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Review

Strategies for Obtaining Stable Enzymes

Štefan Janeček

Department of Biochemical Technology, Faculty of Chemical Technology, Slovak Technical University, Radlinského 9, SQ-81237 Bratislava, Slovak Republic

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Generally, enzymes are only marginally stable. In order to increase their utilization as industrial biocatalysts, it is necessary to obtain enzymes with enhanced operational as well as survival stability. Two principal ways exist to fulfil this task: (i) the use of enzymes which possess natural extremostability, or (ii) stabilization of naturally unstable enzymes. These are reviewed separately in order to describe the individual approaches, mainly immobilization, chemical modification and protein engineering. Some prospective trends (solvent engineering and protein design) are suggested as being potentially useful to provide a wider insight into enzymes designed for industrial processes.

INTRODUCTION

The ability to obtain stable enzymes is crucial for their application as biocatalysts. Catalytic protein molecules, like all other proteins, are only marginally stable due to the delicate balance of stabilizing and destabilizing interactions.[1] This balance can be expressed by the difference in Gibbs free energy between the native and the unfolded state of the protein, with values of about -40 kJ/mol being generally found.[2] An understanding of enzyme stability is therefore not easy, since it needs to take into account the size and complexity of enzymes as macromolecules, as well as very small energy changes in both the folded and the unfolded forms.[3]

Corresponding author: Štefan Janeček

Detailed elucidation of the mechanisms responsible for stabilization and destabilization of enzymes especially at elevated temperatures is of special importance from both a scientific and a commercial point of view.[4] The enzymes from thermophilic organisms, growing at temperatures from 55°C to the boiling point of water,[5] are usually more stable than those from mesophiles; nevertheless, no additional amino acid residue or interaction has been found in their structures. This indicates that the intrinsic stability of thermophilic enzymes is probably achieved by strengthening or increasing the number of existing interactions via the replacement of amino acid residue(s) either throughout the whole enzyme molecule or at key position(s).[6] The physical forces that participate in the stabilization of native enzyme structure (hydrophobic effects, van der

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Waals forces, electrostatic interactions, hydrogen bonds, covalent crosslinks) were qualitatively identified in the 1950s.[7] The current task is to determine the contribution of individual amino acids and physical forces to the stability of a specific enzyme.[8]

In principle, there are three ways to obtain stable enzymes:[9] (i) isolation from extremophilic microorganisms; (ii) production in genetically manipulated non-extremophilic hosts; and (iii) stabilization of unstable enzymes by modification. The last approach can be realized in three ways: (i) by immobilization; (ii) by chemical modification; and (iii) by protein engineering. The empirical use of various additives which have the ability to enhance the stability of protein molecules, has been described recently[4,10] and will not be reviewed here. In order to stabilize enzymes, the knowledge of their structure–stability relationships[11–15] is indispensable, especially for protein engineering.[6]

The aim of this review is to outline the above strategies for obtaining stable enzymes, with a brief description of results achieved in an attempt to answer the question: how can you obtain stable enzymes?

NATURALLY EXTREMOSTABLE ENZYMES

Interest in extremostable enzymes is due mainly to the fact that most industrial enzyme processes are carried out under abnormal physiological conditions, such as higher temperatures, higher pressures, extreme values of pH, etc.

The first way to obtain biocatalysts for these processes is to isolate enzymes from microorganisms which naturally exist in extreme environments, such as thermophiles from the hot springs of Yellowstone National Park[16] and halophiles from the salt-saturated waters of the Dead Sea.[17] Potential extremophilic microorganisms for the production of extremostable enzymes also include barophiles, acidophiles and alkalophiles. Only thermostable enzymes from thermophilic microorganisms have found application in industry on a large scale.

Thermophiles and thermostable enzymes

Temperature is one of the most important environmental factors controlling the activities and evolution of organisms[16] and is probably the best optimized physical variable in chemical reactions.[18]

Some major advantages are offered by thermophiles or their enzymes in industrial processes,[5,18,19] including an increase in the rate of reaction at elevated temperatures resulting in a decrease of the amount of enzyme required. Furthermore, higher temperatures may reduce the possibility of microbial contamination, increase the solubility of substrates and other chemicals and decrease the viscosity of liquids. The fact that intensive cooling becomes unnecessary is also of importance. The only serious limitation for high-temperature operation is the decrease in oxygen solubility which may influence aerobic processes.[5]

The temperatures commonly used in thermostable enzyme conversions (above 60°C[18]) are far from the upper temperature limit for life in liquid water. This has not yet been defined.[5] The report of Baross and Deming[20] on bacteria isolated from a deep-sea hydrothermal vent and growing at temperatures of at least 250°C (pressure 26·5 MPa) remains very controversial and unconfirmed. Based on results from studies on the hydrolytic destruction of proteins and nucleic acids at 250°C,[21,22] Brock[5] has suggested that the upper temperature limit for life lies between 110 and 200°C.

Of the thermostable enzymes characterized, only bacterial amylases for starch liquefaction and proteases for food processing and detergents have found industrial application.[19] α-Amylase from *Bacillus licheniformis*[23] is the most thermostable of all the industrial enzymes, being capable of liquefying starch up to 110°C. Potential new areas of applications of thermostable enzymes include the production of cyclodextrins using cyclodextrin glycosyl transferase and biobleaching of wood pulps using xylanases.[19]

In the past few years, some novel bacteria genera have been isolated from marine geothermal sites, including hydrothermal vents several kilometers below sea level. These extraordinary 'hyperthermophilic' microorganisms exhibit remarkable optimal growth at temperatures exceeding the boiling point of water.[24] They could provide hyperthermostable enzymes for industrial processes, but their significant biotechnological potential is strongly limited at present due to the absence of physiological and biochemical information.[25] The only exception is *Pyrococcus furiosus*, a strictly anaerobic bacterium that

metabolizes sugars by a fermentative pathway with H_2, CO_2 and organic acids as the products.[26] To date, several proteins and enzymes have been purified from *P. furiosus*. All are extremely thermostable with optimal temperatures for activity above 95°C.[25] Despite the lack of structural information, many researchers believe that proteins and nucleic acids from hyperthermophilic bacteria are stabilized by the same types of mechanism as in mesophiles.[27] The most suitable way to obtain information on the factors responsible for the unusual stability of enzymes from both thermophilic and hyperthermophilic microorganisms is a structural comparison of these enzymes and their mesophilic counterparts. Recently, a study based on this approach has revealed[25] that the *N*-terminus of rubredoxin (one of the simplest redox proteins) from *P. furiosus* appears to be fixed by a salt bridge, which does not occur in rubredoxins from mesophilic organisms. Findings of this type might be useful in determining strategies for the stabilization of related enzymes from mesophiles. However, in order to obtain individual hyperthermostable enzymes in the amounts required for their use in industry, new bioreactor concepts and processing protocols must be developed to provide suitable conditions for microorgansims from deep-sea environments.[28]

Genetically manipulated mesophiles

The second way of obtaining naturally extremostable enzymes involves genetic engineering. The first step is to isolate all or part of the genome of a thermophilic microorganism. This is followed by its introduction into the genetic machinery of a suitable mesophile. This general approach is well established[12] and has found applications in the production of pharmaceutically valuable peptides and proteins, such as insulin, interferon and vaccines against viruses.[29] The ability to clone the genes from thermophiles into mesophilic production strains has increased the potential use of thermostable enzymes in industry. For example, the genes coding for thermostable α-amylase[30] and neutral protease[31] from *B. stearothermophilus* have been cloned and expressed in *B. subtilis*.

The production of thermostable enzymes in *Escherichia coli*,[32] *Bacillus subtilis*[33] or *Saccharomyces cerevisiae*[34] could be highly attractive from the point of view of a decrease in possible health risks.[9] On the other hand, the risks associated with the release of genetically engineered organisms into the environment must be considered[35] together with the possibilities of these organisms surviving in nature and influencing evolutionary mechanisms.[36]

STABILIZED ENZYMES

Stabilization of enzymes from mesophiles is considered[12] to be the best way to obtain stable biocatalysts. The procedures can be divided into three areas: immobilization, chemical modification and protein engineering.

Undesirable denaturation

Many enzymes become unstable and lose activity after isolation[37] and the problem of denaturation is relevant in any discussion on stabilization. It is also important in the understanding of structural and functional aspects of protein molecules.[38]

Tanford[39] has defined denaturation simply as a major change from the original native structure without alteration of the amino acid sequence. Unless no changes in the primary structure of a protein are specifically indicated, it should be termed as inactivation.[37] It usually obeys a two-step process:[40]

$$N \rightleftharpoons D \rightarrow I$$

where N, D and I are the native, reversibly denatured and irreversibly inactivated enzyme forms, respectively. A reversible conformational change (i.e. unfolding of the enzyme) in the first stages of inactivation is followed by irreversible processes, including aggregation or covalent modification.[41] However, an earlier proposal[39] that proteins approach a randomly coiled conformation in strong denaturants is refuted by considerable evidence of some structure remaining in protein chains even in 6 M guanidine hydrochloride and 9 M urea.[42]

The native conformation of an enzyme is stabilized by intramolecular interactions, while the major contribution to the unfolded state comes from the chain entropy of the random coil. As the temperature rises, this contribution exceeds the enthalpy from interactions of the native state and enzyme denaturation results.[38] Shifts in environmental conditions such as temperature or the presence of denaturants (Fig. 1), however, produce different changes in the structure of enzymes.[43] Thermodynamic studies of the denaturation of protein molecules[44,45] have revealed that the protein structure is stabilized by intra-

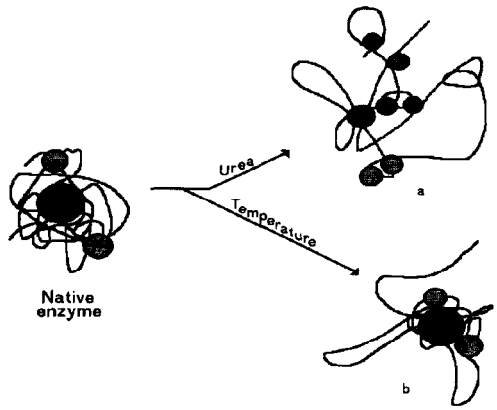

Fig. 1. Denaturation of enzymes. (a) Substantial unfolding of the native conformation of the protein molecule by urea and (b) damaging of the native arrangement of hydrophobic core of the protein globule and its surface hydrophobic clusters at elevating temperature.[43] Dark and dotted circles represent the hydrophobic core of the protein and its surface hydrophobic clusters, respectively.

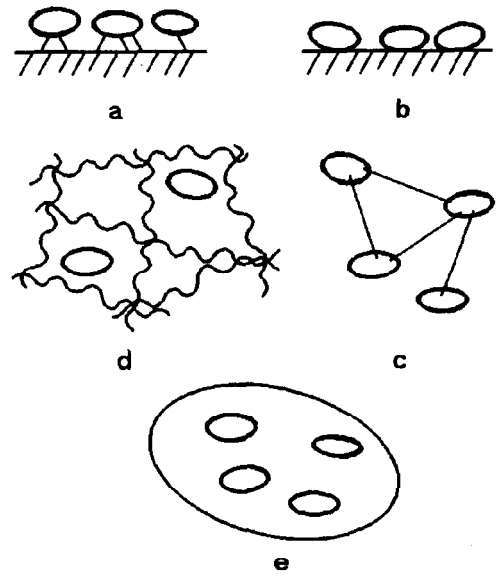

Fig. 2. Methods of enzyme immobilization.[48] (a) Covalent attachment to solid supports; (b) adsorption on solid supports; (c) entrapment in polymeric gels; (d) crosslinking with bifunctional reagents; (e) encapsulation.

molecular hydrogen bonding and van der Waals interactions of nonpolar groups. A second component, hydration of nonpolar groups, destabilizes the compact native state.[44,45] From the point of view of the optimal applications of enzymes in industrial processes, the question of reversibility and irreversibility of inactivation is of special significance because the elucidation of such mechanisms could lead to the solution of problems connected with enzyme reactivation and stabilization.[46]

Immobilization

Developments in the industrial application of enzymes in many cases involves the use of continuous reactors which require biocatalyst immobilization in order to combine efficiency and stability.[47] Klibanov[48] has defined immobilization as the conversion of an enzyme from a water-soluble, mobile state to a water-insoluble, immobile state. The techniques of enzyme immobilization may be divided into five groups (Fig. 2).

While stabilization is sometimes achieved after immobilization of an enzyme[49] there is no way to predict the stability of a given enzyme–matrix complex or to design an enzyme–matrix system with increased stability.[50] Stabilization due to immobilization can be ascribed[49] to mutual spatial fixation of enzyme molecules (against aggregation or autolysis of proteases), an increase in their rigidity (against unfolding, i.e. conformational changes) and their protection from possible inactivators (pH, oxygen, hydrogen peroxide, 'poisons').

One of the most significant criteria applied to immobilized enzymes, however, is stability[50] since heat is the most important reason for inactivation in industrial reactors.[47] According to Ulbrich *et al.*[50] immobilization leads to two enzyme populations with different stabilities. Experimental results from thermal inactivation of soluble and covalently bound α-amylase and chymotrypsin to various supports have been used to propose a model explaining stabilization effects on immobilized enzymes.[51] Each enzyme molecule is characterized by a specific site where the unfolding process begins during inactivation. The protection of this 'unfolding nucleus' due to immobilization is achieved only in one fraction of the enzyme molecules which can block the native unfolding pathway, resulting in enhanced stability.[51] The second fraction with no protection of this nucleus behaves as soluble enzymes.

Although there is no doubt that the use of immobilized enzymes provides more advantages than demerits (Table 1), their practical industrialization is restricted when compared to the extent of research carried out.[52] Moreover, avoiding the use of a polymer support has been suggested to be more useful in enzyme stabilization.[53]

Table 1. Some aspects of the use of immobilized enzymes[52]

Advantages	*Demerits*
(i) Ease of developing continuous processes	(i) Existence of mass transfer resistances
(ii) High stability and resistance to shear stress and contamination	(ii) Necessity of immobilization processes
(iii) Fast reaction rate due to high catalyst concentration	(iii) Additional costs of immobilization reagents
(iv) Easy separation of biocatalysts from fermentation medium	
(v) Repetitive use of biocatalysts	

Chemical modification

The main reason for producing water-soluble stable enzymes is[53] the absence of a polymer matrix which seriously decreases both the binding capacity and the reactivity of immobilized enzymes in the conversion of macromolecular substrates. There is also the possibility of changes of the mechanisms of enzymes used in the clinical treatment of diseases which may be caused by the support matrix during interactions with receptors or other components of cellular membranes. Mozhaev *et al.*[54] have recommended chemical modification of enzymes with low molecular weight compounds as the simplest method from a methodological point of view. This means that the enzyme solution is incubated with a solution of a modifier and, if necessary, the modified enzyme is separated from the reaction mixture simply by dialysis or gel filtration.

The external functional groups located on the surface of an enzyme molecule react most readily with added modifying reagent.[55] On the other hand, the enhanced stability of modified enzymes has been ascribed[56] to the modification of some internal, so-called 'key' functional groups. In order to modify these, a greater molar excess of a modifier or a longer incubation time should be used.[54]

As with immobilization, the prediction of how the thermal stability of an enzyme will be affected by chemical modification is difficult.[53] For instance, the thermostability of *Bacillus subtilis* α-amylase acetylated with *p*-nitrophenyl acetate[57] was increased only at temperatures higher than 70°C, while at lower temperatures the enzyme became less stable. This so-called isokinetic effect has also been observed for the thermoinactivation kinetics of *B. subtilis* α-amylase stabilized by modification with D-glucono-δ-lactone.[58]

In the majority of modification studies, the ε-amino groups of lysine residues are involved as

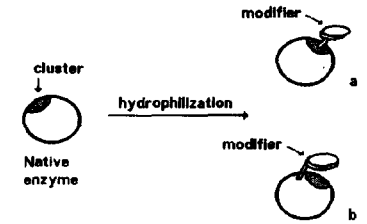

Fig. 3. Approach to hydrophilization of a nonpolar surface area in a protein globule.[61] The hydrophobic cluster can be directly modified with a hydrophilic modifier (a) or the modifier can shield the cluster from a nearly localized site (b).

they are the most abundant and the most accessible groups of amino acid side chains in enzymes.[59]

Mozhaev *et al.*[54] have divided the strategies for obtaining stabilized enzymes by covalent modification into four following groups of methods: (i) modifications with bifunctional reagents (crosslinking of surface functional groups); (ii) modifications with nonpolar reagents (strengthening of hydrophobic interactions); (iii) introduction of new polar or charged groups (formation of additional hydrogen or ionic bonds); and (iv) hydrophilization of the protein surface (prevention from unfavourable hydrophobic contact with water). All of these have their own advantages and problems which have to be overcome if the approach is to be used successfully.[54] The above methods have been improved and new features introduced into them, such as the use of a photogenerated heterobifunctional reagent (based on an aryl azide compound), for intramolecular crosslinking of the lipase from *Candida cylindracea*.[60] Nevertheless, the largest stabilizing effect without the use of a polymeric support has been achieved with hydrophilization.[54] This can be carried out in one of two ways (Fig. 3). In the first case, the hydrophobic amino acid residues which form the surface hydrophobic cluster destabilizing the protein

molecule by contact with water are directly modified with a hydrophilic modifier or, in the second case, any (not necessarily hydrophobic) residue located near the cluster is hydrophilized by the modifier. This results in the shielding of the cluster from the aqueous environment.[61] A 1000-fold increased thermostability of α-chrymotrypsin (in comparison with the native enzyme) prepared by its hydrophilization with glyoxylic acid and cyclic anhydrides of aromatic acids has been reported.[61,62] This increases the stability of this enzyme to a level similar to the proteinases from extremely thermophilic bacteria.

Protein engineering

The introduction of protein engineering in the early 1980s[63] evolved from a knowledge of gene cloning, DNA sequencing and in-vitro DNA manipulation.[64] Site-directed mutagenesis has enabled engineered proteins to be produced that differ from their wild counterparts only in one or several predefined amino acids. This approach has brought the possibility of stabilizing mesophilic enzymes by changes in the amino acid sequences. This is done either by copying the naturally extremostable enzymes or by predicting stabilizing effects.

Table 2. Enzyme properties to be tailored to industrial processes[65]

(i)	Kinetic properties
(ii)	Thermostability and temperature optimum
(iii)	Stability and activity in nonaqueous solvents
(iv)	Substrate and reaction specificity
(v)	Cofactor requirements
(vi)	pH optimum
(vii)	Protease resistance
(viii)	Allosteric regulation
(ix)	Molecular weight and subunit structure

Ulmer[65] has summarized the properties of enzymes (Table 2) whose control in a predictable fashion could be realized by protein engineering. In order to enhance the stability of enzyme structure (Fig. 4), protein engineers can usually explore the following:[67] (i) introduction of internal or surface disulfide bridges; (ii) improvement of internal hydrophobic packing; (iii) increase in internal hydrogen bonding; and (iv) increase of surface salt bridges. Based on a study of aromatic–aromatic interaction, Burley and Petsko[68] concluded that proteins can also derive substantial stabilization from the inclusion of one or more aromatic residues.

Disulfide bonds in globular proteins provide conformational stability.[69] In general,[70] the disulfide bridge must be introduced at positions which are stereochemically optimal for the formation of a crosslink. Perry and Wetzel[71] have replaced isoleucine at position 3 with a cysteine (Ile 3→Cys) in phage T4 lysozyme, and under mild oxidation they have generated a disulfide bond between the new Cys 3 and Cys 97, one of the two unpaired cysteines of the native lysozyme. The disulfide mutant protein retained full enzymatic activity and was more stable against thermal in-

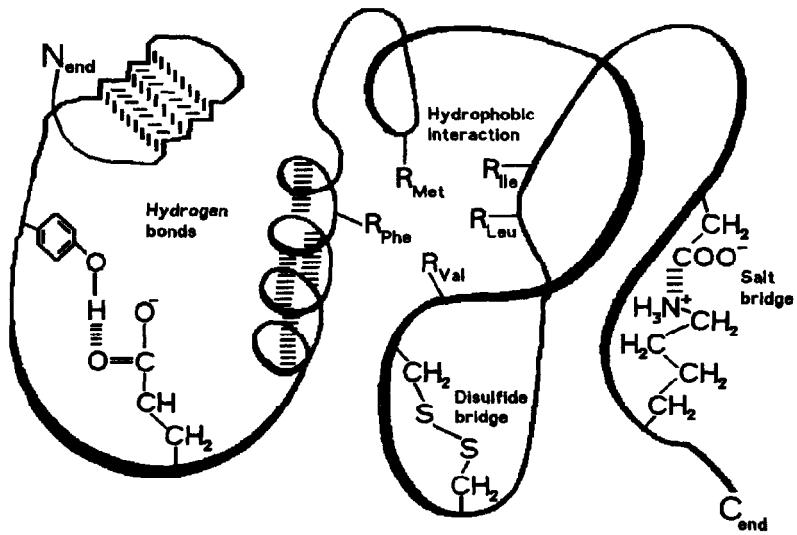

Fig. 4. Bonds and interactions contributing to the stability of the protein structure.[66]

activation than the wild-type protein.[71] The addition of new disulfides has not always led to increased stability.[72] Based on the examination of four disulfide mutant T4 lysozymes, Matsumura *et al.*[73] have suggested that for the successful improvement of protein stability by disulfide bonds: (i) the introduction of the cysteine(s) should minimize the disruption or loss of interactions stabilizing the native structure; (ii) the size of the loop formed by the crosslink should be as large as possible; and (iii) the strain energy introduced by the disulfide bond should be kept as low as possible.

The hydrophobic effect is considered to be the major driving force in protein folding and stability.[74,75] Although the partitioning of amino acid side chains between the protein interior and its exposed surface is far from perfect, it has important effects on every type of conformation.[76] It has been demonstrated[77] that the creation of a cavity the size of a $-CH_2-$ group (truncation Ile 96 → Val) in barnase destabilizes the enzyme by 4·6 kJ/mol. Similar results have been described[78] for five Leu → Ala cavity-creating mutants of phage T4 lysozyme where the substitutions decreased the stability of the protein from 11·3 kJ/mol to 20·9 kJ/mol. From the studies on 15 mutants of barnase with deleted hydrophobic groups, Serrano *et al.*[79] have concluded that the contribution to protein stability of a buried methyl/methylene group is on average 6·3 kJ/mol, being additive for long side chains of amino acids.

Hydrogen bonding is a ubiquitous interaction contributing to protein stability. It was one of the first interactions to be analysed systematically by protein engineering.[79] The destruction of a hydrogen bond by a single amino acid substitution often reduces protein stability.[80] Alber *et al.*[81] have shown a strong correlation between a major reduction in stability and the replacement of Thr 175 in phage T4 lysozyme with other amino acids unable to form a hydrogen bond with the amide of Asp 159. Although the urea and thermal unfolding studies of twelve mutants of ribonuclease T1 have suggested[82] that hydrogen bonding and the hydrophobic effect make comparable contributions to the conformational stability of the enzyme, in general hydrogen bonds seem to be quite variable in their contribution to protein stability.[79]

The salt bridge contributing to enhanced enzyme stability has been constructed, for instance, by site-directed mutagenesis in a Gln 19 → Glu mutant in subtilisin BPN.[83] Generally, however, long-range (solvent-exposed medium-range) as well as short-range (ion pairs) electrostatic interactions do not make large individual contributions to protein stability (1·3–4·1 kJ/mol).[79,84] Fersht and coworkers[79,85,86] have shown, from studies on barnase, that for long-range electrostatic interactions, the effective dielectric constant can be very high (around 150), and the interactions are screened by salt. On the other hand, a buried charge without the partner will destabilize the protein by 12·6 kJ/mol to 16·8 kJ/mol.[87] An unusually large electrostatic effect of the salt bridge between Asp 70 and His 31, contributed 12·6–20·9 kJ/mol to the stability of phage T4 lysozyme.[88] Yang and Honig[84] have suggested that some combination of salt-bridge-favouring interactions, such as hydrophobic interactions between the hydrocarbon side chains of amino acid residues, help to fix the side chains in the favoured salt-bridge conformation.

Recently, some new factors that may contribute to the stabilization of protein structure have been examined. Based on the investigation of the interaction between His 18 and aromatic residues at position 94 in barnase, Loewenthal *et al.*[89] have shown that histidine–aromatic interactions are stabilized on protonation of this histidine residue and this consequently stabilizes the protein. The strength of the interactions observed for barnase decreases in the series His–Trp > His–Tyr > His–Phe.[89] On the other hand, the replacements of Asn 55 and Lys 124 (so-called 'left-handed helical' residues) with two glycines (due to that lack of a β-carbon Gly occurs with approximately equal frequencies as the left-handed and right-handed residue) in phage T4 lysozyme have revealed[90] that even though left-handed nonglycine residues are rare, they do not play an indispensable role in protein stability, since the difference between the conformation energy of a glycine and nonglycine residue in the left-handed helical configuration does not exceed 2·1 kJ/mol. In another study, Eijsink *et al.*[91] have engineered the 10 residue β-hairpin characteristic for naturally thermostable *Bacillus* neutral protease into the thermolabile neutral protease of *Bacillus subtilis*. After the introduction of two additional point mutations designed to improve the interactions between the enzyme surface and the engineered β-hairpin, the increase in thermostability of the loop-containing mutant of 1·1°C has been achieved.[91]

PROSPECTIVE TRENDS

Non-aqueous enzymology

As Klibanov[92] has pointed out, everything we know about enzymes have been supposed to function only in media rich in water.[93] Recently, this belief has been overcome and it is clear that enzymes can function in organic as well as aqueous media.[94] On the other hand, enzymes cannot operate under totally anhydrous conditions,[93] but Zaks and Klibanov,[95] studying porcine pancreatic lipase, have found that the amount of water can be extremely small. This fact can be easily explained[92] by the ability of the enzyme to 'see' no more than a monolayer or so of water around it. Therefore, if this layer of 'essential' water is somehow localized and maintained on the surface of the enzyme, then all the remaining water should be replaceable with organic solvents with no adverse effect on the enzyme.[92] Moreover, in nearly anhydrous organic media (water concentration below 0·3%), lipase not only withstands heating at 100°C for many hours and exhibits a catalytic activity at this temperature, but also changes its substrate specificity.[95] From a technological point of view some other advantages,[96] such as (i) removing the problem of dissolving hydrophobic reagents; (ii) enabling a shift in the thermodynamic equilibrium of many enzymic reactions; and (iii) suppressing bacterial contamination of industrial equipment, could be of importance. In any case, in order to maintain enzymic activity in organic solvents, certain rules have to be followed:[92] (i) the best solvent is a hydrophobic one; (ii) enzymes to be used in organic solvents should be lyophilized from aqueous solutions of the pH optimal for their activity; and (iii) enzymes are suspended, not dissolved (as in water), in nonaqueous solvents.

Protein design

Although changing the reaction medium from water to organic solvent ('solvent engineering') can be used to control the same features of enzymes (Table 2) as protein engineering,[92] the latter seems to remain the most powerful tool for obtaining stabilized enzymes. This view could be supported by the new area which has emerged recently. This is based on inverting the problem of the correct folding of protein molecules into their stable native state.[97] This approach not only tests how the protein structure has been understood, but also allows the construction of novel proteins with structures and properties unprecendented in nature.[98] One of the results of this protein design is the amino acid sequence template representing the sequence of the protein family with a given fold.[97] It should be pointed out that such an approach to protein modelling can be completely automated with all decisions being rule based.[99] Protein design thus gives protein engineers considerable latitude in the design of new structures[97] which consequently could be the structures of enzymes with enhanced stability.

CONCLUDING REMARKS

At present there are several approaches leading to the production of stable enzymes for industrial processes. In principle, either naturally extremostable, mainly thermostable enzymes or stabilized unstable ones may be considered. Thermostable enzymes from thermophiles can be obtained by direct isolation from their origins or after introduction of their genes into suitable mesophilic hosts. Approaches to produce more stable enzymes (in addition to the empirical use of various additives) are based on immobilization and chemical modification of enzymes as well as protein engineering. All of these individual methods have their own advantages, and demerits. Since each enzyme behaves as an individual, no one approach can be recommended unambiguously as the best for all enzymes. Nevertheless, one generalization can be deduced: the claims for understanding the structural laws of protein molecules have been always increased as a new approach has emerged. On the other hand, an insight into the structural world of proteins has been mediated also by individual approaches for the production of stable enzymes.

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