, NADH-oxidase from *B. stearothermophilus*, grown at different temperatures.[128] It can be seen clearly that the higher the temperature of the culture growth, i.e., the more rigid the membrane into which the enzyme is incorporated, the higher the temperature optimum of the catalytic activity. Similar temperature dependencies have been obtained recently[131] with artificially immobilized trypsin, whose molecules had been attached to the support (polyacrylamide gel) via different numbers of bonds (Figure 2). The coincidence of the regularities shown in Figures 1 and 2 is obviously not accidental since it reflects the same tendency, i.e., an increase of enzyme stability caused by rigidification of its structure. The only difference we should notice is that the rigidification occurs in vivo (Figure 1) as a consequence of the structuralization of the natural protein-lipid membrane matrix, whereas in vitro (Figure 2) it is the result of numerous covalent bonds between the enzyme molecule and the polymer matrix.

C. Salt Bridges, and Dipole-Dipole and Other Electrostatic Interactions

Dipole-dipole and dispersion interactions are difficult to consider in studies on protein stability since their energy is small (only a few calories per mole). On the other hand, the

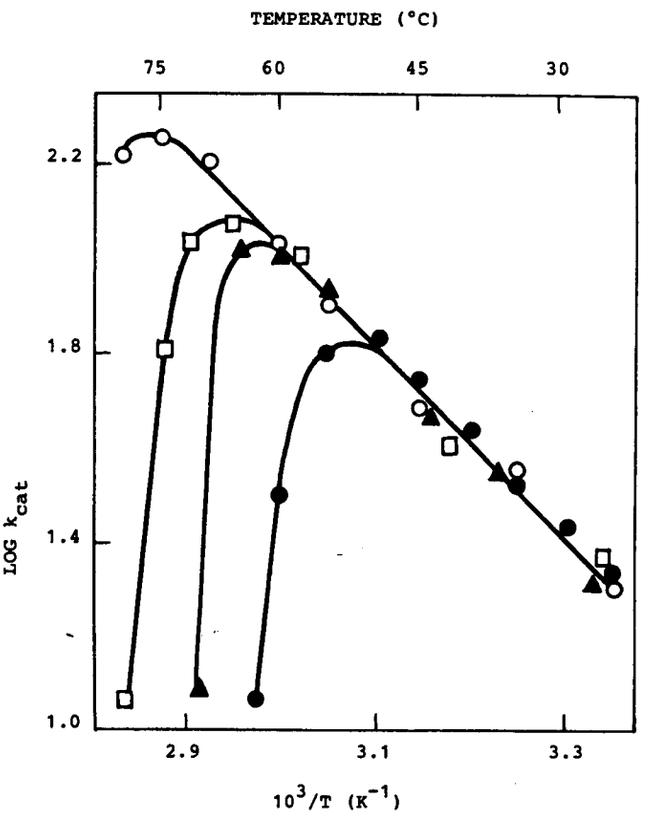


FIGURE 2. Temperature dependence of enzyme activity (Log k_{cat}) for hydrolysis of specific substrate (N-benzoyl-L-arginine ethyl ester) catalyzed by native trypsin (-●-) and trypsin covalently immobilized in polyacrylamide gel by 5 bonds (-△-), 8 bonds (-□-), and 15 bonds (-○-).[131]

number of such interactions in proteins is huge.[1] Some new ideas for more precisely evaluating these weak interactions have been suggested recently,[132,133] yet the calculation of their stabilizing effects so far has been mostly of only theoretical interest.

In contrast, the number of salt bridges in proteins is rather small.[134,135] However, they make a significant contribution to protein stability: up to 5 kcal/mol when they are localized inside the globule, and 1 to 2 kcal/mol when they are on its surface.[1,134] Salt bridges are therefore widely used by nature for the stabilization of thermophilic proteins.[19] Let us consider only one example. The three-dimensional structure of glyceraldehyde 3-phosphate dehydrogenase from the moderately thermophilic strain of *B. stearothermophilus* is very similar to that of the enzyme from rabbit muscle.[136] However, there is a small but very important difference in these structures: the thermophilic dehydrogenase has a cooperative system of salt bridges in its intersubunit region which is absent in the mesophilic enzyme. As a result, both the denaturation and optimum temperatures of the catalytic activity of the thermophilic enzyme are higher (by about 20°C) compared with glyceraldehyde 3-phosphate dehydrogenase from rabbit muscle.

Additional salt bridges can be artificially introduced into the protein molecule (by site-directed mutagenesis or chemical modification). In our opinion, this approach is becoming more and more promising as new data on protein structures are being obtained, mainly by X-ray crystallography.[137]

Hydrogen bonds play an important role in proteins since they support secondary structures, i.e., α-helixes, β-sheets, turns, etc. Their importance for protein stability, however, should not be overestimated. Hydrogen bonding is usually considered a sum of two processes: (1) the rupture of hydrogen bonds between the polar groups of protein and water and (2) the subsequent formation of internal hydrogen bonds in the protein, a process which is undoubtedly energetically advantageous. Schulz and Schirmer[1] believe, however, that it is insufficient to consider only these two processes. To evaluate the energy of hydrogen bonding, the penetration of a polar group (e.g., an amide group) into the hydrophobic core of the globule also should be taken into account. Since the latter process is thermodynamically unfavorable, the true value of free energy of formation of a hydrogen bond in the protein seems to be close to zero. Therefore, in our opinion, there is no use in introducing new hydrogen bonds into the protein globule, e.g., by site-directed mutagenesis.

D. Disulfide Bonds

The idea of protein stabilization by S–S bonds came about from polymer chemistry. In the mid-1950s it was shown[138,139] that the intramolecular cross-linking of macromolecules increases their rigidity and enhances their stability in solution. The stabilization was regarded[138] as being of entropic character: as a result of the cross-linking, i.e., of the formation of S–S bonds in the protein, the entropy of the unfolded protein dramatically decreases and therefore the difference between the free energy of the native and the denatured protein increases. The value of this stabilizing effect increases with the increasing number of amino acid residues localized in the loop[138,139] and becomes 4 to 5 kcal/mol *per* S–S bond.[140]

The intramolecular cross-linking of proteins is, in our opinion, very promising for protein stabilization. There exist a number of techniques for protein cross-linking. First, bifunctional reagents can be used for this purpose.[141] Their action results mainly in cross-linking of the functional groups that are localized on the surface of the protein molecule and leads to a significant stabilization of both monomeric (α-chymotrypsin) and oligomeric enzyme (glyceraldehyde 3-phosphate dehydrogenase).[141] The second approach makes use of site-directed mutagenesis, which offers unique possibilities for introducing cross-links into the protein globule. This technique has been used[142] for the insertion of an S–S bond into the molecule of lysozyme T4, which normally has no S–S bonds (for details, see Section IV.I).

E. Low Content of Amino Acids Sensitive to Oxidative Modification

The oxidation of the structurally important amino acid residues (e.g., those of the active sites) is one of the most frequently occurring mechanisms of protein inactivation.[12,13] The SH group of cysteine and the indole ring of tryptophan are especially sensitive to oxidation. The number of these labile amino acids (cysteines) is therefore often significantly lower in highly stable thermophilic proteins compared with proteins from mesophiles.[19]

There are at least two methods of stabilizing proteins sensitive to oxidation. The first method imitates the manner in which nature synthesized thermophilic enzymes, i.e., by decreasing the number of cysteines.[143] Cysteine residues (unless they are involved in catalysis) are replaced (e.g., by site-directed mutagenesis) by other amino acids, which are less sensitive to oxidation but very similar to cysteine (having a similar geometry, hydrophobicity, etc.); for details, see Section IV.I. The other method is simpler: the microenvironment of the enzyme is changed to protect the enzyme from contact with the inactivating factor (oxygen). A polyelectrolyte support has been employed successfully[144] for this purpose; it envelops the enzyme and slows down the diffusion of oxygen. "Salting out" oxygen by using a polyelectrolyte matrix permits the stability of a number of labile hydrogenases to be increased many thousand times.[144]

F. Compact Packing of Amino Acid Residues

Proteins in solution appear to be densely packed; the compactness of packing in the protein

globule is similar to that of crystals of low molecular weight compounds.[1] Nevertheless, there are cavities in the protein structure. According to Chothia[145] about 25% of the volume of the protein globule remains unfilled, i.e., not occupied by amino acids. Solute molecules, however, can be entrapped in these cavities.[146] The cavities are usually filled with water molecules (5 to 15 molecules *per* protein of 20,000 to 30,000 mol wt). The contact of the polar water molecules with the hydrophobic core of the globule mediated by Brownian motion results, of course, in protein destabilization.

The stability of the protein seems to increase as its structure becomes more compact with the simultaneous removal of water molecules from the cavities. This is how nature stabilizes the structure of thermophilic enzymes: some of the amino acids of the core are replaced (compared with the corresponding mesophilic counterpart) by amino acids of larger volume.[19,181] Hence, protein "compactization" can be proposed as a method of artificial stabilization of proteins. For this purpose, some amino acid residues can be replaced by bulkier ones by site-directed mutagenesis. The following rules should be obeyed:

1. The chemical nature of the two amino acid side chains should be similar (i.e., a charged amino acid is replaced by a charged one, a polar residue is replaced by another polar residue, etc.).
2. The rotational angles in the polypeptide chain (ϕ and ψ) should not be too different, and the conformation of the polypeptide backbone should remain almost the same after the amino acids have been replaced.
3. The volume of the two amino acids exchanged should not differ much. For example, the replacement of glycine by tryptophan is forbidden since the volume of the latter exceeds by three times the volume of the former.[143]

These rules, in addition to statistical data on homologous proteins, enabled Kolaskar and Ramabrahman[147] to find for each amino acid residue its counterparts by which it can be replaced without a major interference with the protein structure.

G. Hydrophobic Interactions

The importance of hydrophobic interactions for the structure and stability of proteins was reported first by Bresler and Talmud.[23] Since then, ideas about the nature of hydrophobic interactions and about their role in protein stability have combined into a harmonious theory.[148-150] Amino acids with nonpolar side chains occupy about one half of the total volume of protein molecules. Their contact with water is thermodynamically disadvantageous since the incorporation of nonpolar fragments into water makes its structure significantly more ordered. Indeed, a pentagonal cluster formed of water molecules near the nonpolar fragment of the protein surface has been revealed recently[151] by X-ray crystallography.

Such a structural rearrangement of water obviously causes a decrease in the entropy of the system and a change in protein folding: nonpolar fragments of the protein have a tendency to release themselves free of contacts with water and thus to become hidden inside the globule as much as possible.[152] As a result, the unfavorable entropic factor becomes weaker, the hydrophobic interactions intensify, and the protein stability increases.*

Shortly after the hypothesis of the stabilizing role of hydrophobic interactions in protein structures had been voiced, the first attempts were made to correlate protein stability with their hydrophobicity. Of course, a quantitative criterion of hydrophobicity was needed. Tanford[154] and Nozaki and Tanford[155] suggested the use of the free energy of the transfer of amino acids from water to an organic phase as such a criterion. According to Tanford's

* The entropic nature of the hydrophobic interactions is generally accepted;[148-150] however, other opinions[153] have also been reported.

Table 6
HYDROPHOBICITY SCALES OF AMINO ACIDS

Amino acid	Free energy of transfer from water into organic solvent (kcal/mol)[156]	Proportion of residues located inside protein globule (%)[157]	Hydrophobicity of microenvironment of residues in proteins (arbitrary units)[158]
Trp	−3.77	0.27	12.95
Phe	−2.87	0.50	13.43
Tyr	−2.67	0.15	13.29
Ile	−3.15	0.60	14.77
Leu	−2.17	0.45	14.10
Val	−1.87	0.54	15.07
Pro	−2.77	0.18	11.19
Ala	−0.87	0.38	12.28
Met	−1.67	0.40	14.33
Cys	−1.52	0.50	14.93
His	−0.87	0.17	12.84
Thr	−0.07	0.23	11.65
Ser	−0.07	0.22	11.26
Asn	−0.09	0.12	11.00
Gln	0.00	0.18	11.28
Asp	−0.66[a]	0.15	10.97
Glu	−0.67[a]	0.07	11.19
Lys	−1.64[a]	0.03	10.80
Arg	−0.85[a]	0.01	11.49
Gly	−0.10	0.36	12.01

[a] The values of free energies of transfer for uncharged forms of amino acid residues are given. The values corresponding to the charged forms are larger by several kilocalories per mole.

classification, the more negative the ΔG value of this process, the more hydrophobic the amino acid. This quantitative concept of hydrophobicity (see Table 6) stimulated efforts to correlate the stability of proteins with their total hydrophobicity calculated as a sum of the hydrophobicities of the constituent amino acids. However, no unambiguous conclusions resulted from either a study of homologous mesophilic proteins[28-30,159] or a comparison of functionally related thermo- and mesophilic proteins[160,161] (for a review, see Reference 19).

What are the possible reasons for failure to correlate hydrophobicity with the stability of proteins? We discussed one of them previously, namely, that big differences in structure result in only minor differences in stability.

The second reason is that ambiguities still exist in the determination of the amino acid composition of proteins. As a rule, the content of aspartic acid and asparagine and of glutamic acid and glutamine is reported as the sum of the amide and the acid form. However, the difference in hydrophobicity of the two forms, e.g., Gln and negatively charged Glu, which exists at neutral pH, is large and equal to several kilocalories per mole (Table 6).

The comparison[162] of the highly stable ferredoxin from *Clostridum thermosaccharolyticus* with the labile ferredoxin from *C. tartarivorum* may serve as an example. The three-dimensional structure and conformation of the two proteins are identical; moreover, their primary structures differ in only two positions (positions 31 and 44 in the stable ferredoxin are occupied by glutamic acid instead of glutamine, which is present in the labile protein). If routine amino acid analysis failed to detect this difference, a sensational conclusion could be made that two proteins with the same primary and tertiary structures differ dramatically in thermostability. The authors[162] were able to solve this discrepancy only after they had shown the difference between glutamic acid and glutamine by X-ray crystallography.

Another reason for failure to correlate total hydrophobicity and stability is the incorrect choice of proteins compared. Such a mistake is made when the hydrophobicity of a stable

thermophilic protein (e.g., ferredoxin from a thermophilic strain) is compared with the hydrophobicity of a "statistically" mesophilic protein (i.e., a mesophilic protein showing average hydrophobicity)[163] rather than with the hydrophobicity of a mesophilic protein that has the same function (mesophilic ferredoxin). This problem is discussed in detail in Reference 19.

Hence, we are faced with the problem of whether we should try to correlate stability and total hydrophobicity of proteins at all. Such a correlation appears to be justified with some "ideal" proteins only, proteins which we can imagine as an oil drop separated from the solvent by a barrier of polar and charged groups.[164] In such a hypothetical protein, all the nonpolar amino acids should be involved in hydrophobic interactions. However, this concept of protein structure is rather simplified since, according to X-ray crystallography data, nonpolar amino acids occupy 50% of the surface area of real proteins.[2,114,150,152] Very often, the nonpolar residues are organized as hydrophobic surface clusters.[150] These clusters are functionally very significant since they enable proteins to bind via hydrophobic interactions to other proteins (forming multienzyme complexes), to lipids in biological membranes, to polysaccharides in cell walls, to substrates and effectors during enzymatic catalysis, in short, to function in the optimal manner.[166,167] However, the location of nonpolar amino acids on the surface of unbound proteins in aqueous solution (enzymologists more often deal with such objects) is harmful to their stability. It is therefore obvious that many regularities of protein folding cannot be explained correctly on the basis of Tanford's extraction model of hydrophobicity of amino acids.[154,155] This discrepancy stimulated a search for new principles of determining the hydrophobicity of amino acids.

It has been suggested[157,158,168-170] that the hydrophobicity of amino acids be estimated on the basis of statistical data of their distribution in the spatial structure of proteins. According to Chothia's[157] statistical scale (based on the data obtained with 12 proteins), the greater the number of the amino acid residues inside the protein globule, compared with those located on the surface, the more hydrophobic the amino acid.

Hydrophobicity in terms of another statistical scale proposed by Ponnuswamy et al.[158] (based on the data obtained with 21 proteins) is determined mainly by the microenvironment of the amino acids in proteins. The hydrophobicity of the microenvironment is calculated as the sum of the hydrophobicities (using Tanford's scale[154-156]) of the number of neighboring amino acids in the tertiary structure. The comparison of these two statistical hydrophobicity scales[157,158] (see Table 6) shows good agreement despite the fact that they are based on different principles. In both scales, Lys, Arg, Asp, Glu, Asn, and Gln belong to the least hydrophobic ones. On the other hand, there is a marked divergence of the data of the statistical scales[157,158] and the data[156] of Tanford's extraction scale. For example, tryptophan, the most hydrophobic amino acid according to Tanford, occupies an intermediate position in the two statistical scales; moreover, Tyr and Pro, which are very close to Trp in their hydrophobicities in Tanford's scale,[156] would most likely be described as hydrophilic amino acids using Chothia's[157] scale and the scale proposed by Ponnuswamy et al.[158] (Table 6).

Chothia[114,157] was the first to analyze the reasons of this discrepancy. He came to the conclusion that the incorporation of a polar atom in the amino acid decreased its hydrophobicity by 1 to 1.5 kcal/mol. This explains why most hydrophobic (according to Tanford) amino acids, such as Trp and Tyr, are not among the most hydrophobic ones in Chothia's scale (Table 6). Although the large surface areas of Trp and Tyr guarantee their high hydrophobicity according to Tanford, nevertheless the presence of a polar atom significantly decreases the advantage of their localization inside the protein globule.

There exist other reasons for the discrepancy between Tanford's extraction scale[154-156] and the statistical[157,158] hydrophobicity scales. These reasons have been analyzed in detail elsewhere,[171] and we discuss them only briefly. Tanford's classification[154-156] is based on model experiments only, i.e., on the transfer of free amino acids from water into an organic phase;

this does not therefore account for the fact that amino acids are components of polypeptide chains. In other words, in real proteins these residues are in contact with other amino acids which influence their geometry and energy.[172-174] In addition to this, the amino acid residues must be packed compactly in the protein molecule during its folding.[1,143,152] As a result, some of the bulky residues, such as Trp and Tyr, remain exposed on the surface of the protein globule.

Another reason for the discrepancy observed when different hydrophobicity scales are used is that ethanol or dioxane,[154,155] octanol,[176] or vacuum[175] used as media in the extraction experiments is not a proper model of the interior of the protein globule. For example, organic solvents can form hydrogen bonds with polar atoms of amino acids.[176,177]

Summarizing these comments, we can conclude that there are three main factors that prevent hydrophobic interactions in proteins from their most advantageous materialization (in terms of stability of the three-dimensional structure). These are (1) the necessity of a rather compact packing of amino acids in the protein globule, (2) the microenvironment of amino acid residues affecting their geometry and energy, and (3) the requirement that hydrophobic clusters remain on the protein surface during folding since in vivo they are responsible for hydrophobic interactions of proteins with other molecules. Hence, there is hardly any sense in seeking a relation between protein stability and total hydrophobicity; it is often absent.[178]

Singleton[160] proposed the use of the content of aliphatic amino acids as a more appropriate measure of protein hydrophobicity since these residues are more often localized inside the protein globule than on its surface. Ikai[178] determined a so-called aliphatic index of proteins as follows:

$$A = X_A + aX_V + b(X_I + X_L)$$

where X_A, X_V, X_I, and X_L are the molar ratios of Ala, Val, Ile, and Leu in proteins, respectively, and a and b are the numerical coefficients determined by the size of the amino acids. Figure 3 shows that stable proteins from thermophilic microorganisms have significantly higher aliphatic indexes than mesophilic proteins.

Both the principle outlined earlier and the experimental data lead us to conclude that the increased stability of proteins in vivo (e.g., thermophilic enzymes) is due not only to an increase in their total hydrophobicity,[179] but also to an even higher degree to a more "regular" arrangement of nonpolar amino acids in the protein globule. In other words, the greater the number of nonpolar residues localized inside the protein globule and the less the number of residues exposed to the solvent, the more stable the protein, while its total hydrophobicity can remain the same.

Lee and Richards[152] considered this problem and introduced the idea of "the accessible surface area". Chothia and Janin[114] showed this characteristic to be related to the total free energy of hydrophobic interactions in the protein according to the following equation:

$$\Delta G_h = \sigma \cdot \Delta A$$

where ΔG_h is the change in the total hydrophobic energy caused by a change in the accessible surface area equal to ΔA, and σ is the proportionality coefficient equal to 25 kcal/mol/Å^2. Hence, a reduction of the surface area responsible for hydrophobic contacts with water leads to an intensification of the internal hydrophobic interactions in the protein and therefore to its stabilization. This phenomenon is observed when comparing meso- and thermophilic[180-182] enzymes, mesophilic enzymes with one another,[183] or enzymes from wild and mutant strains.[167,184]

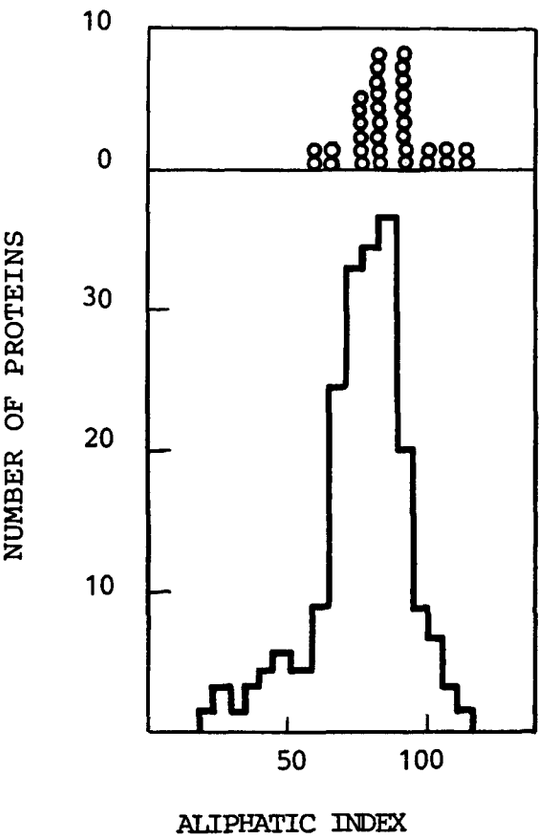


FIGURE 3. Values of aliphatic index for 34 proteins from thermophilic bacteria (top part of figure) and 208 proteins from mesophilic organisms (bottom part of figure). For calculation of aliphatic indexes of proteins, see text.[178]

Let us consider[136] as an example in detail the stability of glyceraldehyde 3-phosphate dehydrogenase from two thermophilic strains: the obligate *B. stearothermophilus* and the extreme *Thermus aquaticus*. The enzyme from the extreme thermophile shows optimum activity at a temperature by 25°C higher than the enzyme from the obligate thermophile. At the same time, the tertiary structure of both enzymes is very similar. The main reason for this difference in stability is that in the more stable enzyme a great number of hydrophobic residues are localized on the surface of the molecule and hence are in contact with water, whereas in the labile enzyme these residues are replaced by polar ones. Moreover, the stable enzyme contains a higher number of hydrophobic amino acids localized in the intersubunit region, which is inaccessible to water. As a result, the enzyme from the extreme thermophilic bacterium has almost the same *total* hydrophobicity as the enzyme from *B. stearothermophilus*. However, the redistribution of hydrophobic amino acids between the inner and outer regions of the protein increases the number of hydrophobic interactions and thus leads to the stabilization of the protein.[136]

In summarizing the data presented in this part of the review, we should emphasize that the stabilization of proteins by increasing the number of their hydrophobic interactions seems to be a very promising practical approach. There are at least two ways in which it can be effected: (1) by decreasing the hydrophobic nature of the protein surface or (2) by increasing the internal hydrophobicity of the protein. Experimentally, this can be achieved either by site-directed mutagenesis[167] or chemical modification[19] (see Sections IV.I. and IV.J).

IV. METHODS OF OBTAINING STABLE BIOCATALYSTS

A. Does Biotechnology Really Need Thermostable Biocatalysts?

The use of thermostable biocatalysts in biotechnology has many advantages.[14-17,185]

1. Acceleration of reaction rate — according to Vant Hoff's rule, an increase of temperature by 10°C results in a two- to threefold rise in the rate of chemical reactions, including those catalyzed by enzymes.
2. Shift of thermodynamic equilibrium — according to Le Shatelie's principle, the equilibrium of any reaction proceeding with heat consumption (i.e., an endothermic process) is shifted toward the end products by elevating temperature. Unfortunately, the thermochemistry of those enzyme reactions that are of practical importance has been studied very little so far.
3. Prolongation of viability of biocatalysts — in modern biotechnological processes, the application of enzymes is rather expensive.[186,187] A great operational stability, which often is due to a higher thermostability[131] of the enzyme, prolongs the time for which they can be used and thus decreases the cost of enzyme preparations.
4. High stability vs. other denaturing effects — the enzymes artificially thermostabilized are, as a rule, highly resistant to other denaturing agents as well, such as concentrated solutions of urea or guanidine chloride, extreme pH values, or to digestion by proteinases. This also holds true for thermostable enzymes from thermophilic microorganisms.[188,189] It is not clear as yet, however, whether thermostability also correlates with the stability of the proteins to the denaturing action of organic solvents. This problem is of great practical importance since the enzymatic conversion of many natural compounds as well as enzyme-catalyzed organic synthesis of a number of substances proceed only in organic media or in aqueous mixtures with a high content of the organic component.[190-198]
5. Increased efficiency of enzymatic processes as a result of increased solubility of reagents and volatility of end products and decreased viscosity of solutions at elevated temperatures.
6. Sterilization of end products in the course of reaction.
7. Simple termination of reaction by cooling.

The work at elevated temperatures, however, has its disadvantages as well. One of the most serious drawbacks is the acceleration of some side reactions such as high-temperature oxidation[13] and racemization of amino acids and their derivatives,[199] which can damage both the reagents and the catalysts. Therefore, the temperature for each biotechnological process must be selected individually, evaluating all the pros and cons.[200]

B. Search for Enzymes with High Thermostability in Mesophilic Sources

This is the oldest approach and needs no special comment. It methodically paved the way to a search for thermostable enzymes in the microorganisms equipped by nature for high temperatures.

C. Isolation of Enzymes from Thermophilic Microorganisms

A homogeneous enzyme preparation from a thermophilic microorganism was obtained first in crystalline form 25 years ago.[201] Since then, the merits of the use of thermophilic enzymes in biotechnology have been discussed repeatedly (for a review, see References 185 and 202). Most of these merits are listed in the previous section. It should be noted that the thermophilic microorganisms are superior to mesophilic organisms from the viewpoint of technology. The industrial-scale cultivation of thermophilic strains is cheaper because of lower energy costs since the growing biomass does not need cooling.[185] Moreover, the loss of activity of a thermophilic enzyme during its isolation and storage is lower.[185]

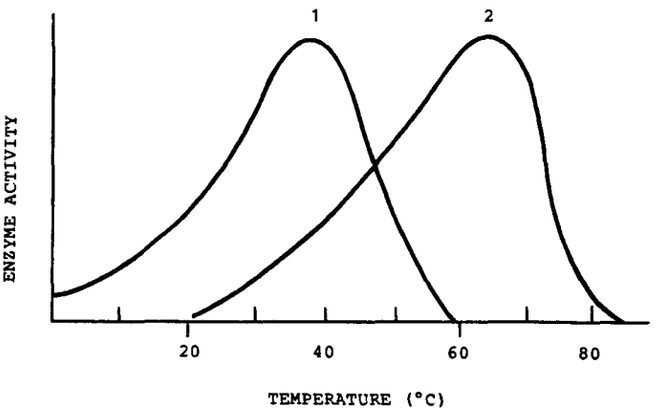


FIGURE 4. Temperature dependence of enzyme activity of mesophilic (curve 1) and thermophilic (curve 2) enzymes.[182]

It is noteworthy that thermophilic enzymes have not reached the upper limit of thermostability and can undergo further stabilization. Hence, a highly stable thermophilic asparaginase[185] and a proteinase[203] were additionally stabilized ten times by immobilization.

In spite of all these obvious advantages, the only example of practical application of a thermophilic enzyme in biotechnology is the production of sweet syrup of a high fructose concentration catalyzed by thermophilic xylose isomerase (for a review, see Reference 49). In our opinion, this limited use is due to the fact that although thermophilic enzymes show maximal catalytic activity at temperatures higher by 20 to 40°C than those of mesophilic enzymes, the activity of both enzyme groups at optimal temperature is often the same.[47,182,204-207] Cases where a thermophilic enzyme shows a higher catalytic activity at its temperature optimum than the corresponding mesophilic enzyme at its optimum are of rare occurrence.[208] Hence, the situation shown in Figure 4 is typical.

As shown recently,[182] at temperature optima thermo- and mesophilic enzymes not only show the same catalytic activities, but also almost the same thermostabilities. Hence, the susceptibility to irreversible thermal inactivation of a mesophilic enzyme at 38°C and a thermophilic enzyme at 63°C is almost identical. The comparison of thermo- and mesophilic enzymes indicates the same relation as that observed[209-213] with mesophilic proteins: the higher the catalytic activity, the lower the protein stability. In other words, to increase their stability, thermophilic enzymes must sacrifice their catalytic activity.[206]

The perspectives of thermophilic enzymes in biotechnology are nevertheless hardly being overestimated. For example, the possibilities of using thermophilic cells and enzymes for conversion of biomass into ethanol and acetic acid, the production of amino acids and lactic acid, and the modification of antibiotics and alkaloids seem promising.[49] In Brook's[49] opinion, the use of thermophilic enzymes in genuine technology, such as hydrolysis of starch to glucose, conversion of glucose into fructose-glucose syrup, and treatment of waste via methanogenesis, can be foreseen in the near future.

Moreover, a new characteristic feature of thermophilic enzymes has been discovered recently[214] which makes these enzymes very valuable for biotechnological purposes. Keinan et al.[214] have shown that alcohol dehydrogenase from *Thermoanaerobium brockii* shows substrate specificity that is not characteristic of its mesophilic counterparts (i.e., of alcohol dehydrogenase from horse liver and yeast). This specific property of thermophilic alcohol dehydrogenase has been used[214] for asymmetric reduction of aliphatic ketones giving optically pure products in high yields.

These results demonstrate that the screening of thermophilic strains should be aimed at

enzymes possessing not only a high activity and stability,[215] but also new types of activity and substrate specificity.

Thermophilic enzymes possess still another unique feature, namely, the ability to change their substrate specificity with temperature. This feature can be seen clearly with enzymes such as proteinases and nucleases, which act on high molecular weight substrates. These substrates (proteins, RNA, DNA, etc.) undergo temperature-dependent conformational transitions.[216] Hence, when the temperature is raised, additional peptide and nucleotide bonds may become exposed in solution and available for enzymatic attack. The enzymes acting on high molecular weight substrates thus attain an apparently new specificity, which has been observed with thermophilic proteinases and restriction nucleases.[217] The latter are of special practical importance since the rapidly developing field of genetic engineering needs restrictases of very different specificities.[218] It is quite possible, therefore, that thermophilic enzymes will become a new and highly efficient tool of genetic engineering.

D. Introduction of Thermophilic DNA (or Gene) into Cells of a Mesophilic Microorganism

It was reported[219-221] in the late 1970s that the introduction of DNA from a thermophilic microorganism, e.g., *B. caldolyticus,* into cells of a related mesophile such as *B. subtilis* resulted in transformation of the mesophilic culture into a thermophilic one. The resultant transformant lost its ability to grow at 37°C, a temperature optimal for the corresponding mesophilic microorganism, but efficiently grew at 65°C.[219] Simultaneously, the apparatus of the cell underwent several changes: the ribosomal proteins and DNA of the transformant became more thermostable,[219,220] and the cells began to synthesize highly stable enzymes.[221]

Still another genetic approach toward thermophilic enzymes was proposed in the early 1980s.[222-224] Instead of using the whole thermophilic DNA molecule, only its fragment (i.e., the gene coding for a certain thermophilic protein) was introduced into the corresponding mesophilic cell. The principal advantage of the approach is that all the proteins produced by the mesophilic strain, except for the highly stable protein, denature at elevated temperatures. This permits the highly purified protein to be obtained in a short time and at a low cost.

It is very difficult to predict at present all the benefits of the genetic approach for biotechnology. The application of genetic methods to thermophilic bacteria has of course some limitations, and "thermophilic genetics is still at the level of arts".[220]

E. Introduction of Mesophilic Gene into Cells of a Thermophilic Microorganism

One more genetic approach towards the rapid obtaining of thermostable enzymes by cloning of a mesophilic gene in a thermophilic cell at elevated temperature has been proposed recently by Liao et al.[279] According to this procedure, new stabilized forms of kanamycin nucleotidyltransferase which can be sustained in prolonged heating at 63°C (and even 70°C) were obtained, whereas native enzyme inactivates rapidly at 55°C. The authors[279] made sure that the enhanced thermostability of the enzyme resulted from one-point mutations, i.e., substitution of Asp 80 by tyrosine in a 63°C transformant or of Thr 130 by lysine in a 70°C transformant.

This approach seems to be highly promising for biotechnology, provided it has a general character — the matter to be proved. It has a significant advantage compared to the genetic approach discussed previously (Section IV.D), namely, greater availability of mesophilic genes compared with thermophilic ones.

F. Free or Immobilized Cells and Organelles

The idea of using cells and organelles as catalysts in biotechnological processes was voiced a long time ago. The prospects and limitations of such biocatalysts have been exhaustively discussed elsewhere.[11,225] It should be noted that, in addition to other advantages, the higher

Table 7
MECHANISMS OF INACTIVATION OF ENZYMES

Inactivation mechanism	Denaturing conditions	Ref.
Aggregation (sometimes followed by formation of intermolecular S–S bonds)	Heating, guanidine chloride, urea, sodium dodecyl sulfate, shaking	226—229
Alteration of primary structure		
H^+- and OH^--catalyzed hydrolysis of peptide bonds, proteolysis, autolysis	Extreme pH values, heating, proteinases	230, 231
Oxidation of functional groups (the SH group of cysteine and the indole ring of tryptophan)	Oxygen (especially during heating) and the products of its metabolism, radiation	81
Reduction of S–S bonds, intramolecular thiol-disulfide exchange	Heating, high pH values, thiols, disulfides	232, 233
Chemical modification of essential SH groups	Metal ions, disulfides	234
Protein phosphorylation	Protein kinases	235
"Suicide" inactivation in the course of catalysis due to the action of reactive intermediates (mainly free radicals)	Substrates	236
Racemization of amino acids	Heating, extreme pH values	237
Scission of S–S bonds with subsequent formation of new amino acids (lysinoalanine, lanthionine, ornithinoalanine)	Heating, high pH values	238, 239
Deamination of Asn	Heating, high pH values	240, 241
Dissociation of coenzyme molecule from the active site	Chelating agents, thiols, dialysis, metal ions, heating	242
Dissociation of oligomeric protein into subunits	Chemical modification, extreme pH values, urea, surfactants, high or low temperature	243
Adsorption to the surface of the vessel	Small concentration of protein, heating	231, 244
"Irreversible" conformational change	Heating, extreme pH values, organic solvents, guanidine chloride	229, 245—247
Shear inactivation in stream	Hydrodynamic deformations	248

stability of enzymes in their natural cellular microenvironment is believed to be one of the reasons for using cells or organelles rather than individual enzymes in biotechnology. However, efforts to verify this assumption experimentally have not yielded unambiguous results, and the perspective that cells and organelles could be used to solve the problem of enzyme instability is still far away.

In summarizing the lines of approach discussed here (Section IV.B-F), it should be noted that none of them can help us to solve two problems: *why* the native structure of proteins is so labile, and *how* enzymes can be made more stable. The techniques of enzyme stabilization, i.e., the methods that provide them with additional stability, are analyzed in the following sections. But before we tackle this problem, we should investigate first why proteins lose their activity, i.e., we should cast light on the molecular mechanisms or at least on the reasons for the inactivation of enzymes.

G. Molecular Mechanisms of Enzyme Inactivation

A comprehensive review of the molecular mechanisms of protein inactivation has been published recently.[12,13] The possible mechanisms (summarized in Table 7) share a common feature. Under denaturing conditions, a conformational change usually occurs first and is

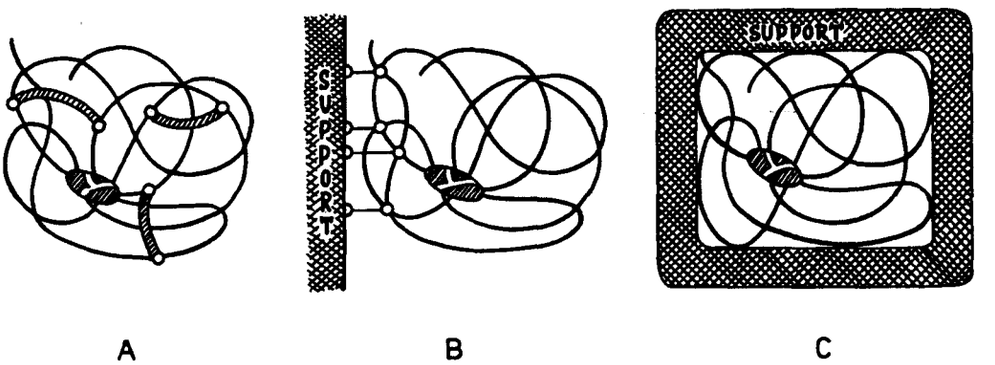


FIGURE 5. Schematic representation of immobilization methods in multipoint fixation of enzyme structure: (A) cross-linking with bifunctional reagents;[141] (B) covalent[252] or noncovalent[253] attachment to a support; and (C) entrapment into "tight pores"[14] of a support. For review, see References 14 and 15.

followed by secondary changes. This characteristic feature of protein inactivation was reported first by Lumry and Eyring[249] back in 1954. They suggested[249] that the inactivation of enzymes be considered a two-step process:

$$N \rightleftarrows D \rightarrow I$$

where N, D, and I are the native, reversibly denatured, and irreversibly denatured forms of the enzyme, respectively. This conception seemed to be true until now.[12,13,165] The inactivation usually starts by a reversible conformational change, i.e., by unfolding. The unfolding of the protein globule is the rate-limiting step of both reversible[250] and irreversible denaturation (for a review, see References 12 to 16). The reversible step of protein unfolding is followed as a rule by some secondary irreversible processes (step $D \rightarrow I$ in the noted scheme) such as aggregation, covalent modification resulting in a change of the primary structure of the protein, or "irreversible" conformational changes, etc. (see Table 7 for examples).

The most general approach to enzyme stabilization should be directed therefore toward the inhibition (or suppression) of the unfolding of the protein molecule, i.e., the first step of the inactivation mechanism ($N \rightleftarrows D$).[14,15,251-253] This can be done mainly by increasing the conformational stability of proteins or, generally speaking, by strengthening one or more of the molecular mechanisms of protein stability discussed in detail in Section III. In the last part of the review (Sections IV.H to J), we concentrate mainly on the methodical aspects of this problem. Some examples of inhibition of other inactivation mechanisms (e.g., oxidation of functional groups of enzymes) are also discussed (Sections IV.H. and IV.I).

H. Immobilization of Enzymes

A number of special reviews[14-19] describe in detail the methods of obtaining stabilized enzyme preparations based on immobilization techniques. In the present review, we therefore merely summarize the main achievements of this approach.

Among the numerous immobilization procedures aimed at the suppression of protein unfolding, the multipoint attachment of the protein molecule to the surface of a support[252,253] is the most promising one. Figure 5 shows the most commonly used experimental methods of multipoint fixation of enzyme structures. This type of immobilization has made it possible to slow down by several hundred to a thousand times both the reversible[131,254] and irreversible[131,252,253] thermoinactivation of enzymes, as well as their reversible unfolding caused by denaturants such as urea,[255] or the dissociation of oligomeric enzymes into active subunits (for a review, see Reference 251).

Immobilization can be used for the suppression of certain secondary irreversible processes (step D → I). Immobilization prevents enzymes from inactivation caused, e.g., by protein-protein interactions (autolysis or aggregation; for a review, see References 14 to 17 and 62). Immobilization inhibits chemical inactivation as well. For this purpose, the enzyme should be immobilized on a support that will shield it from contact with the inactivator. The stabilization of hydrogenase against inactivation by oxygen, performed by Klibanov et al.,[144] is an excellent example. In the immobilized system, free diffusion of oxygen to the enzyme is hindered by the salting-out effect of the polyelectrolyte matrix, which thus stabilizes to a high degree the oxygen-labile hydrogenase. Stabilization also can be achieved by selecting a support that either competes with the enzyme for the inactivating agent or decomposes it. If, for example, inactivation of the enzyme is caused by hydrogen peroxide or superoxide radicals, then it should be immobilized on a support together with peroxidase or catalase as catalysts of the decomposition of the inactivators (for a review, see Reference 15).

I. Protein Engineering

In the beginning of the 1970s, the first successful experiments with the preparation of recombinant DNA molecules were performed,[256] and a new powerful tool was thus obtained which permitted the synthesis of new biological molecules not existing in nature to be carried out. These experiments paved the way to a new scientific field — genetic engineering — i.e., the in vitro construction of functionally active genetic structures (recombinant DNAs), their incorporation into cells, and the subsequent biosynthesis (by the exogenous cells) of biopolymers. The advances of genetic engineering we have witnessed during the past few years[257-259] make the future of this field very promising.

We wish to discuss here only one application of the method in the so-called site-specific mutagenesis[21,22,54-58] (or protein engineering[20]). This method enables us to obtain proteins whose structures differ from those of the parent molecules in only one or a few amino acid residues. From the viewpoint of biotechnology, the main advantage of this method is that it provides us with possibilities for purposefully changing not only the structures of enzymes, but also their catalytic activity (specificity)[260] and stability as well. A brief outline of the procedure is given below.

As a first step, it is necessary to determine the primary structure of the enzyme[103] and then its tertiary structure by X-ray diffraction analysis.[137] Next the mutation site, i.e., the position at which a certain amino acid residue will be replaced by another one, is chosen. This is naturally less elaborate than the preceding procedures (determination of the protein sequence, preparation of protein crystals, interpretation of X-ray diffraction patterns, etc.). Nevertheless, the choice of the proper position is most important since it determines the properties of the end product. In the next step, the selected amino acid residue is replaced by another one as follows. For this purpose, an oligopeptide sequence consisting of four to six amino acid residues is chosen in the primary structure; it contains in its middle part the amino acid to be replaced. An oligodeoxyribonucleotide composed of 12 to 18 bases and encoding the amino acid sequence of the selected oligopeptide is then synthesized by chemical methods. The base triplet coding for the "native" amino acid is replaced in the oligonucleotide by a new triplet coding for the amino acid residue chosen in the previous step. If the length of the synthetic oligonucleotide is of the size of about 15 bases, one can be reasonably sure that its splicing with the gene will occur at only one site, namely, where all the nucleotides except for the mutated site are complementary to the corresponding nucleotides of mRNA. In the subsequent gene engineering manipulations, it is necessary first to find (or artificially construct) a single-stranded plasmid containing the gene coding for the required protein. Second, a double-stranded heteroduplex plasmid must be prepared from the single-stranded plasmid and the oligonucleotide synthesized using various DNA polymerases and DNA ligases; the oligonucleotide serves as a primer. Since homoduplex

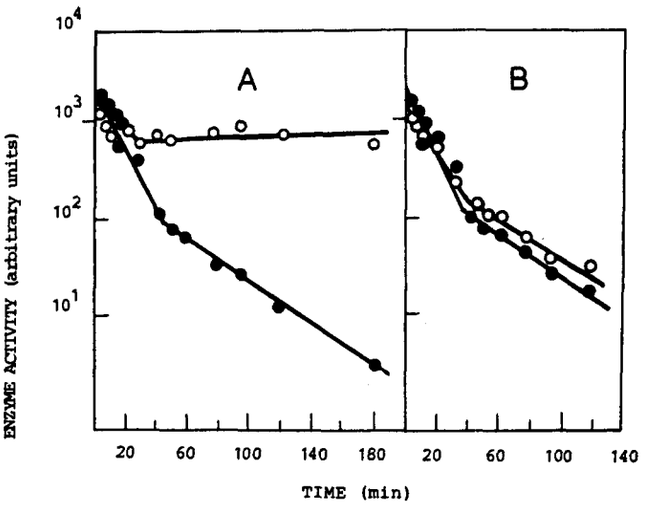


FIGURE 6. Thermoinactivation kinetics of lysozyme T4 from wild strain (-●-) and of mutant lysozyme (-○-) in which a new S–S bond was formed by protein engineering. Thermoinactivation was performed in the absence (A) of thiol compounds or in the presence (B) of 10 m*M* β-mercaptoethanol.[142]

plasmids are synthesized together with heteroduplex plasmids, their separation is necessary. The routine separation technique is based on the difference in the thermostability of the plasmids (heteroduplex plasmids melt at lower temperatures than homoduplex plasmids). Heteroduplex double-stranded plasmids are then introduced into the host cell, e.g., of *Escherichia coli*. In the cell, the heteroduplex plasmid is converted into two homoduplex plasmids: one inherits the gene coding for the native protein and the other one for the mutant protein. The two genes are then separated and cloned. The final product of this multistep process are cell transformants able to synthesize the mutant protein that differs from the parent protein in only one amino acid residue, which occupies the selected site.

The method of site-directed mutagenesis was reported first in the early 1980s and is coming of age.[20,261] Its first achievements are impressive. A few examples are considered here from the viewpoint of production of stabilized proteins.

There are no S–S bonds in the molecule of lysozyme from bacteriophage T4, but its molecule contains two SH groups (Cys 54 and Cys 97), one of which (Cys 97) is localized near the N-terminus in the tertiary structure. In addition to these two residues, another cysteine was exchanged[142] for Ile 3 by site-directed mutagenesis. Since Cys 3 and Cys 97 are localized very close to one another in the tertiary structure of the mutant lysozyme, an S–S bond is formed under mild oxidative conditions. Perry and Wetzel[142] demonstrated that the conformation of the native and the mutant enzyme is identical and that both enzymes have almost the same catalytic properties. However, mutant lysozyme was significantly more thermostable than native lysozyme (Figure 6). This is the result of rigidification of the structure by introduction of a new S–S bond since the scission of this bond decreased the thermostability of mutant lysozyme to the level of the native enzyme (Figure 6). The stability of mutant lysozyme increased even more after Cys 54, which interacts with the engineered S–S bond via thiol-disulfide exchange reactions and diminishes the stabilizing effect, had been replaced[262] by Thr or Val after the second artificial mutation.

The study of Wells and Powers[280] gives an example of when the introduction of the S–S bond in protein molecule does not inevitably increase its stability. Using site-directed mutagenesis, in subtilisin, Ser 87 and Thr 22 and/or Ser 24 were substituted by cysteines, and

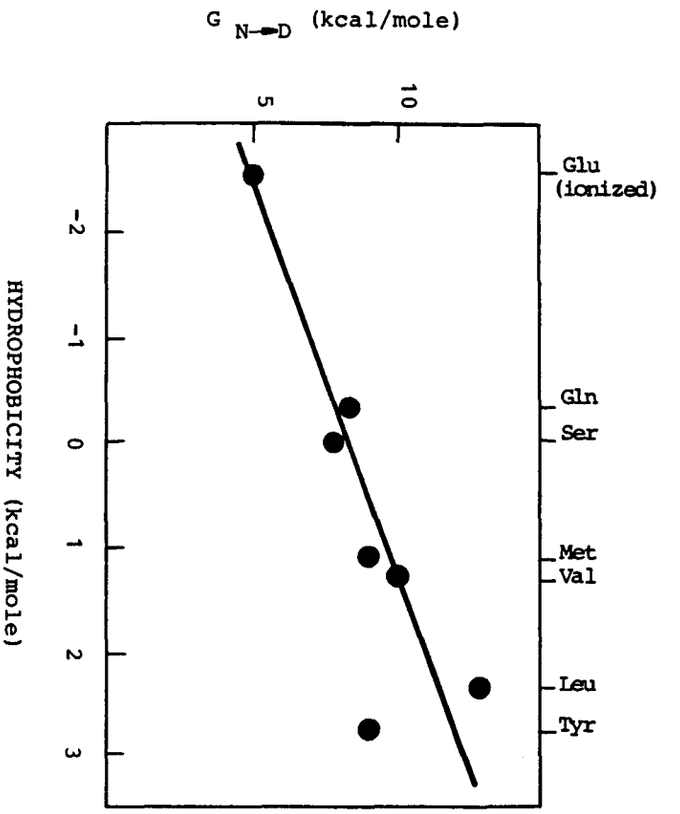


FIGURE 7. Dependence of free energy of stabilization ($G_{N\to D}$) on hydrophobicity of amino acid residue localized in position 49 in primary structure of tryptophan synthase α-subunit from *Escherichia coli*.[263]

the S–S bonds formed in the mutationally changed enzymes between Cys 87 and Cys 22, or Cys 87 and Cys 24, respectively. The stability of both enzymes with an additional S–S bond to proteolysis did not exceed, however, the stability of native subtilisin. The authors explained[280] this fact by destruction, as a result of mutagenesis, of some important interactions in the subtilisin molecule, in particular, a number of hydrogen bonds formed by Thr 22 and Ser 87. In the recent work of Pantoliano et al.,[299] it was shown, however, that these muteins of subtilisin with the engineered S–S bonds were much more thermostable than the native enzyme. An increase in stability was also observed[304] for dihydrofolate reductase, in which molecule an S–S bond was engineered.

Recently, mutation was used[263] to stabilize the α-subunit of tryptophan synthase. A number of forms of the protein were obtained in which Glu 49 localized in the hydrophobic nucleus of the enzyme from the wild strain was replaced by other amino acids. The authors[263] were able to show that neither the conformation nor the functional activity of the α-subunit of tryptophan synthase was affected by such transformations. An interesting observation was made (see Figure 7) that the more hydrophobic the amino acid introduced into the interior of the protein globule instead of Glu 49, the more stable the enzyme. These results[263] show that site-directed mutagenesis offers a unique possibility for increasing the internal hydrophobicity of proteins by replacing polar amino acids by more hydrophobic residues and thereby stabilizing the enzymes. Such a substitution (Gly 144 inside the protein globule by Ala) was performed[300] for protease and the stability of the enzyme actually increased. Some

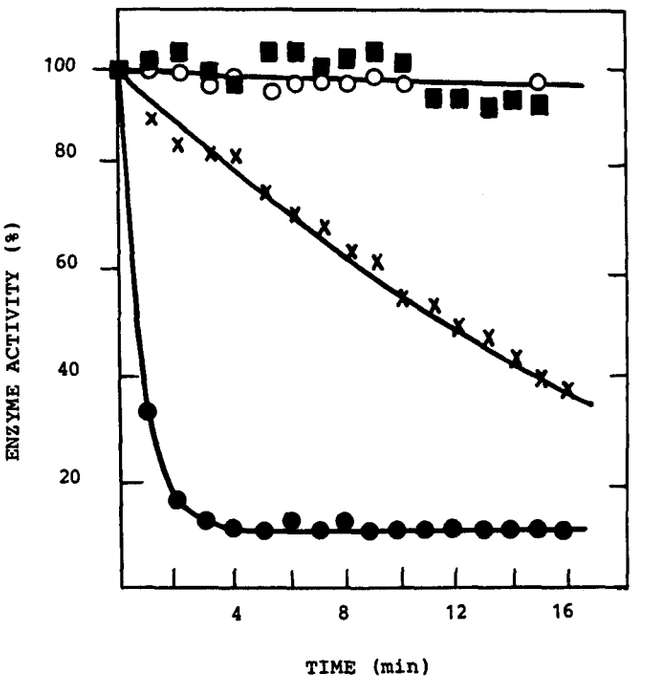


FIGURE 8. Kinetics of inactivation by 1 *M* H_2O_2 of subtilisin from wild strain (-●-) and of subtilisin mutants into which Cys (x), Ala (-■-), and Ser (-○-) were introduced instead of Met 222 by protein engineering.[264]

more muteins of protease were obtained with two or three amino acid residues substituted at once, for which the rule "the higher the hydrophobicity, the greater the stability" did not work. The reason for this is probably[300] that substitutions of amino acid residues which increase hydrophobicity on the one hand, destroy some molecular interactions essential for enzyme stability on the other hand.

An example of strengthening the intersubunit contacts in oligomeric proteins is given by the work of Ward et al.[301] Using site-directed mutagenesis, they obtained[301] a set of isoforms of tyrosyl-tRNA synthase which differs in one amino acid residue located in the intersubunit region; all of them were, however, less stable than the native enzyme with phenylalanine in this position. So, the importance of the hydrophobic interaction involving phenylalanine in the intersubunit region for stability of the enzyme was proved.[301]

The work performed by Estell et al.[264] is a good example of the use of site-directed mutagenesis for the preparation of proteins stabilized against oxidative inactivation. Met 222 in subtilisin is located in the active center and is involved in oxidative reactions which lead to inactivation of the enzyme. By site-directed mutagenesis, Met 222 was replaced by other amino acids less sensitive to oxidation, such as Ser, Ala, and Leu. The catalytic activity of the modified forms of the enzyme decreased less than ten times, however, their resistance to oxidative inactivation increased dramatically (Figure 8).

In the recent works of Klibanov's laboratory,[241,302] it was shown that irreversible inactivation of enzymes at enhanced temperatures (90 to 100°C) resulted from some chemical changes in the protein primary structure. One of them — the hydrolysis of Asn residues — is responsible for thermoinactivation of proteins at acid pH values. Ahern et al.[303] engineered triose phosphate isomerase, which lacks Asn residues in the intersubunit region of the enzyme (they were substituted by threonines). Indeed, the engineered triose phosphate isomerase was more stable towards irreversible thermoinactivation than the native enzyme.

There are still some problems that prevent protein engineering from receiving a wider application in biotechnology. These problems are understandable if we consider the experimental part of site-directed mutagenesis. First, it is necessary to start with a homogeneous protein preparation. This is often a problem in itself. Second, the protein must be crystallized; difficulties may be expected, e.g., in the case of glycoproteins. Third, the subsequent procedures, such as the determination of the amino acid sequence[103] or the interpretation of the X-ray patterns,[137] are very complicated as well.

As far as the genetic part of the program is concerned, it is necessary first to find a plasmid or another molecular vector containing the gene coding for the required protein. If there is no such vector, which is very often the case with animal and plant DNAs, it must be constructed from known bacterial plasmids or bacteriophages. This involves additional problems.[257-259] After they have been solved, the chemical synthesis of the oligodeoxyribonucleotide can be performed. The rapid advance in this field, especially in regard to solid-phase synthesis,[265] makes it possible to efficiently produce the necessary oligonucleotides composed of 12 to 18 bases. The subsequent operations, i.e., the production of a heteroduplex plasmid, its incorporation into the host cell, and its cloning, are well developed, although there are still some other pitfalls.[257-259]

It is evident that the techniques of protein engineering are far from simple. Successful work in this field requires close cooperation of workers specialized in structure studies of proteins, in chemistry of proteins and nucleic acids, in molecular and cellular genetics, and in many other fields of modern biochemistry and molecular biology. Moreover, these experiments should be performed at the highest experimental level, making use of the most recent achievements in these fields. The methods of protein engineering are very expensive at present and therefore the other, traditional methods of enzyme stabilization can still successfully compete with protein engineering.

J. Chemical Modification of Enzymes

The first successful experiments with the stabilization of enzymes by chemical modification were carried out in the laboratory of Nord et al.[266] in the 1950s. Since then, much experimental data on covalent modification of proteins by low molecular weight reagents have been reported which have led to either an increase or a decrease in their stability. From the analysis of numerous studies performed on this subject (for a review, see References 64 to 66), we outline here the main reasons for protein stabilization by covalent modification:

1. The modification sometimes yields a protein with a more stable conformation, which differs from the conformation of the native protein.[104,105]
2. Stabilization may also occur as a result of modification of "key functional groups"[64] (also called "functional groups of type II" elsewhere[66]).
3. New functional groups introduced into the protein by chemical modification can form additional hydrogen or salt bonds.[267]
4. Chemical modification by nonpolar reagents strengthens hydrophobic interactions in the protein.[268]
5. Hydrophilization of the protein surface groups diminishes the surface area responsible for unfavorable hydrophobic contact with water.[19,305]

Which of these mechanisms should be chosen as the basis of a general approach to the stabilization of enzymes?

Consideration 1

There is hardly any point in transforming the enzyme conformation to a more stable one since the character of conformational changes caused by chemical modification is usually unpredictable.

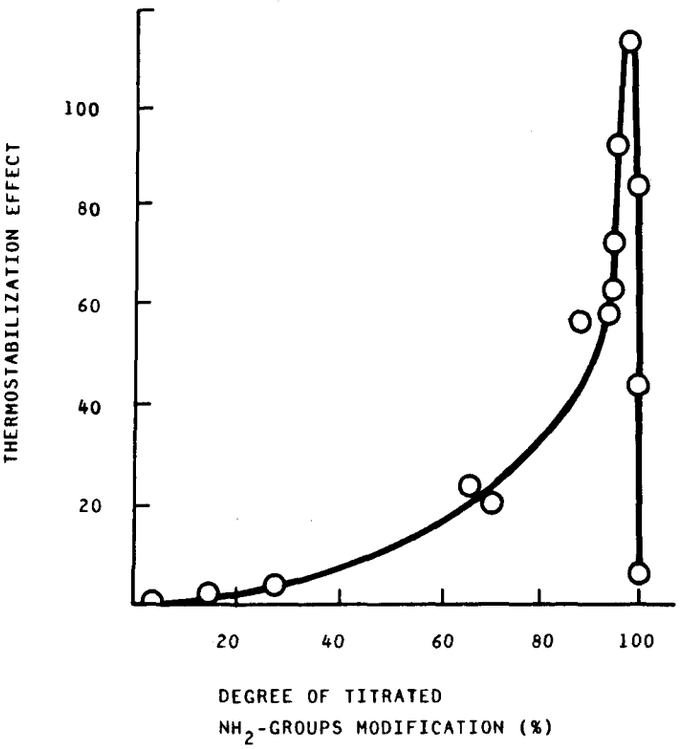


FIGURE 9. Dependence of stabilizing effect against thermoinactivation of modified α-chymotrypsin on the number of alkylated NH_2 groups of the enzyme.[269]

Consideration 2

Considering the "key" functional groups,[64] it should be noted that the chemical modification of proteins very often does not lead to a significant change in stability with the increasing number of modified functional groups until a certain critical value has been reached at which the stability increases abruptly (see Figure 9).[269] This can be explained by assuming that at low modification degrees, i.e., at a little excess of the modifying reagent, only those functional groups are modified which are localized on the surface of a protein and whose role in the protein structure is inessential. If an excess of the modifying reagent is used, some functional groups localized inside the protein globule are also modified. The modification of these groups appears to improve the balance of intramolecular interactions in the protein and results in its stabilization.

There are, however, some problems with the modification of the key functional groups. The first and also the main problem is to determine which of the functional groups is the key one. This can hardly be assessed *a priori*, e.g., from the known three-dimensional structure of the protein. The key groups must be established empirically by investigating the dependence of the protein stability on the degree of modification (Figure 9). The empirical search for the key functional groups must be performed very cautiously since after the modification of the key functional groups some amino acid residues buried more deeply in the protein globule may be modified as well. The latter process usually results in protein destabilization (Figure 9).[64,66] It is not surprising therefore that very often the key functional groups are overlooked in these experiments and the stabilizing effect is not observed at all. There is, however, one more problem with the method of covalent modification concerning

oligomeric enzymes. One should keep in mind that modification of some key functional group in the intersubunit region very often results in destruction of intersubunit contacts and dissociation of enzyme into monomers.

Consideration 3

Polar or charged groups can be introduced into the protein molecule by chemical modification to form new hydrogen bonds or salt bridges. It is very difficult, however, to realize such experiments in practice. The most suitable way is the following: the three-dimensional structure of the protein is determined and a search is made for noncompensated (e.g., charged) groups that could be involved in new electrostatic interactions. Then an appropriate "anchor" functional group is selected which is not too far from the charged group in the tertiary structure of the protein. Finally, a suitable chemical reagent bearing a group specifically reacting with the anchor group of the protein and having a charged fragment localized at a given distance is chosen or synthesized. These experiments are clearly quite elaborate.

Consideration 4

It has also been postulated[268] that protein stability can be increased by its hydrophobization. However, the experimental data are conflicting. In contrast to the observed stabilizing effects,[268] examples can be presented of hydrophobized proteins whose stability is significantly lower than the stability of the native ones.[270-272] This apparent contradiction can be easily explained. The modification of a hydrophilic residue by a hydrophobic reagent (e.g., of the ϵ-NH_2 group of Lys by methyl iodide) should destabilize the protein. After such modification of the protein, in fact, there occurs a thermodynamically disadvantageous (destabilizing) contact of the introduced nonpolar CH_3-groups of the protein with water. There is an exception, however, from this general rule. As shown earlier, many hydrophobic residues localized on the surface of proteins (along with polar and charged amino acids) very often accumulate to form surface hydrophobic clusters.[145,273,274] If a residue to be modified is localized in the vicinity of such a cluster, then a modifier with a suitable chain length could come with it into contact, thus increasing the stability of the protein because of additional hydrophobic interactions.[268]

The increased stability of the proteins which are in complexes with nonpolar molecules (benzene, and lauric and stearic acid, etc.)[275,276] supports the validity of this stabilization mechanism as well.

In terms of hydrophobic stabilization, the most efficient mechanism should be a modification which permits us to introduce nonpolar molecules *inside* the hydrophobic nucleus of the protein.[19] The gist of this approach (schematically represented in Figure 10) is that the protein to be modified has in its initial state an unfolded conformation (random coil) rather than a folded native conformation. As follows from Anfinsen's studies (e.g., see Reference 277), the easiest way to obtain an unfolded protein is to treat the native protein with a strong denaturant (e.g., urea) together with thiol reagents which bring about the scission of S–S bridges (if any). After the unfolding, the structure of the protein should be changed by one of the three following procedures (see Figure 10).[19] First, the protein can be refolded under "non-native" conditions, i.e., in the presence of concentrated salt solutions, organic solvents, or at elevated temperatures. Under such conditions, the protein may adopt another conformation (different from the native one), a conformation which retains catalytic activity and has a higher stability. If the refolding is performed under the conditions mentioned previously which favor hydrophobic interactions, the interior of the protein molecule will become more hydrophobic and, on the contrary, its surface more hydrophilic; this leads to stabilization. Second, the protein can be refolded in the presence of substances interacting with it noncovalently in a multipoint fashion. In our opinion,[19] compounds of nonpolar and diphilic nature are the most promising ones for this purpose.

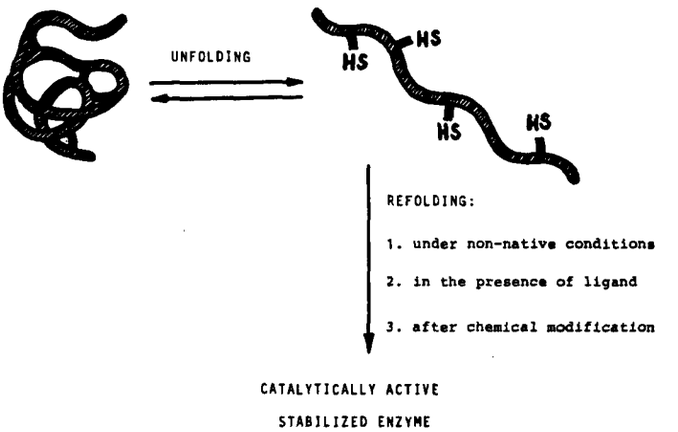


FIGURE 10. Schematic representation of one line of approach to enzyme stabilization: the protein molecule is first unfolded and then refolded in one of three ways.[19]

The former can be entrapped into the hydrophobic nucleus of the protein in the course of refolding, while the latter can be incorporated into the protein in a manner which permits their nonpolar moiety to contact the hydrophobic areas of the protein, leaving polar or charged fragments exposed to the solvent. Third, the unfolded protein can be modified with a chemical reagent and then refolded.

This approach (Figure 10) is being actively developed now.[278] In the present review, only one experimental result will be discussed. Immobilized trypsin was unfolded and then refolded at elevated temperatures. It has been found[19] that trypsin refolded at 50°C, and even higher temperatures, is more stable than the starting enzyme (before unfolding) or the enzyme refolded at normal temperatures (20 to 35°C). These in vitro results are in good agreement with the data obtained in in vivo experiments with thermophilic microorganisms.[45,46,279-281] These microorganisms usually synthesize stable and labile enzymes which are both isoforms of the same enzymes (having the same primary structures). With raising the temperature, the ratio of stable-to-labile enzymes greatly increases. Therefore, depending on the temperature of biosynthesis in vivo[45,46,279-281] and the temperature of refolding in a model system,[19] proteins adopt conformations of different stability.

Consideration 5

Finally, we will consider the approach to the stabilization of enzymes which was recently proposed by our laboratory[19] and is being further developed.[282,283] It is based on the assumption (discussed earlier in detail) that the contact of the hydrophobic surface area of a protein with water is thermodynamically disadvantageous and thus destabilizes the protein. Therefore, we use chemical modification to decrease the hydrophobic character of the protein surface.[282,283,305]

The experimental procedure of hydrophilization involves several principal problems. First, a criterion of hydrophobicity (hydrophilicity) of chemical compounds must be chosen. The role of this factor, i.e., the evaluation of the hydrophilicity of the modifying reagent, has usually been underestimated when the effect of chemical modification of protein stability was considered (e.g., see References 64 to 66). Sometimes, it is very easy to decide whether chemical modification leads to hydrophobization of the protein or to its hydrophilization and no quantitative measurements are required. The modification of α-chymotrypsin by treatment with aliphatic aldehydes is evidently a good example of hydrophobization.[269] In

fact, in this case, the amino groups of the enzyme are transformed to $-NH-(CH_2)_n-CH_3$. However, it is hardly possible to estimate qualitatively how the hydrophilicity of an amino group will change after modification by succinic anhydride transforming $-NH_2$ to $-NH-CO-(CH_2)_2-COOH$. Nevertheless, this type of modification is sometimes regarded[66] as hydrophilization.

The partition coefficient (P) of the compound in the system *water-immiscible* organic solvent (usually octanol) can serve as a quantitative criterion of hydrophobicity.[284-286] According to Hansch, the more positive the value of P, i.e., the higher the preference of a substance for the organic phase than for the aqueous phase, the more hydrophobic the substance. In agreement with the principle of additivity of free energies,[287] the hydrophobicity of any compound is a sum of hydrophobicities of its atoms and functional groups (the so-called hydrophobic increments designated π). Hence, the hydrophobic increment of a chlorine atom (π_{Cl}) can be easily calculated after the partition coefficients between water and octanol of the two compounds, benzene chloride and benzene, have been experimentally determined:

$$\pi_{Cl} = \log P_{C_6H_5Cl} - \log P_{C_6H_6} = 2.84 - 2.13 = 0.71$$

Using this method, Hansch and co-workers[284,285] were able to calculate the hydrophobic increments of the individual atoms and functional groups most common in biochemistry (about 150 π-values). Some examples are given in Table 8. The same method of calculation can be used to solve the opposite problem, i.e., to evaluate the hydrophobicity of complex compounds from the known hydrophobic increments (π-values) of atoms and functional groups. The principle of additivity of free energies[287] and some of the empirical rules discussed in detail by Hansch and Leo[285] should be employed for this purpose.

Now we suggest using the procedure developed by Hansch and co-workers[284,285] for estimating the contribution of a modifying reagent to protein hydrophobicity.

Let us consider some of the previous experimental data on chemical modification of proteins from this aspect. Stoetz and Lumry[288] showed that the oxidation of Met 192 in α-chymotrypsin which gives rise to the hydrophilic $SOCH_3$ group increases the conformational stability of the protein by 2.1 kcal/mol. On the other hand, the difference between the increments of the free energies of extraction of the two groups from water into the organic solvent, RT ($\pi_{SOCH_3} - \pi_{SCH_3}$), calculated from the data of Hansch and Leo[285] is -2.2 kcal/mol. Thus, we may conclude that the stabilization of α-chymotrypsin observed[288] during the oxidation of a single Met residue is caused by hydrophilization of a hydrophobic residue on the surface of the protein molecule.

The modification of the amino groups of proteins by o-methyl-*iso*-urea and its analogs to arginine-like structures may serve as another example. It can be seen from Table 6 that Arg is more hydrophilic than Lys. The numerous examples of protein stabilization observed[289-294] after guanidination of the Lys residues can therefore be accounted for by a decrease of the surface hydrophobicity of proteins.

Covalent modification of enzymes by polysaccharides which very often results in their stabilization[295] can also be regarded as hydrophilization. If the hydrophobic increment of the polysaccharide moiety introduced by this type of modification is calculated using the data of Hansch and Leo[285] (Table 8), it can be seen that the modification leads to a certain hydrophilization of the protein surface area. This is not, however, the only explanation since the stabilizing effect of saccharides is mainly ascribed[296] to their preferential binding to the native form of the protein (rather than the denatured form) and is caused by perturbation of the water structure by saccharides.

A marked stabilizing effect was observed[282,305] to result from the amination of the tyrosine

Table 8
HYDROPHOBIC INCREMENTS (π-VALUES) FOR FUNCTIONAL GROUPS[285]

Group	H	CH_3	C_2H_5	$CH(CH_3)_2$	C_3H_7	C_6H_{11}	C_6H_5	OH	SH	OCH_3	$NHCH_3$
In aromatic ring	0.00	0.56	1.02	1.53	1.55	2.51	1.96	−0.67	0.36	−0.02	−0.47
In aliphatic compound	0.23	0.77	1.43	1.84	1.97	—	1.90	−1.64	−0.23	−1.54	−1.38

Group	CN	NO_2	NH_2	COOH	COO^-	$COCH_3$	$CO(O)CH_3$	$C_2H_4CO(O)H$	$N(CH_3)_2$	$N^+(CH_3)_3$
In aromatic ring	−0.57	−0.28	−1.23	−0.32	−4.36	−0.55	−0.01	−0.29	0.18	−5.96
In aliphatic compound	−1.27	−1.16	−1.54	−1.11	−5.19	−1.13	−0.72	—	−0.64	—

residues in trypsin. Four tyrosine residues are localized on the surface of this enzyme.[297] It is clear from Table 8 that the introduction of an amino group into the aromatic ring of tyrosine decreases its hydrophobicity by a value of −1.24 kcal/mol. The stability of trypsin in which two, or up to four, tyrosine residues have been modified increases more than 100 times.[282] Such a large stabilization seems to be the result of an essential hydrophilization of the nonpolar surface area of the enzyme molecule.

The stabilization via hydrophilization was very successful in the case of acylation of α-chymotrypsin by pyromellitic dianhydride.[283,305] The amino groups of the protein were mainly modified by this reagent, although the dianhydride can also react with the OH groups of Ser and Thr. The modification of any group in the enzyme molecule introduces three new carboxylic groups (Figure 11), hence, an enzyme preparation with the highest modification degree bears at least 50 new COOH groups.[283] At slightly alkaline pH (i.e., under the conditions of thermal inactivation of the enzyme), all the carboxylic groups are ionized and the protein surface is therefore hydrophilized to a higher degree (see Table 8 for the degree of hydrophilization). Since the modified enzyme preparation is significantly more stable than the native one, it is practically impossible to select a temperature at which the kinetics of inactivation of the two preparations could be directly compared. To evaluate the difference in their stabilities, the experimental data (Figure 12) were extrapolated to an intermediate temperature. Thus, at 60°C the stabilizing effect is more than 10^3 times higher and it increases even more with the increasing temperature (see Figure 12).

So, the hydrophilization of the surface of α-chymotrypsin by a large number of carboxylic groups results in dramatic stabilization of the enzyme.[283,305] Such marked stabilizing effects have been previously observed only after multipoint binding of enzymes to a support[131,252,253] (for a review, see References 11 to 19). Thousand-fold (and even higher) stabilizing effects[283] are the highest achieved up to now by chemical modification of proteins by low molecular weight reagents (for a review, see References 64 to 66). The stability of α-chymotrypsin modified by pyromellitic dianhydride[283,305] practically equals the stability of proteinases from extreme thermophilic microorganisms,[298] which are the most stable proteolytic enzymes known to date.

V. CONCLUSION

In the present review, we attempted to answer two major questions concerning the relationship between protein structure and stability: first, how to ascertain the structural determinants of protein stability, i.e., those structural features (or inter- and intramolecular interactions) which are responsible for the higher stability of proteins; and second, what approaches and techniques should be used to produce stabilized enzyme preparations (utilizing the known structural determinants of protein stability). We believe that the new methods and concepts for increasing the stability of enzymes, some of which have been discussed or suggested in the present review, will stimulate further research directed toward this goal and will also receive application in practice.

ADDENDUM

Some important communications appeared after the authors finished the review.

The aspects of thermodynamic stability of proteins are discussed in a review by Schellman.[306]

Chothia and colleagues[307] considered the role of hydrophobic contacts in stabilization of oligomeric proteins.

The role of hydrophobicity in conformational stability of proteins was further evaluated by Yutani et al.[308] by example of tryptophan synthase α-subunit.

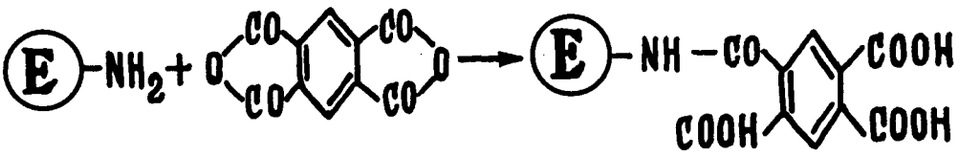


FIGURE 11. Scheme of the main reaction which occurs as a result of modification of α-chymotrypsin by pyromellitic dianhydride.[283]

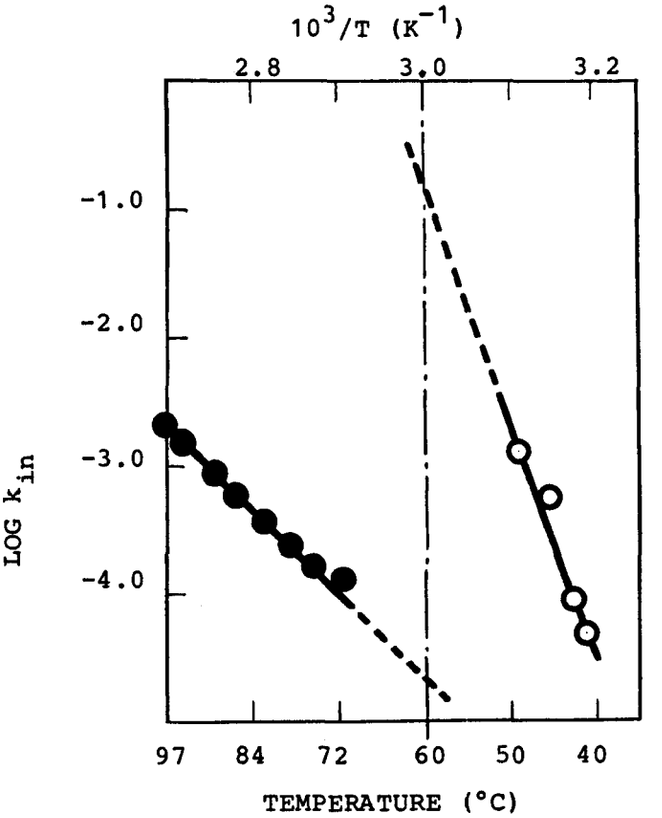


FIGURE 12. Temperature dependence of the first-order rate constants of thermal inactivation of native α-chymotrypsin (-○-) and α-chymotrypsin modified by pyromellitic dianhydride (-●-).[283]

Volkin and Klibanov[309] emphasized the contribution of high temperature destruction of S–S bonds and the subsequent thiol-disulfide exchange reactions in the thermal destruction of proteins.

A number of studies on protein stability were carried out using site-directed mutagenesis. Some recent achievements on the topic are summarized by Shaw.[310] Matthews et al.[311] have realized the idea of enzyme stabilization by amino acid substitutions resulting in a decrease in the conformational entropy of unfolding. Using the method of temperature-sensitive mutations, it was shown[312] in the same laboratory that the replacements of amino acid residues lead to dramatic alterations in protein stability if they occur in the internal regions of the protein molecule characterized by low mobility and low solvent accessibility. Experiments on protein engineering pointed out that introduction of new S–S bonds in the protein molecule can either increase or decrease its stability.[314] The latter is explained by

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