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Enzyme Thermostabilization: the State of the Art

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Introduction

Enzymes form a class of natural catalysts with interesting features. They can be selective for the substrate and specific with regard to the catalysed reaction. In addition they may be stereospecific, which renders them useful in the manufacture of fine chemicals. Another important feature is that they usually require mild conditions, which can be considered as an advantage, but is at the same time a limitation for their use in the organic solvents, extremes of pH and high salt concentrations that might be encountered in industrial processes.

From the commercial point of view, annual sales of bulk enzymes (1994) represent US$ 600 million (see *Table 1*). There are other enzymes of industrial importance in addition to polymer degrading enzymes: penicillin acylase, used to produce 6-APA from penicillin G or V, enzymes used in animal nutrition, and enzymes with high added values used in DNA technology. More information about industrial applications of enzymes can be found in Walsh and Headon (1994) and Godfrey and Reichelt (1983).

However, the greatest problems that prevent the widespread use of enzymes in industry are their cost and their stability against various denaturants. Stability at high temperature is one of the most significant parameters. Often when an enzyme is thermostable it is likely also to be stable against any of the other agents that tend to unfold its tertiary structure. Further, working at high temperature presents some major advantages: (1) for each increase of 10°C in temperature, the reaction rate doubles; (2) temperatures greater than 60°C inhibit microbial growth; (3) in some cases, solubility of substrates increases with temperature and viscosity of solution decreases; (4) in the case of endothermic reactions, the equilibrium is shifted toward the final products. The conquest of the challenge of using enzymes in industry requires a multidisciplinary approach from chemistry to microbiology; there has been research in four major areas: chemical modification, enzyme immobilization, protein engineering and the use of additives.

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Table 1. Annual worldwide sales of major enzymes (from Walsh and Headon 1994)

Enzyme type	Market value (US $ million)
Proteases	330
Carbohydrates	150
Isomerases	50
Lipases	20

The origins of protein stability

Stability in a folded protein is the result of the balance between stabilizing interactions and the tendency towards destabilization caused by the loss of conformational entropy associated with protein folding (Dill, 1985). There is, in fact, a small difference between two large values, 5–20 kcal/mol (Vanhove *et al.*, 1995). So small modifications affecting either stabilizing interactions in the folded state or destabilizing interactions in the unfolded state, can significantly increase the stability of the protein (Jaenicke, 1991; Matthews, 1987a; Matsumura *et al.*, 1986; Yutani *et al.*, 1977).

The denaturation process of proteins can be represented by a two-step model (Lumry and Eyring, 1954).

$$N \overset{K}{\rightleftharpoons} U \xrightarrow{k} I \tag{1}$$

where the enzyme in its folded state [N] is reversibly unfolded [U], and then irreversibly inactivated [I] often by aggregation or covalent alteration. In non-denaturing conditions, [N] has a lower free energy than [U]. The conformational change is characterized by an equilibrium constant K and the irreversible process by the kinetic constant k. The inactivation rate, v_{in} is expressed by the following equation:

$$v_{in} = -\frac{d[N]}{dt} = k_{in}.[N]_0 \tag{2}$$

where k_{in} is the apparent rate constant of inactivation, $[N]_0$ and $[N]$ are the initial and actual concentrations of native enzyme respectively. k_{in} is related to K and k by the following equation:

$$k_{in} = \frac{k.K}{1+K} \tag{3}$$

Thus stability against inactivation can be gained either by reducing K or k (Martinek and Klibanov, 1993). To reduce K, anything that will stabilize N (such as additional hydrogen bonding) or destabilize U (such as protein crosslinking) will increase the free energy change for unfolding and thereby displace the equilibrium towards N. Irreversible inactivation may result from several different alterations (see *Table 2*; Gupta 1991). Each alteration can be prevented by a specific treatment to the protein. Globally irreversible inactivation depends upon the conformation of the unfolded form [U]; more compact forms of [U] will be inactivated more slowly and will thus also be more stable (Nosoh and Sekiguchi, 1990).

Table 2. Processes responsible for irreversible protein inactivation (Gupta, 1991 and Cleland and Langer, 1994)

1. Aggregation: When the protein is in the unfolded state [U], internal hydrophobic side chain residues are exposed to water and to avoid this entropically unfavorable interaction, above a limit of concentration, aggregation occurs.
2. 'Scrambled' structures: In the pathway from the [U] to [N] state, the folding process may lead to incorrectly folded inactive structures.
3. Alteration of disulfide bridges. At high temperature, cysteine residues can be degraded to dehydroalanine and thiocysteine. Dehydroalanine can be involved into new lysine-alanine crosslinks, while thiocysteine catalyses the disulfide exchange reactions. Decreasing pH can slow down the reaction rate since it is in fact the thiolate species that actually catalyses the reaction. (Liu and Klibanov, 1991).
4. Peptide bond hydrolysis, particularly by proteases.
5. Deamidation of Gln and especially Asn (Gln deamidation is a slow reaction).
6. Racemisation of amino acid residues.
7. The Maillard reaction.
8. Dissociation of prosthetic group.
9. Isopeptide bond formation between the amino group of Lys and carboxylates of Asp and Glu.
10. Oxidation of the SH group of cysteine and the indole group of tryptophan.

Thermophilic enzymes

USE OF NATURALLY THERMOSTABLE ENZYMES

Once it has been established that a thermostable enzyme is needed, one should first look for an already naturally existing homologous thermostable equivalent. Thermostable enzymes are mostly found in thermophilic microorganisms that live above 60°C, such as *Thermus aquaticus* and *Bacillus stearothermophilus* or other hyperthermophilic microorganisms (Rees and Adams, 1995). Almost all thermophilic strains can be obtained from culture collections such as the DSM, Braunschweig D-3300 Germany. Mesophiles and moderate thermophile organisms possess enzymes stable at temperatures higher than the optimal growth temperature of the organism. For example, catalase from *Aspergillus niger* resists temperatures above 60°C while the bacterium grows optimally in the temperature range from 30°C to 40°C. We should note that there is no well-defined method to predict if an enzyme will be thermostable. The only way is to measure experimentally individual temperature-activity-stability profiles for each enzyme, regardless of source.

For bulk production, once the enzyme has been found in suitable amounts in some organism, one can start the fermentation process. Details of various methods are given by Nolan and Davies (1990). Otherwise, the approach is to look for the gene, insert it into a vector then transform a host bacterium. The criterion for host selection is to find bacteria whose genetic system is well known and controlled. Using gene-splicing techniques, structural genes may be attached to regulatory genes. With the added feature of gene amplification, genetic engineering techniques should result in development of strains overproducing the enzyme. Care should be taken to select bacteria accepted by the legal authorities of the country for the production of healthcare products. Some examples include:

The transformation of cDNA of neutral protease from *Bacillus stearothermophilus* CU-21 into *Bacillus subtili* – the resulting strain secreted an enzyme with identical thermostability to the original strain (Fuji *et al.*, 1983);

The transformation of the gene for α-amylase from an unknown thermophilic bacteria into *Bacillus subtilis* (Schinomiya *et al.*, 1980);

The purification and cloning of the gene for halostable and thermostable leucine dehydrogenase from *Thermoactinomyces intermedius* in *E. coli* to lead to overproduction of the enzyme (Oshima *et al.*, 1994a).

A number of other examples are also known, such as DNA polymerase and carbohydrate metabolizing enzymes.

Once the gene has been cloned, it may be possible, by using site directed mutagenesis, to produce recombinant enzymes with properties that fit the initial requirement better and to study the origin of their stability. Usually the optimum temperature of reaction for such an enzyme is higher than that from mesophiles, and at 37°C often the mesophilic enzyme is more active. This might be interpreted by lack of flexibility of the thermostable enzyme due to the required rigidity to ensure thermostability (Daniel *et al.*, 1996). Large scale genome sequencing has been undertaken for several hyperthermophilic microorganisms and this will be a valuable source of both proteins and protein sequence information (Kim *et al.*, 1993).

COMPARISON OF ENZYMES FROM THERMOPHILIC AND MESOPHILIC MICROORGANISMS

Statistically, when we compare enzymes from thermophilic organisms to their homologues from mesophilic sources, there are some recurrences in the amino-acid replacements, the following being the most frequently encountered (Argos, 1979): Gly→Ala, Ser→Ala, Ser→Thr, Lys→Arg, Asp→Glu, Ser→Gly, Lys→Ala, Val→Ala, Asp→Asn and Ile→Val.

Analysed in terms of their effect on the protein structure, the ways in which thermophilic organisms obtain relative stabilization of their proteins can be classified as follows:

Increase of compactness and better packing

Minimization of the ratio of surface area to volume is correlated with increased stability (Chan *et al.*, 1995). An extensive comparison of the structure of D-glyceraldehyde-3-phosphate dehydrogenase (GADPH) from *Thermus aquaticus* with four others that span a broad range of thermostability supports this observation (Tanner *et al.*, 1996).

Increase in electrostatic interactions

The formation of additional ion pairs also increases thermostability. For example between two tyrosinases from two different strains of *Neurospora crassa*, a change from Asn_{201} to Asp, resulting in an additional ion pair, increases the half life of the enzyme at 60°C by 66 minutes (Rüegg *et al.*, 1982). Between the structure of rubredoxin from *P. furiosus* and mesophilic bacteria the changes are mostly additional electrostatic interactions between groups near the protein surface, and a more extensive hydrogen-bond network in the secondary structure (Day *et al.*, 1992; Blake *et al.*, 1992). Nirasawa *et al.* (1994) showed that between the heat-stable sweet protein

malabin II isolated from the seeds of *Capparis masaikai Lev I* and its heat labile homologue only one amino acid replacement is responsible for the difference in thermal stability. It is in fact an Arg residue that replaces a Gln in the latter thereby providing possibilities for an additional salt bridge with a carboxylic group. The change from Lys to Arg is one of the commonest when we compare mesophilic and thermophilic organisms (Merkler *et al.*, 1981; Menendez-Arias and Argos, 1989). The reasons might lie in the facts that (1) the hydration of Arg is better compared to Lys: (a) there is a better distribution of the charge over the guanidinium function where it is located on three atoms rather than over the single nitrogen of the lysine ammonium function, enabling it to have more interactions with water or hydrogen bonding with neighbouring residues; (b) the guanidinium group provides a better shielding of the hydrophobic hydrocarbon fragment from water; (c) this hydrophobic chain is one methylene shorter in Arg thus creating less unfavorable interactions with water. These considerations are quantified by the ΔG of transfer from the interior to the exterior of the protein: –2.71 kcal/mol for Arg and –2.05 for Lys. (2) The repartition of the positive charge on two nitrogen atoms of the guanidinium, is complementary to the repartition of the negative charge on the carboxylic function of Asp and Glu, thus producing stronger salt bridges than the ammonium of Lys. (3) As the side chain is bulkier for Arg, there is less possibility of movement: Bhaskaran and Ponnuswamy (1988) have calculated flexibility. Arg is better defined by X-ray crystallography than Lys (Anderson, 1978a, b), hence we can expect that it will locally rigidify the structure. Cai *et al.* (1996), showed that the presence of two Arg in the binding loop of *Cucurbita maxima* trypsin inhibitor-V modulates its flexibility, provides hydrogen bonding possibilities between the binding loop and the protein core, and is correlated with its stability and resistance to protease attacks. (4) The pKa of Arg is 12.5 and Lys is 10.5, so it should remain charged at higher pH. Closely connected is the experiment of del Rio *et al.* (1995): these workers replaced Trp 431 by Arg in penicillin acylase from *E. coli*. The resulting enzyme was clearly more resistant at alkaline pH than the wild type.

In a comparative study of β-lactamases from various sources, Vanhove *et al.* (1995) showed that the global balance between charged residues is more equilibrated in the case of thermostable enzymes. Indeed, proteins are generally more stable near their isoelectric points (Pace, 1990a; Vanhove *et al.*, 1995); Pace and colleagues have also established a correlation between the number of salt bridges and hydrogen bonds with the observed stability.

Additional hydrogen bonds

The presence of charged groups can be correlated not only to the formation of salt bridges, but also to charged-neutral hydrogen bonds. The Gibbs energy of interaction is higher than that for neutral-neutral hydrogen bonds as measured by Huyghues-Despointe *et al.* (1995): the contribution to stability of an engineered hydrogen bond between Gln-Asp (i, i+4) in an α-helical peptide is 1 kcal/mol when Asp is charged and 0.4 when it is uncharged. We have pointed out the importance of H bondings in the stability of β-lactamases, but they play a determining role in numerous thermophilic enzymes (Chan *et al.*, 1995). For example, Tanner *et al.* (1996) have correlated the ranking thermostability of five GADPH enzymes from mesophilic sources to the

extreme thermophile *Thermus aquaticus*, to the number of salt links and hydrogen bonds, especially charged-neutral ones.

Additional disulphide bridges

The formation of additional disulfide bridges also reinforces the structure, although the engineering of disulfide bonds does not always have the expected stabilizing effect.

Increasing hydrophobic interactions

Hydrophobic interactions are also a determinant parameter for thermostability. Between glyceraldehyde-3-phosphate dehydrogenase (GADPH) from lobster and GADPH of *Bacillus stearothermophilus* 130 substitutions resulted in new intra- and intersubunit ion pairs and new intersubunit hydrophobic interactions (Harris *et al.*, 1980). Furthermore, the amino acid composition does not change a lot from GADPH of *Bacillus stearothermophillus* to that of *Thermus aquaticus*. But a relocation of charged residues to the protein's exterior results in the formation of additional surface ion pairs, and a greater proportion of buried hydrophobic residues increasing internal hydrophobicity that finally induce a shift of $T_{1/2\,life}^{20\min}$ from 75°C to 100°C.

The protein microenvironment

Michels *et al.* (1995) demonstrated on a luciferase expressed in mammalian cells that the microenvironment of an intracellular compartment can modulate the thermal stability. Osmolytes that are usually polyols, amino acids and their derivatives can be accumulated in the cytoplasm and protect the enzyme against various stresses like temperature, desiccation (Bernard *et al.*, 1993). Different chaperones might promote resolubilization, reactivation or proteolysis of damaged proteins (Parsell and Lindquist, 1993; Georgopoulos and Welch, 1993; Craig *et al.*, 1994). The binding of some metal ions (Frommel and Hohne, 1981; Imanaka *et al.*, 1986), heme groups (Dolla *et al.*, 1995) or substrate binding (Pace and McGrath, 1980; Gray, 1988) is also encountered as a way of stabilizing protein. There are a large number of enzymes like thermolysin and α-amylase from thermophiles which require calcium for their high thermostability (Ward and Moo-King, 1988).

Glycosylation

Carbohydrates may contribute to the stability of glycoproteins by different mechanisms. (1) Blomhoff and Christensen (1983) suggested that the glycan may preserve the hydration shield surrounding the protein and form hydrogen bonds with surface hydrophilic amino acids. Mer *et al.* (1996) provided evidence on the fucosylated proteinase inhibitor of hydrogen bonding and hydrophobic interactions between the protein surface and the glycan, as did Wiss *et al.* (1995a, b) for the N-linked glycan of human CD2. (2) Barker and Gray (1983) proposed a stabilization of the 3D structure. Klibanov (1979) invoked a multipoint attachment. (3) Carbohydrates may also stabilize protein comformation simply by steric interactions of the carbohydrate with the adjacent peptide residues (Rudd *et al.*, 1994; Gerken *et al.*, 1989). Markus (1965) has indicated that the binding of any ligand to a polypeptide is likely to diminish the

tendency for oscillation between different conformational states. (4) The carbohydrate moieties also render the denatured or partially denatured state more soluble, preventing aggregation, and thus help in protein renaturation after thermal unfolding. Similarly glycosylation is involved in early stages in the folding of nascent glycopolypeptides (Imperiali and Rickert, 1995; Wang *et al.*, 1996). Powell and Pain (1992) studied in detail the conformational equilibria and folding of the non-glycosylated recombinant α_1-antitrypsin (AT) and compared the results with those for the human protein (glycosylated). They showed that the more rapid turnover of the recombinant protein *in vivo* (8 h for the recombinant AT and 50 h for the human AT) is not due to a lower thermodynamic stability, but may be associated with a lower kinetic stability arising from the increased tendency to aggregation. (5) Protection against the action of proteases, directly by steric shielding of the cleavage site and indirectly by reduction of the conformational changes necessary for proteolysis (Saravena*et al.*, 1995; Janbon *et al.*, 1995; van Berkel *et al.*, 1995).

For other reviews, see Rees*et al.* (1995), Chan*et al.* (1995), Wasserman (1984) and Adams *et al.* (1993, 1995).

Protein engineering

ADDITIVITY OF MUTATION EFFECTS

If we consider that the ΔG responsible for protein stability is a small value (several kcal mol^{-1}), it follows that only a small number of amino acid substitutions are necessary to impart substantial increase in stability (Pantoliano*et al.*, 1989; Yutani*et al.*, 1977). Further, the effects of mutations are to some extent additive (Declerck *et al.*, 1995; Zhang, *et al.*, 1995; Wells 1990; Merutka and Stellwagen, 1990; Matsumura *et al.*, 1986) although some restrictions apply to this concept. It has been suggested that long-range and small magnitude effects of mutations are not additive (Licata and Ackers, 1995). So we should consider that the effects on stability of combinations of mutations that do not interact directly with each other in the 3D structure are expected to be additive (Blaber *et al.*, 1995; Merutka and Stellwagen, 1990; Serrano *et al.*, 1993; Zhang *et al.*, 1992).

ENGINEERING ENZYMES FOR THERMOSTABILITY

Refinements of the techniques of genetic engineering have transformed it in the last few years into a powerful tool for the enzymologist, allowing: (1) the production of large quantities of enzymes by the cloning and expression of their cDNA; (2) the determination of their primary structures more easily and faster than by direct protein sequencing; (3) the possibility of undertaking investigations on protein structure/function and stability by site directed mutagenesis. The details of the technique are outside the scope of this article (for more information, see Crabbe (1990)).

To introduce changes in the primary structure of the protein, genetic manipulations are carried out on the coding sequence of the cloned gene. Such changes may be random or site specific. Random mutagenesis has the advantage that large numbers of mutations may be produced. This can be very useful, especially in the early stages of analysis, provided that a rapid and specific screening method is available. By contrast,

site directed mutagenesis has the advantage that specific mutations can be induced. However, as the number of possible mutations for the gene of a protein is large, this approach requires some structural information about the protein in order to perform a rational elaboration of the strategy that will be employed.

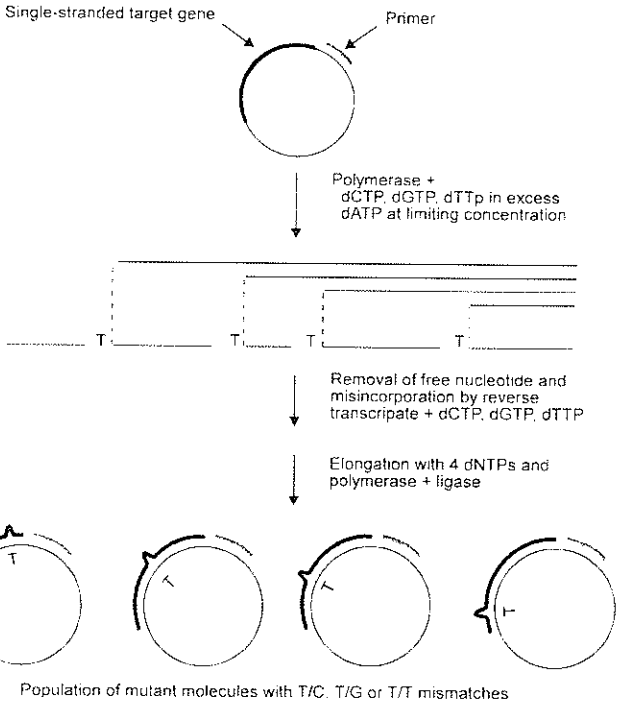
Random mutagenesis

In the absence of detailed structural information, random mutagenesis combined with selection or screening can be a useful alternative to site directed mutagenesis for generating both the desired improvements and a database for future rational approaches to protein design. Mutations can be induced by a variety of methods, some of which (Lethovaara *et al.*, 1988), allow the generation of complete random mutant libraries containing all possible single base substitutions (see *Scheme 1*).

The applications are varied. Yamashita *et al.* (1994), using a method involving random mutagenesis of the entire gene for pullulanase with an appropriate screening procedure, created several mutants with increased stability and recovered information useful for designing a strategy for site directed mutagenesis. Other cases of increased thermostability by random mutagenesis have often been reported in the literature (Lopez-Camacho *et al.*, 1996; Asano and Yamagushi, 1995; Alber and Wozniak, 1985; Matsumura *et al.*, 1985; Lio *et al.*, 1986; Makino *et al.*, 1989; Joyet *et al.*, 1992). Random mutagenesis has also been used to obtain other features such as stability to alkali (del Rio *et al.*, 1995; Cunningham and Wells, 1987) and changes in substrate specificity (Olphant and Struhl, 1989).

A closely related technique is evolutionary molecular engineering. It consists, first, of the induction of random mutations in the desired gene and, secondly, of a procedure for reproduction under strong selective pressure. The two steps can be perfomed either *in vivo* or *in vitro* (Szostak, 1992). This technique, introduced by Eigen and Gardiner (1984), involves the following procedures (1) the gene coding for the desired protein is used to transform an extreme thermophile organism; (2) culture conditions are selected so that the desired protein is essential for growth of the transformant; (3) the temperature is raised; (4) mutants capable of growing at high temperatures are selected and the mutant gene and its product analysed. Steps 3 and 4 can be repeated to reach the desired temperature gradually (Oshima, 1994b). The choice of the *in vivo* selection system is crucial for the improvement in protein thermostability since the maximum temperature we can reach is primarily limited by the growth temperature limit of the host cell, so extreme thermophile or hyperthermophile microorganisms are preferred to moderate thermophiles. The efficiency of the method is illustrated by some successes. Matsumura and Aiba (1985) isolated and cloned from a mesophile the gene encoding for kanamycin nucleotidyltransferase. The cloned DNA was treated with mutagens, introduced into *Bacillus stearothermophilus* and kanamycin-resistant transformants were selected at 61°C.

Tamakoshi *et al.* (1995) replaced the leuB gene coding for 3-isopropylmalate dehydrogenase (IPMDH) in *Thermus thermophilus*, an extreme thermophile with a temperature-sensitive chimeric leuB gene. The resultant transformant was adapted to high temperature, and a thermostable mutant strain obtained. A single base substitution that replaces isoleucine at 93 with leucine was found in the chimeric leuB gene of the thermostable mutant. The resultant amino acid residue coincided with the corres-



Scheme 1. Generation of complete random mutant libraries (Lethovaara *et al.*, 1988). An oligonucleotide primer is synthesized, and incubated with polymerase, three deoxynucleotides in excess, one at limiting concentration and a single stranded vector containing the gene to be mutagenized. In such conditions, the elongation will stop at different points along the sequence where the nucleotide at limiting concentration is required. Misincorporation to the 3' ends is then forced using reverse transcriptase and the three wrong nucleotides. The molecules are finally completed to covalently closed double-stranded forms using the four nucleotides, polymerase and ligase.

sponding residue of the *Thermus thermophilus* enzyme. For more examples refer to the review of Oshima (1994).

The work of Kotsuka *et al.* (1996) is a good example of the use of random mutagenesis as a first shot before performing site directed mutagenesis. Kotsuka *et al.* have also built a chimeric enzyme of IPMDH consisting of portions of thermophile enzyme from *Thermus thermophilus* and the mesophile one that they submitted to random mutagenesis and integrated into *Thermus thermophilus*. After screening of stable mutants, isolation, cloning and analysis, they have identified the Ala 172 Val mutation. They eventually introduced this mutation in the gene of the thermophile IMPDH with site directed mutagenesis, and further improved its stability.

Site directed mutagenesis

The most common technique for introducing a site specific mutation is oligonucleotide-directed mutagenesis (*Scheme 2*) but this is neither the only one, nor the best one. For example 'mutagenesis by overlap extension' based on PCR technology (Horton *et al.*, 1989), has the advantage of not requiring the generation of single-stranded DNA, resulting in a high efficiency of mutation (98%), so that often there is no need to screen

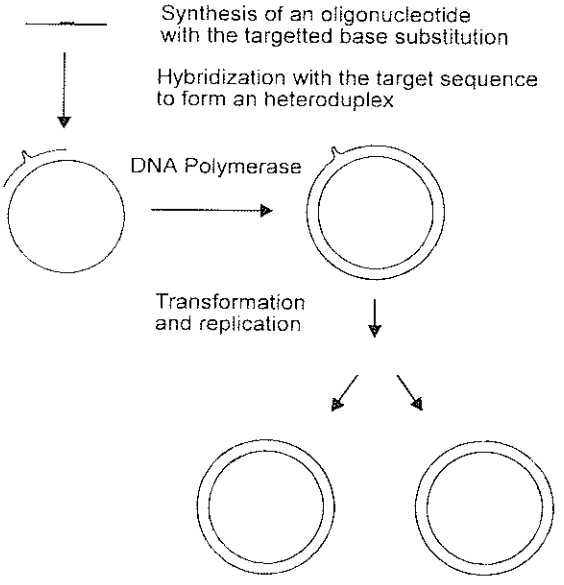
to eliminate wild-type transformants. A panel of variations in the technique employed exists, often commercially available as kits easy to handle and run.

We have chosen to present the possible strategies for protein stabilization with examples classified according to the type of interaction engineered. However we should emphasize that single amino acid replacements can induce changes by several effects.

Electrostatic interactions Charged groups in proteins are not distributed uniformly but tend to be surrounded by groups of opposite charge, suggesting that some electrostatic interactions between such groups (including the apparent charge of the α-helix dipole) contribute to protein stability (Wada and Nakamura, 1981; Oliveberg and Fersht, 1996a,b; Tissot *et al.*, 1996). Other studies suggest that electrostatic interactions contribute little to protein stability (Serrano *et al.*, 1990; Pace *et al.*, 1990; Dao-pin *et al.*, 1991; Hendsch and Tidor, 1994) and even more that ionizable amino acids might, in some cases, contribute to stability mainly through packing and bonding interactions that do not depend on their electrostatic charge (Meeker *et al.*, 1996).

Engineering interactions with α-helix dipoles

The α-helix possesses a dipole with a positive moment at the *N*-terminus and a negative one at the C-terminus. This might account for the high frequency with which negatively-charged amino acids are present at the N-terminus of helices in known protein structures (Blagdon and Goodman, 1975; Richardson and Richardson, 1988).



Scheme 2. Oligonucleotide site directed mutagenesis. An oligonucleotide partly complementary to the nucleotide sequence in the target DNA is synthesized. At an appropriate temperature, it can hybridize with the target sequence to form heteroduplex, even though one or more bases are mismatched. The synthetic oligonucleotide is then used as a primer for the Klenow fragment of DNA polymerase I to produce a plasmid with a specific mispaired region. This plasmid is used to transform a bacteria. There its replication will produce two matched duplexes, one from each of the heteroduplex strands.

So insertion of charges at the end of helices which previously did not have them could be a good approach to stabilizing enzymes. Nicholson *et al.*, (1988) identified two sites suitable for this approach at the N-termini of two helices. The double mutant Ser38→Asp and Asn144→Asp in T4 lysozyme presents an increase of 1.6 kcal mol^{-1} of $\Delta\Delta G$, the free energy of stabilization relative to wild-type. The effects of those two amino acid substitutions were additive. The choice of the substitution site is very restrictive; out of 11 helices, only 2 were suitable. Also, Nicholson *et al.* only tested Asp substitution at the N and not the C termini because (1) Arg, Lys (for C-termini), and Glu (for N termini), residues have longer side chains and consequently their conformations are harder to predict, and (2) the Cα-Cβ bonds in a helix project away from the helix axis and toward the N terminus so that it is more difficult to find potentially useful substitution sites at the C termini of α-helices. More recently, the same team identified two additional positions in the T4 lysozyme sequence for stabilizing mutations Thr109→Asp and Asn116→Asp (Nicholson *et al.*, 1991), hence providing more credibility to this strategy for stabilization. Some other cases are also reported in the literature (Sali *et al.*, 1988; Pickersgill *et al.*, 1991). At this point we would like to refer to a recent publication from Fiori *et al.*, (1994) reporting the substitutions Lys→Arg near the C-terminus of an alanine based peptide. The result is an increase in α-helicity throughout the whole of the helix up to the N-terminus. Nevertheless, this effect is more the result of the more effective hydrogen bonding permitted by Arg than an electrostatic effect since it seems to be independent of ionic strength. This finding provides new insights and a better understanding of the basis of arginine stabilization in thermophilic proteins.

Engineering interactions at subunit interfaces

Analysis of the irreversible thermal denaturation pathway of D-xylose (glucose) isomerase reveals chemical modifications by sugar components of the high-fructose syrups and in particular the nonenzymatic glycosylation of lysines (Bookchin and Gallop, 1968; Bunn *et al.*, 1978). To prevent this problem Mrabet *et al.* (1993) have undertaken the replacement of lysine residues 253, 309, 319 and 323 by Arg. The results are mutants with not only significantly improved resistance to glucose-induced inactivation, but also an increased thermostability in the absence of glucose. Among those mutations, K253R located at the buried inter-dimer interface diplays the largest effect, while K309R and K319R, located on the surface, provide modest improvement in stability and K323R has no effect at all. Detailed biochemical, structural and model building analysis shows that the improved thermostability of the K253R mutant originates from strengthening existing electrostatic interactions at intersubunit contacts and by forming new ones.

Biesecker *et al.*, (1977), compared the structure of D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), holoenzyme from *Bacillus stearothermophilus* with that of the lobster muscle enzyme. Stabilization of the former is attributed to hydrogen bonding and mainly to three additional intersubunit salt bridges. The *B. stearothermophilus* enzyme contains more arginines. Ser 281 and Gln 201 in lobster are replaced in the thermophile by Arg and Glu which form salt bridges between symmetry-related subunits in a cavity shielded from solvent. Another buried ion pair is formed in the thermophile enzyme between Arg 194 of one subunit and Asp 293

in a symmetry-related one. The substitution of Gly-281 in GPDH from *Bacillus subtilis*, a closely related enzyme, by a positively charged residue (Lys or Arg) resulted in a substantial increase in stability, comparable in size to the stability differences observed between proteins from mesophiles and thermophiles (Mrabet *et al.*, 1993). Arg substitution increased stability more than Lys. Fairman *et al.* (1996) designed heterotetrameric coiled coils stabilized by Glu Lys ion pair interactions distributed at strategic locations, each potential Glu-Lys pair contributing about 0.6 kcal/mol of stabilizing free energy. The contribution of ion pairs in coiled coil stability has also recently been demonstrated by Yu *et al.* (1996).

These experiments show that electrostatic interactions at intersubunit interfaces provide important contributions to stability and reveal the important role of Arg in this respect. Generally, macromolecular association can be divided into two phases (Schreiber and Fersht, 1996; Pontius 1993). In the first phase, the two proteins or subunits form a low affinity non-specific complex held together by long-range electrostatic interactions. The higher the favourable electrostatic forces, the earlier is the transition state. The second phase is the docking to give the final high affinity complex. Inversely, subunit dissociation occurs as an early step in the denaturation of multimeric proteins (Friedman and Beychok 1979; Mrabet *et al.*, 1986) and thus improved electrostatic interactions between subunits may retard denaturation.

Engineering salt bridges on the protein surface

Engineering salt bridges on the protein surface is quite unpredictable with regard to its effect on stability. Mrabet *et al.* (1993) have been able to give evidence that it could work: (1) the replacement of surface-exposed Lys 9 by Arg in human Cu Zn superoxide dismutase resulted in a significant increase in heat stability, the half life at 85°C being increased 6 times. (2) K309R and K319R mutations in D-xylose isomerase also provided some stabilization.

On the other hand, K323R mutation of xylose isomerase provides no increase in stability. Although it has been shown in T4 lysozyme that the salt bridge between His 31 and Asp 70 stabilizes the protein by 3–5 kcal mol^{-1} (Anderson *et al.*, 1990), attempts to create putative salt bridges on the surface of the protein resulted in enzymes where the effects of mutations ranged from no change in stability to a 1.5°C (0.5 kcal mol^{-1} for $\Delta\Delta G$) increase in melting temperature. Further, double mutants were created in order to introduce His-Asp charge pairs on the protein surface and they resulted in a destabilization of the protein by 1–3 kcal mol^{-1}. X-ray analysis showed that the introduction of His and Asp probably induced strain into the folded protein which destabilized this variant. It was concluded that pairs of oppositely charged residues located on the protein surface which have freedom to adopt different conformations do not necessarily tend to come together to form struturally localized salt bridges. Rather, such residues tend to remain mobile, interacting weakly if at all, and do not contribute significantly to protein stability. Probably the entropic cost of localizing a pair of solvent exposed charged groups on the surface largely offsets the interaction energy expected from the formation of a defined salt bridge. And if there are examples of strong salt bridges, such interactions require that the folding of the protein provides the requisite driving energy to hold the interacting partners in the correct rigid alignment (Dao-Pin *et al.*, 1991). Another example of the small contribution of surface electrostatic interactions to stability is given by Sali *et al.* (1991) with barnase.

Engineering buried salt bridges

Inside the protein the dielectric constant is lower so that the enthalpy of formation of a solvent protected salt bridge is higher. However it may not be enough to offset the considerable energy required to desolvate the charged groups during folding (Waldburger *et al.*, 1995).

In the case of barnase, ionic interactions within clusters of hydrophobic residues can be important for the stability and the folding pathway. Tissot *et al.* (1996) have estimated the contribution of existing salt bridges to 3.0–3.5 kcal/mol. This is not due to the inherent stability of the salt link, but because the presence of unpaired charges in the core of the protein is very unfavourable. However, the contribution of this interaction may be more pronounced for the folding of the protein than its stability as it is also the case for Anthopleurin B toxin (Khera and Blumenthal 1996). By their specificity, ionic interactions may act early on the folding pathway by restricting the number of available conformations within the substructure and thus accelerate the transition from the unfolded state to the native state, even if it does not stabilize the structure *per se* (Tissot *et al.*, 1996; Oliveberg and Fersht, 1996a, b).

Theoretically, the replacement of buried nonpolar residues by charged polar ones should be possible for a limited subset of residues so long as all electrostatic and hydrogen bonding potential is satisfied, but practically, it remains a challenge that has still to be realized (Matthews, 1995).

Disulfide bridging Disulfide bridges are the most common examples of naturally occurring crosslinks. If we refer to the two-step model for denaturation, the thermodynamic explanation for the origin of stabilization is the following (Schellman, 1955): when the cross-linked protein is in the unfolded state there are many fewer possible conformations it can adopt than when it is not crosslinked. This is translated in thermodynamic terms by a decrease in the entropy of the unfolded state (S^u), causing a destabilization of the unfolded state of the cross-linked protein and hence a reduction in the free energy difference between the folded and unfolded states.

$$\Delta S_x = S^u \text{ cross-linked} - S^u \text{ not cross-linked} = R \ln(P) = -R\,(1.73 + 1.5 \ln(n)) \quad (4)$$

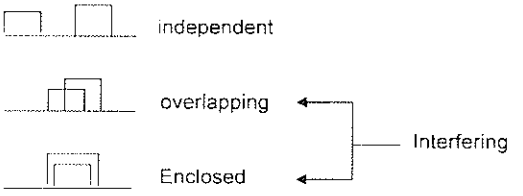
ΔS_x is the molar change in entropy of the unfolded state due to the cross-link (in entropy units). R is the gas constant. P is the probability of the formation of an isolated closed loop from the chain. The entropic effect hence depends upon the loop length (n is the number of amino acids enclosed in the loop), larger loops making larger contributions to the reduction in ΔS_{conf} and also upon the amino acid sequence determining the flexibility of the unfolded chain especially the ratio Gly/Pro and Gly/Ala and the hydrophobicity of the sequence segment involved in the loop (Zhang *et al.*, 1994; Matthews *et al.*, 1987b; Harrison and Sternberg, 1994; Stites and Pranata, 1995). In the case of multiple disulfide bonds added, the connectivity (relation between the disulfide bonds, i.e., independent, overlapping or enclosed (*Scheme 3*)) will also be a determining factor, and this point is well treated in a relevant statistical study from Harrison and Sternberg (1994). To the change in entropy should be added the ΔH of the newly created chemical bond (Betz, 1993). But the contribution to stability of cross-linkage is primarily the chain entropic effect (Cooper *et al.*, 1992). The stabilization energy (ΔG) that could be gained is

about 1–5 kcal mol^{-1}, which is quite substantial (Matsumura *et al.*, 1989a, b; Pace *et al.*, 1988; Johnson *et al.*, 1978). Disulfide bonds also affect protein stability through local interactions in the folded and unfolded state and other interpretations are also possible (Betz, 1993; Doig and Williams, 1991; Creighton 1988). Doig and Williams (1991), on the basis of data from the thermal unfolding of six proteins, attributed the destabilization of the unfolded state to the burial of nonpolar groups in the denatured state, inducing a decrease in the hydrophobic effect; here the destabilization of U is primarily enthalpic due to unfavourable hydrogen bonding networks. Betz (1993) considers that neither the entropic interpretation, nor the hypothesis of Doig and Williams is fully satisfactory in explaining the observed energetics. In addition to thermodynamic action, the disulfide bridges can have kinetic effects and affect the rate of unfolding, and therefore the stability towards inactivation (Clarke and Fersht 1993; Wetzel *et al.*, 1988). In the case of multimeric protein inter-subunits disulfide bonds can substantially prevent dissociation by covalently locking weakly interacting regions of the subunits interface and thus stabilizing the quaternary structure of the protein (Gokhale *et al.*, 1996).

With the help of precise information about the structure it is possible to insert cysteine residues to establish disulfide bridging. Matsumura *et al.* (1989b) provide a very elegant demonstration of this possibility on the phage T4 lysozyme, a naturally disulfide free-enzyme. They introduced by mutation three disulfide bridges which resulted in a temperature of unfolding 23.4°C higher than that for the wild-type lysozyme. Gokhale *et al.* (1996) introduced four Cys symmetrically across the dimer interface of thymidylate synthase. These intersubunit disulfide bridges stabilized the protein against the effect of chaotropic agents and prevented aggregation. Reiter *et al.* (1994) produced recombinant Fv fragments of antibodies in which the unstable variable heavy (V_H) and variable light (V_L) heterodimers are stabilized by disulfide bridges located at specific sites that lie between structurally conserved framework positions of V_H and V_L. As it is a structurally conserved part of the molecule they also showed that stabilization by disulfide bridges located at these positions is generally applicable to other Fv-containing 'fragments'. In the literature, insertion of disulfide bridges has also been reported to increase the stability of some other enzymes: barnase (Clarke and Fersht, 1993), subtilisin BPN' (Pantoliano *et al.*, 1987) and dihydrofolate reductase (VillaFranca *et al.*, 1983).

But in some other cases the introduction of disulfide bridges destabilises the protein, for example D-xylose isomerase (Varsani, 1993) and cytochrome *c* (Betz and Pielak, 1992; Betz *et al.*, 1996). This effect is generally attributed to dihedral strain in the disulfide and the neighbouring residues, counteracting the benefit of the cross-link from the non-local entropic effect (Katz and Kassiakoff 1986; Matsumura *et al.*, 1989a, b). Two important considerations in the design of stabilizing disulfide bridges are, therefore, the use of large loops to maximize the entropic effect on the unfolded state and the choice of flexible sites to avoid the introduction of strain into the folded state. Finally, we should add that the disulfide bridge is a potentially weak link at high temperatures because of β-elimination reactions and subsequent disulfide interchange reactions (Liu and Klibanov, 1991; Gupta, 1991).

Improving α-helix propensity Data on the helical propensity of each amino acid residue are useful for improving α-helix propensity. Chou and Fasman (1974) first



Scheme 3. Disulfide connectivity.

published a very detailed evaluation of conformation probabilities for amino acids in secondary structure and the rules that guide prediction (Chou and Fasman, 1978), but refinements and updates have been published regularly (Richardson and Richardson (1988), O'Neil and DeGrado (1990), Munoz and Serrano (1994a, b, 1995), Perez-Paya *et al.*, 1996)). However we should be cautious when using and interpreting such data. In fact, the actual helical propensities of residues is strongly affected by the environment of the helix. Although in aqueous solution it matches the values calculated by Chou and Fasman, the helicity of a peptide trapped in a membrane or similar environments is to a large extent controlled by the side chain hydrophobicity of surrounding residues (Shun-Cheng and Deber, 1994). Minor and Kim (1996) designed an 11 amino acid sequence that folds as an α-helix when it is located at one position and as β-sheet when it is located in another position of the primary sequence of the IgG binding domain of protein G. Munoz and Serrano (1994a, b) detected an α-helical tendency, in solution, of a peptide corresponding to a β-strand of ubiquitin. This demonstrates that the secondary structure present in the protein does not always reflect the intrinsic tendency of the amino acids. To get a reliable prediction, Qian (1996) proposes integration of solution thermodynamic studies with protein structural analysis. Lastly, we should add that the effects of amino acid replacements on helix stability can be additive in monomeric peptides (Merutka and Stellwagen, 1990) and depend on their position in the sequence (Padmanabhan *et al.*, 1996; Perez-Paya *et al.*, 1996).

In general, Ala is energetically preferred to Gly in helices, except at the N-cap (i.e., the first residue in the helix) either because of the greater entropic freedom of glycine-containing peptides in the unfolded state, or because of hydrophobic interactions of the alanine β-methyl group within the helix (Chakrabartty *et al.*, 1994; Strehlow and Baldwin, 1989). Imanaka *et al.* (1986) give an example of this approach for protein stabilization, identifying in the neutral protease of *Bacillus stearothermophilus* two Gly (144 and 61) involved in α-helices for which replacement by Ala resulted in an increased thermostability. Kotik and Zuber (1993) replaced Thr 29 and Ser 39, two residues present in α-helices of lactate dehydrogenase from *Bacillus megaterium* by Ala, with substantial increase in stability. This supports the idea that the decrease in flexibility and increased hydrophobicity in α-helices is a valuable stabilizing principle which is consistent with the results of comparison of protein sequences of phylogenetically related mesophilic and thermophilic organisms (Menendez-Arias and Argos, 1989). It is also possible to engineer interactions between the side chains of residues in positions *i* and *i*+4. Those can be of various natures, for example hydrogen bonds (Huygues-Despointes *et al.*, 1995), or hydrophobic interactions.Viguera and Serrano (1995) engineered

interactions in alanine based peptides between (1) Phe and Met and (2) Phe and Cys that provided an increase in stability of up to 2 kcal mol^{-1}. An extensive study of interactions between hydrophobic side chains within α-helices by Creamer and Rose (1995) has revealed that the amino acids with aromatic rings are most involved in such types of stabilizing interactions.

Paradoxically, at the N-cap, Gly should be preferred to Ala, because the β-methyl group of Ala can sterically interfere with solvation of the NH groups at the N-terminus of the helix (Serrano and Fersht, 1989). A recent estimation of Gly *versus* Ala N-capping stabilization would be 1.2 kcal/mol (Petukhov *et al.*, 1996).

Provided that they are compatible with the local stereochemical environment, residues able to form hydrogen bonds or electrostatic interactions can also be advantageously inserted. This point has already been discussed before with the electrostatic interactions, from which we can conclude that Asp is probably the best residue for an exposed N-terminus that has no adjacent negative charges (Serrano and Fersht, 1989) and that the C-terminal can also be the object of stabilizing substitutions (Fiori *et al.*, 1994; Yumoto *et al.*, 1993; Jimenez *et al.*, 1993; Sancho *et al.*, 1993). Doig and Baldwin (1995) recently published N- and C-capping preferences for all 20 amino acids in α-helical peptides, which could be very useful in rationalizing mutations at capping sites in proteins.

Actually, the N-cap is also important for stability and has recently been the object of particular attention. Substitution of selected residues can induce energy changes of over 2 kcal mol^{-1}. Munõz *et al.* (1995) found a recurrent structural motif at the amino terminus of α-helices that consists of hydrophobic interaction between the residue located before the N-cap with a residue within the helix (*i*, *i*+5) and which is important not only for the stability of the helix but also determinant for the direction of the polypeptide chain.

Petukhov *et al.* (1996) bring new elements and clarify the situation at N-termini. They assume that the most determining factors are: (1) hydrogen bonding at the capping box; (2) side chain-side chain interactions between N-cap and near terminal residues (N2, N3); (3) long range electrostatic interactions between the side chains of N4 and N-cap; (4) interactions between on one side helix macrodipole and on the other side the charged and polar side chains near termini; (5) capping interactions of the α-amino group at N-cap: if it is protonated, via electrostatic interactions with the helix macrodipole and with charged side chains, if it is uncharged via hydrogen bonding with side chains of N3 and N4.

To conclude, Munoz and Serrano (1994a) summarize very well the major known contributions to helix stability that should be taken into account to design a stabilization strategy: (1) amino acid entropic cost; (2) *n*-4 hydrogen bonds (n is the length of the helix) between main chain groups; (3) *i*+3 and *i*+4 side chain-side chain interactions; (4) capping and dipole effects. The diversified approach of Mainfroid *et al.* (1996) is a good illustration of what is possible in stabilizing an α-helix in a rational way. Among the different mutations introduced into the sequence of triosephosphate isomerase the effective ones were proceeding through (1) increase of the α-helix propensity by introducing Ala (K193A), (2) reduction of conformational entropy of unfolding (A215P) and (3) introduction of favorable electrostatic interactions with α-helix dipoles (Q179D).

Hydrophobic interactions It is generally recognized that the hydrophobic effect is one of the major factors that stabilize the folded structure of globular proteins (Dill, 1990; Pace, 1992). The logic of hydrophobic interactions as being very important in the process of stabilization is that the formation of a hydrophobic interactions involves the gain of a full hydrogen bond between water molecules rather than simply a change in the strength of a hydrogen bond upon folding (Kauzmann, 1959). Proteins gain 1.3 kcal mol^{-1} for each methylene group buried during the folding (Pace, 1992). Changes in the accessible surface area (a concept of Lee and Richards, 1971) that occur during protein folding can be used to calculate the contribution of hydrophobic interactions to the free energy of folding, with which it has been correlated by Chothia (1974). The large heat capacity changes observed in protein folding provide a quantitative evaluation of the reduction in water-accessible non-polar surface area and thus of the contribution of the hydrophobic effect to the stability of the native state (Spolar *et al.*, 1989; Livingstone *et al.*, 1991). Globally, a good correlation between the change in the buried hydrophobic surface area and the change in the free energy of unfolding due to mutation have been established (Yu *et al.*, 1995; Matsumura *et al.*, 1988; Kellis *et al.*, 1988), although sometimes specific studies have found poor correlations (Milla *et al.*, 1994; Serrano *et al.*, 1992).

Substitutions in thermophilic enzymes tend to increase hydrophobicity and decrease flexibility, especially in α-helices. (Menendez-Arias and Argos, 1989; Argos 1987, 1979). This is not surprising since among the various stabilizing interactions which are all temperature dependent, hydrophobic interactions are the only ones which increase with rising temperature (Mozhaev and Martinek, 1984). To classify the amino acids, there are several scales of hydrophobicity that can be used, based either on solubility data where hydrophobicity is a function of the energy of transfer of the residues from water to an organic solvent or a concentrated urea solution (Nozaki and Tanford, 1971; Radzicka and Wolfenden, 1988; Wimley *et al.*, 1996), or statistical scales where the distribution of the amino acids in the protein (surface, core, helix etc) and their tendencies to be the nearest protein neighbors of bound water molecules is analysed in a relatively large number of different proteins (Kuhn *et al.*, 1995; Ponnuswamy *et al.*, 1980).

The systematic replacement of one amino acid at a specific buried location by others enables correlations to be drawn between hydrophobicity and stability (Linske-O'Connel *et al.*, 1995; Matsumura *et al.*, 1988; Yutani 1987). More precisely, side chain-side chain interactions between hydrophobic residues are determining for protein stability at various levels of the structure. They are involved in the following ways:

1. Secondary structure. Hydrophobic clustering of side-chain atoms has been shown to be essential for β-sheet nucleation (Graciani *et al.*, 1994) and important for their stability (Otzen and Fersht, 1995). We have already discussed their role in the stabilization of α-helices.
2. Tertiary structure. The interdigitation of nonpolar side chains is a major factor contributing to the packing of α-helices and β-sheets (Kellis *et al.*, 1988). They constitute hydrophobic clusters that link distinct secondary structural elements and determine their relative spatial disposition. Aromatic residues are particularly good contributors to side chain-side chain interactions and good participants in

forming hydrophobic clusters (Yu *et al.*, 1995; Viguera and Serrano, 1995; Burley and Petsko, 1985; Hetch *et al.*, 1984; Thomas *et al.*, 1982).

Hydrophobic cores are very important to protein folding as well as to native protein structures in which small alterations can affect stability by several kcal mol^{-1} (Cordes *et al.*, 1996; Kay and Baldwin, 1996; Ladbury *et al.*, 1995; Kwon and Kim, 1995; Kellis *et al.*, 1988). In the remarkable work of Waldburger *et al.*, (1995) on the Arc repressor dimer, a complementary set of charged residues responsible for a salt-bridge network (Arg 31-Glu 36-Arg 40) has been replaced by hydrophobic residues in combinatorial randomization experiments. The result is active mutants with side chains packing against each other, efficiently filling the space formerly occupied by the salt bridge triad and that are significantly more stable than the wild type. This experiment supports the concept that hydrophobic interactions are one of the most important contributors to protein stability since simple hydrophobic interactions provide more stabilizing energy than the buried salt bridges with a folding comparable to the wild type.

3. Quaternary structure at the intersubunit interface (Kirino *et al.*, 1994).
4. Their implication in surface interactions is somewhat more controversial. On one hand, we know that contact of hydrophobic residue with water is thermodynamically unfavourable (Kauzmann, 1959; Tanford, 1979). Pakula and Sauer (1990) showed that substitution of Tyr 26, an exposed surface residue of λ Cro protein, with less hydrophobic amino acids increased the stability. On the other hand, half of the protein surface is hydrophobic (Chothia, 1984) and stabilizing hydrophobic interactions within these hydrophobic surface clusters are possible. Van Den Berg *et al.*, (1994) have been able to increase $T_{1/2\,life}^{30\,min}$ *of* the *Bacillus* neutral protease by 1.5°C–7.1°C by replacing Thr 63, a surface residue partially trapped in a hydrophobic pocket by other residues (Met, Tyr, Ile, Phe, Lys and Arg), and able to realize hydrophobic interactions with nearby residues. Lys and Arg are responsible for the greatest stabilization effects, respectively 6.7°C and 7.1°C. This might be due to the fact that, in addition to the hydrophobic interactions with their hydrocarbon fragment, they are also able to provide good interactions with the solvent and to shield the hydrophobic pocket from unfavourable contact with water via their charged moities.

Finally we should add that in the case of membrane proteins, hydrophobic surface residues are important for their solubilization and stability.

Packing interactions Packing interactions in the folded state are important contributors to overall stability (Sneddon and Tobias, 1992). We distinguish the packing effect from the hydrophobic interactions by referring with the term 'packing' to a class of effects due to the differences between protein interior which is more like a molecular crystal than an apolar liquid like a drop of oil. These effects are generally related to the rigidity and tight packing of the protein. According to the definition provided by Sandberg and Terwilliger (1989) packing encompasses the combined effects of close packing, distortion of the remainder of the protein and the polarity of the protein interior on protein stability. Combinations of complementary shaped residues, which can pack together without steric overlaps and efficiently fill the available core space, may be required to specify the correct native structure (Ponder and Richards, 1987).

Chan *et al.* (1995) in an extensive study with a large number of proteins, showed that there is a correlation between increasing stability and decreasing surface/volume ratio (i.e., packing). Indeed reducing the surface/volume ratio simultaneously reduces the unfavorable surface energy and increases the attractive interior packing. Packing interactions are also critical in the folding process and the stabilization of intermediate structures (Dill, 1990; Kellis, 1988; Kay and Baldwin, 1996).

Although proteins in solution have a rather compact structure, the interior of the globule may contain cavities that are sometimes filled with water molecules (Ernst *et al.*, 1995; Buckle *et al.*, 1996). Such water molecules are not easily detected by crystallographic analysis, especially in the case of large cavities, because they are too mobile. The presence of small spaces which are responsible for the dynamics essential for enzyme function would be expected to be reduced by altering the sizes of nonpolar side chains, with consequent stabilization of the protein. We have seen that the five most frequent amino acid exchanges when going from mesophiles to thermophiles are Gly→Ala, Ser→Ala, Ser→Thr, Lys→Arg and Asp→Glu (Argos, 1979). In almost all cases, the volume of thermophilic to mesophilic amino acid increases.

Such replacements are efficient for protein stabilization if they meet three requirements: (1) the side chains of the introduced residue should be chemically similar to the substituted one, such as Ser→Thr; (2) the replacement should not distort the configuration of the polypeptide backbone; (3) there should not be too much discrepancy in size with the substituted amino acid. For example, the replacement Gly→Ala is conceivable but Gly→Trp is clearly not feasible.

Based on these rules and on statistical data, Kolaskar and Amelunxen (1981) deduced pairs of amino acids that could replace each other, which can be a helpful base in planning a site directed mutagenesis strategy to stabilize a protein. For example, Sandberg and Terwilliger (1991) showed that the replacement in mutant gene V protein, of Ala 47 by Val, Ile or Leu stabilizes the protein by respectively 4.5, 7.0 and 6.4 kcal mol^{-1}. Mollah *et al.* (1996) succeeded in increasing Tm of the protein by about ±5°C by combinatorial mutagenesis to correct packing defects in Cro monomer with the F58W mutation. Another 1.3 C increase in Tm had also been achieved in conjunction with an A29I substitution. This increase in thermostability could be explained by the compensation of the deficit between the volume occupied by the former residues and the accessible volume. In *Bacillus licheniformis* α-amylase Declerck *et al.* (1995) replaced Ala 209 located in the bottom of a small cavity by alternatively Val and Ile that could fill this cavity and thus increase intra and interhelical compactness and hydrophobic interactions. The resulting enzymes display a lengthening in the half life at 80°C by a factor of 3.

Inversely, creation of a cavity usually leads to destabilization (Kellis *et al.*, 1988; Jackson *et al.*, 1993; Eriksson *et al.*, 1992). Kellis *et al.* (1988) estimated that the creation of a cavity the size of a $-CH_2-$ group destabilizes ribonuclease from *Bacillus amyloliquefaciens* by 1.1 kcal mol^{-1}, and a cavity the size of three such groups by 4.0 kcal mol^{-1}. According to Sneddon and Tobias (1992) the free energy change observed when replacing an amino acid by a smaller one cannot be explained simply on the basis of the energy of leaving a cavity in the protein core: the surrounding core residues relax to pack favorably around the smaller side chain, without really leaving a cavity, and the free energy change for mutation is ascribed to the loss of a set of net favorable packing interactions compensated, however, by new interactions created by the

structural rearrangement. In the core of barnase, in cavities created by mutation, Buckle *et al.* (1996) recently discovered buried water involved in hydrogen bonding with some polar residues of the cavity which compensate for the unfavorable entropy cost of removing the water molecules from bulk solvent.

Hydrogen bonding If H-bonding is essential for the formation of secondary structure (Pauling and Corey, 1951; Pauling *et al.*, 1951; Kabsch and Sander, 1983) it is also involved in stabilization of the protein tertiary structure. To give an idea of its importance, Shirley *et al.* (1992) estimated that the formation of an intramolecular hydrogen bond contributes 1.3 ±0.6 kcal mol^{-1} to protein stability. The contribution of Thr 198 involved in hydrogen bonding with Glu 194 and Ser 318 in lactate dehydrogenase is estimated at 17.2 kcal mol^{-1} (Nicholls *et al.*, 1993). Yu *et al.* (1995), by scanning alanine mutagenesis, revealed the extreme sensitivity of the stability to changes in the internal hydrogen bonding network, small changes inducing a decrease of 10°C in the melting temperature (±1.3 kcal mol^{-1}) of bovine trypsin pancreatic inhibitor. For Pace (1996, 1992) hydrogen bonding and hydrophobic interactions make comparable contributions to protein folding. Engineering amino acid substitutions to improve the hydrogen bonding network, therefore, is a good approach to increase protein stability.

Eijsink *et al.* (1992) have moderately increased $T_{1/2life}^{30min}$ of *Bacillus stearothermophilus* neutral proteinase by 0.7°C, with a single substitution (Ala170→Ser) that simply improved the quality of existing hydrogen bonding but did not introduce new ones. However we should mention that this substitution also fills a pre-existing cavity. The experiment of Gryk and Jardetzky (1996) indicates that the replacement Ala 77→Val in a helix-turn-helix domain of *trp* repressesor strengthens all the backbone hydrogen bonds in both helices and is responsible for the increased stability and the related alteration of the protein dynamics.

Substitution of residues susceptible to degradation Replacement of cysteine residues (Cys85→Ala and Cys152→Ser) in dihydrofolate reductase from *Escherichia coli* improved its resistance to the irreversible loss of activity at high temperature (Iwakura *et al.*, 1995). Replacement of selected lysine residues with arginine can prevent non enzymatic glycosylation (Mrabet *et al.*, 1993). Substitution of Asn to avoid deamination is also possible (Ahern *et al.*, 1987; Casal *et al.*, 1987). In hen egg white lysozyme Tomizawa *et al.* (1995b) replaced some Asp.Gly and Asn.Gly sequences that are susceptible to chemical degradation (respectively isomerization for the Asp Gly sequence and deamination of Asn). Surprisingly, each substitution resulted in a reversible destabilization of 1 to 2 kcal/mol, but the mutant enzymes were much more resistant to irreversible inactivation at pH 4 and 100°C. Several similar examples of amino acid substitutions to prevent protein inactivation from enzymatic or chemical modifications are reported in the literature (Estell *et al.*, 1985; McRee *et al.*, 1990; Lepock *et al.*, 1990). To conclude we must stress that replacement of chemically sensitive amino acids is more effective in protecting enzyme against irreversible thermoinactivation than in strengthening reversible conformation stability (Tomizawa *et al.*, 1995a, b).

Engineering proteins for other purposes

Resistance to proteolytic action

For the digestion of a protein to be efficient, it is presumed that it should be unfolded, at least partially, to provide easy access to the cleavage site. Any of the modifications mentioned above that will tend to stabilize the 3D structure will, therefore, also increase stabilization against proteolytic cleavage. However, more specifically adapted strategies to counteract their action can also be used. The most evident possibility is to change the primary autolysis site of the protease. This method has turned out to be very successful (Bae *et al.*, 1995; Eijsink *et al.*, 1995).

In a remarkable work Newsted *et al.* (1995) have demonstrated that a trypsin sensitive enzyme such as L-asparaginase can be rendered trypsin resistant by genetically fusing its gene with that of a single-chain antibody derived from a preselected monoclonal antibody capable of providing protection against trypsin. The chimeric L-asparaginase retained 75% of its original activity upon exposure to trypsin, whereas the native unprotected L-asparaginase was totally inactivated.

Preparing enzymes for use in organic solvents

The preparation of enzymes for use in organic solvents is also within the reach of protein engineering. However since it is outside the scope of this article, the reader is simply referred to some reviews on the topic (Gupta, 1992; Chen *et al.*, 1991; Dordick, 1992; Arnold, 1990).

ADDITIONAL CONSIDERATIONS

Two relevant considerations have been proposed by Imanaka *et al.* (1986).

1. A comparison of sequences of known mesophilic and thermophilic proteins can be used to suggest changes likely to increase thermostability. Some good broad or specific studies to help to design such a strategy are available: Argos (1979, 1987), Menendez-Arias and Argos (1989), Vanhove *et al.*, (1995), Warren and Petsko (1995) and Volk *et al.*, (1994).
2. Residues which are highly conserved throughout evolution should be left unchanged. An illustration of this point is the recent experiment of Peilak *et al.* (1995). They created random mutant libraries with substitutions at one of the most highly conserved regions of *Saccharomyces cerevisiae* iso-1-cytochrome *c*, the interface between the N- and C-terminal helices. After analysis of the stability changes, they reach the conclusion that in relation to protein evolution, interface variants possessing residue combinations found in naturally occuring cytochrome *c* sequences are the most stable. The catalytic activity might also be affected by mutations in the conserved regions (Yamashita *et al.*, 1994).

In addition to these two points, numerous statistical or systematic analyses of various types are available in the literature that can be very useful in planning site directed mutagenesis. They can concern: (1) the primary sequence. Brocchieri and Karlin (1995) have developed an interesting analysis. They have correlated the distance separating residues in the primary sequence that are close in the structure; (2) structural aspects. Predki *et al.* (1996) tried to deconvolute intrinsic preferences of

amino acids from local environmental effects in a surface turn and the way it modulates protein stability.

Molecular modelling can also be an helpful tool. A very workable system is the SWISS PROT (http://expasy.hcuge.ch/swissmod/swiss-model.html) server on internet to which one can submit a sequence for a defined protein and receive a file containing the structure extrapolated from known structures of homologous sequences (Peitsch, 1996). However, one must be careful to keep in mind that even if the prediction results look very consistent and coherent it is solely a simulation that needs to be confirmed by experimental data.

In conclusion, precise structural information is necessary to design a strategy rationally and specifically for a protein. The nature of each residue and its location will determine the result. 'The context is everything' (Matthews, 1993).

Chemical modification

Most current efforts in chemical modification are aimed at identifying residues involved in the catalytic and binding sites of proteins. However, well before the arrival of molecular biology to resolve the challenge of protein stability, chemical modification was one of the first and most efficient way of stabilizing proteins.

Even now that site directed mutagenesis is more widely used, chemical modification still holds some important advantages compared to site directed mutagenesis:

- little or no information about the protein is essential;
- the methods are comparatively simple;
- reagents are relatively inexpensive;
- radiolabels can be incorporated.

Nevertheless it has some severe limitations. Tyagi and Gupta (1993) exposed some interesting considerations to be taken into account before carrying out chemical modification:

- A reagent which is known to react with a specific functional group may not react with all free functional groups of that kind in a protein. Often the most accessible free residues will react first while the buried ones may not, depending on their degree of exposure, the steric hindrance of the reagent used for modification and its concentration.

  Polar amino acids tend to be located at the protein surface, thus being the most accessible for chemical modification. Hydrophobic amino acids may also be present on the protein surface, quite often as part of hydrophobic clusters. In fact about 50% of the surface area of globular proteins are occupied by hydrophobic amino acids (Mozhaev *et al.*, 1988; Burley and Petsko, 1985; Chothia 1984).
- Usually the most reactive form of a protein side chain residue is the unprotonated one (Means and Feeney, 1971). So the reactivity of the functional groups depends upon their pKa. Their pKa may vary not only according to the type of residue, but also on its location in the protein: the polarity of its microenvironment, the presence of electrostatic effects, the formation of hydrogen bonds and steric effects (Cohen,

1971). These factors control not only the individual reactivity of the residues, but might also influence the reactivity of a chemical agent approaching the protein.

- The reaction products are generally heterogenous in nature and may involve protein molecules with varying degrees of modification or even with different kinds of amino acid residues modified. Measurement of the number of modified amino acids can only give an average of a heterogeneous population ranging from unmodified to fully modified enzyme. Care must be taken, therefore, in establishing any correlation between the number of amino acids modified with any property (Germain *et al.*, 1989). Often the chemically modified protein preparations need to be subjected to standard bioseparation processes (Schummer, 1991).
- The biological activity of the protein may change (generally decrease) as a result of chemical modification. Limited modification may lead to drastic changes in activity if the residue involved is part of the active site. In this case, the presence of a competitive inhibitor or substrate analogue during the chemical modification generally protects the active site and leads to a product with greater retention of biological activity (Brandt, 1964).
- Possibly the biggest drawback of chemical modification is the potential toxicity of some of the reagents that prevent their use for protein preparations designed for human health care or food (Branner-Jorgensen, 1983).

Despite the diversity of the factors which determine the reactivity of a protein towards a chemical agent, it is possible to control approximately the level and site of a chemical modification (Cohen, 1971). The major parameters we can tune are the choice of reagent, solution parameters such as pH, ionic strength, temperature, buffer, mode of addition of the reagent, etc. There are also methods to control the procedure of modification, such as the protection of the active site, reversible blocking of one type of function, etc. Means and Feeney (1971) and Lundblad and Noyes (1985) have written extensive accounts of chemical modifications for protein.

MONOFUNCTIONAL REAGENTS

We can divide modifications into groups depending upon the kind of physico-chemical changes they induce in the residue: (1) there are modifications with retention of the initial charge of the residue, such as guanidination and alkylation of lysine residues or the modification of non-charged residues with chemical agents, which results in no charge change like the iodination of tyrosine or tryptophan residues; (2) modification inducing a reversal or elimination of the charge of the residue, such as succylination or acetylation of lysine residues respectively; (3) modifications which introduce hydrophobic functions on residues such as aspartate or glutamate in their reaction with a water soluble carbodiimide and a hydrophobic amine.

The lysine residue is one of the best targets for stabilizing proteins by chemical modification. It is a polar residue preferentially exposed at the protein surface and rarely involved in the catalytic site. It is also often located at the subunit interface of multimeric proteins involved in salt bridges and it presents a reactive free amino function.

Guanidination and amidination

Guanidination of lysine residues produces homoarginine, which is quite similar to Arg, the only difference being the length of the hydrocarbon chain. As pointed out above in discussing the importance of the Arg to Lys ratio for protein stability, this modification should stabilize the protein, and indeed it does so. Mainly from studies around 1980, many cases of enzyme stabilization by guanidination have been reported (Tuengler and Pfleiderer, 1977; Cupo *et al.*, 1980, 1982; Shibuya *et al.*, 1982; Abe *et al.*, 1983; Quaw and Brewer, 1986; Wolfenden *et al.*, 1981). One important contribution is the work of Cupo *et al.* (1980) who stabilized bovine carbonic anhydrase, chymotrypsinogen, α-lactalbumin, serum albumin, ribonuclease and horse heart cytochrome C. They only failed in their attempt to stabilize hen egg lysozyme which already has a high Arg to Lys ratio. An important hypothesis which they demonstrated by hydrogen isotope exchange experiments is that the conversion from lysine to homoarginine rigidifies the local segment of protein structure and reduces conformational changes. They also showed that stabilization by guanidination may not involve so many groups since superguanidination after amination of carboxylic groups, did not bring any further stability, but led to destabilization; added positive charges may cause backbone fluctuations because of repulsion of excessive surface charges.

Guanidination may be very efficient on multimeric proteins, where it can reinforce the inter-subunit salt bridges. Abe *et al.* (1983), with hydrogen-deuterium exchange studies on LDH, also supported the hypothesis of rigidification, establishing a link between (1) the degree of guanidination, (2) thermostabilization and (3) decrease in flexibility. They confirmed the two steps in stabilization depending on the degree of modification that Shibuya *et al.* (1982) had already found and interpreted as follows: the first five Lys modified mainly stabilize the monomer and the next 10 Lys modified strengthen the interactions between the subunits.

Acetamidination of lysine produces a residue with a structure close to that of homoarginine. This modification has also been shown to stabilize proteins against heat, alkaline pH (due to the increase of pKa from 10.5 to 12.5) and tryptic digestion (Tuengler and Pfleiderer, 1977), the use of a bi-functional reagent for acetamidination can also stabilize by cross-linking between neighbouring lysine residues (Minotani *et al.*, 1979). Although homoarginine or acetamidinated lysine have a structure close to that of Arg, they are no longer recognized as cleavage sites for trypsin which is another aspect of enzyme stabilization (Tuengler and Pfleiderer, 1977; Minotani *et al.*, 1979; Muller, 1981). One drawback of the method is that a high pH is required for an efficient reaction rate (Means and Feeney, 1971).

The concept of stabilization by hydrophilization is also sustained by a counter-argument whereby the charge removal by chemical modification such as acetylation destabilizes the protein (Cupo *et al.*, 1982; Muller, 1981). This suggests that neutralization of the amino charge produces a less hydrophilic residue side chain with an increased ability to enter the protein core, inducing larger fluctuations in the backbone conformation. The destabilization is amplified by the electrostatic repulsion of the remaining negative charges, and also the breakage of former salt bridges (Batra and Uetrecht, 1990).

Acylation

Acylation can increase or decrease the conformational stability depending on the protein, on the amount of substitution and the type of acylating agent. Stability is likely to be a result of inductive and mesomeric effects rather than due to steric effects (Hora, 1973). Hydrophobicity plays an important role, so that the longer the side chain of the acyl group the less the stabilization, to the point where palmitylation leads to a reduced thermal stability (Schmidt, 1979).

We have already mentioned that more or less half of the protein surface is occupied by non-polar residues. If in their natural environment, these hydrophobic clusters are useful, for example to maintain the enzyme trapped across a membrane (Haltia and Freire, 1995), or to promote favourable interactions between proteins in multi-enzyme chain reactions: in solution they tend to promote protein aggregation in order to escape the entropically unfavourable contact with water molecules. Mozhaev *et al.*, (1988) attempted the hydrophilization of the protein surface by chemical modification with the two following approaches: (1) hydrophilization of hydrophobic residues located inside the surface hydrophobic cluster by amination of the tyrosine residues of trypsin. A gain in stability of 100 fold is recorded; (2) hydrophilization of residues known to be located around hydrophobic clusters to obtain a shielding of the cluster from the aqueous medium by acylation of lysine residues of α-chymotrypsin with anhydrides of aromatic carboxylic acids. The thermostability of the modified enzyme is near that of proteolytic enzymes from extremely thermophilic bacteria. They also set up a stabilization scale depending on the hydrophilicity of the acylating reagent: benzoyl choride < phthalic anhydride < trimellitic anhydride ≅ pyromellitic anhydride ≅ mellitic ≅ anhydride.

Acylation with hydrophobic reagents may be interesting for enzymes engineered to work in non-aqueous media, such as the improvement of the efficiency of lipase (Takahashi *et al.*, 1995).

Succinylation usually leads to protein destabilization. The repulsion between the added negative charges is mainly responsible for the expansion and unfolding of the native conformation. An experiment by Batra and Uetrecht (1990) lends additional support to this point by showing that it can even induce helix coil transition of the secondary structure of ovalbumin, an effect reduced in high ionic strength media. Hollecker and Creighton (1982) pointed out that destabilization by succinylation of cytochrome c, ribonuclease and β-lactoglobulins A and B, is more the result of the breakage of specific electrostatic interactions leading to abrupt decreases in stability, rather than electrostatic interactions of a general Coulombic nature responsible for a progressive decrease in stability with the degree of modification.

But succinylation has also sometimes led to stabilization: for example Maneepun and Klibanov (1982) have taken advantage of the repulsion between negative charges to propose an interesting way for protecting proteases from autolysis. They introduced negative charges using succinic and maleic anhydrides, hence reducing intermolecular interactions as a result of electrostatic repulsion between protein molecules and modifying some of the cleavage sites (many of them being adjacent to positively charged amino acids).

Alanine aminotransferase has also been stabilized for storage by succinylation (O'Fagain, 1991). He suggested that the addition of the anhydride must have modified the labile amino groups whose loss would cause denaturation.

Reaction of amino groups of a protein with N-carboxyamino acid anhydride should also be considered. It forms polyamino-acylated proteins with various degrees of substitution and length of side chain (Schmid, 1979). Stabilization of proteins against proteolysis can also be achieved by this way. The polarity of the amino acid side chain is very important, polar amino acids being able to prevent aggregation of α-chymotrypsin up to temperatures of 100°C (Becker, 1964).

Reductive alkylation

Reductive alkylation is a good tool for either protein stabilization (Torchilin *et al.*, 1979) or labelling using tritiated sodium or potassium borohydride (Kumarasamy and Symons, 1979, Keul *et al.*, 1979). It is a modification that does not affect the distribution of the charge on the protein.

According to Shatsky *et al.* (1973) low degree of substitution and moderate hydrophobicity of the alkylating agent led to highest resistance with regard to heat and cold denaturation, while hydrophilic alkylation had little effect on the stability. Mozhaev and Melik-Nubarov (1990), who hold to the concept of stabilization by surface hydrophilization, interpreted the possibility of stabilization when adding hydrophobic residues on the protein surface, to the fact that in these cases the modifications may occur in the neighbourhood of hydrophobic clusters realizing additional stabilizing hydrophobic interactions. They showed that alkylation with hydrophilic reagent can also stabilize with the modification of α-chymotrypsin by glyoxylic acid converting the lysine amino groups into $NHCH_2COOH$ (Melik-Nubarov *et al.*, 1987).

Torchilin *et al.* (1979), when modifying α-chymotrypsin with acrolein, observed that thermal stability is largely influenced by the degree of substitution. The results show that the modification of 80% of amino groups has only a slight effect on the denaturation rate constant, but for a degree of modification higher than 80% the stability strongly increases. However when all the amine functions are modified, destabilization occurs. This lends additional support to the hypothesis that there are key functional groups for the protein 3D structure, the modification of which leads to thermostabilization.

Finally concerning alkylation, we mention the work of Maras *et al.* (1992), who showed that methylation of lysines of glutamate dehydrogenase from the thermophilic microorganism *Sulfolobus solfataricus* occurs as a natural way of enzyme thermostabilization.

Reduction of Cysteine

Moisture induced instability of dried enzymes may in some cases be correlated to the content of cysteine residues which tend to form undesirable inter- and intramolecular disulfide bridges after oxidation of their SH-groups, especially at alkaline pH (Liu *et al.*, 1991). Reduction with dithiothreitol is therefore necessary to avoid the problem. However, addition of SH reagents in the reaction medium may be undesirable, therefore chemical modification of the cysteine groups with iodoacetic acid may be preferred (Lundblad and Noyes, 1985; Oakes 1976).

Table 3. Some common stabilizing chemical modifications of proteins (Lundblad and Noyes, 1985; Means and Feeney, 1972)

	Residue	Reaction Reagent
Lys	Acylation	Acetic anhydride
	Guanidination	O-methyl-isourea
	Amidination	Imidoesters
	Alkylation	Formaldehyde
Cys	Reduction	Dithiotréitol
	S-alkylation	
	Carboxyamidation	Bromoacetic acid
	Iodoacetamide	
	Carboxymethylation	Iodoacetic acid
	Formation of mercaptide bonds	p-Mercuribenzoate
Asp & Glu	Amination + secondary reaction of amine	
	Amidination	Carbodiimide + Primary amine
His	Alkylation	Iodoacetic acid
Trp	Oxidation	N-Bromosuccinimide
Tyr	Amination (nitration + reduction)	Tetranitromethane + sodium dithionite
Arg	Condensation	Phenylglyoxal

Glycosylation

The covalent attachment of carbohydrate to the polypeptide may confer stabilization of protein conformation and protease resistance, that are closely related to the extent of glycosylation as well as playing a role in molecular recognition and solubilization of the protein (Lis and Sharon, 1993). The origin of stabilization with carbohydrate has already been discussed, and the grafting of polymeric glycan is treated as a type of immobilization. Mer *et al.* (1996) fucosylated a proteinase inhibitor. The result is an increase in thermal stability of about 1kcal/mol, correlated with a decrease in flexibility and the creation of hydrophobic interactions and hydrogen bonding between the protein and the glycan. Ueda *et al.* (1996) designed mutant lysozymes with N-glycosylation signal sequences which, when expressed, were 0.4 to 1.6 kcal/mol more stable than the corresponding unglycosylated lysozyme. Kato *et al.* (1995) improved heat stability and emulsifying activity of ovalbumin by chemical modification with glucose and glucose 6-phosphate; Wartchow *et al.* (1995) synthesized a series of carbohydrate protease conjugates that were able to work in organic solvents. Inversely glycoproteins treated with endo- and exo-glucosidases in native conditions have a decreased thermostability, ability to renature and tend to aggregate during thermal inactivation, suggesting that carbohydrate moieties prevent the unfolded protein from aggregation and supporting the hypothesis that the general function of protein glycosylation is to aid in folding of the nascent polypeptide chain and in stabilization of the conformation of the mature glycoprotein (Wang *et al.*, 1996; Lis and Sharon, 1993). Some common stabilizing chemical modifications are summarized in *Table 3*.

CHEMICAL CROSSLINKING

Principle

The principle of stabilization by crosslinking is to create a bridge between two parts of the molecule, which is then expected to induce an increase in the rigidity of the structure which is often correlated with a decrease in activity and an increase in stability (Torchilin and Martinek, 1979; Fernandez-Lafluente *et al.*, 1995). This type of stabilization is effective against all denaturing agents, such as temperature, urea, guanidinium hydrochloride, that tend to unfold the structure or dissociate subunits of proteins. As is the case for disulfide bridges, the stabilization by crosslinking incorporates an important entropic term, resulting from the reduction of the possible conformations of the unfolded state. Pace (1990b) has proposed an equation for predicting the effect of a crosslink on the conformational entropy (ΔS_{conf}). of a protein: ΔS_{conf}=–2.1–(3/2). R ln n, where n is the number of residues in the loop forming the crosslink. The stabilization energy that could be gained is about 5 kcal mol^{-1}, which is quite substantial (Johnson *et al.*, 1978). So if the reactive groups are appropriately distributed on the protein surface, introduction of even one crosslink can lead to substantial stabilization. This is the case with lectin cross-linked with dimethyl adipimidate, where one bridge per subunit led to appreciable thermostabilization (Kamra and Gupta, 1988b).

Modulation of the reaction

The efficiency of the method will depend mainly on the strategic location of the bridge anchors in the structure and the length of the bridge. From this point of view we can anticipate that there will not be any general method, each protein requiring a specific approach for its stabilization, depending on its structure, the availability of reactive residue side chains and their location.

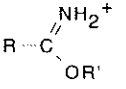
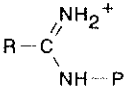
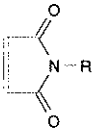
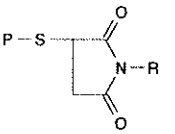
The first parameters to optimize are the length of the crosslinking agent and the way to connect it to the protein surface, followed by the conditions of reaction.

Influence of chain length The chain length of a cross-linker is one of the major parameters which will determine its ability to stabilize a defined protein. In fact its length should match the distance between the residues that are located at a weak part of the protein or between an intersubunit gap. If it is too short the bridging will not be realized, if it is too long it will not be able to limit the increasing conformation fluctuations due to increase of temperature. We discuss here some examples of the incidence of this parameter on the efficiency of stabilization:

The modification of catalase with dimethyl suberimidate decreases stability while dimethyl adipimidate increases thermal stability (Shaked and Wolfe, 1988). The crosslinking of chicken egg white lysozyme with bis(bromoacetamide) derivatives of various lengths between His 15 and Lys 1, can bring about stabilization ranging from 1.6 to 2.3 kcal mol^{-1} (Ueda *et al.*, 1985). Torchilin *et al.* (1978) carried out an experiment showing that the thermostabilization obtained by using diamines of $NH_2(CH_2)_nNH_2$ on activated α-chymotrypsin varies with the number of methylene groups, the combination giving the best results being succinylated α-chymotrypsin

crosslinked with ethylenediamine. O'Fagáin *et al.* (1991) observed that the degree of stabilization for storage conferred on alanine aminotransferase by bis-imidates is related to the length of the bifunctional cross-linker, the most efficient being dimethyladipimidate. Finally, Minotani *et al.* (1979) created very short bridges in lactate dehydrogenase (direct amide formation between neighboring lysine and carboxyl residues by modification with carbodiimide).

Table 4. Some functions commonly employed for crosslinking

Function	Structure	Target function	Product
Dialdehyde	R—CHO	H_2N—P	R—CHOH—NH—P
Halide	R—X	H_2N—P	R—NH—P
		HS—P	R—S—P
Imidoester		H_2N—P	
Isocyanate	R—N=C=O	H_2N—P	R—NH—CO—NH—P
Maleimide		HS—P	
Sulfonyl chloride	R—SO_2Cl	H_2N—P	R—SO_2—NH—P

The chemical function and the targeted residues The nature, availability and distribution on the protein surface of the reactive residue side chains will determine the nature of chemical functions of the reagent used. Often the ε-amino groups of lysine are the preferred targets for the anchor of the bridge since they are mostly exposed on the protein surface and seldom involved in the catalytic site. Glutaraldehyde is for this purpose one of the most widely used bifunctional reagents (Tyagi and Gupta, 1993). Its capacity to polymerize makes the preparation of different types of crosslinkages possible: amine-(reagent)$_n$-amine (Zaborsky, 1973). The reduction of the aldehyde-amine bond is necessary to prevent any reversal of the reaction. Bis-imidate reagents are also widely used for crosslinking with amines, inducing no change in the charge of the protein. An example is the crosslinking of alanine aminotransferase by a bis-imidate reagent cited above (O'Fagáin *et al.*, 1991). But it is not the only solution, other functions than amine can be targeted and a great variety of reagents can be used. A good example for the importance of the nature of the reagent is the experience of Kamra and Gupta (1988a, b): when concavalin A is modified with glutaraldehyde no stabilization occurs, while modification with dimethyl adipimidate results in a great enhancement in stability. Heterofunctional reagents can also be used.

The conditions of reaction The conditions of reaction are very important for the success of the modification. The use of inappropriate conditions can lead to several undesirable results.

High protein concentration and a pH corresponding to the minimum net charge favour intermolecular crosslinking leading to protein aggregation. So dilute protein solutions should be used.

The problem of aggregation can also be treated by first immobilizing the enzyme before proceeding to the chemical modification. Since the immobilization involves a small percentage of the protein surface (15%–30%), the rest remains available for the chemical crosslinking. This combination of techniques presents some appreciable advantages: (1) immobilization drastically reduces the possibilities of intermolecular covalent crosslinking or precipitation promoted by changes in the charge of the protein surface; (2) it enables a better control of the conditions of reaction and its end point; (3) immobilization might bring to the enzyme a better resistance to some deleterious effects of the chemical reagent, and thus enable it to withstand more drastic experimental conditions (Fernandez-Lafluente *et al.*, 1992).

We should note that even if extensive crosslinking can lead to aggregation, the enzyme aggregates may retain enzyme activity with an added value in terms of enhancement of thermostability. Although enzyme aggregates are difficult to handle, some applications are possible (Chawla *et al.*, 1991) and their entrapment in gels (Husain *et al.*, 1985) can transform them into useful products.

A high reagent/protein ratio during prolonged time of reaction leads to extreme crosslinking and may also result in insoluble aggregates. A too high reagent/protein ratio can also lead to a rapid saturation of the reactive protein functions and the impossibility for the crosslinker to realize the second link, which ends up behaving like a monofunctional reagent. Fernandez-Lafluente *et al.* (1995) proposed an ingenious procedure in three steps to avoid this problem and improve the efficiency of the reaction: (1) the enzyme is partially modified in a very controlled fashion; (2) the excess of reagent is removed; and (3) the modified enzyme is incubated in reactive conditions for a long time to allow the crosslinking reaction to go on without the competition of additional single-point modifications.

The use of a heterofunctional reagent targetting two different types of residues at each end can also avoid this problem.

Another possible consequence of extensive crosslinking is inactivation of the catalytic site. To prevent this problem a chemically oriented modification i.e., in the presence of substrate or an analogue, can be carried out, thus protecting access to the catalytic site (Tatsumoto *et al.*, 1989; Broun, 1977).

Applications

Although it has proved to be an efficient tool for protein stabilization, crosslinking is now not widely used compared to site directed mutagenesis or immobilization techniques, mainly because of the lack of unified strategy. For each enzyme there are methods that will work and others that will not, and this with very little predictability. However, additional information about the structure can help to introduce some rationality.

The major applications of stabilization by crosslinking are in the sugar transforma-

tion industry, on enzymes like glucoamylase from *Aspergillus niger* (Tatsumoto *et al.*, 1989), and β-galacosidase from *E. coli* (Gekas and Lopez-Levia, 1985).

Like disulfide bridges, crosslinking is also a method particularly well adapted for the stabilization of multimeric enzymes. A recent example is the covalently linked dimers of alamethicin which form stabilized ion channels (You *et al.*, 1996).

Crosslinking, and, more generally, chemical modifications combined with other methods can also be used to identify amino acids important in structure/function relationships. For example Godavarti *et al.* (1996) used site directed mutagenesis in combination with chemical modification of His with diethyl pyrocarbonate to determine His residues essential for catalysis of heparinase I from *Flavobacterium heparinium*. Krehan *et al.* (1996) carried out investigations on the catalytic and regulatory subunits of human protein kinase CK2 combining crosslinking, immunological assay and site directed mutagenesis.

The crosslinking of an enzyme, followed by its immobilization on a carrier usually increases the level of stabilization. A bifunctional reagent can be used here to couple the enzyme covalently to a support. The bridge made by the crosslinking agent keeps the enzyme at a defined distance from the carrier, hence diminishing the problem of steric hindrance between high molecular weight substrates and the carrier. Alanine aminotransferase consecutively modified by dimethylsuberimidate and immobilized on a preactivated agarose gel exhibit greater stability than the native, modified or immobilized enzymes (Moreno and O'Fagain, 1996). The same pattern is observed for invertase from baker's yeast treated with periodate + ethylenediamine and immobilized on Sepharose (Husain *et al.*, 1996).

CONCLUSION

The same observations as those presented above for crosslinking can be generalized in conclusion to the whole field of chemical modification, with the additional consideration that most of the stabilizing modifications on folded proteins are directed to the amino function of lysine residues. Activation of protein by amination of other residues like Asp, Glu, Tyr, is also often encountered. Alternatively a variety of other modifications are also possible (see *Table 3*). Often stabilization by chemical modification proceeds by hydrophilization, but this point is sometimes controversial and other types of interaction are also involved in the stabilization. According to Mozhaev *et al.* (1990) 'the most reliable approach' for stabilization, is hydrophilizing the protein surface. This is, however, not consistent with the thermostabilization frequently observed following acetylation and reductive alkylation with reagents of moderate hydrophobicity.

Functional groups of the same type in the protein globule do, as a rule, exhibit high heterogeneity in the degree of protein stabilization conferred by reaction with a given reagent. Usually we can only very approximately target the residues by selectively tuning the pH, the steric or electrostatic hindrance of the reagent in function of the accessibility and the environment of the target residues.

In conclusion, there are some general recipes that can be rationally selected based upon the knowledge we have of the considered protein. Thereafter the conditions and the level of modification must be optimized for each case.

Some relevant reviews on chemical modifications of proteins are Roig and Kennedy

(1995), Tyagi and Gupta (1993), O'Fagain *et al.* (1988), Lundblad and Noyes (1985), and Means and Feeney (1971).

Enzyme Immobilization

If globally chemical modification has lost its primacy in the domain of protein stabilization, enzyme immobilization is still employed in industry, thanks mainly to the advantages it brings: (1) the enzyme can be recovered after the reaction and the product easily separated; (2) the possibility of setting up a continuous flow system; (3) better control of the conditions of reaction; (4) it does not require a thorough knowledge of the structural properties of the enzyme; (5) stability is often improved and sometimes optimal conditions of the enzyme reaction can be changed (pH shift, salt, etc)..

ENZYME STABILIZATION BY IMMOBILIZATION

Before going deeper into the subject, we should warn that it is difficult to interpret data from stability assay experiments on immobilized enzymes. Often an important part of the observed stability is the result of artefacts generated by diffusional problems of the products and substrates and a partial inactivation by chemical modification of the less stable subpopulation of enzyme (Klibanov, 1979).

Stabilization against unfolding and subunit dissociation

An enzyme immobilized at multiple sites has a significantly reduced conformational flexibility, and while it is still capable of the degree of movement required for efficient catalysis, gross distortions of its structure that would normally cause denaturation are prevented by its attachment to the carrier (*Scheme 7a*). As in the case of crosslinking, this results in an increased thermostability and resistance against other denaturing agents like guanidinium hydrochloride, surfactants, organic solvents.

For the same reason as presented in the case of cross-linking, oligomeric enzymes are good subjects for stabilization by immobilization. Subunit dissociation, one of the first steps in the denaturation of such enzymes, is prevented by linking to the support (*Scheme 7b*).

Stabilization against proteolysis

The first experiments on enzyme immobilization around 1950 were carried out on proteases. Immobilization is very efficient for stabilizing against autolysis in several ways: (1) indirectly, preventing unfolding decreases accessibility to cleavable bonds for proteases (Shami *et al.*, 1989; Daniel *et al.*, 1982; Klibanov, 1979). Coffey and de Duve (1968) showed that denaturation is a prerequisite for hydrolysis by lysosomal extracts; (2) the presence of the carrier strongly hinders access for the protease to cleavage sites; (3) the immobilization of proteases on insoluble carriers protects them strongly against autolysis, their static position impedes the movement of protease molecules towards each other (Shah *et al.*, 1995): however it can strongly affect their

catalytic activity because of steric hindrance. For the same reason immobilization is also effective against microbial attack (Klibanov, 1979).

Stabilization for other purposes

Stabilization against unfavourable pH pH variation induces changes in the ratio of positive and negative charges that alters the enzyme in several ways: (1) breakage of salt bridges; (2) repulsion between the remaining charges distorts the protein conformation and finally unfolds the polypeptide chain; (3) loss of activity because of the change in the charge of residues involved in catalysis; (4) hydrolysis of peptide bonds in case of extreme pH. Immobilization can stabilize against these deleterious effects at several levels excepted for extreme pH, first by preventing unfolding, and secondly by microenvironmental effects from the carrier. When the enzyme is immobilized it is partially extracted out of the solution, and its environmental parameters may also depend upon the features of the carrier. Hence, polyelectrolytic surfaces can change the local pH and consequently the optimal pH of the enzyme. It is possible to design carriers with a buffered microenvironment. This is also possible for non-electrolytic carriers if the H^+ partition coefficient between the microenvironment and the solution is different from 1. Guisan *et al.* (1994) immobilized penicillin G acylase (PGA) on a porous support. The system has two optimal pH values: pH 5.5 inside the particles of the biocatalyst and pH 8 in the bulk solution. The result is a system with interesting industrial parameters: (1) high hydrolytic yields (higher than 97%); (2) an increase in the stability of PGA derivatives (a 50-fold factor); and (3) a small decrease in operational activity with no diffusional hindrance compared to the soluble enzyme at pH 8.0. The covalent attachment of subtilisin to PEG significantly stabilizes the enzyme against both thermal denaturation and pH (Yang *et al.*, 1996).

Stabilization against organic solvents Some enzymes need to operate in organic media when the solubility of the substrate in water is low, or to shift the thermodynamic equilibrium of the reaction towards the products. However, many enzymes are inactivated in organic solvents. One cause is enzyme aggregation, which can be prevented by immobilization. Another is the change in conformation induced by the solvent. Here also immobilization can help by (1) rigidification of the enzyme structure, and (2) very hydrophilic carriers which can exclude the organic solvent molecules from the enzyme microenvironment. Attachment of subtilisin to PEG provides stability and solubilization of the enzyme in water and organic solvents (Yang *et al.*, 1995a). Entrapment of enzymes in reverse micelles is also very efficient in the design of catalytic systems for non aqueous media (Ayagari, 1995). Lipase entrapment in treated silica gels enhances its esterification activity by a factor of 88 and its stability in both aqueous and organic media (Reetz *et al.*, 1996).

Stabilization against hydrogen peroxide Hydrogen peroxide can irreversibly oxidize some amino acids involved in the catalytic site. This can be a major problem if peroxide is a product of the reaction as in the case of oxidases. One solution is the immobilization of such enzyme on metal oxide such as Manganese oxides, or activated carbon that can catalyse the chemical decomposition of the peroxide.

Another possibility is to coimmobilize the enzyme producing the peroxide with catalase ($H_2O_2 \rightarrow O_2 + H_2O$) which will also destroy the peroxide. (Klibanov *et al.*, 1979).

TECHNIQUES OF ENZYME IMMOBILIZATION

We classify the various types of immobilization on the basis of the nature of the interaction between the protein and the carrier and the nature of the carrier. We distinguish five types of immobilization: covalent coupling, adsorption, reticulation, matrix entrapment and inclusion in microspheres (*Scheme 4*).

Covalent attachment on soluble or insoluble carriers

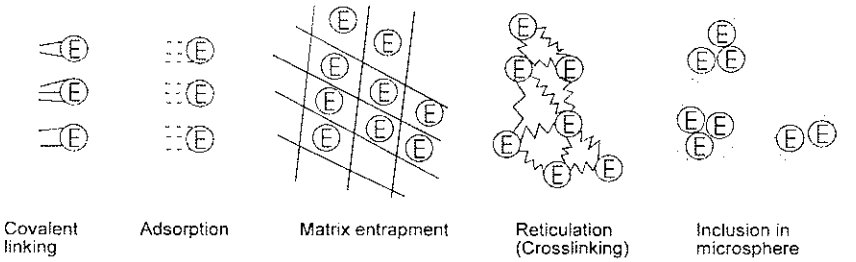
Most covalent immobilization procedures rely on the reactivity of Lys ε-amino functions. The most frequently used reactions are reductive alkylation and acylation. There are a wide variety of carriers. *Table 5* lists the main ones. The choice will be determined by important features such as ability to be functionalized, resistance to microbial attack, dynamic and structural stability, hydrophilicity (a lot of enzymes are denatured in contact with hydrophobic surfaces), the possibility to be regenerated, accessibility to solvent etc.

We have developed in our laboratory a technique for covalent immobilization of enzymes on soluble polysaccharides (Lenders and Crichton, 1984). It consists in the multipoint covalent coupling of a protein through the amine functions of its exposed lysine residues with aldehyde functions of a pre-oxidized glycan (*Figure 1, Scheme 5*). To stabilize the link and avoid any rearrangement, a subsequent reduction of the

Table 5. Main types of carrier for enzyme immobilization

	Material	Examples
A.	**Organic**	
	Polysaccharides	Cellulose, agarose, starch, dextran. . .
	Vinyl polymers	Polyacrylamide, polymetacrylic acid, polyethylene/ maleic anhydride. . .
	amino acid polymers and their derivals.	Poly(p-amino-D, L-phenyl-alanine/L-leucine)
	polyamides	nylon. . .
B.	**Inorganic**	
	Glass	Porous glass, beads. . .
	Metal	Nickel based alloy. . .
	Miscellaneous	Colloïdal solutions of silicate, alluminate . . .

Schiff base (imine) is necessary (Bonneaux and Dellacherie, 1995). $NaBH_3CN$ is the most convenient reducing agent we have found for this purpose, since it is very selective for imines and does not attack the hydrated aldehyde function of the glycan thus ensuring that we get more complete coupling (Lenders and Crichton, 1984, 1988). We have shown with cellobiase and glucose oxidase that there is a relation



Scheme 4. Techniques of immobilization.

between stabilization and rigidification of the 3D structure estimated by the mean number of links established between the carrier and the enzyme (*Figure 2*, Germain *et al.*, 1989; Burteau, 1993). If there are not enough lysine residues exposed on the surface to get a dense enough reticulation, as is the case for glucoamylase, we can undertake amination by coupling the diamines to carboxyl groups of the enzyme prior to immobilization (Lenders and Crichton, 1988). For enzymes working on high molecular weight substrates, immobilization often leads to an important loss of activity mainly because of steric hindrance limiting access to the catalytic site (Germain and Crichton, 1988). To resolve this problem for pullulanase, we have tried chemically oriented immobilization performed in the presence of the substrate, and get an increase of 12% to 16% of residual activity after the coupling compared to statistically immolized pullulanase (Germain *et al.*, 1988).

We have also developed a method for immobilizing enzymes on pre-functionalized insoluble inorganic carriers (silica particles from synthetic calcium silicate) with a spacer such as glutaraldehyde (*Scheme 6*, Anton *et al.*, 1987). This technique can be coupled with the stabilizing immobilization on soluble polysaccharides. The oxidized polysaccharide presents free aldehyde functions, so that it can link the polysaccharide-enzyme conjugate to the aminated silica in the same way that glutaraldehyde does. Thus we can combine the advantages of an enzyme immobilized on an insoluble carrier and a stabilized soluble one, the polysaccharide acting as a spacer maintaining the enzyme at a defined distance from the carrier (Germain and Crichton, 1988; Lenders *et al.*, 1985). *Figure 3* shows the incremental stabilization of penicillin acylase involved in such an immobilization procedure (Burteau *et al.*, 1989). An extensive review of the use of polymeric cabohydrates with proteins has been recently published and covers the whole field of immobilization from covalent attachment to microencapsulation (Chen *et al.*, 1995).

Many applications of covalent immobilization have been reported in the literature, the following being an interesting case: Rosell *et al.*, (1995) immobilized penicillin acylase in the presence of various inhibitors (penicillin sulfoxide, phenylacetic acid etc). Each inhibitor, when it is adsorbed at the active centre of penicillin acylase promotes a specific enzymic conformation which remains fixed after the stabilization process by multipoint covalent attachment to supports. The stability and activity (in hydrolysis and in synthesis) towards different substrates was determined. The result is a broad spectrum of enzymatic derivatives with a range of activity/stability depending on the inhibitors used in their stabilization. The resulting choice offers a considerable

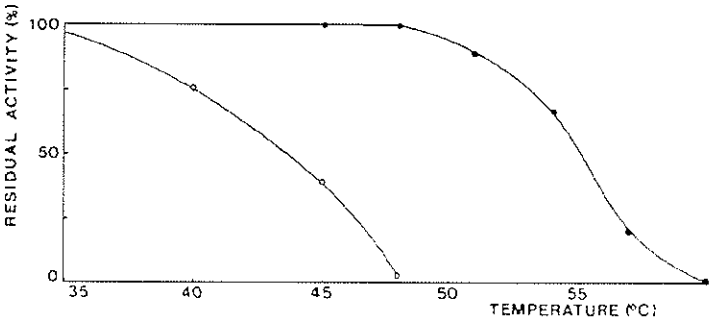
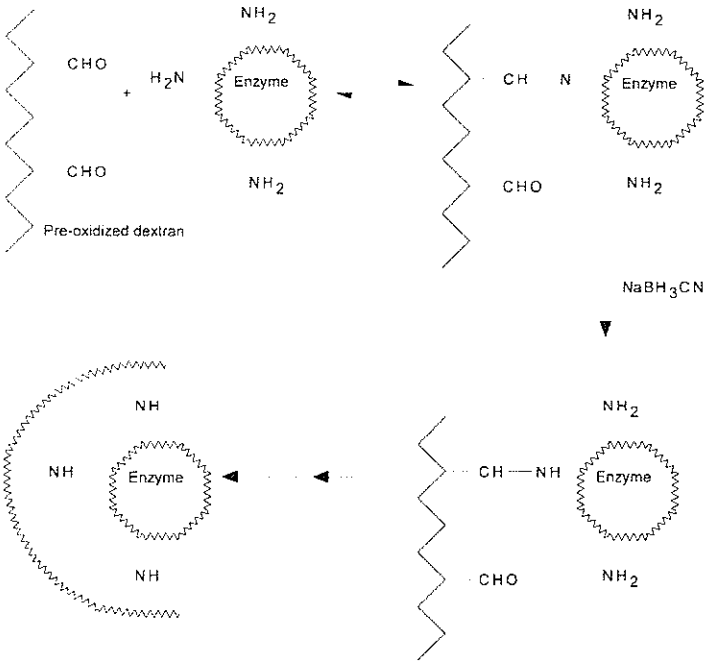


Figure 1. Stabilization of pullulanase by covalent coupling on a polysaccharide Heat stability of (o). native pullulanase and (•). pullulanase-amylose conjugate (Lenders and Crichton 1984).



Scheme 5. Covalent coupling of a protein to a polysaccharide.

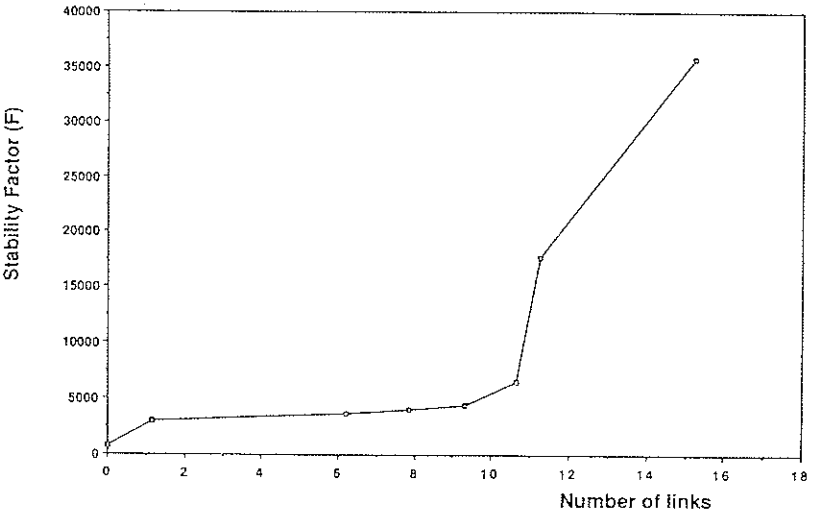


Figure 2. Relation between stability and number of links introduced between dextran and cellobiase. (Germain *et al.*, 1989).

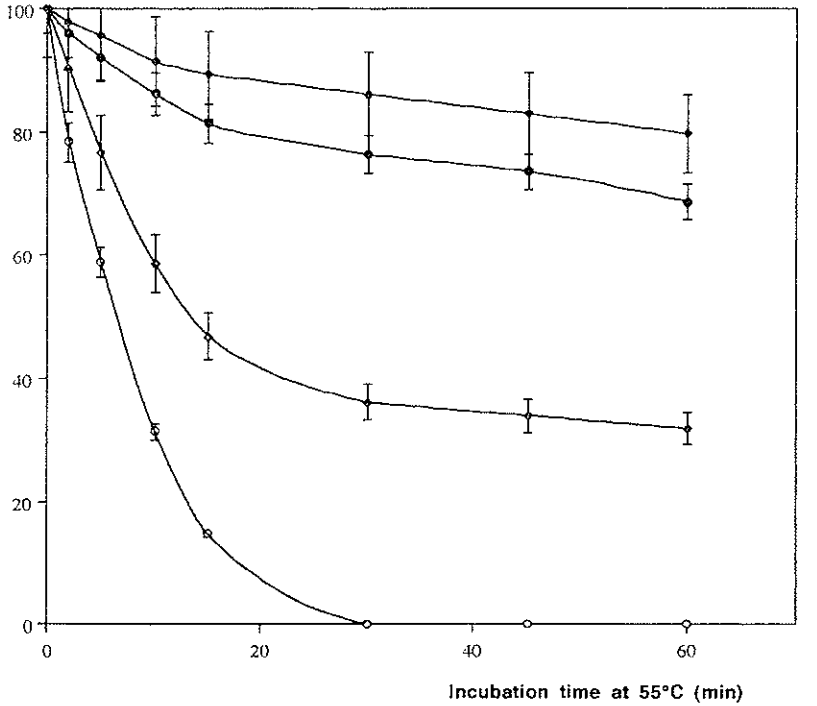
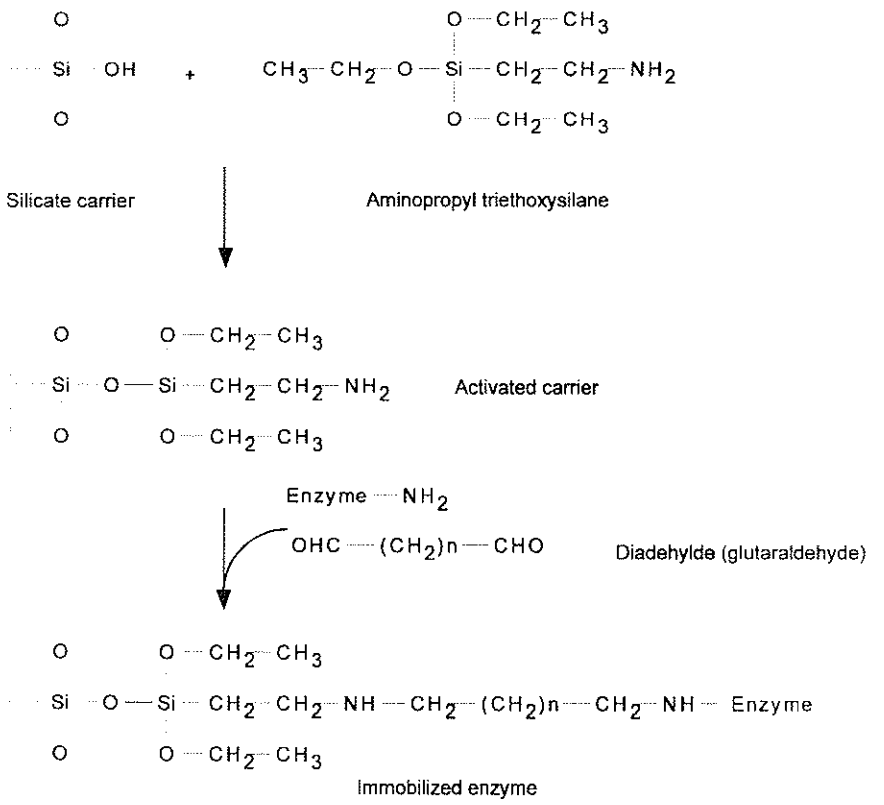


Figure 3. Immobilization of soluble stabilized and non stabilized penicillin acylase (P.A.). (Burteau *et al.*, 1989). Native soluble P.A. (○)., native immobilized on Promaxon[1] (◇)., stabilized soluble P.A. (♦). stabilized and immobilized P.A. (●). (Promaxon® is the commercial name of the mineral xonotlite ($CA_6Si_6O_{17}(OH)_2$). Promaxon is a registered trade mark of Promat.

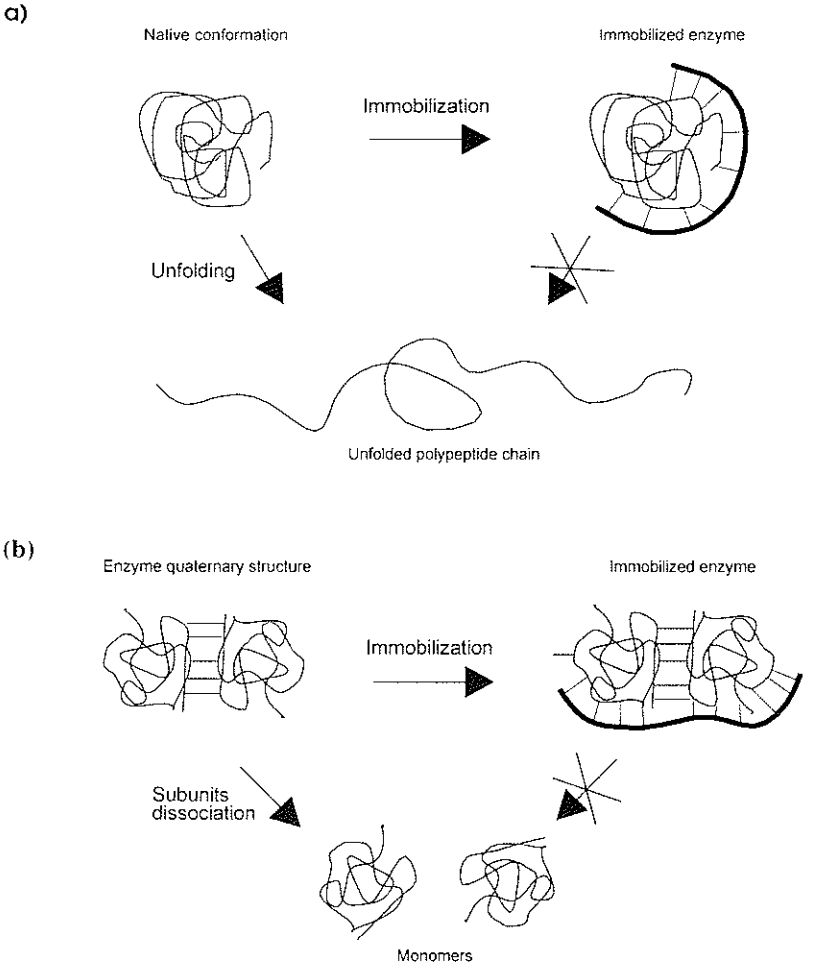


Scheme 6. Immobilization of an enzyme on a prefunctionalized inorganic carrier.

increased potential for the use of the enzyme since one can select a derivative which will be more specifically designed to catalyse the reaction of interest.

Adsorption on various types of surface

We have already seen that the protein surface presents more or less 50% of hydrophobic clusters and 50% of hydrophilic residues (Chothia, 1974). Immobilization by adsorption takes advantage of the physico-chemical properties of those residues without any covalent linkage. The carrier can be either (1) ion exchange resins such as DEAE-Sephadex, CM-cellulose; (2) classical reverse phase supports used in chromatography such as hexyl-Sephadex, octyl-Sepharose; (3) polyvalent carriers able to realize both types of interactions i.e. a concomitant juxtaposition of hydrophilic interactions with hydrophobic interactions as for example phenoxylacetyl-cellulose. The latter offers the possibility of tuning several parameters such as solvent polarity and pH ionic strength. (Hartmeier, 1988; Klibanov, 1979, 1983). Kleijn and Norde (1995) have reviewed the general principles governing the process of protein absorption from aqueous solution onto a solid surface.



Scheme 7. (a) Preventing unfolding by rigidification of the native conformation of the enzyme. (b) Preventing subunit dissociation by immobilization.

We present three interesting applications:

(1) Matoba *et al.* (1995) designed a novel 'tentacle-type' porous membrane that allows adsorption of enzymes in multilayers in amounts about 50 times those permitted by monolayer adsorption. Diethylamino (DEA) groups as an anion-exchanger were attached to a polymer chain grafted onto the pores of a hollow-fiber membrane. The binding capacity exceeded one gram of urease per gram of the membrane at DEA group densities higher than 1.6 mmol per gram.

(2) Ampon *et al.* (1994) elaborated a procedure intermediate between covalent attachment and adsorption for attaching lipase to certain kinds of hydrophobic surfaces. It involves covalent derivatization of the protein molecule by reaction in solution with a hydrophobic imidoester, aldehyde or activated polyethyleneglycol.

The resulting protein derivative is then allowed to adsorb onto an insoluble hydrophobic surface.

(3) Carriers designed for a specific protein can also be prepared on the basis of specific ligand-protein interactions. Anspach and Altmannhaase (1994) grafted Cu(II) chelate sorbents on Sepharose 4B and silica based carrier. The treated carriers were thereafter able to immobilize Penicillin G amidohydrolase (PGA) and could be regenerated with penicillin G which competes for the protein binding site of the Cu(II) chelate. Fibronectin, can also be adsorbed on such a carrier bearing metal chelates (Gmeiner *et al.*, 1995).

Intra and intermolecular crosslinking

Intra and intermolecular reticulation immobilization does not necessarily require a carrier. Indeed, it is a covalent immobilization leading to the formation of either soluble or insoluble agregates where the enzyme is its own carrier. The polymerization of the enzyme can be performed in the presence of another protein which is inert with regard to the catalyzed reaction. This technique is cheap and is applicable to most enzymes. However, it often brings little stabilization and the induced diffusional constraint leads to a great loss of catalytic activity (Hartmeier, 1988; Kilbanov, 1979). Examples are numerous, and we simply mention a recent one: Ron *et al.* (1995) stabilized antibody by a two step method ('bi-layer encagement'). The protein was first treated with glutaraldehyde and polyglutaradehyde, and then crosslinking was performed with polyamine.

Matrix entrapment and co-polymerisation

Similarly to reticulation, the entrapment of an enzyme in a polyacrylamide gel, introduces diffusional problems. However, great stabilization can be expected, and the gel form is very convenient for preparing columns, membranes or beads. We report three recent representative cases:

The incorporation of chymotrypsin into a polyacrylamide matrix by azide coupling resulted in extreme stabilization of the enzyme towards heat (about 20% of its original activity withstands the treatment at 100°C for 2 h), organic solvents and urea (Ulbrichhofmann *et al.*, 1995). Two enzymes (subtilisin and thermolysin) have been modified with a polyethylene glycol (PEG, MW 3400) having an acrylate group at one terminus and an active ester at the other and then subsequently incorporated into polyacrylates in the course of free-radical initiated polymerization in a variety of organic solvents. The enzyme's tolerance toward both heat and a miscible organic solvent was also enhanced by incorporation into the polymer (Yang *et al.*, 1995b).

Matsukata *et al.* (1996) recently provided some refinements in the technique changing the molecular architecture of trypsin-poly(N-isopropylacrylamide) conjugates. The best one with regard to stability, retention of biological activity and solution properties involved the enzyme conjugation to the polymer at a single end.

Another technique of inclusion consists in the entrapment of the enzyme in a natural preformed polymer such as collagen, alginate and completion of the reaction with a

crosslinker. This technique provides the advantage of a great bio-compatibility between the enzyme and the carrier, and usually leads to good stabilization. For example, alginate entrapment has been shown to increase thermostability as well as broadening the pH optimum of naringinase with application at debittering of kinnow and orange juice (Puri *et al.*, 1996); the pH stability range of cathepsin immobilized in calcium alginate beads is extended towards both acidic and alkaline environments (Kamboj *et al.*, 1996); the entrapment of β-glucosidase on chitosan and the subsequent crosslinking with glutaraldehyde yields a product with high stability in acidic media (Martino *et al.*, 1996).

Inclusion in microcapsules, hollow fibers, reverse micelles, liposomes

The basis of inclusion techniques depends on the semi-permeability of the membrane. Hence they are only adapted to small sized substrates and reaction products. An important problem is the disruption of the fiber under the action of osmotic pressure. This method provides poor stabilization. Its main advantages are very high retention of activity, for enzymes requiring a macromolecular coenzyme the possibility to co-immobilize it and ease of realization. Microencapsulation can be achieved through either physical or chemical means. In the physical method, the enzyme solution is suspended in droplets in an organic solvent (another protein inert for the reaction is also added to limit denaturation). Then to obtain the microcapsules, cellulose nitrate or an analogous product is added. In the chemical method, polar synthetic polymers are used to form beads or microparticles containing the enzyme (Hartmeier, 1988).

There are many other techniques of encapsulation, such as the use of reverse micelles. These consist of an array of amphiphilic molecules in a predominantly organic phase enclosing an aqueous phase. It is possible to obtain reverse micelles as micro-emulsions in a water-immiscible solvent with enzymes trapped within the water phase. They are convenient for making water soluble enzymes functional in an organic solvent. (Roig and Kennedy, 1995). This method exploits its potential fully with enzymes working with non-water-soluble substrates such as lipases (Ayagari, 1995: Hayes and Gulari, 1994), but also penicillinases (Chakravarty *et al.*, 1995), and chymotrypsin (Rairy *et al.*, 1995).

We can also carry out microencapsulation by formation of liposomes. In this case, we take advantage of the amphipathic nature of some lipids such as phosphatidyl ethanolamine. Liposomes are very useful for the targeted delivery of drug products (Kupcu *et al.*, 1995; Kukarni *et al.*, 1995; Grimaldi *et al.*, 1995; Lo and Rahman, 1995).

Another refinement of membrane confinement is to trap the enzyme in a reactor made of hollow-fiber membrane where reaction products can pass through the membrane and be removed continuously while the biocatalysts are retained (*Scheme 8*, Hartmeier, 1988). Ju *et al.* (1995) designed a hollow-fiber hemodialyzer with alpha-amylase immobilized on the lumen and outer surface of hollow fibers employed to hydrolyze high-concentration starch slurry. The immobilization of urease in fiber by Matoba *et al.* (1995), already described, is another example. Finally, we mention a recent review by Gerbsch and Buchholz (1995) on the topic of hollow microspheres, which provides additional information.

Use of additives

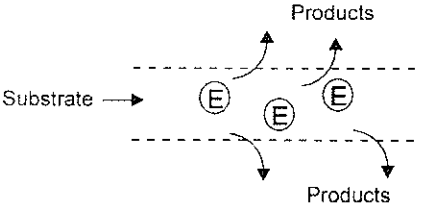
The last way to stabilize proteins, but by no means the least important, is to put them in the presence of some well chosen additives. By this term we mean any molecule that will not react covalently with the enzyme. This method presents several advantages over the others. Most importantly it is simple to undertake, with immediate results, does not require a lot of information about the protein, generally costs very little and its scope extends beyond operational stability to long term storage stabilization.

SUBSTRATE AND ANALOGUES

According to our definition, substrate is not properly an additive since it will be transformed in the catalytic site of the enzyme. However, since at the completion of the reaction the enzyme has returned to its initial state and the presence of the substrate displays stabilizing properties, it is worth mentioning here. If we take into account the two steps model for enzyme denaturation and inactivation we have previously described, the first point in the explanation of the stabilizing effect of the substrate is that it mobilizes the free native enzyme [N] to form the enzyme substrate complex [NS], so that it shifts the equilibrium of the denaturation process from U toward N.

$$NS \overset{S}{\rightleftharpoons} N \rightleftharpoons U \longrightarrow I \qquad (5)$$

Secondly when the substrate is located at the catalytic site, it realizes interactions with amino acids located on different segments of the polypeptide chain which are brought close together by folding. By doing so the substrate acts as a node for crosslinking between different parts of the structure and thus stabilizes the native protein conformation.



Scheme 8. Hollow fiber reactor.

The same explanation is also relevant for any molecule presenting analogous structural features to those responsible for substrate binding, such as competitive inhibitors and reaction products.

Many cases of enzymes stabilized by their substrate have been reported in the literature, for example enzymes processing carbohydrates, such as invertase (Combes *et al.*, 1987). NAD or NADP dependent dehydrogenases are often stabilized by the presence of NAD (Citri 1973; Hoenes 1985).

CONTRIBUTION OF THE SURFACE FREE ENERGY PERTURBATION TO PROTEIN-SOLVENT INTERACTIONS (KITA *ET AL.*, 1994)

The presence in solution of substances such as organic molecules, salts, amino acid salts or polymers may have profound effects on the stability of proteins.

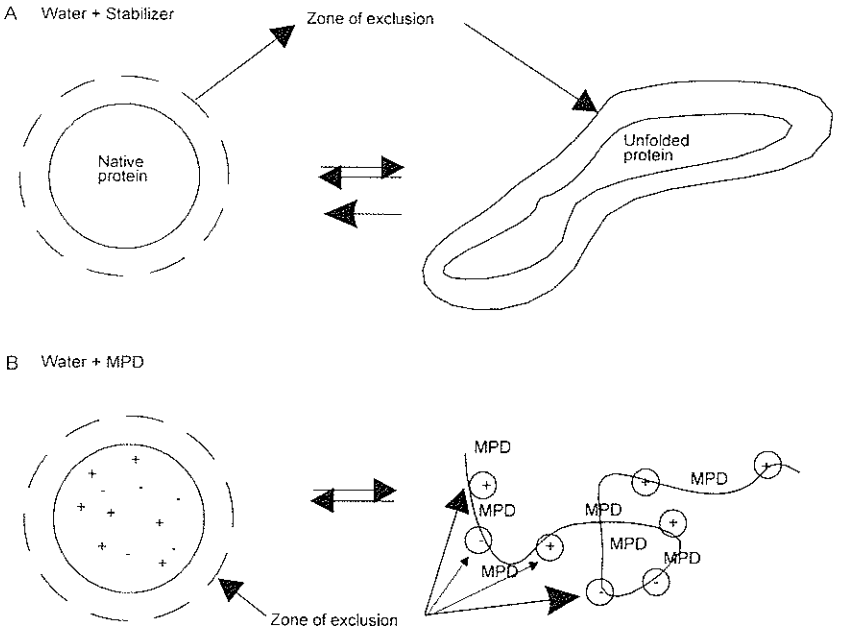
The first point to consider is that many of those substances exert cohesive forces on water molecules, resulting in an increase in the surface free energy of water. The surface free energy of the formation of a cavity to accommodate a solute molecule in a solvent is important for the stability and self-association of macromolecules. More schematically, the contact surface of a protein with the solvent is greater in the unfolded state than in the native state, so that an additive that will increase the surface free energy of water will increase the chemical potential of the unfolded state more than it will for the native state and consequently will shift the thermodynamic equilibrium toward the N state.

There are, however, many exceptions to this rule such as glycerol, the presence of which stabilizes the protein although it is known to decrease the surface tension of water, or the opposite case of urea, usually employed to unfold proteins although it increases the surface free energy of water.

The second point to consider is the affinity of the cosolvent (additive) for the protein surface. It is important to note that the situation at the interface protein/water is not as simple as that at the water-oil interface. In the first place the protein surface is not flat and above all it is not homogeneous so that some parts may provide good interactions with the mixed solvent while others may not. In any event, the important factor to consider is the relative importance of the protein-cosolvent interactions compared to the protein-water interactions. We can distinguish two cases: (1) the protein-cosolvent interactions are weaker than the protein-water interactions so that the exclusion of those solutes from the protein surface and its preferential hydration occur (positive free energy of interaction) or (2) the solute has a relatively good affinity for the protein compared to water-protein interactions and binding of the solute to the protein occurs. The negative free energy of interaction will compensate the effect of the increase in the surface free energy of water and consequently lead to a lesser stabilization, or even to destabilization.

More precisely, the stabilizing or destabilizing effect of an additive on the protein structure will depend upon the balance between preferential interactions of the mixed solvent with the protein in the unfolded and the native state. Unfolding first exposes residues that were previously buried and secondly increases the overall surface of the protein. If those residues have little affinity for the cosolvent molecule relative to water, an increase in preferential exclusion occurs, the unfolded structure will be destabilized and consequently the native structure will be favoured. On the other hand, if they have good interactions with the cosolvent molecules (as it is the case for urea) the effects due to the increase in surface tension could be overwhelmed and the denatured state rendered more thermodynamically favourable that the native form (*Scheme 9*).

In conclusion, the entire pattern of interactions at the protein-solvent interface is a continuum from strong stabilization to strong unfolding and each cosolvent must be analyzed in terms of the complex balance between binding and exclusion and the expected contribution of the surface tension perturbation finds its place along this



Scheme 9. Protein-solvent interactions. **(A)** The predominant interaction with proteins is non specific exclusion. Since in the denatured state, it is greater per protein molecule than in the native state, the equilibrium is shifted to the left. **(B)** In the denatured state, the exclusion of 2-methyl-2,4-pentanediol (MPD) due to the high charge density on the native protein is replaced by MPD exclusion from individual charged sites and MPD binding to non-polar regions in the denatured state. (Arakawa *et al.*, 1990b).

spectrum. In addition, preferential hydrating systems can be classified in two categories: (1) those for which the preferential hydration is dominant and does not vary with solution conditions. In this case protein salting out and stabilization always follow. (2) Those for which the preferential hydration varies with the conditions and which do not always stabilize. In the latter case the specific nature of the protein surface may be a determining factor (Arakawa and Timasheff, 1990a,b).

LOW MOLECULAR WEIGHT SOLUTES

Sugars are good stabilizers of proteins (Williams *et al.*, 1995; De Cordt *et al.*, 1994). The studies of Arakawa and Timasheff (1982a) and Lee and Timasheff (1981) strongly suggest that the increase in the surface tension due to the presence of sugar constitutes a very important factor governing the preferential interaction of proteins with solvent components. Proteins are preferentially hydrated and hence stabilization occurs.

More generally, polyols are good additives for stabilization (Cioci, 1995; De Cordt *et al.*, 1994; Back *et al.*, 1979) in contrast to monohydric alcohols that generally decrease T_m for the reversible denaturation of proteins. This effect is enhanced with the increasing number of methylene groups on the alcohol, in other words the hydrophobic character of the cosolvent (Gray, 1993).

The case of glycerol is somewhat particular. Here the contribution of the free energy of cavity formation to macromolecule stabilization cannot play a significant role since glycerol decreases the surface tension of water. In fact, the increase in free energy of denaturation appears to be due principally to a decrease in the entropy change during denaturation: (1) proteins are preferentially hydrated in aqueous glycerol solution. Preferential exclusion of glycerol is related to enhanced solvent ordering so that it is a thermodynamically unfavourable phenomenon. (2) The exclusion of glycerol from protein domains increases on denaturation. Consequently the tendency of the system to minimize the unfavourable contact, i.e., the extent of the protein-solvent interface induces stabilization of the protein by favouring the folded state. (Gekko and Timasheff, 1981). Recently some details have been established by Priev *et al.* (1996): they have shown a decrease in volume and compressibility of the protein interior in the presence of glycerol. The interpretation they propose is that the compression of protein, by reducing its surface of contact with the solvent, due to the preferential hydration, would squeeze out water from the protein interior that was previously involved at keeping apart neighboring segments of the polypeptide chain that would otherwise stick. The loss of that water would induce the collapse of voids and cavities, leading to the creation of intramolecular bonding that locally rigidifies the protein and decreases the amplitude of thermal motions.

Organisms submitted to environmental stresses such as high or low temperatures, desiccation, high concentrations of salt or urea, concentrate certain substances in their cytosol. These molecules are responsible for the osmotic balance and are so called osmolytes (Bernard*et al.*, 1993; Imhoff and Rodrigues-Valera, 1986; Robertson*et al.*, 1990; Wegmann, 1986). They are often accumulated to cytoplasmic concentrations above 1 mol/kg of water. *In vitro* studies of these substances show that their presence stabilizes proteins from thermal denaturation (Taneja and Ahmad, 1994; Santoro *et al.*, 1992; Arakawa and Timasheff, 1985; Delpino and Sanchezruiz, 1995). Chemically they belong to three major classes of compounds: polyols, amino acids and their derivatives, and methylamines (Yancey *et al.*, 1982). Galinski (1993), using infrared spectroscopy, has demonstrated that they are strong water-structure formers and as such are probably excluded from the hydration shell of proteins. This effect might account for the stabilization of the hydration shell of native proteins.

Salts may influence stability either by specific or non-specific interactions with protein. (1) In the case of specific ionic effects, the situation is very similar to that described for enzyme-substrate complexes. Many enzymes require the binding of metal ions, particularly calcium at some specific binding sites for their stability. An example is the subtilisin-like serine proteinase secreted by *Ophiostma piceae* (Abraham and Breuil, 1995). Electrostatic interactions with negatively charged residues (Asp, Glu) located on different segments of the polypeptide chain realize a kind of crosslinking that rigidifies the folded conformation. The affinity in this kind of binding is strong (dissociation constants are of the order of 10^{-3} to 10^{-4}M) (Gray, 1993). Pantoliano *et al.* (1988) introduced Asp in place of Pro 172 and Gly 131 at the binding site of Ca^{++} of bacterial subtilisin (BPN'). By so doing, they increased the binding affinity by a factor 6 and consequently increased its Ca^{++} dependent kinetic thermal stability.

(2) Non specific ionic effects require much higher salt concentrations than specific effects. The stabilizing or destabilizing effect of a specific ion, correlates well

with the Hofmeister series of salts and the effects of the anion and cation are additive. Stabilization of native protein structure and salting out are related, and inversely, ions that increase protein solubility destabilize protein structure. The model of preferential hydration of proteins, provides a good frame of interpretation. Ions with stabilizing effects are those that are preferentially excluded from the protein surface, particularly when the protein is unfolded, so that the native conformation is preferred. The observed salting out is the response chosen by the system to the positive change in the chemical potential of the protein due to the increase in the energy of cavity formation, itself due to the increase in water surface tension. Inversely, ions that bind preferentially to the buried hydrophobic residues will stabilize the unfolded state and induce the salting in of the protein. Finally, the situation is intermediate in the case of ions where the preferential hydration depends upon the conditions and which will or will not stabilize, according to the particular nature of the surface of the protein (Gray, 1993; Arakawa and Timasheff, 1982a; Arakawa *et al.*, 1990a, b).

POLYMERIC SOLUTES

Several types of polymer have been reported to stabilize proteins. Invertase is stabilized by the presence of PEG, and the effect increases with the polymer length and its concentration (Combes *et al.*, 1987; Monsan and Combes, 1984). PEG strongly lowers the surface tension of water, however it is preferentially excluded from proteins, mainly due to its great size, which leads to preferential hydration (Kita *et al.*, 1994). Polyvinyl alcohols have been reported to reduce by half the inactivation constant of lipase at 49°C (Battistel *et al.*, 1995). The entrapment of an enzyme in linear polymers that will restrain the possibility of unfolding is also possible (this point has already been discussed in the immobilization section).

STABILIZATION AGAINST OTHER STRESS FACTORS

Stabilization against oxygen inactivation

The way in which enzymes are inactivated by oxygen is very different from thermoinactivation. It involves an oxidation reaction of protein functional groups either by O_2 or its very reactive radical derived species. Klibanov *et al.* (1979) prevented inactivation of *Clostridium pasteurianum* hydrogenase with classical chelating agents such as EDTA, sulphosalicylic acid and chelex resin. It is also possible to stabilize by adding ligands that shield the oxidizable groups from oxygen (Khan *et al.*, 1981). Various anti-oxidant molecules can also be used such as vitamin E and ascorbic acid.

Stabilization against proteolysis

Abraham and Breuil (1995) identified some factors affecting autolysis of a subtilisin-like serine proteinase secreted by *Ophiostma piceae,* such as calcium ions and ammonium sulfate that increased the temperature of unfolding and consequently reduced autolysis.

Stabilization against deamination

Tomizawa*et al.* (1995a) showed that phosphate ions could accelerate the deamination of Asn as well as racemization of Asp and Asn, while the presence of trifluoroethanol could drastically reduce deamination rate but not suppress it completely.

Table 6. Some additives with stabilizing properties for proteins

Type of Additive	Protein	References
Substrate and similar ligands		
Penicillin G		
	Penicillin acylase	Illanes *et al.*, (1996); Bianchi *et al.*, (1996)
Protein RNase inhibitor	Bovine seminal RNase	Murthy *et al.*, (1996)
Phosphoribose-PP	Hypoxanthine guanine phosphoribosyltransferase	Montero *et al.*, (1996)
UTP	UMP kinase	Serina *et al.*, (1996)
Progesterone, testosterone, ketoconazole	Cyt P450c17 (stabilization against subtilisin action).	Kuhnvelten and Lohr, (1996)
Low M.W. organic molecules		
Dithiothreitol	Pyruvate decarboxylase from *Zymomonas mobilis*	Phol *et al.*, (1995)
Trehalose, glucose, raffinose, sucrose, N, N' dimethyl glycine		Urokinase Foster *et al.*, (1996)
Glycerol, N acetyl glucosamine	Lysozyme	Maeda *et al.*, (1996)
Glycine	Xylanase for thermophilic *Bacillus NCIM 59*	Nath and Rao, (1995)
Salts		
Specific effect		
Zn++	Yeast alcohol dehydrogenase	Le *et al.*, (1996)
Ca++	rhDNase	Chan *et al.*, (1996)
Non specific effect		
NaCl	HIV 1 protease	Szeltner and Polgar, (1996)
$(NH_4)_2SO_4$	Pyruvate decarboxylase from *Zymomonas mobilis*	Pohl *et al.*, (1995)
$(NH_4)_2SO_4$ and $MgCl_2$	Urokinase	Foster *et al.*, (1996)
Polymers natural and synthetic		
Polyvinyl alcohols	Lipase from *Candida rugosa*	Battistel *et al.*, (1995)
Polyethyleneglycol	Invertase	Combes *et al.*, (1987)
Heparin	Human keratinocyte growth factor	Chen and Arakawa, (1996)
Bovine serum albumin	α glucosidase from *Thermococcus* AN1	Piller *et al.*, (1996)

Conclusion

THE CONVERGENCE OF THE EFFECTS

A recurrent theme in thermostabilized enzymes, is the correlation between the stability, a decrease in flexibility of the folded structure and consequently a decrease of the activity for a defined temperature of reaction. Flexibility is necessary in the catalytic mechanism of the enzyme for the geometric fit, to allow versatility in making specific contacts with more than one partner and to break those contacts (Gryk and

Jardetzky, 1996; Shoichet *et al.*, 1995; Wiseman 1983). So thermostable enzymes are generally less active than their homologous mesophilic counterpart at the optimum temperature of the latter. However this is not due to net loss in the specific activity (turnover), but rather a shift upwards of the optimum temperature (Brock, 1985) and as the optimum temperature is higher, the reaction kinetics are accelerated. Additional evidence of this correlation is the typical counter-example of enzymes coming from organisms living in a cold environment where the unavoidable reduction of reaction rates at low temperature is prevented by increasing flexibility at the expense of the stability. A very good example is the case of α-amylase from the antartic psychrophile *Alteromonas haloplanctis A 23*. The comparison with its homologous porcine pancreatic α-amylase reveals several features assumed to be responsible for a more flexible and heat-labile conformation, such as the lack of several salt bridges, the reduction of the number of weakly polar interactions involving aromatic side chains, a decrease of the hydrophobicity of amino acid involved in hydrophobic clusters and the alteration of its compactness (Feller *et al.*, 1994).

THE CHOICE OF THE METHOD OF STABILIZATION

We have reviewed of a wide panel of methods for enzyme stabilization among which we need to choose the one that will best meet our requirements. The first steps in the approach to the problem are to collect maximum information about the protein and precisely define the objectives as well as the means we can employ. Sometimes a simple additive will better fit our requirements than a thorough comprehensive study by site directed mutagenesis. The relevance of a method will depend on the following equation: interest=residual activity × longevity. However it is also important to take into account the price of the enzyme and the price of the stabilization procedure as a function of the 'added value' of the product.

Immobilization of enzymes is a method which is occasionally used in industrial processes. It presents the great advantage that the enzyme is easily recovered from the products of the reaction, brings substantial stability, does not require any precise information about the enzyme structure and facilitates continuous processes. It has the major disadvantage that relatively highly purified enzymes are needed, since these are rarely used in industry.

Random mutagenesis occupies an intermediate position between immobilization techniques and site directed mutagenesis. Here also, we do not need much information about the protein except the cDNA. We have seen that it enables us to change important features of the enzyme. At the end of the procedure we have at our disposal a cloned gene which, once transformed and over-produced in bacteria, might constitute a good and cheap source of the enzyme. This might be why it has become a technique which is invested in by most of the companies that produce and sell enzymes. The search for a thermophilic enzyme is also possible but not always successful and sometimes it could be more interesting to modify the gene of a mesophilic enzyme by evolutionary molecular engineering.

Site directed mutagenesis enables a more comprehensive approach to the stability problem but generally of marginal use. It has the advantage of producing exactly the desired modification in the sequence of the protein. It is often used to understand the

interactions important for structural stability of protein and more widely used in academic research.

Enzyme formulation (additives) is a significant parameter for the operational and storage stability of enzymes, and hence constitutes a point to which industry attaches a lot of importance. Additives also constitute a quick way to resolve some problems of instability.

Chemical modification has proved to be an efficient way for thermostabilization of proteins, but it lacks predictibility. However it can be useful when we know a little bit about an enzyme as 'a one shot in the dark' technique (to use the expression of Tyagi and Gupta, 1993). It can reveal the involvement of an amino acid in the catalytic site more easily and more quickly than site directed mutagenesis. It can provide some important information about a protein that can be useful to assess whether it is worthwhile to undertake a site directed mutagenesis. For example the ability of guanidination to stabilize a protein is a good indication that the replacement of some well defined lysine residues by Arg might be successful in stabilizing the enzyme.

To conclude, despite all the efforts, research and work performed by biochemists at stabilizing enzymes, we have the feeling that nothing new has really been invented, but rather there has been a decoding and subsequent application of the principles that Nature has set up throughout evolution in creating thermostable enzymes. These principles converge in the maximization of the cohesive forces inside the protein and, to a lesser extent, improvement of its interaction with the solvent.

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