Stability and Stabilization of Biocatalysts
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RIGIDITY OF THERMOPHILIC ENZYMES

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Enzymes and proteins isolated from thermophilic microorganisms are not only unusually stable to heat and protein denaturants, but also display enhanced protein rigidity in respect to that of their mesophilic counterparts. The molecular rigidity of thermophilic enzymes appears to explain why their specific activity at room temperature often is less than that of the corresponding mesophilic enzymes, considering that an appropriate level of protein mobility is required for catalysis. Evidence of protein rigidity can be obtained from hydrogen exchange measurements, molecular dynamics simulations, by computing flexibility indices based on crystallographic data, as well as by proteolysis experiments. Although the structural and functional complexity of proteins likely does not allow firm generalizations, it can be proposed that thermophilic enzymes are rigid molecules, but not optimally active at ambient temperature. Considering that extremophiles appeared earlier on hearth in a hotter environment, it can be suggested that present-day mesophilic enzymes evolved to be more flexible, and thus more labile, in order to optimize their catalytic function.

1. INTRODUCTION

The structural, functional and stability properties of enzymes isolated from thermophilic bacteria have attracted the interest of many investigators in the last two decades (Perutz & Raidt, 1975; Jaenicke, 1981, 1991, 1996; Fontana, 1990; Adams, 1993; Gupta, 1993; Vieille & Zeikus, 1996; Vieille et al., 1996; Arnold, 1998). Thermophilic enzymes possess unusual stability towards the denaturing action of heat and protein denaturants and therefore can be used as biocatalysts under rather harsh environmental conditions (Sonnleitner & Fiechter, 1983; Zamost et al., 1991; Adams et al., 1995; Fágáin, 1995). Moreover, the unusual properties of thermophilic enzymes and proteins prompted their use as suitable protein models for addressing a number of fundamental problems in current protein research (Jaenicke, 1991, 1996). The aims of studies on thermophilic enzymes complement those of modern protein engineering studies by genetic methods, since both studies can ultimately lead to a better and, hopefully, quantitative understanding of the structure-stability-function correlations in

proteins (Matthews, 1991, 1993; Fontana, 1991). Indeed, some useful guidelines for enhancing protein thermostability have been deduced from studies on thermophilic enzymes and successfully applied in protein research (Fersht & Serrano, 1993; Menèndez-Arïas & Argos, 1989; Russell & Taylor, 1995; Fágáin, 1995).

The numerous studies carried out in the past on the molecular properties of thermophilic proteins revealed that quite subtle structural differences between a thermophilic and a mesophilic protein are sufficient to cause the observed unusual stability (Jaenicke, 1981, 1991, 1996; Fontana, 1990). In particular, the analysis of homologous proteins from thermophilic and mesophilic sources in terms of amino acid sequences and three-dimensional structures (Argos et al., 1979; Menéndez-Arias & Argos, 1989; Vogt et al., 1997; Vogt & Argos, 1997) revealed that the enhanced thermostability cannot be attributed to a common determinant, but is the result of a variety of stabilizing effects brought about by hydrophobic interactions, ionic and hydrogen bonding, disulfide bonds, metal binding, and so on. Moreover, the effects of amino acid exchanges in a protein can be cumulative (Wells, 1990), so that few amino acid substitutions, giving each an extra free energy of stabilization of few kcal/mole, can lead to a cumulative effect of significant enhancement of protein stability (Matthews, 1991, 1993; Zhang et al., 1995). Nowadays there is a general consensus that a universal molecular mechanism of thermostability cannot be proposed, since different proteins may be stabilized in different ways. Nevertheless, in a recent study (Vogt et al., 1997), the stabilizing interactions and forces occurring in 16 families of proteins of known three-dimensional structure and different thermal stability have been carefully analyzed. It was concluded that an increased number of hydrogen bonds and salt bridges appears to play a major role in enhancing protein thermostability. The variety of physical and chemical reasons that have been advanced in order to explain the enhanced thermostability of thermophilic enzymes have been recently reviewed (Querol et al., 1996; Vogt et al., 1997).

Why all enzymes in nature have not yet evolved to be more stable, despite the three billion years of evolution? Since this has not occurred, there should be good reasons for it. The hypothesis can be advanced that an enzymatic apparatus constituted by extremely stable and thus rigid enzymes is not required for an organism living in a normal habitat, while thermolabile enzymes are more suited for life under common physiological conditions. Indeed, protein molecules and enzymes exploit their functions via their dynamic nature characterized by backbone and side-chain mobility (Gurd & Rothgeb, 1979; Alber et al., 1983; Fersht, 1985; Kraut, 1988), as well as domain motion (Huber, 1979), and exceedingly stable proteins are therefore too rigid molecules to be optimally functioning (Zuber, 1981; Hochachka & Somero, 1984; Jaenicke et al., 1996; Fontana, 1990, 1997).

In this article we will summarize experimental findings that indicate that the stable thermophilic enzymes are quite rigid molecules at room temperature with respect to their mesophilic counterparts, as a direct consequence of the "clamping" effect of protein structure brought about by the interactions and forces which stabilize thermophilic proteins. No attempt is made here to provide a complete coverage of the

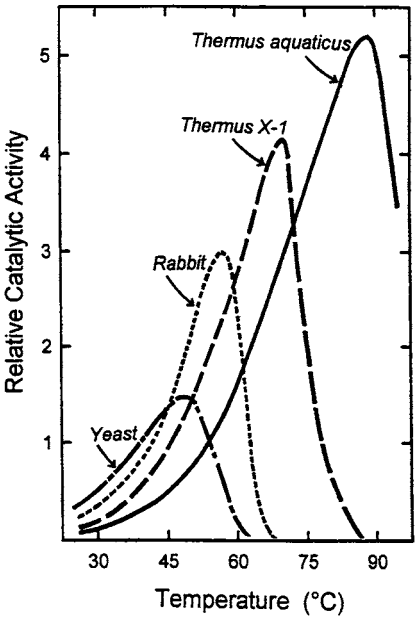

Figure 1. Effect of temperature on the catalytic activity of enolase from rabbit, yeast, *Thermus aquaticus* and *Thermus X-1*. All assay solutions contained 2 mM 2-phosphoglycerate and 1 mM $MgSO_4$. The assay solution was placed in a cuvette and brought to the appropriate temperature in a thermostatted cell holder of the spectrophotometer. The enzymatic reaction was initiated by adding an aliquot of the enzyme solution. Data taken from Stellwagen et al. (1973), Barnes & Stellwagen (1973) and Stellwagen & Barnes (1976).

vast literature dealing with structure, stability, rigidity and functional properties of thermophilic enzymes, so that the reader may find herewith some personal selections and omissions of issues as well. In particular, data will be presented which are in line with the proposed stability-rigidity correlation in thermophilic enzymes, even if we are aware that the complexity of protein molecules does not permit to affirm the strict generality of this correlation. Despite the existence of numerous reviews, monographs and books dealing with the molecular and stability properties of the enzymes isolated from extremophiles, an account which specifically addresses and discusses the rigidity of thermophilic enzymes is missing. The authors therefore hope that their effort will be useful and will prompt new interpretations and ideas, as well as further experimentation.

2. INVERSE CORRELATION BETWEEN PROTEIN STABILITY AND CATALYSIS

Thermostable enzymes isolated from thermophiles are expected to be rigid molecules at room temperature and consequently this rigidity should have an adverse effect on their catalytic efficiency (Zuber, 1981), since it is known that an appropriate degree of flexibility is required for enzyme catalysis (Alber et al., 1983; Fersht, 1985; Kraut, 1988). In fact, thermophilic enzymes are usually poor catalysts at room temperature and assays of their enzymatic activity should be conducted at a temperature close to that of optimum temperature growth of the organism from which the enzyme has been isolated. Often it has been observed that at moderate temperature the specific

activity of a thermophilic enzyme is less than that of its mesophilic counterpart and only at high temperature is sufficiently flexible to be fully active and yet rigid enough not to be denatured (Jaenicke, 1981; Fontana, 1990).

The inverse correlation between enzyme activity and thermostability has been demonstrated in several cases. As shown in Fig. 1, the specific activity (*i.e.*, catalytic efficiency) at 37°C of enolase from rabbit, yeast, *Thermus X-1* and *Thermus aquaticus YT-1* at room temperature is of the inverse order of their thermostability (Stellwagen et al., 1973; Barnes & Stellwagen, 1973; Stellwagen & Barnes, 1976). At high temperature, thermophilic enolases acquire enhanced mobility and catalytic potency, while the mesophilic ones do not resist to the denaturing action of heat.

The relationships between stability, dynamics and activity in 3-phosphoglycerate kinase (PGK) from yeast, the moderate thermophile *B. stearothermophilus* and the extreme thermophile *Thermus thermophilus HB8* have been carefully analyzed by Varley & Pain (1991). This enzyme is a two domain protein operating through a hinge bending mechanism and consequently its activity would be expected to be dependent upon large-scale dynamics due to domain movements (Banks et al., 1979). The thermophilic PGK is more stable than the yeast enzyme, but less active at 25°C. The maximum specific activity of PGK from the three sources reached similar values at the temperature of optimal growth of the organism from which the enzymes are derived. The conclusion of this study was that a proper balance and correlation exist between thermodynamic stability, dynamics and specific activity and, in particular, that increased stability constrains conformational dynamics and enzyme activity (Varley & Pain, 1991). Several other proteins of the same function from mesophilic and thermophilic bacteria exhibit similar levels of activity at the temperature of optimal growth of the organism from which they are derived (Zuber, 1981; Hochachka & Somero, 1984; Somero, 1995; Vihinen, 1987; Rees & Adams, 1995; Jaenicke et al., 1996).

Enhancement of protein stability by site-directed mutagenesis is a major aim of modern protein engineering studies (Alber, 1989; Matthews, 1991, 1993). The proposal that stability/rigidity hampers enzyme catalysis, and protein function in general, predicts that enhanced protein stability would be achieved at the expenses of catalytic potency. Indeed, the inverse correlation between stability and catalysis was documented by measuring stability-activity relationships in four mutants of kanamycin nucleotidyltransferase with single or double amino acid replacement(s) (Matsumura et al., 1986). Matthews and coworkers (Zhang et al., 1995) prepared a series of mutants of T4 lysozyme and demonstrated that the combination of several point mutations in the protein leads to mutant species with an additive increase in thermal stability, but also to a gradual decrease in activity. Moreover, the hypothesis that amino acid residues at the active site of an enzyme are mobile and thus not optimized for protein stability has been tested by analyzing the effects of amino acid replacements at the active site of T4 lysozyme (Shoichet et al., 1995). It has been demonstrated that it is possible to produce active site mutants with improved stability but reduced activity. A variety of protein engineering experiments designed to increase protein stability also resulted the production of more stable mutants at the expenses of catalysis or of other functional

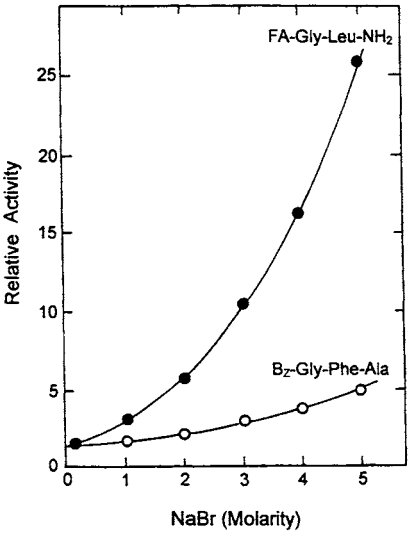

Figure 2. Salt-mediated activation of thermolysin. The enzymatic assays were conducted at 25°C in Tris buffer, pH 7.5, in the presence of the indicated concentrations of NaBr with furylacryloyl-glycyl-L-leucine amide (FAGLA) (●) or benzoyl-glycyl-phenylalanyl-alanine (○). Data taken from Holmquist & Vallee (1976).

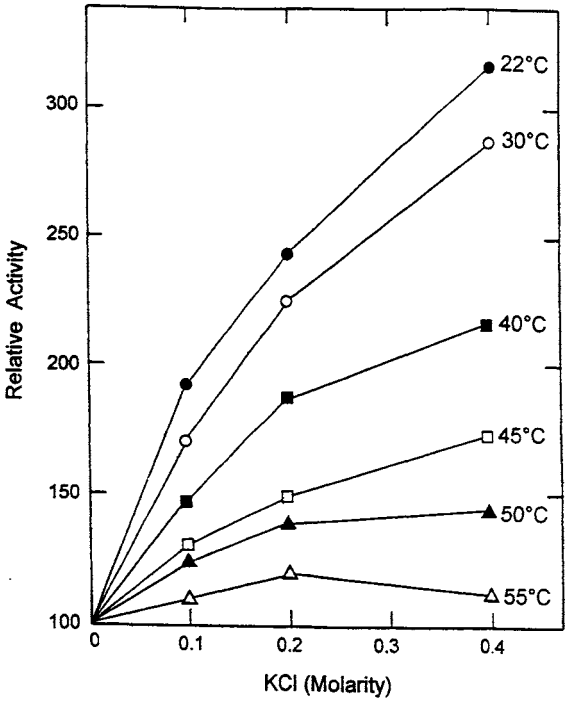

Figure 3. Activation by KCl of isocitrate lyase from *Bacillus stearothermophilus BS1*. The enzymatic assays were conducted under standard conditions in the presence of various concentrations (0-0.4 M) of KCl at 22°C (●), 30°C (○), 40°C (■), 45°C (□), 50°C (▲) and 55°C (△). Data taken from Griffiths & Sundaram (1973).

properties of the proteins (see Shoichet et al., 1995, for a list of these experiments). Of interest, Kidokoro et al. (1995) prepared a series of mutants of thermolysin by replacing the Gln residue in position 119 of the polypeptide chain. All mutants were less thermostable than the wild-type species, but displayed a much improved enzyme activity, in line with the proposed correlation between enzyme lability/flexibility and catalysis.

3. ENZYME ACTIVATION AT LOW DENATURANT CONCENTRATION

Thermophilic enzymes can be activated in the presence of protein denaturants (salts, organic solvents, urea, guanidine hydrochloride) at moderate concentrations. For example, glyceraldehyde-3-phosphate dehydrogenase from *Thermus thermophilus* is activated when salts and ethanol are added to the enzymatic assay solution (Fujita et al.,

1976). 6-Phosphogluconate dehydrogenase from *B. stearothermophilus* is more active in the presence of organic solvents (dioxane, dimethylformamide, acetone) (Veronese et al., 1984). Similarly, a number of organic solvents have been shown to substantially activate the malic enzyme from the extreme thermoacidophilic archaebacterium *Sulfolobus solfataricus* (Guagliardi et al., 1989). The catalytic activity of malate dehydrogenase isolated from several thermophilic bacteria is strongly activated, if the assays are performed in the presence of 3-20% acetone, 4-8 M urea or 0.5-2 M guanidine hydrochloride (Sundaram et al., 1980). Triosephosphate isomerase from *Thermotoga maritima* is activated (up to 180%) when assayed in the presence of 2 M guanidine hydrochloride (Beaucamp et al., 1997).

Fig. 2 and 3 illustrate the salt-mediated activation effects for thermolysin (Holmquist & Vallee, 1976) and isocitrate lyase from *B. stearothermophilus BS1* (Griffiths & Sundaram, 1973), respectively. It is seen that thermolysin can be activated up to about 25-fold and 5-fold if assayed in the presence of 5 M NaBr with furylacryloyl-Gly-Leu-NH_2 or benzoyl-Gly-Phe-Ala as substrate, respectively (see Fig. 2). The data of Fig. 3 indicate that the KCl-mediated activation of thermophilic isocitrate lyase occurs at moderate temperature only, while at 55°C the salt activation is marginal. These data can be interpreted as indicating that at relatively high temperature the thermophilic enzyme becomes flexible enough to be optimally active, while at 22-30°C some salt-mediated weakening of the interaction and forces leading to its rigid structure is required for improving its catalytic potency.

These various observations can be taken as an indication that thermophilic enzymes are activated by some loosening of their structure in the presence of protein perturbants. The denaturant-mediated enhancement of catalysis can result from a very limited change of structure/dynamics of the enzyme. Far-UV circular dichroism or fluorescence emission measurements do not detect changes in secondary and tertiary structure, respectively, with activated *Thermotoga* triosephosphate isomerase dissolved in the presence of 2 M guanidine hydrochloride (Beaucamp et al., 1997).

4. ANALYSIS OF PROTEIN RIGIDITY/FLEXIBILITY

The rigidity of thermophilic proteins has been verified utilizing hydrogen exchange measurements (Wraba et al., 1990; Rehaber & Jaenicke, 1992), fluorescence quenching (Varley & Pain, 1991) and theoretical calculations (Vihinen, 1987; Vihinen et al., 1994; Lazaridis et al., 1997). The H-D exchange rates in globular proteins are a reflection of protein mobility/flexibility (Delepierre et al., 1983), as documented for example by the inverse correlation between the melting temperature (T_m) and H-D exchange rates in a series of derivatives of basic pancreatic trypsin inhibitor (Wüthrich & Wagner, 1979; Wüthrich et al., 1980). The higher the exchange rate, and thus the higher the flexibility, the lower is the denaturation temperature. Elongation factor Tu of *Thermus thermophilus* shows a reduced rate of exchange in respect to the more thermolabile *E. coli* factor (Tsuboi et al., 1978).

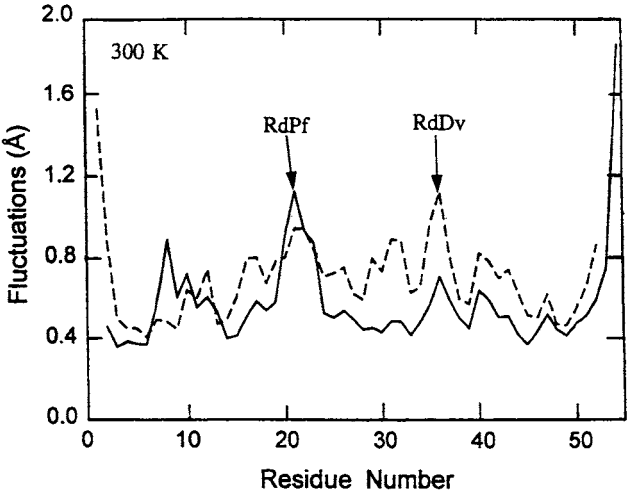

Figure 4. Fluctuations along the polypeptide chain of rubredoxin from *Pyrococcus furiosus* (RdPf) (solid line) and from *Desulfovibrio vulgaris* (RdDv) (dashed line). Molecular dynamics simulations were performed at 300 K and the simulation extended to 400 ps. Data taken from Lazaridis et al. (1997).

Since the rate of quenching of tryptophan fluorescence in a globular protein is dependent upon the fluctuation of protein structure (Eftink & Ghiron, 1976, 1981), Varley & Pain (1991) measured the ability of acrylamide to quench the fluorescence of a buried tryptophan in thermophilic and mesophilic 3-phosphoglycerate kinase. At 25°C the rate of quenching was more than one order of magnitude less in the *Thermus* than in the yeast enzyme.

The increased rigidity of thermophilic proteins in respect to their mesophilic counterparts was also documented by calculating protein flexibility indices (F) derived from normalized *B*-values (temperature factors determined crystallographically) of individual amino acids in several refined three-dimensional structures of globular proteins. The results of these calculations showed that F-values correlate with protein stability, *i.e.*, that rigidity correlates with thermostability of proteins (Vihinen, 1987; Vihinen et al., 1994).

More recently, a comparative analysis of the molecular rigidity of a thermophilic and a mesophilic protein has been conducted utilizing molecular dynamics simulations (Lazaridis et al., 1997). At room temperature, the fluctuations along the polypeptide chain of rubredoxin from the hyperthermophilic archaeon *Pyrococcus furiosus* (RdPf) are somewhat reduced in respect to those of the homologous rubredoxin from the mesophile *Desulfovibrio vulgaris* (RdDv) (see Fig. 4). The average value of fluctuation for all residues is 0.8 Å for RdPf and 0.9 Å for RdDv. Residues 2-25 show similar fluctuations in the two proteins, while residues 25-50 exhibit higher fluctuations in the mesophilic protein.

5. PROBING OVERALL AND LOCAL PROTEIN FLEXIBILITY BY PROTEOLYSIS

The overall rigidity of the stable proteins from thermophilic microorganisms can be evidenced by the use of proteolytic enzymes as probes of protein structure and

dynamics (Fontana et al., 1993). In analogy to all enzymatic reactions, the proteolytic cleavage of a polypeptide chain occurs only if the site of cleavage can bind and adapt itself in a specific way to the stereochemistry of the active site of the protease. This is difficult to achieve with native globular proteins, whereas denatured/unfolded proteins are much more susceptible to proteolysis. On this basis, it is expected that only the unfolded species of a globular protein is attacked by a protease and that the actual equilibrium between the native (N) and denatured (D) form of protein is controlling the rate of protein degradation. The N↔D equilibrium is expected to be shifted towards the N form under physiological conditions with the stable thermophilic proteins, so that proteolysis would be hampered. The correlation between protein stability/rigidity and resistance to proteolysis is also substantiated by the fact that proteins with relatively short half-lives *in vivo* have high protease susceptibility and are generally unstable *in vitro* (Goldberg & Dice, 1974; McLendon & Radany, 1978).

Unusual resistance to proteolysis of thermophilic enzymes has been documented in a number of cases, thus verifying their overall rigidity. 6-Phosphogluconate dehydrogenase from *B. stearothermophilus* is much more resistant at room temperature to proteolysis than the same enzyme from yeast (Veronese et al., 1984). Similarly, the activity loss due to proteolysis of asparaginase and β-galactosidase from thermophilic and mesophilic sources correlates with the thermal instability of the enzymes (Daniel et al., 1982). Oligo-1,6-glucosidase from *B. thermoglucosidicus* was more resistant against proteolysis than its homologous counterpart from *B. cereus* (Suzuki & Imai, 1982). An inverse correlation between protein stability (and thus rigidity) and proteolytic degradation was reported for mutants of the α-subunit of tryptophan synthetase from *E. coli* (Ogasahara et al., 1985). Similar observations were reported for mutants of kanamycin nucleotidyl transferase (Matsumura et al., 1986) and T4 lysozyme (Schellman, 1986).

In a recent study, proteolysis experiments were employed to monitor the stability/rigidity of mutants of neutral protease from *B. stearothermophilus* prepared by introducing Pro residues at the level of the active site helix of the enzyme (Nakamura et al., 1997). As shown in Fig. 5, there is an inverse correlation between protein thermal stability, and thus rigidity, and rate of chymotryptic hydrolysis of the Xaa→Pro mutants.

A systematic study of the behaviour of thermophilic enzymes towards proteolysis was conducted using the metalloendopeptidase thermolysin as a model protein (Fontana et al., 1986). In this case, specific experimental conditions of limited proteolysis by added protease or autolysis of thermolysin permitted the isolation and characterization of several "nicked" thermolysin species constituted by two as well as three fragments associated in stable complexes. The pattern of limited protein fragmentation of thermolysin allowed to infer molecular aspects of the protein-protein recognition process underlying the proteolytic event and, in particular, to establish that flexible loops of the thermolysin molecule are the most susceptible sites of proteolysis. Of note, a striking correlation was shown to exist between sites of proteolysis and sites of high segmental mobility determined crystallographically (*B*-values). The conclusion was

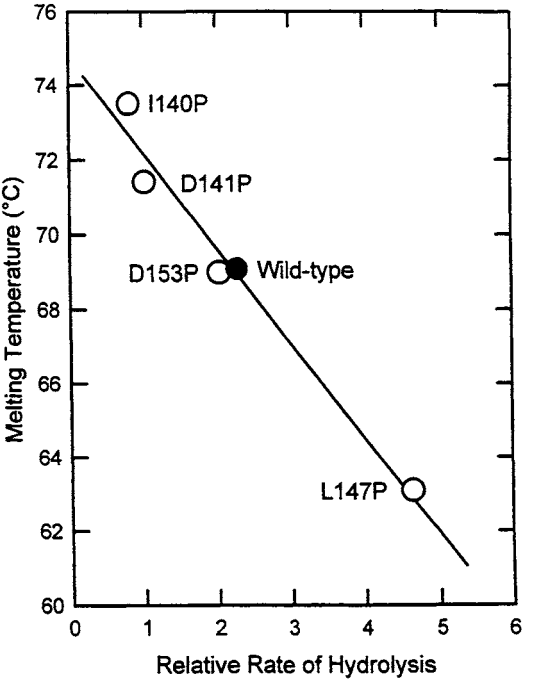

Figure 5. Inverse correlation between thermostability and susceptibility to proteolysis of wild-type (●)and mutant (○) *B. stearothermophilus* neutral protease. The protease was dissolved at 37°C in 50 mM Tris-HCl buffer, pH 7.5, containing 5 mM $CaCl_2$ and 3 mM phosphoramidon and then heated at 70°C for 10 min. Under these solvent conditions the *B. stearothermophilus* protease is inactivated by removal of its functional zinc ion, while the stabilizing calcium ions remain bound to the protein. Proteolytic digestion was conducted at 37°C by adding α-chymotrypsin at an enzyme to substrate ratio of 1:10 (by weight). The relative rate of initial hydrolysis was determined by quantitation of the free amino groups formed during digestion. The thermostability of wild-type and mutant neutral protease was evaluated from the irreversible thermal denaturation curves from 20 to 90°C of the circular dichroism signal at 222 nm *versus* temperature. From these curves the melting temperature (T_m) was calculated. The single site mutants of neutral protease (I140P, D141P, L147P and D153P) are indicated in the figure, while the number refers to the site of the polypeptide chain where the Xaa→Pro exchange has been introduced. Data taken from Nakamura et al. (1997).

reached that a mechanism of local unfolding dictates the phenomenon of limited proteolysis of globular proteins (Fontana et al., 1986). On this basis, a procedure was proposed for probing the sites of flexibility of a protein by proteolysis experiments (Signor et al., 1990), as well as for stabilizing proteins against proteolytic degradation by site-directed mutagenesis of the protein sites most prone to proteolysis (Braxton & Wells, 1992; Fontana et al., 1993; Rosè et al., 1993; Van den Burg et al., 1998a).

6. ENGINEERING PROTEIN STABILITY/RIGIDITY

The advent of site-directed mutagenesis techniques made possible in recent years to produce a large variety of protein mutants and, in particular, to analyze the forces and interactions thought to be important in determining protein stability (Alber, 1989; Matthews, 1991, 1993). These numerous studies allowed a detailed, and often quantitative, analysis of the contributions to protein stability of hydrogen bonds, electrostatic interactions, hydrophobic effects, disulfide bridges, ion binding and so on (Dill, 1990). As a result of these intensive and systematic investigations, some strategies

for improving protein stability by the use of genetic methods have been developed. As a matter of fact, the successful production of engineered variants of proteins that are more stable than the wild-type protein has been often described (Matthews et al., 1987; Matthews, 1991, 1993; Matsumura et al., 1986, 1989; Fontana, 1991; Zhang et al., 1995; Van den Burg et al., 1998b). The results of protein engineering experiments complement those derived from studies on thermophilic enzymes, since both approaches aim to unravel structure-stability relationships in proteins (Fontana, 1991; Fersht & Serrano, 1993; Russell & Taylor, 1995). Indeed, some strategies of protein stabilization developed by protein engineers parallel those followed by nature in engineering by evolution and natural selection the stable enzymes from thermophilic microorganisms. In the following, few selected examples of both stabilization and rigidification of proteins by genetic methods will be briefly mentioned.

The stabilizing role of protein-bond calcium ions has been extensively documented with both mesophilic and thermophilic proteins (Roche & Voordouw, 1978; Fontana, 1991; Tainer et al., 1992; Nayal & Di Cera, 1994, and ref.es cited therein). Calcium binding is expected to induce and stabilize protein structure and consequently also to rigidify a protein molecule at the site of the ion binding, since Ca^{2+} can bind up to nine ligands. Themolysin binds four Ca^{2+} ions, while the thermolabile mesophilic protease from *B. subtilis* only two ions, despite the high sequence similarity between the two proteins (Roche & Voordouw, 1978). Genetic methods have been used by Toma et al. (1991) to replace a surface loop (from residue 188 to residue 194) of *B. subtilis* neutral protease with a 10-residue segment which, in the homologous polypeptide chain of thermolysin, binds Ca-4. The mutant protease was shown to bind an additional calcium ion and, moreover, to display enhanced stability in respect to the wild-type species. Engineering of calcium binding sites into proteins has proved to be aviable procedure for enhancing stability/rigidity of proteins (see Fontana, 1991; Tainer et al., 1992, for ref.es).

A comparative analysis of amino acid sequences and three-dimensional structures of homologous proteins from mesophilic and thermophilic sources indicated that Gly→Ala is the most frequent "cold to hot" amino acid exchange often occurring at helical segments (Argos et al., 1979; Menéndez-Arias & Argos, 1989; Vogt & Argos, 1997; Vogt et al., 1997). In the context of present discussion of rigidity of thermophilic enzymes, it is of interest to observe that Gly is the most flexible amino acid residue among the protein amino acid residues (Yan & Sun, 1997), while Ala is the best helix-inducer (O'Neil & De Grado, 1990; Chakrabartty et al., 1991). Thus, the Gly→Ala exchange appears to be a strategy followed by nature in engineering protein stability, as well as rigidity. The same amino acid replacement was proposed and utilized by Matthews et al. (1987) for enhancing the stability of T4 lysozyme, considering that the Gly→Xaa exchange is expected to indirectly stabilize the folded protein by decreasing the chain entropy of the unfolded state. This strategy of protein stabilization has been successfully employed by Margarit et al. (1992) for enhancing the thermostability of the mesophilic neutral protease from *B. subtilis*. Single and double Gly→Ala mutants of this

protease, which is homologous to thermolysin, were more stable than the wild-type protein. Moreover, it was shown that mutational effects were cumulative (Wells, 1990).

The most rigid amino acid residue in proteins clearly is Pro, as a result of the steric constrains of the pyrolidine ring of this residue. Accepting the view that there is a correlation between rigidity and stability, one would predict that thermophilic enzymes should possess enhanced content of Pro residues. Indeed, Suzuki et al. (1987) has proposed the "proline rule" for thermophilic enzymes, in that they contain an enhanced number of Pro residues in respect to their mesophilic counterparts. The introduction of Pro residues into a protein was therefore utilized as a strategy for stabilizing proteins. Mutants of oligo-1,6-glucosidase from *B. cereus* with multiple Pro exchanges (up to nine residues) were thus produced and shown to be significantly and cumulatively stabilized against thermal denaturation (Watanabe et al., 1994). The same Xaa→Pro replacement for enhancing protein stability was also proposed by Matthews et al. (1987) on the basis of entropic considerations of protein stability (see above). Since the Pro residue is a helix-breaker (O'Neil & De Grado, 1990), the Xaa→Pro replacement should occur at the level of loops in the protein molecule. If the same exchange is placed in the middle of a helical segment, the protein becomes less thermostable (Nakamura et al., 1997).

The concept of protein stabilization by rigidification has been recently applied by De Filippis et al. (1998) introducing by a semi-synthetic procedure α-amino-isobutyric acid (Aib, or α-methylalanine) at selected positions in a folded 62-residue fragment of thermolysin (C-terminal subdomain 255-316). The geminal methyl groups of Aib severely reduce the conformational space of this amino acid residue and thus restrict the peptide backbone torsion angles to those characteristic of the helical configuration. Consequently, the Xaa→Aib exchange is expected to rigidify and stabilize a protein in analogy to the Gly→Ala or Xaa→Pro exchange (Matthews et al., 1987). Indeed, a double Ala→Aib replacement in thermolysin fragment 255-316 enhanced the melting temperature from 63.5 to 71.5°C (ΔT_m= 8°C) (De Filippis et al., 1998).

7. DISCUSSION

The rigidity of the stable enzymes that can be isolated from thermophilic and extremophilic microorganisms is documented by a variety of experimental findings. The major effect of this protein rigidity is in reducing the catalytic efficiency of thermophilic enzymes in respect to that of their mesophilic counterparts and thus thermophilic enzymes are usually poor catalysts at room temperature. It is proposed that a rigid protein structure does not allow correct proximity and positioning of the key residues required for an efficient catalysis, which requires instead a somewhat flexible protein architecture to bind and properly accomodate the substrate at the active site, to perform the catalytic event and to release the product of the enzymatic reaction (Alber et al., 1983; Fersht, 1985). Considering that extremophiles appeared earlier during biological evolution (Stetter et al., 1990; Stetter, 1993), it can be proposed that the more flexible (and allosteric) mesophilic enzymes derived from thermophilic ones and, since at

ambient temperature catalysis is reduced, evolution created more efficient biocatalysts by improving their plasticity (for an alternative view see Danson et al., 1996).

Having proposed that thermophilic enzymes are more rigid molecules, one should expect that psychrophilic enzymes should be even more flexible than the mesophilic ones. Indeed, the thermolabile enzymes isolated from psychrophilic microorganisms have been shown to possess improved catalytic activity in respect to that of their mesophilic or thermophilic counterparts. The molecular basis for this effect has been analyzed utilizing the structural models of some psychrophilic enzymes produced by homology modelling (Feller et al., 1996; Narinx et al., 1997) or by direct determination of their X-ray structure (Aghajari et al., 1996; Feller et al., 1996). In general, it has been observed that psychrophilic enzymes possess enhanced overall flexibility in respect to their mesophilic or thermophilic enzymes, as judged from physicochemical data (Feller & Gerday, 1997). It has been proposed that the structural factors favouring the conformational flexibility of psychrophilic enzymes likely reside in a reduced number of salt bridges, shorter chain loops, less proline residues in loops, low hydrophobicity of protein core(s) and weaker binding of calcium ion(s) (Feller et al., 1996; Narinx et al., 1997). In general, it can be stated that the results of structural, functional and stability studies on psychrophilic enzymes parallel those conducted on thermophilic and extremophilic enzymes, since the results of both studies indicate the same factors and interactions as modulators of protein stability/rigidity/flexibility (see Feller & Gerday, 1997; Gerday et al., 1997, for a review). As an example, a higher or lower content of Pro residues in loops appears to correlate with enhanced or reduced thermostability in thermophilic and psychrophilic enzymes, respectively (Feller & Gerday, 1997). In conclusion protein flexibility appears to be a main reason for the enhanced activity of psychrophilic enzymes, while protein rigidity explains why thermophilic enzymes are poor catalysts at room temperature.

Nevertheless, enzyme catalysis is a complex process involving a proper stereochemistry of the active site, specific binding of the substrate, participation of functional (acid or basic) groups characterized by specific pK values, a microenvironment at the active site of defined dielectric constant, etc. Since proteins must move in order to function, an efficient active site requires some mobility, but it is clear that the molecular mechanism of enzyme catalysis is the result of a subtle balance between a variety of energetic, structural and dynamic effects and, consequently, perhaps it is not appropriate to overemphasise the role of protein mobility in catalysis. We are inclined to consider therefore protein rigidity of thermophilic enzymes an often made observation, but we are aware that exceptions can exist. For example, a laboratory evolution technique has been devised by using an engineered *E. coli* containing the LeuB gene coding for the isopropylmalate dehydrogenase from the extreme thermophile *Thermus thermophilus* (Topt 80°C). Upon prolonged incubation at moderate/ambient temperature, *E. coli* mutants able to grow rapidly were obtained, producing isopropylmalate dehydrogenase mutants which were shown to retain the unusual thermostability of the wild-type enzyme from *Th. thermophilus*, while displaying high catalytic activity even at ambient temperature (Suzuki et al., 1997). Since it is possible

therefore to produce by evolution stable proteins still maintaining high catalytic activity at mesophilic temperatures, it is clear that the inverse correlation between thermostability and functional efficiency of a protein is a common finding and not a rule. It can be concluded therefore that there are several ways to develop a functional protein, as well as to improve protein stability. The major conclusion deriving from the vast amount of experimental data accumulated so far from protein engineering studies (Matthews, 1987, 1993), as well as from studies on thermophilic enzymes (Jaenicke et al., 1996; Fontana, 1990, 1991), is that guidelines only and not generally applicable rules can be advanced for explaining at a molecular level and for modulating protein structure/stability/function, since protein mutations can be strongly context dependent (Russell & Taylor, 1995; Arnold, 1998).

Acknowledgements. This study was conducted with financial support from the Italian National Research Council (CNR) and from the Ministery of University and Scientific Research (MURST).

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