

Adaptation of Proteins from Hyperthermophiles to High Pressure and High Temperature

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Abstract

Further clarification of the adaptations permitting the persistence of life at temperatures above 100 °C depends in part on the analysis of adaptive mechanisms at the protein level. The hyperthermophiles include both Bacteria and Archaea, although the majority of isolates growing at or above 100 °C are Archaea. Newly described adaptive features of hyperthermophiles include proteins whose structural integrity persists at temperatures up to 200 °C, and under elevated hydrostatic pressure, which in some cases adds significant increments of stability.

Introduction

It is now very well established that microbial growth can occur at temperatures well above 100 °C. The recently described microorganism with the highest recorded growth temperature of 113.5 °C, is the chemolithoautotrophic archaeon *Pyrolobus fumarii* (Blochl *et al.*, 1997). Hyperthermophiles, which are defined as organisms with maximal growth temperatures above 90 °C (Adams, 1994), are widely distributed in hydrothermal environments in terrestrial as well marine and abyssal vent systems. The group includes 21 archaeal genera, and two bacterial genera with diverse growth physiology including both heterotrophs and chemoautotrophs (Stetter, 1996; Robb *et al.*, 1995). The archaeal hyperthermophiles have exceptionally high growth temperature limits, unique, molecular characteristics, such as ether-linked lipids and eukaryotic transcription and translation factors (Robb *et al.* 1995), and unusual enzyme chemistry, such as the presence of multiple tungsten-containing enzymes (Kletzin and Adams, 1996). The unusual adaptive responses of the hyperthermophiles include heat shock proteins of the hsp60 family (Trent, 1996; Trent *et al.*, 1997; Kagawa *et al.*, 1995) which are inducible by supraoptimal temperatures, and reverse gyrase which installs positive supercoils in DNA (Guipaud *et al.*, 1997; Borges *et al.*, 1997).

The ongoing release of genomic sequence data from hyperthermophiles will continue to accelerate the discovery of thermostable proteins, however understanding the functional adaptations of these proteins requires the

application of novel methods of analysis. For example, two key enzymes in glycolysis, phosphofructokinase and hexokinase, remained undiscovered in extracts of the hyperthermophile *Pyrococcus furiosus* because it was anticipated that they would require ATP. It transpires that enzymes are active and highly expressed, but require ADP (Kengen *et al.*, 1995), presumably due to the higher stability of ADP compared with ATP. This review focuses on mechanisms of stabilization of enzymes with novel catalytic specificities, unusual thermal stability, and the ability to withstand, and in many cases to gain enhanced thermostability under elevated hydrostatic pressure. The latter is an unusual and intriguing feature of many proteins from thermophiles and recent work suggests that the structural stability of proteins in the 100-130 °C range is achieved by quite generic adaptive mechanisms. We will review the current field of protein stability at very high temperatures and discuss recent findings on the theoretical basis for pressure stabilization of proteins.

Mechanisms of Hyperstability

The known strategies for maintaining stability at high temperatures are summarized in Table 1. Five classes of adaptations are discussed below.

1. Amino Acid Composition

Although many formulae for compositional bias in hyperstable proteins have been suggested, there appear to be few answers to the quest for a thermoadapted amino acid composition. On the contrary, hyperstable proteins, like their counterparts in mesophiles, are composed of the same 20 amino acid set found in mesophiles. These amino acids undergo accelerated covalent modification at the high temperatures, pressures and extremes of pH that many thermophiles and hyperthermophiles can withstand (Hensel *et al.*, 1992). At temperatures beyond 100 °C, the stability of common amino acids declines in the following order: (Val,Leu)>Ile>Tyr>Lys>His>Met>Thr>Ser> Trp>(Asp, Glu, Arg, Cys) (Jaenicke 1991; Jaenicke 1996a; Jaenicke and Bohm, 1998). Since many of the hyperthermophiles are relatively slow growing, the half lives of some of the more labile amino acids such as Cys, Arg, Gln, Asn are considerably shorter than the generation times of the organisms (Bernhardt *et al.*, 1984). Common types of chemical alterations include deamidation, β -oxidation, Maillard reactions, hydrolysis, and disulfide interchange, etc (Jaenicke and Bohm, 1998). Many of the amino acids are more stable upon internalization in the hydrophobic core of the proteins than in free solution (Hensel *et al.*, 1992), however the frequencies of occurrence of Cys, Asn, Gln and Asp is significantly lowered in the hyperstable variants of some common proteins. The genomic sequences that are determined or in progress will continue to make available a flood of deduced protein sequences,

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Table 1. Synopsis of Mechanisms of Extreme Thermostability in Proteins from Hyperthermophiles

Organism (Topt)	Thermostability feature	Protein	Reference
<i>Pyrococcus furiosus</i> (100 °C)	Mg ⁺⁺ requirement KCl (1M)	Acetyl-CoA synthetase (ADP-forming)	Glasemacher <i>et al.</i> , 1997
<i>P. furiosus</i> (100 °C)	Salt bridges	Glutamate dehydrogenase	Klump <i>et al.</i> , 1992; Vetriani <i>et al.</i> , 1998
<i>Sulfolobus acidocaldarius</i> (85 °C)	Lysine monomethylation NOT salt bridges	Sac7d	McCrary <i>et al.</i> , 1996
<i>S. acidocaldarius</i> (85 °C)	The data conforms to none of the sequence based conventions proposed by others	Adenylate kinase Pyrophosphatase Superoxide dismutase	Schafer <i>et al.</i> , 1996
<i>Methanothermus fervidus</i> (65 °C)	Hydrophobic "proline N caps" Interhelical hydrogen bonds Short N- and C-termini	Histone rHMF	Starich <i>et al.</i> , 1996
<i>Aquifex pyrophilus</i> (98 °C)	Salt bridges Hydrophobic packing Polymeric tetramer	Superoxide dismutase (SOD)	Lim <i>et al.</i> , 1997
<i>Thermotoga maritima</i> (77 °C)	Homodimer	Phosphoribosyl anthranilate isomerase	Hennig <i>et al.</i> , 1997
<i>T. maritima</i> (77 °C)	Replace loop with helix Homo-dimer hydrophobic packing N- and C- termini immobilized Salt bridges	Phosphoribosyl anthranilate isomerase	Hennig <i>et al.</i> , 1997

which will lead to additional insights in the area of compositional bias. Compatible solutes, some of which have recently been found to provide generic thermoprotection to proteins, may be acting by incrementally slowing amino acid modification reactions at high temperatures (Hensel *et al.*, 1992).

2. Hydrophobic Packing

An extensive, efficiently packed hydrophobic core is a common feature of stable globular proteins, although it appears to be more dominant in the lesser hyperthermophiles than in those growing at or above 100 °C (Russell *et al.*, 1995; Macedo-Ribeiro, 1997; Pfeil *et al.*, 1997; Jaenicke, 1996b).

At present, the effect of temperature on hydrophobic interactions is still a subject of debate in the literature. The strength of the hydrophobic interaction is most commonly represented by ΔG_{tr}° , the Gibbs free energy of transfer of hydrocarbons from a pure hydrocarbon liquid to water (Schellman, 1997). An alternative is to define the strength of the hydrophobic interaction by $\Delta G_{tr}^{\circ}/T$, which is proportional to the natural log of the equilibrium constant (Schellman, 1997). The temperature dependence of the strength of the hydrophobic interaction depends on which definition is used. In particular, plots of ΔG_{tr}° vs. T for model hydrocarbons exhibit a maximum at around 140 °C (Privalov and Gill, 1988), whereas curves of $\Delta G_{tr}^{\circ}/T$ vs. T have a maximum at around 20 °C (Privalov and Gill, 1988). Thus, it is still unclear whether hydrophobic interactions in proteins become stronger or weaker at temperatures above room temperature, and how important they are to the stability of thermophilic proteins at high temperatures. Interestingly, some of the same constraints appear to operate in limiting the stability of proteins at low temperatures, between -10 and 20 °C (Privalov and Gill, 1988). There is also evidence that hyperstable glutamate dehydrogenase requires high temperatures for assembly, suggesting that specific hydrophobic states are critical to the molecular engagement of subunits, and that partly assembled multimeric proteins may be "frozen" in metastable intermediates. This system may provide insights into the nature of the folding and assembly pathways of hyperstable proteins (DiRuggiero and Robb, 1995; 1996).

3. Ionic Networks

Extensive networks of acidic and basic side chains on the surface of subunits and domains interact to form cooperatively bound assemblages (Russell and Taylor, 1995; Robb and Maeder, 1998; Rice *et al.*, 1996; Britton *et al.*, 1995). Ionic interactions by nature act over much longer ranges than hydrophobic interactions, and are relatively immune to alterations in the structure of water which is compromised at elevated temperatures. Consequently, widespread networks of ionic interactions are observed in the proteins of the more extreme hyperthermophiles compared with homologous proteins in the thermophiles (T_{opt} for growth between 50 and 75 °C) or in mesophiles (Osterdorp *et al.*, 1996; Britton *et al.*, 1995). The ionic networks of *P. furiosus* glutamate dehydrogenase demonstrate extensive clustering of spatially alternating positive and negative charges, which have a component of hydrogen bonding. Recreating a network in the less stable glutamate dehydrogenase from *Thermococcus litoralis* resulted in elevated thermostability without any penalty in catalytic activity (Vetriani *et al.*, 1998).

4. Cooperative Association

In proteins that form oligomers or bind to larger substrates than themselves, dissociation is thought to precede the irreversible unraveling of monomers. The loss of integrity of the protein molecule is dependent on the unfolding of the monomeric protein into the denatured form. It follows that strong intermolecular associations can forestall this process. Consequently, many proteins that are monomeric in mesophiles are found to form oligomers in extreme thermophiles or hyperthermophiles. For example, the chorismate mutase from the hyperthermophile, *M. jannaschii*, appears to have developed a dimeric quaternary organization as an adaptation to stability (MacBeath *et al.*, 1998), and the compact beta-alpha TIM barrel of phosphoribosyl anthranilate isomerase, which is monomeric in enteric bacteria, is dimeric in *T. maritima* (Hennig *et al.*, 1997),

5. Pinning the Loose Ends

It is thought that many N- and C-termini are immobilized in hyperstable proteins, and are therefore not free to fray and initiate unraveling. In the case of phosphoribosyl anthranilate isomerase from *T. maritima* (Hennig *et al.*,

1997), not only are the termini buried in hydrophobic pockets, but a disordered loop found in the homologous protein from *E. coli* is replaced with an alpha helix, thereby inhibiting the nucleation of melting. The C-termini of the dimeric citrate synthases of hyperthermophiles has an intertwined structure which contributes stability by locking the dimer together as well as securing the carboxyl groups by ionic interaction, preventing disengagement of the ends (Russell *et al.*, 1997). The protein with the highest temperature stability on record, *P. furiosus* ferredoxin, which retains structure at temperatures up to 200 °C (Hiller *et al.*, 1997), features an ion-pair at the amino terminus (Cavagnero *et al.*, 1998). Loose ends must be handled somewhat differently in the multiple proteins from hyperthermophiles that have inteins. For example, the ribonucleotide reductase from *Pyrococcus furiosus* has been shown to have two inteins (Riera *et al.*, 1997), and the mechanisms of intein excision have been shown to proceed at optimal growth temperatures (Perler *et al.*, 1997), implying that there is an end-stabilizing mechanism for these protein splicing reactions.

Pressure Stabilization

Many hyperthermophiles isolated from deep sea hydrothermal vents are either indifferent to the effects of pressure on growth at high temperature, or else they are barophilic in terms of maximal growth rate and the upper temperature limits of growth (Miller *et al.*, 1988; Nelson *et al.*, 1992; Pledger *et al.*, 1994; Marteinsson *et al.*, 1997). Although many studies of pressure effects on enzyme stability have appeared since the early 1950's, in nearly all cases the experiments were performed with proteins from mesophilic sources at temperatures below 60 °C, such as lysozyme (Samarasighe *et al.*, 1992). Moreover, much of this early work focused on enzyme denaturation at very high pressures (> 300 MPa) (Jaenicke, 1991; Weber and Drickamer, 1983). More recent work, however, has shown that moderate pressures (100 MPa) below those normally needed for pressure-induced denaturation can in fact stabilize proteins against thermoinactivation (Hei and Clark, 1994; Michels and Clark, 1997; Michels *et al.*, 1996; Mozhaev *et al.*, 1996). Particularly large effects have been observed for thermophilic enzymes at very high temperatures. This behavior has important implications for the adaptation of thermophilic proteins *in extremis*, as discussed more fully below.

In the first report of thermophilic enzyme stabilization by pressure, Hei and Clark (1994) examined the effect of pressure on the thermal stability of four partially purified hydrogenases from methanogens of the genus *Methanococcus*. Only one of these organisms, *M. jannaschii*, was isolated from a deep-sea habitat: a deep-sea hydrothermal vent at a depth of 2500 m (Jones *et al.*, 1983). Notably, hydrogenases from the extreme thermophiles *M. jannaschii* and *M. igneus* were substantially stabilized by pressure whereas hydrogenases from *M. thermolithotrophicus* (a moderate thermophile) and *M. maripaludis* (a mesophile) were destabilized by pressure. These results were the first demonstration of pressure stabilization for thermophilic enzymes, and showed that the effect is not unique to enzymes isolated from high-pressure environments. The work of Summit *et al.* (1998) confirmed that the DNA polymerases from non-

barophilic thermophiles could be stabilized by pressure. Furthermore, the hydrogenase studies, in combination with studies on the effects of pressure on several homologous glyceraldehyde-3-phosphate dehydrogenases from mesophilic and thermophilic sources and a rubredoxin from *P. furiosus*, implicated hydrophobic interactions as an important factor in the stabilization of thermophilic enzymes by pressure.

In follow-up work to the studies of Hei and Clark (1994), Michels and Clark (1997) isolated a proteolytic enzyme from *M. jannaschii* and found that the enzyme was both activated and stabilized by pressure. Even at moderate pressures, the thermal half-life of the enzyme was exceptionally high; for example, $t_{1/2} = 45$ min at 116 °C, and 7 min at 125 °C (at ~10 atm pressure). Moreover, the thermostability of the *M. jannaschii* protease increased with pressure in a physiologically-relevant range. For example, by raising the pressure to 500 atm, the half-life of the enzyme was increased 2.7-fold at 125 °C. The pronounced effect of pressure on the *M. jannaschii* protease stability was unusual for proteolytic enzymes: when 500 atm was applied to trypsin, α -chymotrypsin, and subtilisin Carlsberg, half-lives at the enzyme's reported melting temperature increased by 40%, 30% and 30%, respectively, compared to 170% for *M. jannaschii* protease at 125 °C (Michels and Clark, 1997).

The barophilic behavior of the *M. jannaschii* protease (and of the corresponding hydrogenase; Miller *et al.*, 1989) is especially noteworthy in view of the unusual barophily exhibited by *M. jannaschii* in high-pressure growth experiments (Miller *et al.*, 1988). Whether such pressure-activation and stabilization of key enzymes is responsible for the barophilic growth of *M. jannaschii* remains to be seen; however, the similar effects of pressure on growth and enzyme activity suggests that these phenomena are interrelated and motivates further pressure studies of enzymes from *M. jannaschii* and other deep-sea thermophiles.

In more recent work, Sun *et al.* (1999) examined the thermostability and pressure-induced thermostabilization of glutamate dehydrogenase (GDH) from the hyperthermophilic archaeon *Pyrococcus furiosus* (*Pf*) at temperatures up to 109 °C (*Pf*GDH, a hexamer composed of six identical subunits, has a melting temperature for denaturation of 113 °C). The native GDH from *Pf* was substantially stabilized by 500 atm, up to 18-fold at 109 °C. By comparison, a recombinant GDH mutant containing an extra tetrapeptide at the C-terminus was stabilized to an even greater degree, by 28-fold at 105 °C. Although the presence of the tetrapeptide destabilized the enzyme markedly, the destabilizing effect was largely reversed by pressure. The remarkable degree of pressure stabilization, especially of the recombinant GDH mutant, could not be attributed to hydrophobic interactions alone. However, further insights into the possible mechanism(s) of pressure stabilization were provided by inactivation experiments in the presence of glycerol. Specifically, stabilization was also achieved by adding glycerol, albeit to a lesser extent than effected by pressure, suggesting that compression and/or rigidification of the protein's structure played a role in pressure-induced thermostabilization.

Of the primary intramolecular interactions responsible for maintaining native protein structure, only hydrophobic interactions are expected to be stabilized by elevated

pressure (although this conclusion is not universally accepted in the literature). This effect has been attributed to the relatively open structure of water solvating apolar surfaces exposed during protein unfolding (Hei and Clark, 1994; Michels *et al.*, 1996). In contrast, intraprotein electrostatic interactions ("salt bridges" or ion pairs) are generally believed to be strongly destabilized by increasing pressure (Michels *et al.*, 1996). Hydrogen bonds are slightly stabilized by pressure (Michels *et al.*, 1996), but intramolecular hydrogen bonds readily exchange with solvating water molecules upon protein unfolding. Hydrogen bonds are therefore expected to have little net influence on the stability of proteins under pressure.

So far it appears that pressure-stabilization can result from the unfavorable thermodynamics of hydrophobic solvation and/or stabilizing interactions in the protein induced by compression or rigidification of susceptible structures. Although these effects are not restricted to proteins from organisms of the deep sea or other high-pressure environments, pressure-induced activation and stabilization of proteins might be an underlying characteristic of microbial barophily. Moreover, in some cases of pronounced stabilization, structural interactions within thermophilic proteins seemingly at odds with pressure stabilization, *e.g.* the extensive ionic pairing in GDH from *P. furiosus*, are apparently outweighed by other factors that contribute to net stabilization (Clark *et al.*, 1996). Clearly, much more needs to be learned about the mechanisms of pressure stabilization at high temperatures, and the apparent synergy between pressure and thermostability in proteins from hyperthermophiles. This information will no doubt provide new insights into structure-stability relationships in general, if not the evolutionary pressures and pathways that lead to them.

Conclusions

The discovery of a hyperthermophile that grows at 113 °C has implications for adaptive features of proteins, and confirms that, *in vivo*, enzyme stability and activity may be observed at temperatures above 110 °C. Intrinsically stable proteins are typical of hyperthermophiles and the studies of chemical stability at temperatures higher than 110 °C will become familiar experimental procedures in future. The current focus on ion-pair network formation as a critical mechanism for stabilizing protein structures above 100 °C may explain many instances of stabilization by optimization of subunit interactions. The effects of pressure in stabilizing thermostable proteins are intriguing and a mechanistic explanation may be close at hand.

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