[Frontiers in Bioscience 5, d787-795, September 1, 2000]

PROTEIN STABILITY IN EXTREMOPHILIC ARCHAEA

Roberto Scandurra [1], Valerio Consalvi [1], Roberta Chiaraluce [1], Laura Politi [1], Paul C. Engel [2]

[1] *Dipartimento di Scienze Biochimiche "A.Rossi-Fanelli" Università 'La Sapienza', P.le A.Moro 5 00185 Rome Italy,*
[2] *Department of Biochemistry, Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland*

TABLE OF CONTENTS

1. ABSTRACT

Extremophilic microorganisms have adapted their molecular machinery to grow and thrive under the most adverse enviromental conditions. These microorganisms have found their natural habitat at the boiling and freezing point of water, in high salt concentration and at extreme pH values. The extremophilic proteins, selected by Nature to withstand this evolutionary pressure, represent a wide research field for scientists from different disciplines and the study of the determinants of their stability has been an important task for basic and applied research. A surprising conclusion emerges from these studies: there are no general rules to achieve protein stabilization. Each extremophilic protein adopts various strategies and the outstanding adaptation to extreme temperature and solvent conditions is realized through the same weak electrostatic and hydrophobic interactions among the ordinary amino acid residues which are also responsible for the proper balance between protein stability and flexibility in mesophilic proteins.

2. INTRODUCTION

Microorganisms that live under extreme conditions of life, e.g.close to the freezing point or the boiling point of water, are termed 'extremophiles'. The extreme environments, probably similar, in some cases, to those that existed during early periods of life on earth, have provided this name and are an essential part of the growth conditions for these microorganisms. The extremophilic microorganisms so far known belong to different taxa within the archaeal and bacterial domains, and the majority of these 'exotic' microorganisms are found within the Archaea. Representative among them are the hyperthermophile *Pyrococcus furiosus* (optimal growth temperature above 100°C), the thermoacidophile *Sulfolobus acidocaldarius* (growth temperature 80°C at pH <2.75), the alkaliphile *Natronobacterium pharaonis* (optimal growth at pH > 10), the halophile *Haloferax mediterranei* (living at >10% salt) and the psychrophile *Cenarchaeum symbiosum* (growth only below 25°C).

The phylogenetic tree constructed on the basis of the 16/18 SrRNA sequence (1), shows a primary tripartite division of the living world into the three domains of Bacteria, Archaea and Eukarya (2). The deepest branches and the shortest lineages represent the most extreme hyperthermophiles known so far (*Pyrodictium, Methanopyrus, Aquifex*). These Archaea live in the hottest places on earth and are considered to be the oldest living organisms. To thrive in such adverse conditions evolution has devised some peculiar mechanisms such as the formation of very resistant macromolecular structures able to defend the archaeal cell from the hostile environment. Evolutionary pressure has, for instance, selected for the Archaea particular cell membranes more resistant than those of mesophiles. In particular, the lipid bilayer, whose fatty acids are commonly bound to glycerol molecules through ester linkages in mesophiles, is replaced by a monolayer membrane highly resistant to heat, acid and alkali, with glycerol molecules bound by ether linkages to branched hydrocarbons of the phytanyl or biphytanyl type. Another difference is that the central carbon atom of the glycerol is in the R stereoisomeric form in Bacteria and Eukarya and in the L form in Archaea. Accordingly, not only the cell membrane but all the archaeal macromolecular cellular components are expected to be highly resistant to physical and chemical stresses and possibly assembled from stable chemical compounds.

Proteins from Eukarya and Bacteria, extremophilic or otherwise, are composed of amino acids linked by strong covalent bonds which

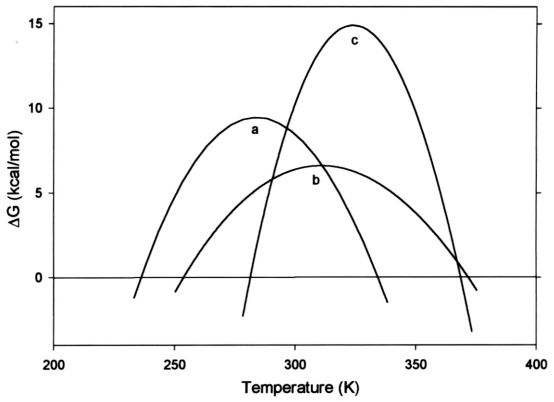

Figure 1. Hypothetical different strategies in thermophilic adaptation. The stability curves of a mesophilic (a) and two thermophilic (b, c) proteins are represented. Protein b is characterized by an increase in melting temperature and by a decrease of thermodynamic stability; protein c is characterized by an increase in melting temperature and in thermodynamic stability. The intercepts at DeltaG=0 correspond to the temperature values for cold and heat denaturation.

confer on the primary sequence resistance to acid, alkali and heat. Proteins, however, gain the native three-dimensional structure essential for their biological functions through the concomitant positive contributions of weak forces such as hydrophobic, electrostatic and Van der Waals interactions whose bonding energies (2-10 kcal/mol) are at least one order of magnitude lower than those (~100 kcal/mol) of any covalent bond. In general, proteins lose their native and biologically active conformation when some or all of these weak interactions are lost. These weak bonds have a profound physiological importance since they can be easily formed and/or broken thus contributing to the protein plasticity and flexibility, essential to function and for adaptation to all the variable conditions during the life of the cell. On the other hand, the three-dimensional structures of proteins from Eukarya and Bacteria are generally poorly resistant to acid, alkali and heat, with a few exceptions, such as the enzyme pepsin which is active at pH 2 and maintains its native three-dimensional structure at this extreme pH value.

In 1972, the discovery of the first hyperthermophilic bacterium living at above 80°C extended the known limits of possible life on earth (3). Scientists from various disciplines were greatly attracted by these microorganisms, and their proteins rapidly became targets for both academic and applied research (4). The proteins extracted from these microorganisms retained their thermal resistance after purification with optimal temperatures for activity close or identical to the growth temperature of the extremophilic microorganism. These results suggested that thermal resistance was an intrinsic property of the protein structure independent of the presence of some particular compounds produced in the microorganism, although some stabilizing factors as polyamines and ectoines (5) have been found occasionally in some extremophiles. A further confirmation of the intrinsic thermostability of the proteins from extremophiles was obtained by the production of a thermoactive and thermostable recombinant protein in a mesophilic host (6, 7). Thermotolerance was thus encoded in the genetic blueprint and could be ascribed either to the presence of some particular amino acid residues or to a different and/or increased number of the weak interactions that are essential for the stability of the native three-dimensional structure. The search for peculiar amino acid residues in the proteins of these archaeal microorganisms responsible of their high stability was fruitless and revealed only the same 20 ordinary amino acids found in mesophilic counterparts, thus indicating the importance of studying three-dimensional structure to understand stability.

3. PROTEIN STABILITY

3.1. Thermodynamic aspects of protein stability

The difference in free energy, Delta*G*, measurable from the reversible transition from the native to the denatured state, is always a small number (5-17 kcal/mol) (8, 9) and results from a sum of all the large stabilizing and destabilizing interactions in the polypeptide chain. Delta*G* is a useful parameter to describe quantitatively a protein's conformational stability. Denaturation, i.e. the loss of tertiary interactions, secondary structure elements and native properties, is always accompanied by the exposure of the hydrophobic residues to the aqueous solvent. This event is considered to be the cause of the large and positive heat capacity change, Delta*C*p, observed upon protein unfolding (10) and measurable by calorimetry. The burial of non polar-surfaces and, in general, the hydrophobic driving force (11, 12) is directly related with this latter thermodynamic property, and about 95 % of the change in *C*p upon denaturation can be attributed to the increase in hydration. The change in protein Delta*G* as a function of temperature is represented by a curve which intersects the temperature axis twice, indicating that protein may denature at low as well as at high temperature, with a maximum usually in the range between 10 and 40 °C for mesophilic proteins (7). Proteins can adapt to exert their functions at the temperature of the psychrophilic, mesophilic or hyperthermophilic environments by changes of their stability curves (figure 1) obtained by selective modifications of those weak interactions responsible for maintaining the compactness of structural elements which form the native structure. These modifications result in higher melting temperatures or in a different temperature resistance by shifting, broadening or raising the stability curves, and in some cases through a combined effect of all these changes. The maximum free energy of stabilization in any protein, independently of its size and structure and of its temperature adaptation and resistance, is always in the range of few weak interactions and it results from the competition between stabilizing and destabilizing interactions. Among these latter one may mention the decrease in conformational entropy, and the removal of peptide and polar groups from the solvent which counteract the stabilizing terms of hydrogen bonding and desolvation of hydrophobic groups (13, 14). The structural analysis of proteins from extremophiles may highlight the differences from mesophilic homologs that provide the basis for increased thermal resistance and decreased flexibility.

3.2. Proteins from (hyper)thermophiles

Thermostable proteins produced by thermophilic and hyperthermophilic microorganisms between 45 and 110°C are generally very resistant also to chemical denaturation and to proteolysis (7). Thermophilic and hyperthermophilic proteins, for example, require higher concentrations of urea and guanidinium chloride for denaturation (4-8M) (15, 16) than their mesophilic (1-2M) (17-19) and psychrophilic counterparts. This resistance may reflect a restriction on the flexibility of thermophilic proteins, which allows them to be functionally competent at elevated temperatures and unusually rigid at mesophilic temperatures (10-45°C). Increased rigidity at room temperature has been demonstrated not only by indirect evidence such as the higher resistance to denaturants but also more directly through the measurement of the exchange rates of amide protons (7, 20). The increased rigidity of proteins from hyperthermophiles at mesophilic temperatures may find a structural determinant in an increased compactness which can be obtained with a decrease in both number and size of internal cavities as compared with mesophilic counterpart (21, 22).

The high intrinsic stability of the thermophilic proteins becomes marginal at their growth temperature where thermal motion induces a decrease of the protein structural rigidity yielding to an increase in flexibility which is essential for function. The adaptation of proteins to extreme temperatures appears then to be the result of a compromise between the increased rigidity responsible for thermal stability and the flexibility required for playing their physiological roles.

Hyperthermophilic archaea, near the upper temperature limits for life, may acquire thermotolerance after a short heat shock by producing a 60-kDa protein with structural and functional features of bacterial chaperonins (23) whose role in archaeal cells is not yet clear. Despite the presence in Archaea of some particular solutes as ectoines, which can increase proteins' thermal resistance, their role in conferring protein thermotolerance in Archaea has not been demonstrated (5). Thus, the only well-established strategy for the archaeal cell to produce thermotolerant proteins is to combine all the canonical weak forces which are the basis of native folding also in mesophilic proteins (electrostatic, Van der Waals and hydrophobic interactions) so that the resulting cumulative effect will yield a protein with a high intrinsic stability. This cumulative effect, induced by minute local interactions of protein structural elements, leads to the stable conformation of the native state. The native three-dimensional structure is essential for the protein's biological functions, such as ligand binding or enzyme catalysis, and is stable over a determined range of physical and chemical conditions. The stability, however, has to find a compromise with functionality, and the protein's native state fluctuates among various preferred conformations to exert its biological functions, despite the maintenance of its unique spatial arrangements (24). The physiological fluctuations of the native structure comprise either small movements of a few amino acids or major structural rearrangements such as repositioning of a large chain segment or changes in the relative interdomain orientation. This is presumably at the origin of protein marginal stabilities and the net result from the balance between stabilizing and destabilizing forces is represented by only few weak intermolecular interactions, as indicated by the free energy change of the denaturation transition which is always in the range 5-17 kcal/mol (8, 9). Notably, proteins from extremophiles do not deviate significantly in this respect from these general rules established for mesophilic proteins. Their temperature adaptation is always accompanied by free energies of stabilization similar to those of their mesophilic counterparts or only slightly increased. An important difference between the mesophiles and thermophiles is the reduced catalytic activity of thermophilic enzymes around 25°C due to the decrease in their flexibility. Adaptation, then, may be considered as a temperature shift of the subtle balance between flexibility and rigidity to the temperature range close to the optimal growth condition of the organism (7, 20).

3.3. Proteins from psychrophiles

Extremophilic microoorganisms living close to the freezing point of water are named psychrophiles. Some of them belong to the Archaea such as the two methanogens *Methanogenium frigidum* (Tmin= -10°C; Topt=15°C) (25) and *Methanococcoides burtonii* (Tmin= -2.5°C; Topt = 23°C) (26), and one halophile *Halorubrum lacusprofundi* (Tmin= 2°C;Topt= 33°C) (26). The potential biotechnological applications of these microorganisms have recently aroused growing interest. The functional properties of the proteins extracted from the psychrophiles are characterized by opposite features to those found in proteins from thermophiles despite the presence of the same ordinary amino acids in their primary structure. Adaptation to low temperatures is achieved also for psychrophilic proteins through a balance of the same interacting weak forces which allow resistance to high temperatures.

The structural features of proteins from psychrophilic microorganisms may be determined from comparative studies of mesophilic and thermophilic proteins, based in some cases on homology modelling, together with analysis of recently determined crystal structures for four psychrophilic proteins: alpha-amylase (27), citrate synthase (28), alkaline protease (29) and triosophosphate isomerase (30). These studies lead to the following conclusions: cold adapted proteins have evolved to be structurally flexible and catalytically efficient at cold temperatures. These properties are obtained through small changes in protein secondary and tertiary structures such as the decrease in the number of disulphide bonds, a decrease of net charge in helix-dipole structures, a decrease of protein-solvent interactions, a decrease in the number of hydrogen bonds at domain interfaces and a general decrease in the number of hydrophobic interactions within the core of the protein (31-33) as compared to mesophilic counterpart.

4. STRUCTURAL DETERMINANTS OF PROTEIN STABILITY

After this general premise we will start to analyze in detail the results obtained from the structural studies of extremophilic proteins to search for those interactions mainly involved in protein stabilization. For this purpose

we will take into account the analysis of primary structure, the structural modifications obtained through electrostatic and hydrophobic interactions and the crystallographic parameters.

4.1. Primary structure analysis

The comparative analysis of the amino acid composition conducted on a restricted number of the same extremophilic and mesophilic proteins (34, 35) indicates some amino acid substitution in thermophilic proteins. The amino acid exchanges, such as Lys to Arg, Ser to Ala, Gly to Ala, Ser to Thr and Val to Ile, found in thermophilic proteins were considered one of the possible determinants of protein thermal stability (35). However a statistical analysis performed on a greater number of mesophilic, thermophilic and halophilic proteins revealed the inconsistency of this assumption (36).

The availability of the complete genome sequence of 14 bacterial and archaeal genomes, including 4 hyperthermophiles, has allowed the examination of a large data set to evaluate the influence of amino acid composition on the features of encoded proteins. The comparative analysis of proteins primary sequences indicated some important differences between the proteins from hyperthermophiles and those from mesophiles (37). In these latter, charged residues (Asp, Glu, Lys, Arg and His) are significantly less abundant (24.11%) than in proteins from thermophiles (29.84%), while polar/uncharged residues (Gly, Ser, Thr, Asn, Gln, Tyr, Cys) are more abundant (31.15%) in mesophilic rather than in thermophilic proteins (26.79%). Hydrophobic residues (Leu, Met, Ile, Val, Trp, Pro, Ala, Phe) are distributed in similar numbers in proteins from mesophiles (44.74%) and in those from thermophiles (43.36%). These results confirmed previous (38) and more recent (39) comparative analyses performed by Vogt and Argos on 16 protein families containing 56 different proteins from thermophilic, mesophilic and thermophobic sources. Proteins from hyperthermophiles contain higher levels of charged residues and lower levels of polar, uncharged residues, and, in addition, residues with a higher side chain volume and higher average hydrophobicity appear to be preferred. The charged residues are used by proteins from thermophiles to increase the number of hydrogen bonds and salt bridges compared to proteins from mesophiles. These major contributions to protein thermal stability are implemented in thermostable proteins by increases in polar surface areas.

4.2. Structural adaptations

A detailed analysis of the structural adaptation of a protein to the environment may be attempted by the comparison of the three-dimensional structures of an extremophilic protein with its mesophilic counterpart. This preliminary study should be followed by site directed mutagenesis experiments to test the role of the hypothetical structural determinants of protein stability identified from the structural comparison. The availability of many three-dimensional structures for proteins extracted from extremophilic microorganisms allows us to define some undoubted structural adaptations of these proteins in comparison to the mesophilic counterparts. The increase in the number of hydrogen bonds and ion pairs are the structural adaptations that are most generally accepted. Furthermore this increased number of electrostatic interactions is responsible for most of the structural modifications observed, such as secondary structure propensity, amino acid replacements, burying of hydrophobic accessible area, and strengthening of intersubunit association, all of which are considered as determinants of protein thermal stability.

An ion-pair is defined as the favourable energetic interaction which occurs when oppositely charged groups are within a distance of 4 Å (40). When the same charged groups are involved in the formation of hydrogen bonds, these ion-pairs are referred to as salt-bridges in order to distinguish between the different electrostatic interactions (41). The increase in hydrogen bonds often parallels the increase in ion-pairs in thermostable proteins. However hydrogen bonds do not always correspond to ion-pairs and may also represent an alternative to ion-pairs. The analysis of a number of crystallographic structures shows that thermostable proteins have an increased number of hydrogen bonds accompanied by an increased number of hydrophobic internal packing (42).

The analysis performed by Vogt *et al*. (38) on the 16 families of proteins containing 56 different proteins from thermophilic, mesophilic and thermophobic sources, revealed that hydrogen bonds and salt bridges are used by thermophilic protein to increase their thermostability. Furthermore 80% of these proteins showed an increase in polar surface due to an increased exposure of oxygen and nitrogen and the fraction of this latter was threefold that of oxygen, mainly through the contribution of Arg residues. Thus the increase in polar surface is related to the increase of protein thermotolerance. The Arg residues responsible for the increase in polar surface are often also involved in the formation of ion pairs.

Pyrococcus furiosus, an archaeal microorganism which leaves at temperatures over 100°C, has been the source for some of the most thermostable proteins purified, studied and crystallized so far. The hexameric glutamate dehydrogenases (GDH) from *P. furiosus* (PfGDH) (43) and from *Thermotoga maritima* (TmGDH) (44) have been crystallized and compared with the three-dimensional structure of the same protein from mesophilic *Clostridium symbiosum* (CsGDH) (45). Compared to the CsGDH the hyperthermophilic PfGDH presents a higher charged fraction (27%) over the average found in mesophilic proteins (19%) (46). This difference in the charged fraction is an important difference between thermophilic and mesophilic proteins. Considering only the ion pairs evaluated at the distance of 4 Å, PfGDH shows 288 ion pairs per hexamer, TmGDH 223 and CsGDH 188. Ion pairs are formed mainly with arginine side chains: 90% of the arginine residues of Pf GDH are involved in the formation of ion-pairs compared to only 55-60% in the mesophilic enzymes. In the thermophilic enzymes, moreover, arginine residues are involved in multiple ion-pairs whereas in the mesophilic enzyme arginine residues are mainly involved in isolated linkages. The largest ion-pairs network in PfGDH, which involves 18 charged residues, is present at the interface between dimers and is reinforced by four more

clusters of three residues and two isolated ion pairs.This extensively ion-pair linked surface extends to meet two further clusters of six residues and two isolated ion-pairs which derive from interactions centred around the interface between monomers. Considering the surface of each subunit which provides charged residues for these ion-pair interactions, a total of 25 out of 33 charged residues are involved in the formation of 19 ion pairs in the PfGDH. In the CsGDH only 15 charged residues out of 32 are involved in the formation of four ion-pairs in the equivalent region. Furthermore in different regions of the thermophilic protein there are other ion-pair networks which are absent in the mesophilic enzymes.The large number of ion-pair clusters formed in PfGDH is the consequence of the high concentration of basic and acidic residues on adjacent regions of the enzyme which makes the formation of such networks almost unavoidable. In PfGDH the highly charged subunits interfaces are neutralised because of the presence of multiple extensive ion-pair networks (43) and the largest network, which comprises 18 residues, cross-links secondary structure elements from four different subunits and is three times present in each hexamer at the trimer interface.

In conclusion, the main tool responsible for the increased thermotolerance in this archaeal enzyme is the presence of ion–pair clusters at the subunit interfaces forming a large number of salt bridges. This strategy has been observed in other enzymes from the same archaeon *P. furiosus* such as citrate synthase (47) and aldehyde oxydoreductase (48), and in GDH from the archaeon *Pyrococcus kodakarensis* (49). However this use of ion–pair clusters is not peculiar to Archaea because it has been reported also for the bacterial superoxide dismutase from *Aquifex pyrophilus* (50), in TmGDH (44), in *T. maritima* glyceraldehyde-3-phosphate dehydrogenase (51) and in malate dehydrogenase from *Thermus flavus* (52) and in DNA-polymerase from *Thermus aquaticus* (53). In particular in TmGDH (*T. maritima* growing temperature 80°C) the 18 residues network found in the same position in the PfGDH is much decreased and fragmented. After the introduction of four new charged amino acid residues into the subunit interface of TmGDH by site-directed mutagenesis, a 16-residues ion-pair network is formed with a concomitant increase of protein thermotolerance (54). These findings confirm the hypothesis that large ion-pair networks do indeed stabilize enzymes against high temperature.

The three-dimensional structure for a halophilic GDH is not available. However, the primary sequence analysis of the GDH from the halophilic archaeon *Halobacterium salinarum* has revealed in this protein the general adaptive structural modifications of halophilic proteins: an excess of acidic over basic residues, an increase of serine and threonine residues and a reduction of strongly hydrophobic residues (55). Homology- based modeling of this GDH indicated that the acidic residues cluster on the outer surface (55). These features have been found in other proteins from halophilic archaea, such as the elongation factor EF-Tu from the *Halobacterium marismortui* (26) and in malate dehydrogenase from *Haloarcula marismortui* (56). The crystal structure of this latter enzyme, similarly to other archaeal halophilic proteins, revealed a large increase of ion-pair networks similar to those found in proteins from hyperthermophilic archaea, and they are considered to be the structural determinants of the outstanding thermostability of several halophilic proteins. In halophilic microorganisms protein stability is generally achieved by the formation of hydrated salt ion networks coordinated by the acidic groups of the protein surface (56). In halophilic GDH, for example, the 18 residues network of PfGDH, considered an important determinant for its hyperthermostability (43), is only partially conserved but may nevertheless give a real contribution to the good thermotolerance of this protein compared to its mesophilic counterpart (55). The abundance of ion-pair networks is less pronounced in moderately halophilic proteins such as the dihydrofolate reductase from the archaeon *Haloferax volcanii* (57), which lacks all the characteristics of such extreme halophilic proteins as the malate dehydrogenase from *H. marismortui* (56) and ferredoxin (58). However also in this moderately halophilic enzyme some clusters of non-interacting negatively charged residues are found on the surface of the protein (57).

In summary, the most remarkable characteristic of halophilic proteins is the predominance at the protein-solvent interface of a large number of negatively charged residues which may coordinate and compete, for water hydration, with the hydrated salt ion networks present at the high salt concentrations of the environment. Mesophilic proteins, without this protective shell, tend to aggregate at high salt concentration (59). The instability of halophilic proteins at low salt concentrations may be explained by the repulsion between charged residues. In selected halophilic proteins some of these negatively charged residues are also effective in the formation of clusters of intramolecular ion-pair networks responsible of the outstanding thermotolerance of these halophilic proteins.

In psychrophilic microorganisms adapted to survive to low temperatures, evolution has selected different strategies in their proteins, which are structurally flexible and catalytically efficient at low temperatures. The availability of the crystal structures of three psychrophilic bacterial proteins as alpha-amylase from *Alteromonas haloplanctis* (27), triosophosphate isomerase from *Vibrio marinus* (30) and citrate synthase from *Antarctic* strain DS2-3R (DsCS) (60) offers insights into the adaptations of psychrophilic proteins to low temperatures. The cold-active citrate synthase compared to the hyperthermophilic enzyme from *P. furiosus* (47) showed a reduced number of interactions at the subunit interface with no intersubunit ion-pair networks and an increased number of intramolecular ion-pairs (42 against 27 in PfCS). Furthermore several loops, with a larger number of charged residues, are increased in length and present a decreased number of proline residues than in the PfCS enzyme. The increased amount of hydrophobic residues exposed to solvent in DsCS was considered a distinct signature of thermolabile enzymes and is found also in the cold-active 3-isopropylmalate dehydrogenase from *Vibrio* sp.15 (61). Protein destabilization is obtained when the hydrophobic residues are exposed to solvent due to the ordering of water molecules. The complex ion-pairs networks, isoleucine clusters and tyrosine clusters found in PfCS were not found

Table 1. Structural determinants of thermal adaptations in some thermophilic archaeal enzymes

Enzyme	MjAK[a]	PfGDH	TaCS	PfCS
Increased compactness	•	•	•	•
Increased number and/or volume of internal cavities	•	•	•	•
Increased hydrophobic interactions	•	•	•	•
Increased number of ion pairs		•		•
Increased number of ion-pair networks		•		•
Amino acids exchange	•	•	•	
Buried polar surface areas		•		
Decrease in sulphur content	•	•	•	
Strengthening intersubunit association		•		•
Decrease Gly and/or Pro in loops		•		
Increased aromatic interactions	•		•	
Helix-dipole stabilization	•		•	
Shorter loops	•		•	•
Isoleucine clusters				•

[a]Abbreviations: MjAK, adenylate kinase from *Methanococcus jannaschii* (64); PfGDH, glutamate dehydrogenase from *P. furiosus*; TaCS, citrate synthase from *Thermoplasma acidophilus*; PfCS, citrate synthase from *P. furiosus*

Table 2. Structural determinants of salt adaptation in some halophilic archaeal enzymes

Enzyme	HmMDH[a]	HsGDH	HvDFR
Increase of negatively charged residues	•	•	•
Decrease of positively charged residues	•	•	
Increased number of ion-pairs	•	•	
Increased number of salt bridges	•	•	
Acidic residues (clusters) on the enzyme surface	•		•
Reduction in surface exposed Lys residues		•	
Increased number of Arg residues			•

[a]Abbreviations:HmMDH, malate dehydrogenase from *H. marismortui*; HsGDH, glutamate dehydrogenase from *H. salinarum*; HvDFR, dehydrofolate reductase from *H. volcanii*.

in the psychrophilic enzyme. The high flexibility of the psychrophilic enzyme may be achieved by the decrease of proline residues in the loops and by their increase in length and charge. Three-dimensional homology based modeling of elongation factor 2 from the archaeal psychrophile *M. burtonii* (26) on the same protein from archaeon *Thermus thermophilus* (62) clearly suggested that the cold adapted archaeal protein have a greater flexibility, obtained through a reduced number of salt bridges, less packed hydrophobic cores and the reduction of proline in loops, confirming the results obtained in other psychrophilic proteins.

Enhanced secondary structure propensity, helix - dipole stabilization, amino acid replacements, burying of hydrophobic accessible area, strengthening of intersubunit association have all been indicated as structural determinants of protein stability and they are attributable, totally or in a significant part, to the increased number of hydrogen bonds and salt links and are reported in tables 1, 2, and 3.

5. CONCLUSIONS

The recent studies on proteins from extremophiles and the rational attempt to emulate Nature in modulating protein stability have provided a general structural framework for understanding protein resistance. The conclusion and the general rule derived from these studies is that there is not a unique way to gain protein stability and/or to find a proper balance between protein rigidity and flexibility over the wide temperature range where life is possible. Proteins individually adapt to environmental conditions by accumulation of different stabilizing interactions at all the protein structure levels. More importantly, the determinants of stability are those same weak intermolecular interactions used to stabilize the native structure in mesophilic proteins: networks of electrostatic interactions such as hydrogen bonds and/or salt bridges, optimization of local packing and hydrophobic interactions.

Nature has procured thermophilic proteins' structural resistance to high temperature at the expense of a decreased flexibility, giving poor activity at mesophilic temperature.This adaptation has involved selection of those proteins with a large number of charged amino acid residuesable to interact to give ion-pairs and to form hydrogen bonds and salt bridges with an optimal local packing and extended hydrophobic interactions (63). Proteins with extended surface loops, a large number of charged residues with a reduced number of proline and salt bridges and less packed hydrophobic cores, are preferred by evolution in a psychrophilic environment because of their high flexibility and high catalytic efficiency at low temperature obtained at the expense of their thermostability.

Table 3. Structural determinants of cold adaptation in some psychrophilic bacterial and archaeal enzymes

Enzyme	AhaA[a]	DS2-3RCS	Vsp15IPMDH	VmTIM	MbEF-2
Reduced number of salt bridges	•		•		•
Increase in size of loops	•	•	•		
Reduced number of Pro residues in loops	•	•	•		•
Reduced compactness	•	•			•
Increase in intramolecular ion-pairs		•			
Exposure of non polar groups to solvent	•	•	•		
Reduced number of buried non polar groups	•				•
Reduced number of charged residues	•	•			
Weaker interdomain interactions	•	•			
Reduction of aromatic-aromatic interactions			•		
Increased resilience of the molecular surface	•				
Decrease in Arg residues content	•				
Increase in Ala content				•	

[a]Abbreviations are: AhaA, alpha-amylase from *A. haloplanctis*; DS2-3RCS, recombinant citrate synthase from *Antartic bacterium strain DS2-3R*; Vsp15IPMDH, 3-isopropylmalate dehydrogenase from *Vibrio sp* 15; VmTIM, triose-phosphate isomerase from *Vibrio marinus*; EF-2, elongation factor EF-2 from *Methanococcoides burtonii*.

The presence on a protein of clusters of non-interacting negatively charged surface groups able to coordinate hydrated salt ion network in the solvent, a reduced number of hydrophobic residues exposed to solvent and an increased number of ion-pair clusters, can result in a halophilic structure with a good thermostability and high salt tolerance.

However these general observations cannot be considered rules but only structural trends. Evolutionary pressure has exerted a fine tuning on similar common structures, selecting the most appropriate amino acid residues to produce a final protein structure that gives best catalytic efficiency at the growth temperature of each organism.

6. PERSPECTIVES

Most of the extremophilic proteins are found in archaeal microorganisms. These proteins are very interesting for their potential biotechnological use. Engineering of these proteins may further increase their potential use. The results obtained so far allow to predict a larger use of these proteins in the future in many industrial production processes in order to reduce the environmental pollution due to the chemical pathways actually used.

7. REFERENCES

1. Woese, C.R.& G.F.Fox: Phylogenetic structure of the prokariotic domain: the primary kingdoms. *Proc Natl Acad Sci USA* 74, 5088-5090 (1977)

2. Stetter, K.O.: Hyperthermophilic procaryotes. *FEMS Microbiol Rev* 18, 149-158 (1996)

3. Brock, T.D., K.M.Brock, R..T.Belly & R.L.Weiss: *Sulfolobus*: a new genus of sulfur-oxidizing bacteria living at low pH and high temperature. *Arch Microbiol* 84, 54-68(1972)

4. J.A. Baross & J.F. Holden: Overview of hyperthermophiles and their heat-shock proteins. *Adv Protein Chem* 48, 1-34 (1996)

5. Lippert, K. & E.A.Galinski: Enzyme stabilization by ectoin-type compatible solute interaction, protection against heating and drying. *Appl Microbiol Biotech* 37, 61-65 (1992)

6. Tanaka T., N. Kawano & T. Oshima: Cloning of 3-isopropylmalate dehydrogenase gene of an extreme thermophile and partial purification of the gene product. *J Biochem (Tokyo)* 89, 677-82 (1981)

7. Jaenicke, R.: Protein stability and molecular adaptation to extreme conditions. *Eur J Biochem* 202,715-728 (1991)

8. Pace, C.N., L.M. Fisher & J.F. Cupo: Globular protein stability: aspects of interest in protein turnover. *Acta Biol Med Ger* 40, 1385-1392 (1981)

9. Pfeil, W. : Unfolding of proteins.In: Thermodynamic Data for Biochemistry and Biotechnology .Ed.: Hinz H J, Springer-Verlag, Berlin, New York 349-376 (1986)

10. Sturtevant, J.M.: Heat capacity and entropy changes in processes involving proteins. *Proc Natl Acad Sci USA* 74, 2236-2240 (1977)

11. Tanford, C.: 'The Hydrophobic Effect' Wiley, New York. (1973).

12. Privalov, P.L. & S.J. Gill: Stability of protein structure and hydrophobic interaction. *Adv Prot Chem* 39, 193-231 (1988)

13. Stickle, D.F., L.G. Presta, K.A. Dill, , & G.D. Rose: Hydrogen bonding in globular proteins. *J Mol Biol* 226, 1143-1159 (1992)

14. Fauchere, J.L. & V.E Pliska: Hydrophobic parameters of amino acid side chains from the partitioning of N-acetyl-amino-acid amides. *Eur J Med Chem* 18, 369-375 (1983)

15. Consalvi, V., R. Chiaraluce, L. Politi, A. Pasquo, M. De Rosa & R. Scandurra: Glutamate dehydrogenase from the thermoacidophilic archaebacterium *Sulfolobus solfataricus*: studies on thermal and guanidine-dependent inactivation. *Biochim Biophys Acta* 1202, 207-215(1993)

16. Consalvi, V., R. Chiaraluce, S. Millevoi, A. Pasquo, P. Vecchini, E. Chiancone & R. Scandurra: Refolding pathway and association intermediates of glutamate dehydrogenase from the hyperthermophile *Pyrococcus furiosus*. *Eur J Biochem* 239, 679-685 (1996)

17. West, S.M. & N.C. Price: The unfolding and refolding of glutamate dehydrogenase from bovine liver, baker's yeast and *Clostridium symbiosum*. *Biochem J* 251, 135-139. (1988)

18. Lebbink, J.H.G., R.I.L. Eggen, A.C.M. Geerling, V. Consalvi, R. Chiaraluce, R. Scandurra & W.M. de Vos: Exchange of domains of glutamate dehydrogenase from the hyperthermophilic archaeon *Pyrococcus furiosus* and the mesophilic bacterium *Clostridium difficile*: effects on catalysis, thermoactivity and stability. *Protein Eng* 8, 1287-1294 (1995)

19. Aghajanian S.A., S.R. Martin & P.C. Engel: Urea-induced inactivation and denaturation of clostridial glutamate dehydrogenase: the absence of stable dimeric or trimeric intermediates. *Biochem J* 311, 905-910 (1995)

20. Jaenicke, R. & G.Bohm: The stability of proteins in extreme environments. *Curr Opin Struct Biol* 8, 738-748 (1998)

21. Richards, F.M.: The interpretation of protein structures: total volume, group volume distribution and packing density. *J Mol Biol* 82, 1-14 (1974)

22. Russel, R.J.M., D.W. Hough, M.J. Danson & G.L.Taylor: The crystal structure of citrate synthase from the thermophilic Archaeon *Thermoplasma acidophilum*. *Structure* 2, 1157-1167 (1994)

23. Trent, J.D., J.Osipuk & T.Pinkau: Acquired thermotolerance and heat shock in the extremely thermophilic archaebacterium *Sulfolobus* spl.strain B12 .*J Bacteriol* 172,1478-1484 (1990)

24. Stillman, T.J., P.J. Baker, K.L. Britton & D.W Rice: Conformational flexibility in glutamate dehydrogenase. Role of water in substrate recognition and catalysis. *J Mol Biol* 234, 1131-1139 (1993)

25. Franzman, P.D., Y. Liu, D.L. Aldrich, E. Conway de Macario & D.R. Boone: *Methanogenium frigidum* sp.nov., a psychrophilic H2-using methanogen from Ace Lake, Antarctica. *Int J Syst Bacteriol* 47, 1068-1072 (1997)

26. Thomas T. & R. Cavicchioli: Archaeal cold-adapted proteins: structural and evolutionary analysis of the elongation factor 2 proteins from psychrophilic, mesophilic and thermophilic methanogens. *FEBS Lett* 439, 281-286 (1998)

27. Aghajari, N., G. Feller, C. Gerday & R.Haser: Structures of the psychrophilic *Alteromonas haloplanctis* alpha-amylase give insights into cold adaptation at a molecular level. *Structure* 6, 1503-1516 (1998)

28.Gerike, U., M.J.Danson, N.J.Russell & D.W.Hough: Sequencing and expression of the gene encoding a cold –active citrate synthase from an Antartic bacterium, strain DS2-3R. *Eur J Biochem* 248, 49-57 (1997)

29. Villeret, V., J. P. Chessa, C.Gerday & J.Van Beeumen:Preliminary crystal structure determination of the alkaline protease from the Antartic psychrophile *Pseudomonas aeruginosa*. *Protein Sci* 6, 2462-2464 (1997)

30. Alvarez, M., J.P. Zeelen, V. Mainfroid, F. Rentier-Delrue, J.A. Martial, L.Wyns, R.K.Wierenga & D. Maes:Triose-phosphate isomerase (TIM) of the psychrophilic bacterium *Vibrio marinus*. *J Biol Chem* 273, 2199-2206 (1998)

31. Feller, G., E. Narinx, J. L. Arpigny, M. Aittaleb, E. Baise, S.Genicot & C.Gerday: Enzymes from psychrophilic organisms. *FEMS Microbiol Rev* 18, 189-202 (1996)

32. Marshall, C.J.: Cold adapted enzymes. *Trends Biotechnol* 15, 359-363 (1997)

33. Russell, N.J.: Molecular adaptations in psychrophilic bacteria: potential for biotechnological applications. *Adv Biochem Eng Biotechnol* 61, 1-21 (1998)

34. Argos, P., M. Rossmann, U. Grau, H. Zuber & G. Tratschin: Thermal stability and protein structure. *Biochemistry* 25, 5698-5703 (1979)

35. Menéndez-Arias, L. & P. Argos: Engineering protein thermal stability. *J Mol Biol* 206, 397-406 (1988)

36. Bohm, G. & R. Jaenicke: Relevance of sequence statistics for the properties of extremophilic proteins. *Int J Pept Protein Res* 43, 97-106 (1994)

37. Deckert G, P.V. Warren, T. Gaasterland, W.G. Young, A.L. Lenox, D.E. Graham, R. Overbeek, M.A. Snead, M. Keller, M. Aujay, R. Huber, R.A. Feldman, J.M. Short, G.J. Olsen & R.V. Swanson: The complete genome of the hyperthermophilic bacterium *Aquifex aeolicus*. *Nature* 392, 353-358 (1998)

38. Vogt, G., S. Woell & P. Argos: Protein thermal stability, hydrogen bonds and ion pairs. *J Mol Biol* 269, 631-643 (1997)
39. Vogt, G. & P.Argos: Protein thermal stability: hydrogen bonds or internal packing? *Fold Des* 2, S40-S46 (1997)

40. Barlow, D.J. & J.M. Thornton: Ion-pairs in proteins. *J Mol Biol* 168, 867-885 (1983)

41. Marqusee, S. & R.L.Baldwin: Helix stabilization by Glu…Lys+ salt bridges in short peptides of *de novo* design. *Proc Natl Acad Sci* USA 84, 8898-8902(1987)

42. Querol, E., J.A. Perez-Pons, & A. Mozo-Villarias: Analysis of protein conformational characteristics related to thermostability. *Protein Eng* 9, 265-271 (1996)

43. Yip, K.S.P., T.J. Stillman, K.L. Britton, P.J. Artymiuk, P.J.Baker, S.E. Sedelnikova, P.C. Engel, A. Pasquo, R.Chiaraluce, V.Consalvi, R. Scandurra & D.W. Rice: The structure of *Pyrococcus furiosus* glutamate dehydrogenase reveals a key role for ion-pairs networks in maintaining

enzyme stability at extreme temperatures. *Structure* 3, 1147-1158 (1995)

44. Knapp, S., W. de Vos, D.W.Rice & R. Ladenstein: Crystal structure of glutamate dehydrogenase from hyperthermophilic eubacterium *Thermotoga maritima* at 3.0 Å resolution. *J Mol Biol* 267, 916-932 (1997)

45. Rice, D.W., K.S.P. Yip, T.J. Stllman, K.L. Britton, A. Fuentes, I. Connerton, A.Pasquo, R. Scandurra & P. C. Engel : Insights into the molecular basis of thermal stability from the structure determination of *Pyrococcus furiosus* glutamate dehydrogenase. *FEMS Microbiol Rev* 18, 105-117 (1996)

46. Miller, S., J. Janin, A.M. Lesk & C. Chothia: Interior surface of monomeric proteins. *J Mol Biol* 196, 641-656 (1987)

47. Russel, R.J.M., J.M.C.Ferguson, D.W.Hough, M.J.Danson & G.L.Taylor: The crystal structure of citrate synthase from the hyperthermophilic archaeon *Pyrococcus furiosus* at 1.9 Å resolution. *Biochemistry* 36, 9983-9994 (1997)

48. Chan, M.K., S.Mukund, A. Kletzin, M.W.W.Adams & D.C.Rees: Structure of hyperthermophilic tungstenopterin enzyme, aldehyde ferredoxin oxidoreductase. *Science* 267,1463-1469 (1995)

49. Rahman, R., S.Fujiwara, H.Nakamura, M.Takagi & T.Imanaka: Ion pairs involved in maintaining a thermostable structure of glutamate dehydrogenase from a hyperthermophilic archaeon. *Biochem Biophys Res Commun* 248, 920-926 (1998)

50. Lim J.H., Y.G. Yu, Y.S. Han, S. Cho, B. Y. Ahn, S. H. Kim & Y. Cho: The crystal structure of an Fe-Superoxide dismutase from the hyperthermophile *Aquifex pyrophilus* at 1.9 Å resolution: structural basis for thermostability. *J Mol Biol* 270, 259-274 (1997)

51. Korndörfer, I., B. Steipe, R. Huber, A.Tomschy & R. Jaenicke: The crystal structure of holo-glyceraldehyde-3-phosphate dehydrogenase from the hyperthermophilic bacterium *Thermotoga maritima* at 2.5 Å resolution. *J Mol Biol* 246, 511-521 (1995)

52. Kelly, C., M. Nishiyama, Y.Ohnishi, T. Beppu & J.J. Birktoft: Determinants of protein thermostability observed in the 1.9 Å crystal structure of malate dehydrogenase from the thermophilic bacterium *Thermus flavus*. *Biochemistry* 32, 3913-3922 (1993)

53. Korolev, S., N. Murad, W.M. Barnes, E. Di Cera & G.Waksman: Crystal structure of the large fragment of *Thermus aquaticus* DNA polymerase I at 2.5 Å resolution: structural basis for thermostability. *Proc Natl Acad Sci USA* 92, 9264-9268 (1995)

54. Lebbink, J.H.G., S. Knapp, J. van der Oost, D.W. Rice, R. Ladenstein & W. de Vos: Engineering activity and stability of *Thermotoga maritima* glutamate dehydrogenase.II: Construction of a 16 residue ion-pair network at the subunit interface. *J Mol Biol* 289,357-369 (1999)

55. Britton, K.L., T.J.Stillman, K.S.P.Yip, P. Forterre, P.C.Engel & D. W. Rice: Insights into the molecular basis of salt tolerance from the study of glutamate dehydrogenase from *Halobacterium salinarum. J Biol Chem* 273, 9023-9030 (1998)

56. Dym, O., M. Mevarech & J.L. Sussman: Structural features that stabilize halophilic malate dehydrogenase from an Archaebacterium. *Science* 267,1344-1346 (1995)

57. Pieper, U., G. Kapadia, M.Mevarech & O.Herzberg: Structural features of halophilicity derived from the crystal structure of dihydrofolate reductase from the dead sea halophilic archaeon, *Haloferax volcanii*. *Structure* 6, 75-88 (1998)

58. Frolow, F., M.Harel, J.L. Sussman, M.Mevarech & M. Shoham: Insights into protein adaptation to a saturated salt environment from the crystal structure of a halophilic 2Fe-2S ferredoxin. *Nat Struct Biol* 3, 452-458 (1996)

59. Bonneté, F., D.Madern & G. Zaccai: Stability against denaturation mechanisms in halophilic malate dehydrogenase "adapt" to solvent conditions. *J Mol Biol* 244, 436-447 (1994)

60. Russel, R.J.M, U.Gerike, M.J.Danson, D.W.Hough & G.L.Taylor: Structural adaptations of the cold-active citrate synthase from an Antartic bacterium. *Structure* 6,351-361(1998)

61. Wallon, G., S. T.Lovett, C. Magyar, A. Svingor, A. Szilagyi, P. Zavodsky, D. Ringe & G.A.Petsko: Sequence and homology model of IPMDH from the psychrophilic bacterium *Vibrio* sp.15 suggest reason for thermal instability. *Protein Eng* 10, 665-672 (1997)

62. Evarrson, A., E. Brazhinikov, M.Garber, Zheltonosova, J.Chirgadze, S.Al-Karadaghi, L.A.Svensson & A.Liljas: Three-dimensional structure of the ribosomal translocase:elongation factor G from *Thermus thermophilus.EMBO J* 13, 3669-3677 (1994)

63. Scandurra R, V. Consalvi, R. Chiaraluce, L. Politi & P.C. Engel PC: Protein thermostability in extremophiles. *Biochimie* 80, 933-941 (1998)

64. Haney P., J. Konisky, K.K Koretke, Z. Luthey-Schulten Z & P.G. Wolynes: Structural basis for thermostability and identification of potential active site residues for adenylate kinases from the archaeal genus *Methanococcus*. *Proteins* 28, 117-130 (1997)

Key Words: Protein Stability, Extremophiles, Electrostatic Interactions, Halophiles, Psychrophiles, Thermophiles, Review

Send the correspondence to: Prof. Roberto Scandurra, Dipartimento di Scienze Biochimiche "A. Rossi-Fanelli" Università "La Sapienza", Piazzale Aldo Moro, 5 00185 Roma, Italy, Tel.: +39-0649910947, Fax: +39-064440062, E-mail: scandurra@caspur.it

This manuscript is available on line at:

http://www.bioscience.org/2000/d/scan/fulltext.htm