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INTERNATIONAL UNION OF PURE
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PHYSICAL CHEMISTRY DIVISION
STEERING COMMITTEE ON BIOPHYSICAL CHEMISTRY*

PRACTICAL IMPORTANCE OF ENZYME STABILITY†

I : Natural Sources of More Stable Enzymes

II : Increase of Enzyme Stability by Immobilization and Treatment with Low Molecular Weight Reagents

III : Increase of Enzyme Stability by Protein Engineering

(Technical Report)

Prepared for publication by

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†The series is intended to direct the attention of physicochemists and biophysicists to stability studies on enzymes which are of practical importance in biotechnology.

Practical importance of enzyme stability – I: Natural sources of more stable enzymes; II: Increase of enzyme stability by immobilization and treatment with low molecular weight reagents; III: Increase of enzyme stability by protein engineering

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I. NATURAL SOURCES OF MORE STABLE ENZYMES

Abstract.The most frequently used sources of more stable enzymes are aerobic thermophilic microorganisms. They rapidly and effectively produce enzymes having a higher thermal stability than those derived from mesophilic microorganisms. They have also a higher resistance to most chemical denaturants. This is of importance for enzyme application especially in biocatalytic reactions.

Introduction

An industrial disadvantage of the most commercially used biocatalysts - enzymes and enzyme complexes - is their relatively low stability. Therefore, microorganisms which require extreme physico-chemical conditions for their growth and proliferation - and enzymes derived from them - bear a potential for overcoming this situation (refs. 1 and 2). In particular, enzymes derived from thermophilic microorganisms show an increased thermal stability compared to those of mesophilic microorganisms (refs. 3 - 7). They are called thermostable if they have a maximum reaction temperature above that of the optimum growth temperature for the microorganism (refs. 3 and 8). Clearly, such a definition is invalid in view of the fact that enzymes from microorganisms growing at temperatures above 90 °C are "thermostable" regardless of whether their optimum activity temperature is below or above the optimum growth temperature. It becomes obvious that thermostability of an enzyme is a relative term. Thermostability is a function of the stabilizing forces which include hydrogen bonding, hydrophobic bonding, ionic interactions, metal binding, and/or disulfide bridges(ref. 3). Such stabilizing effects contribute to the long-term stability of an enzyme. Thermostability is also connected with a higher resistance to most chemical denaturants (refs. 9 and 10).
A suitable method for characterizing the enzyme stability is the scanning calorimetry (ref. 11).

Thermophilic microorganisms as a source of more stable enzymes

Mixed populations of bacteria which seemed to occur in the ocean near the Galapagos Island in a depth of 2500 metres were purported to have an optimum growth temperature of 250 °C at a pressure of 250 atm (ref. 12). Trent et al. (ref. 13) subsequently attempted to refute this result by physico-chemical experiments showing the formation of artefacts in complex media under such extreme conditions.
In contrast to this there is reliable verification of the existence of microorganisms at temperatures approaching the boiling point of water (Table 1).

Table 1. Optimum growth temperature (t_{opt}) of thermophilic bacteria (refs. 2, 5, 14, and 15)

Species	t_{opt} (°C)
Bacillus acidocaldarius	60 - 65
Bacillus stearothermophilus	55 - 70
Caldarobacterium hydrogenophilum	74 - 76
Clostridium thermohydrosulfuricum	67 - 70
Methanobacterium thermolithotrophicum	65 - 70
Pyrococcus furiosus	100
Pyrodictium occultum	105
Sulfolobus acidocaldarius	70 - 75
Thermoanaerobacter ethanolicus	69
Thermoplasma acidophilum	59
Thermoproteus tenax	88
Thermotoga maritima	80
Thermus aquaticus	70

These bacteria occur in natural and/or artificial habitats. As an example the original isolations of Thermus aquaticus were from hot spring algal mats. Incubation in aerobic liquid medium at 70 up to 75 °C led to the formation of visible turbidity, often with clumps or a surface pellice (ref. 16). Brock and Freeze isolated one strain of Thermus aquaticus from a hot water tap in Indiana. Brock and Yoder (ref. 17) found another strain of Thermus aquaticus in a creek receiving thermal pollution. Ramely and Hixson (ref. 18) as well as Brock and Boylen (ref. 19) and Heinritz et al. (ref. 20) obtained strains of Thermus aquaticus in a creek receiving thermal pollution and domestic hot water reservoirs, respectively. It thus seems likely that Thermus is capable of growing in manmade habitats of high temperature.
Table 2 shows the specific growth rate and the specific yield coefficient of selected thermophilic bacteria utilizing glucose as the carbon and energy sources at temperatures up to 80 °C.
It seems that the specific growth rate is in the range of the values of mesophilic bacteria. On the other hand, the specific yield coefficient is obviously lower compared to mesophiles. As an example the mesophilic strain Escherichia coli C 600 utilizes glucose in continuous cultivation with a specific growth rate of 0.67 h^{-1} and a specific yield coefficient of 0.47 up to 0.56 $g \cdot g^{-1}$ (ref. 26).
The specific growth rate and/or the specific yield coefficient of thermophilic bacteria could be improved by
- continuous instead of batch cultivation preventing inhibition of bacterial growth and proliferation due to carbon substrate and/or metabolites (refs. 2 and 20),
- increasing system pressure in fermenters (ref. 22).

However, in no case did the specific yield coefficient realized by thermophiles under extreme physico-chemical conditions approach to the values of mesophilic microorganisms (ref. 27).
In order to establish rapid and efficient biotechnical processes on the base of thermophiles for commercial use the by-products of biomass production should be taken into consideration. As an example thermophilic Bacilli growing and proliferating aerobically on cheap synthetic media produce rapidly and effectively intracellular and extracellular thermostable enzymes (refs. 28 and 29).

Table 2. Specific growth rate (μ), specific yield coefficient ($Y_{x/s}$), and optimum growth temperature (t_{opt}) of selected thermophilic microorganisms utilizing glucose

Microorganism	μ (h^{-1})	$Y_{x/s}$ ($g \cdot g^{-1}$)	t_{opt} (°C)	Ref.
Bacillus stearothermophilus	1.16	0.3	60	21
Bacillus stearothermophilus TP 5	1.2	0.1-0.4	68	22
Thermus aquaticus	1.6	0.4	70	23
Clostridium thermocellum	0.3	0.11	65	24
Clostridium thermohydrosulfuricum	0.7	0.09	65	24
Thermotoga maritima	0.55[1]	0.11[1]	80	25

1) calculated from ref. 25

Table 3. Major application of thermostable enzymes

Enzyme	Operating temperature (°C)	Major application
α -Amylase	60 - 90	Starch processing Brewing Baking Synthesis of detergents Waste-water treatment
β -Galactosidase	55 - 75	Lactose hydrolysis Food processing
Cellulase	70	Cellulose hydrolysis
Protease	60 - 90	Baking Brewing Food processing Production of detergents
Oxidoreductase	70 -100	Construction of biosensors
DNA polymerase and RNA polymerase	70 -100	Molecular biology and genetic engineering

More stable enzymes from thermophilic microorganisms

Thermostable enzymes have several advantages over their counterparts from mesophilic microorganisms (refs. 2 and 3):

- higher thermal stability and resistance to most of the chemical denaturants, e.g. organic solvents,
- higher storage stability,
- increased reaction rate and comparable catalytic activity,
- lower viscosity of reaction mixture and improved mass-transfer,
- lower danger of contamination in microbial enzyme production as well as biocatalytic reactions.

Because of these facts thermostable carbohydratases, proteases, and oxidoreductases were introduced recently into starch processing, hydrolysis of cellulose and lactose, brewing, baking, food processing, waste water treatment, biosensors and/or other applications(Table 3).

Some of these enzymes are also of great interest as biocatalysts in organic synthesis because of their high thermal stability as well as their high resistance to chemical denaturants , e.g. several organic solvents. As an example oxidoreductases from Caldariella acidophila have been studied using intact resting cells, acetonized cells or entrapped cells for transformation of progesterone, resulting in a conversion efficiency between 4 and 24 % at 85 °C. The product spectrum in this system depends on temperature and oxygen availability. The hydroxylative and oxydative system are both inactive below 70 °C and the hydroxylative system is activated by oxygen (ref. 30).
Furthermore, thermostable and highly specific enzymes, e.g. DNA polymerases and RNA polymerases open up new dimensions in molecular biology and genetic engineering (refs. 31 - 33). As an example the DNA polymerase of Thermus aquaticus made possible the polymerase chain reaction (PCR) technology.

Table 4. Enzymes derived from thermophilic microorganisms according to Sonnleitner (ref. 2), Ng and Kenealy (ref. 3), and Heinritz (ref. 6)

Thermostable Enzyme	$t_{opt,enzyme}$ (°C)	Microorganism	Ref.
α -Amylase	60 - 80	Bacillus acidocaldarius	34
α -Amylase	90	Bacillus stearothermophilus	35
β -Galactosidase	75	Bacillus stearothermophilus TP 32	28
β -Galactosidase	55	Bacillus stearothermophilus	36
β -Galactosidase	70	Caldariella acidophila	37
Cellulase	70	Clostridium thermocellum	38
Glucoseisomerase	70	Bacillus stearothermophilus	39
Hydrogenase	90 -100	Caldarobacterium hydrogenophilum	40 41
ADH	70	Thermoanaerobacter ethanolicus	42
Protease (Thermolysin)	70	Bacillus thermoproteolyticus	43
Protease (Thermitase)	60 - 80	Thermoactinomyces vulgaris	44
Protease (Caldolysin)	65 - 85	Thermus T-351	45
Protease	70	Bacillus stearothermophilus YG 185	46
Protease	75 - 80	Bacillus stearothermophilus TP 26	29
Protease	90	Thermus caldolyticus GK 24	53
RNA polymerase	100	Thermoproteus tenax	31
DNA polymerase	70	Sulfolobus acidocaldarius	32
DNA polymerase	70	Thermus aquaticus	33

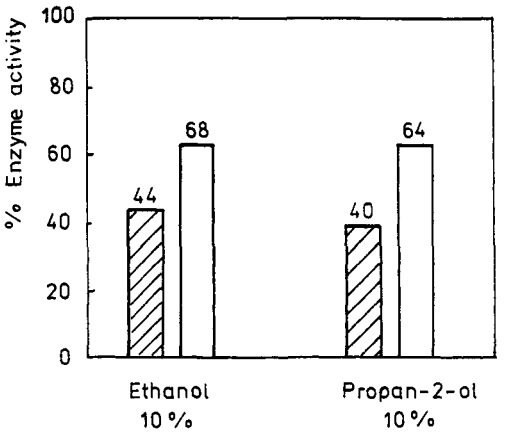

Fig 1.

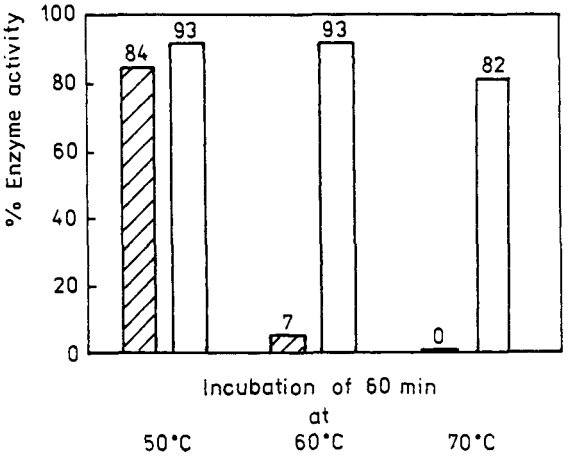

Fig 2.

Fig 1. Thermal stability of a thermostable β-galactosidase of Bacillus stearothermophilus TP32 ▭ compared to the mesophilic β-galactosidase of Escherichia coli ▨

Fig 2. Resistance of the thermostable β-galactosidase of Bacillus stearothermophilus TP 32 ▭ and the mesophilic β-galactosidase of Escherichia coli ▨ to ethanol and propan-2-ol (incubation of the enzyme for 60 min at 50 °C

In Table 4 selected enzymes derived from thermophilic microorganisms together with the temperature optimum of the enzyme and the enzyme-producing microorganism are shown (refs. 2, 3, and 6). As an example Thermoproteus tenax contains a DNA dependent RNA polymerase with the highest known temperature optimum of 100 °C (ref. 31). We assess our results on a continuous unprotected synthesis of intracellular thermostable β-galactosidase using the strain Bacillus stearothermophilus TP 32 as most promising for commercial production. The Bacillus strain produces the enzyme at a specific growth rate of 0.17 up to 0.28 h^{-1}, temperatures of 68 up to 70 °C and pH values of 6.8 up to 7.5. The β-galactosidase activity of the biomass is about 15000 U per gram of biomass dry weight (ONPG as a substrate). The purified thermostable β-galactosidase shows a higher thermal stability than the β-galactosidase of a mesophilic Escherichia coli strain (Fa. Boehringer), Fig. 1. It is also more resistant to ethanol or propan-2-ol compared to this enzyme, Fig. 2. These properties favour the enzyme for biocatalytic reactions, such as transgalactosylation of sugars, alcohols,and steroids (refs. 47 - 52).

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II. INCREASE OF ENZYME STABILITY BY IMMOBILIZATION AND TREATMENT WITH LOW MOLECULAR WEIGHT REAGENTS

Abstract. Increase of enzyme stability is possible by multi-point-interaction with a support and treatment with low molecular weight reagents. There seems to be a correlation between the degree of stabilizing and the number of covalent bonds between the enzyme and the polymeric support until a critical number of bonds is attained. On the other hand low molecular weight reagents cause stabilizing covalent modifications of enzymes. For instance, hydrophilization of surface residues of enzymes results in a stability increase.

Introduction

Enzyme inactivation under various denaturing actions is usually considered a two-step process (ref. 1):

$$N \rightleftharpoons D \longrightarrow I$$

where N,D and I are the native, reversibly denatured and irreversibly inactivated forms of an enzyme, respectively.
This concept has remained a useful, testable model of inactivation processes (ref. 2 - 8). Inactivation usually begins with a reversible conformational change (i.e. unfolding of the protein globule followed by some secondary irreversible processes such as aggregation or covalent modification (resulting

in a change of the primary structure of the protein) or "irreversible" conformational changes in the protein molecule. In the last case, for instance, after returning from the elevated temperature resulting in inactivation, the protein is "trapped" in a metastable denatured state that cannot return spontaneously to a structural state corresponding to the native conformation of the protein (ref. 9 - 11); see also some recent reviews (refs. 7 and 8). Hence, a general strategy of enzyme stabilization should be based on inhibition of the first step of the inactivation mechanism ($N \rightleftarrows D$), i.e. unfolding of the protein molecule (ref. 12 and 13). To this end, some chemical approaches can be used to prevent these types of destabilizing mechanisms (refs. 14 and 15). Let us analyse two of the most promising methods in more detail.

Increasing stability of enzymes via their multi-point interaction with support

After the pioneering work by Gabel in 1973, it became evident that multi-point attachment of a protein molecule to a support is capable of suppressing its unfolding. A correlation: "The greater the number of covalent bonds between enzyme and polymeric support, the higher is the stability", was demonstrated for denaturation of immobilized enzymes by concentrated urea solution (ref. 16), irreversible thermoinactivation of both monomeric (refs. 3, 17, and 18) and oligomeric enzymes (ref. 19), reversible thermally induced inactivation (refs 3 and 20), denaturation by surfactants (ref. 37) or by organic solvents (refs. 21 and 22).
The reviewed potential of such an immobilization approach has been reviewed many times (ref. 4, 8, 12, 13, 14, 23, and 24). In this article we would like to emphasize an important but not properly appreciated aspect of previously published results, namely that the stabilizing effect increases with the number of covalent bonds between the enzymes and the polymeric support until some critical value (a limit) is achieved (Fig. 1), and further increase in the number of bonds does not lead to further stabilization (refs. 3, 17, and 25). Such a phenomenon seems to have a general validity, since it takes place for enzymes stabilized by other methods as well; cf. below.

Increasing enzyme stability by chemical modification with low molecular weight reagents

Covalent modification of proteins has been widely used for enzyme stabilization (refs. 8, 12, 15, and 26). For instance, hydrophilization of the surface residues in proteins results in essential increase in stability of α-chymotrypsin, the stabilizing effects being thousand-fold and greater (ref. 27 and 28).

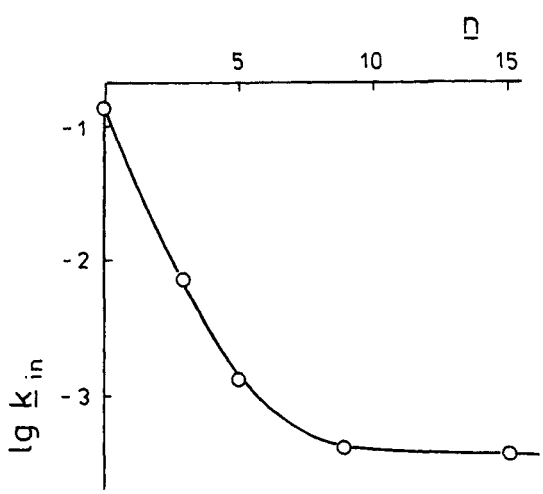

Fig. 1. The inactivation (60 °C, pH 8.0) rate constants (k_{in}, min^{-1}) of trypsin covalently immobilized in polyacrylamide gels as a function of the number (n) of bonds between the enzyme and gel matrix (ref. 3)

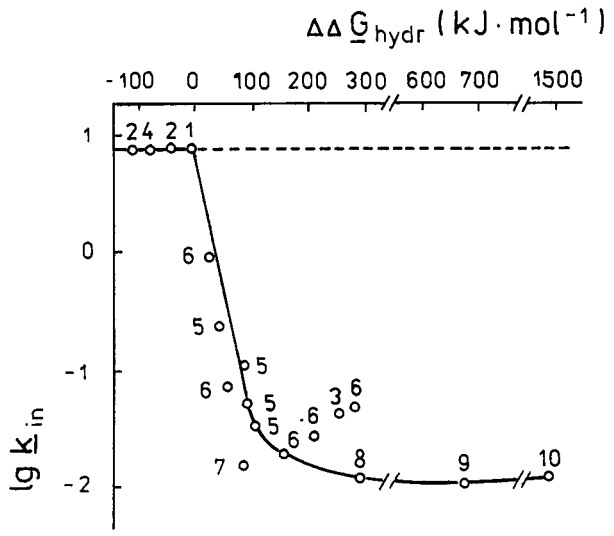

Fig. 2. Dependence of the rate constant of thermoinactivation (k_{in}, min^{-1}) of α-chymotrypsin preparations on the change of hydrophilicity of protein ($\Delta\Delta G_{hydr}$) as a result of modification of its NH_2 - groups (ref. 15) was calculated according to the formula: $\Delta\Delta G_{hydr} = -2.3\ R\ \overline{\pi}\ T\ n$ where $\overline{\pi}$ is hydrophobic increment of modifying reagent (refs. 34 and 35) and n modification degree (the number of the modification functional groups of the protein).
Thermoinactivation conditions: 76 °C, pH 8.0 (3 mmol/l KH_2PO_4), 0.1 mol/l KCl, enzyme concentration 0.1 - 1 μmol/l. The limit value of thermoinactivation 0.1 - 1 μmol/l.The limit value of thermoinactivation rate which is possible to detect by the experimental methods used (ref. 28), is shown by the dotted line.
1- native enzyme; the enzyme modified with:
2 - acetaldehyde; 3 - formylmethylmalonic acid;
4 - its diethyl ester and 5 - glyceraldehyde (ref. 36);
6 - glyoxylic acid (ref. 27), 7 - succinic acid (ref. 36); 8 - trimellitic anhydride (ref. 28); 9 - pyromellitic anhydride and 10 - mellitic anhydride (ref. 28).
The 2, 5, and 6 preparations were studied with different modification degrees.

Fig. 2 summarizes the data on thermoinactivation rate constants(k_{in}) of different α-chymotrypsin preparations in which amino groups are either acylated or alkylated. The tendency in Fig. 2 is unambiguous: the more hydrophilic groups are introduced into α-chymotrypsin molecule, the greater is the stabilizing effect. However, enzyme stability does not increase further after some critical value of hydrophilic increment is achieved (Fig. 2).

What is the nature of the irreversible process that we have failed to suppress?

The results of our research (Figs. 1 and 2) clarify the fact that there is a limit in conformational stability of the enzyme which cannot be exceeded by suppressing its unfolding only. In other words, the residual irreversible inactivation process (definitely conformational) but in parallel with them:

$$\begin{array}{ccc} E & \longrightarrow & I \\ \downarrow\uparrow & & \\ D & & \end{array}$$

To increase the enzyme stability above such a "conformational limit" (Figs. 1 and 2), it is reasonable to suppose one should inhibit some other inactivation mechanisms, for instance chemical ones, such as oxidation of protein

functional groups, splitting peptide bonds, etc. as was stated earlier (ref. 3) and then demonstrated for oxidative inactivation of lysozyme and ribonuclease at enhanced temperatures (90 to 100 °C) which also results from some chemical changes in the primary structure of the protein. One such process - the hydrolysis of Asn residues - is responsible for thermoinactivation of proteins. Furthermore, the contribution of high temperature destruction of S - S bonds and the subsequent thiol-disulfide exchange reactions are significant in the thermal destruction of proteins involving cysteine residues (ref. 32).

Stabilizing effect on the conformational stability of α-chymotrypsin

In conclusion, it should be emphasized that the value of stabilizing effect does not depend on what approach is used for stabilizing enzymes. For instance, the limit values for k_{in} are equal to about 10^{-2} for chemically modified (hydrophilized) α -chymotrypsin (Fig. 2), and 5×10^{-3} min^{-1} and 8×10^{-3} min^{-1} for the enzyme entrapped into polyacrylate (refs. 17 and 25) gels, respectively. These values characterizing the stabilized enzymatic preparations are about one thousand times lower than the value of $k_{in} \approx 10$ min^{-1} for the free enzyme incubated in the same conditions: 76 °C, pH 8.0, 0.1 M KCl (ref. 17).

The 1000-fold stabilizing effects are the highest ever achieved by treatment of protein with low-molecular-mass compounds. The thermostability of α-chymotrypsin modified with hydrophilic reagents (anhydrides of some aromatic acids; Fig. 2, see points 8 - 10) is practically equal to the stability of proteolytic enzymes from extremely thermophilic bacteria (ref. 33), the most stable proteinases known to date.
It is the task of future investigations to find out whether the correlations of thermostability are observed also for other enzymes.

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III. INCREASE OF ENZYME STABILITY BY PROTEIN ENGINEERING

Abstract. Protein engineering can be used to prevent destabilizing processes that normally result in enzyme inactivation. The structural integrity of a protein can be strengthened via mutagenesis guided either by rational design or by selection pressure. This article reviews recent applications of both methods for the stabilization of enzymes.

Introduction

It is conceptually useful to classify the general mechanisms disrupting protein stability as reversible, potentially reversible, or strictly irreversible processes (for a review see ref. 1). The first two classes of mechanisms involve the denaturation and/or aggregation of the molecule, whereas the third consists principally of irreversible covalent changes in the structure of the protein. The rate of such covalent changes may be augmented by denaturation of the enzyme.
Protein engineering can be used to prevent each of these types of destabilizing mechanisms from occurring; recent applications are summarized below.

Stabilizing the structural integrity of an enzyme by design

All proteins maintain their native conformation by a complex balance of noncovalent means, which include hydrogen bonds and hydrophobic, ionic, and van der Waals interactions. It is reasonable to assume that the overall stability of the protein can be increased by replacing those amino acid residues providing only weak contributions to the conformational stability of the molecule with other providing stronger interactions. The superposition of hydrogen bond dipoles in alpha helices, resulting in opposite charges at the ends of helical segments, was originally believed to be a relatively small electrostatic contribution to the sum of energetic interactions maintaining the native structures of proteins (ref. 2). Recently, however, Matthews et al. (ref. 3) have demonstrated that amino acid substitutions designed to increase stabilization via the helix dipoles in T 4 lysozyme increased the melting transition temperature by as much as 4 °C, reflecting a stabilization of the protein conformation of approximately 1.6 kcal/mol. The mutations introduced charged aspartyl residues that interacted electrostatically with the positively charged N-termini of helices in the protein. Earlier work by Mitchinson and Baldwin had demonstrated that increasing the charge from +2 to -1 on the N-terminal residue of a helix in analogs of the S-peptide of ribonuclease S resulted in an increase of the melting temperature of the reconstituted enzyme by as much as 6 °C compared to the protein containing the native S-peptide (ref. 4). Secreted proteins are further stabilized by the presence of disulfide bonds. Engineering novel disulfide bonds into dihydrofolate reductase (ref. 5), T 4 lysozyme (ref. 6), and subtilisin BPN' (ref. 7) have stabilized the active enzymes with respect to reversible unfolding.

Another approach that enhances the stability of a protein is to replace a flexible residue such as glycine, which requires greater free energy than other, sterically constrained residues in order to restrict its conformation. Provided the substitutions do not introduce undesirable steric interactions, the mutated protein will have a decreased entropy of unfolding, resulting in a higher temperature of denaturation. In accordance with this theory, substitution of selected glycine and alanine residues in T4 lysozyme (Ala82Pro and Gly87Ala) increased the melting transition temperature by as much as 2 °C, reflecting an increase of approximately 1 kcal/mol to the free energy of folding (ref. 8). Similarly, substitutions of glycines in λ repressor (Gly46Ala and Gly48Ala) increased the melting temperature of the N-terminal domain by 3-6 °C (ref. 9).

Discrepancies between the amino acid sequences of enzymes having high degrees of overall sequence similarly but widely varying thermostability, can be

"corrected" in order to increase the thermostability of the less stable enzymes within the family. This technique has yielded a mutant form of the neutral protease from Bacillus stearothermophilus of enhanced thermostability (Gly44 → Ala), although many mutants designed by this criterion exhibited decreased thermostability (ref. 10).

Stabilizing the structural integrity of an enzyme by selection

The examples cited above illustrate rational approaches to stabilizing the conformation of proteins; by means of structural and functional data, the researchers conceived of specific changes that would theoretically improve the stability of a given enzyme. A second approach selects for improvement in the stability of an enzyme without prior knowledge of its structure. Mutants of kanamycin nucleotidyl transferase having increased thermostability were discovered by cloning and expressing the enzyme originally from a mesophilic organism into a thermophilic bacteria host, and selecting for colonies containing the activity of the enzyme at temperatures higher than the normal melting transition temperature of the enzyme (refs. 11 and 12). Such screening techniques also have yielded variants of T4 lysozyme (ref. 13) and subtilisin BPN' (ref. 14) having enhanced thermostability.
Shortle and Lin (ref. 15) have identified several random mutations in staphylococcal nuclease that stabilize the enzyme against unfolding by guanidine hydrochloride. For example, whereas approximately 0.85 M guanidine hydrochloride results in the denaturation of the wild type enzyme, as determined by the relative fluorescent intensity of Trp140, as much as 1.3 M denaturant is required for a similar quenching in one of the mutants. Furthermore, three such mutations, those encoding His124 → (Leu or Arg) and Val66 → Leu, were found to "correct" other mutations that had resulted in colonies having greatly reduced nuclease activity. One hypothesis of the authors is that residues 66 and 124 may confer a global stability upon the enzyme.
Recently, Das et al. discovered that the activities of non-functional and presumably unstable mutants of yeast cytochrome c were partially restored by a second-site mutation, Asn57Ile. Subsequent construction of the Asn57Ile mutant by site directed mutagenesis resulted in an extraordinary 17 °C increase in the transition temperature, corresponding to a greater than 2 - fold increase in the free energy change for thermal unfolding (ref. 16).

Preventing inactivation of enzymes due to covalent mechanisms

Several of the common amino acids comprising proteins are known to be especially susceptible to degradative processes causing the loss of biological activity. Irreversible thermoinactivation of enzymes in aqueous solutions is largely due to deamidation of asparagines (and to a lesser degree, glutamines), destruction of disulfide bonds, and hydrolysis of peptide bonds (ref. 17 and 18).

Replacement of specific asparagine residues in triosephosphate isomerase resulted in a significant decrease in the rate of irreversible thermoinactivation of the enzyme (ref. 19). Exposure to the oxidative environments can result in the degradation of methionine. Subtilisin was stabilized against such oxidation by replacing a methionine (residue 222) important for catalysis with serine, alanine, or leucine (ref. 20). Conformation plays a significant role in the rates of these degradative processes. For example, at temperatures below the transition temperature of an enzyme, relatively rigid, buried asparagine residues are less likely to deamidate than asparagine residues within flexible portions of the protein exposed to solvent. For this reason, the overall rates of deamidation of native, oxidized ribonuclease A are 10 to 30-fold lower than the rereduced enzyme (ref. 21). Above the transition temperature, the entire protein is considered to be in a flexible, hydrated state in which each individual residue can come in contact with water while freely sampling many conformations. Under such conditions, proteins deamidate at roughly similar rates (ref. 22).

Conclusion

From the foregoing discussion, it is now clear that the appropriate techniques for stabilizing proteins against such process are to (1) increase the transition temperature (by mutagenesis, chemical crosslinking or immobilization), (2) eliminate or drastically reduce the amount of water in the reaction system (refs. 23 and 24), or (3) eliminate the labile amino acid residues from the protein by mutagenesis (ref.19). The technique chosen will depend upon the nature of the protein and its application.

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