Extremophiles in Astrobiology*: Per Ardua ad Astra*

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ABSTRACT

As we consider the possibilities of finding life on other planets, it behooves us to evaluate what we know about the limits for life on planet Earth. In our continued exploration of Earth, we are finding microbes in a variety of unexpected habitats. In geothermal hot springs, we have discovered organisms thriving at temperatures near the boiling point of water and at pH values down to 0.5; in the deepest parts of the oceans, those that grow optimally at pressures above 1000 bars and die at pressures below 500 bars; and at the poles, those that grow below the freezing point of water and die at temperatures above 10°C. All of these organisms are living proof that the biochemical "machinery" of life can be adapted to conditions that, from our anthropocentric perspective, appear to be extreme.

By studying the molecular adaptations of extremophiles, we begin to identify the critical cellular components that expand the envelope for life. As an example, I will discuss what we have learned about the role of the proteins we call "heat shock proteins" in pushing the upper temperature limit of life and how our studies have provided a new perspective on the function of these proteins.

ASTRO-EXTREMO-BIOLOGY

One of the prominent goals of astrobiology is to discover life or signs of life on planets beyond Earth. To approach this goal, it will be useful to know the physical and chemical limits for life on Earth and, perhaps more importantly, to understand the underlying biophysical characteristics of life that set these limits. Such knowledge would allow us to make educated guesses— based on remote measurements of physical and chemical parameters alone— about the likelihood of finding life on other planets.

Although an inventory of life on Earth is far from complete, it is clear that microbes (bacteria and archaea) dominate most habitats, especially extreme habitats. In this context, *extreme* means habitats that radically deviate from the very limited physical and chemical conditions that a human being would consider normal. In cold, deep-sea habitats, with temperatures between 2-4°C and pressures up to 1100 bars, specially adapted microbes known as "barophiles" or "piazophiles" thrive (Yayanos, 1995). These organisms require low temperatures and high pressures to grow, and they die at temperatures above 15°C and pressures below 200 bars (Yayanos et al., 1981). Other extreme habitats of microbes include:

- sulfuric acid springs that emerge from mines associated with pyrite deposits, where acidophiles thrive by iron-sulfur metabolism at pH 0 (Edwards et al., 2000);
- salt deposits of evaporation ponds, where the halophiles grow in saturated salt solutions and withstand desiccation for long periods of time (Kates, 1993; Vreeland, 1993);
- the Siberian permafrost, where psychrophiles metabolize at temperatures down to –20°C (Mazur, 1980);
- terrestrial and submarine hot springs, where thermophiles grow at temperatures above 100°C (the current highest temperature observed is 113°C) (Blöchl et al., 1997).

Microbes have also ingeniously adapted to extremes of radiation, toxin concentrations, low nutrients (starvation), water activity, longevity, and other seemingly bizarre conditions (Kushner, 1981; Scheie, 1970).

Here the focus will not be on cataloging the esoteric extremes to which life has adapted, but rather on how research on extremophiles has provided insights into fundamental problems in biology; in particular, how studying the hyperthermophilic acidophile *Sulfolobus shibatae* (an organism that grows at 85°C and pH 3) has contributed to an understanding of protein folding and membrane stabilization.

HOW TO BEAT THE HEAT

Thermophiles is the name given to organisms that grow optimally at temperatures above 50°C; *hyperthermo-philes* to those that grow optimally above 80°C (Figure 1). Although the existence of these high- temperature organisms has been known since the early twentieth century (Brock, 1978), research on their structural and functional adaptations to these extreme temperatures is still in its early stages. There is currently considerable interest in applying

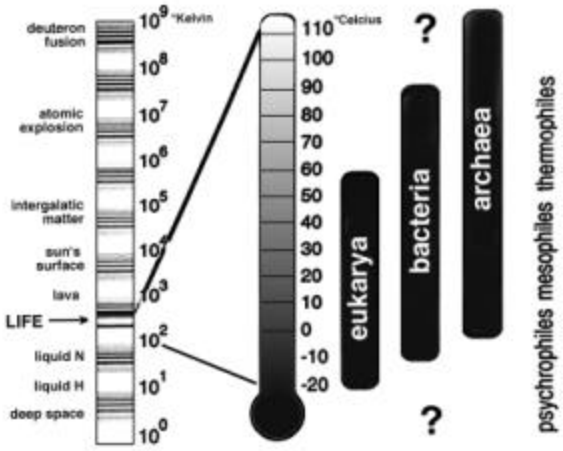

Figure 1. The Temperature Range in the Universe (°K), the Envelope in Which Life Is Known to Be Metabolically Active (°C), and the Current Ranges for Each of the Recognized Phylogenetic Divisions of Life—Eukarya, Bacteria, and Archaea. *The terms used to distinguish groups of organisms based on their temperatures for optimum growth:*

- *psychrophiles = 15°C or below*
- *mesophiles = between 15 and 50 °C*
- *thermophiles = above 50°C*
- *hyperthermophile = above 80°C*

what we learn about the molecular basis of thermophily to research in biotechnology, bioremediation, and molecular biology; and, more recently, in astrobiology (Clark and Kelly, 1989; Kelly and Deming, 1988). In biotechnology, interest is based in part on the need to discover or develop more stable and effective enzyme systems (Adams and Kelly, 1995). In bioremediation, research focuses on understanding and engineering microbes that can thrive in the harsh conditions associated with environmental restoration. In molecular and astrobiology, the interest is twofold: (1) to determine the repertoire of critical molecular adaptations that transform the familiar heat-labile biomolecules into thermostable variants, and (2) to understand how the integrated living system can cope with temperatures near boiling (Jaenicke, 1991).

Thermophiles are living proof that all fundamental life processes can be adapted to high temperatures. How are the macromolecules (e.g., nucleic acids, lipids, and proteins) stabilized? How does the living system as a whole remain coordinated and balanced? How far can biological systems be pushed before kinetic energy tears them apart? These are critical questions that current research is attempting to address. Two partial answers are emerging:

- Macromolecules in thermophiles are modified in a variety of subtle ways that intrinsically increase their thermostability (natural selection is working at the level of function, so there are many possibilities for thermostabilizing structures as long as the function is uncompromised).
- Intracellular conditions can be modified in ways that extrinsically increase thermostability (Hensel, 1993; Perutz, 1978).

These modifications include changes in intracellular salts, production of compatible solutes, and the synthesis of specific proteins. Research is being conducted on a class of proteins known as “heat shock proteins” (HSPs), so called because their synthesis markedly increases with increasing temperatures (Lindquist and Craig, 1988; Watson, 1990). HSPs, abundantly produced by hyperthermophiles, appear to be essential for the thermophilic lifestyle (Trent, 1996). Investigation has provided insights into the function of thermophilic HSPs *in vivo* (Trent et al., 1998).

HSPs, THERMOTOLERANCE, AND MOLECULAR CHAPERONES

All organisms produce HSPs. It has been known for many years that the production of HSPs correlates with a physiological phenomenon known as “acquired thermotolerance.” This designation derives from the observation that an organism exposed to a lethal temperature tends to survive much better if it has first been exposed to a *near*-lethal temperature for a short time (Gerner and Schneider, 1975). Enhanced survival after a heat shock is observed in nearly all organisms, including thermophiles, and it correlates with the increased synthesis of HSPs (Laszlo, 1988). In different species, different HSPs appear to be critical. In the bacterium *Escherichia coli*, 20 different HSPs are correlated to thermotolerance, but their respective roles haven’t yet been clarified (VanBogelen et al., 1987). In the yeast *Saccharomyces cerevisiae*, thermotolerance depends on the synthesis of a 104-kDa protein (HSP104) (Sanchez and Lindquist, 1990); and in the fruit fly *Drosophila melanogaster*, it depends on a 70 kDa protein (HSP70) (Sanders et al., 1986). In mammalian cells, a variety of HSPs, especially the small HSPs, appear to be important (Welch and Mizzen, 1988). In hyperthermophilic archaea, in particular the hyperthermophilic/acidophilic archaeon *Sulfolobus shibatae*, it seems that only two 60 kDa HSPs (HSP60s á & â) are critical for thermotolerance (Trent, 1996).

The actual role of HSPs in acquired thermotolerance is not yet clear. Since the early 1980s, most research has focused on the hypothesis that HSPs interact with damaged proteins. Hightower, who first observed that the heat and chemical agents inducing HSP synthesis also cause proteins to unfold and aggregate, suggested that HSPs may help cells cope with unfolded proteins—either by binding to them to prevent aggregation, marking them for proteolysis, or assisting in their refolding (Hightower, 1991). *In vitro* experiments with pure proteins indicate that some HSPs can indeed recognize and bind unfolded proteins, thereby preventing aggregation and assisting in the process of refolding (Georgopoulos et al., 1994; Hendrick and Hartl, 1993). These *in vitro* observations of protein folding, combined with *in vivo* observations that some HSPs (in particular HSP60 and HSP70) are present in cells under normal growth conditions, led to the hypothesis that these HSPs may be involved in *de novo* protein folding (Pelham, 1986). In this role, HSPs have been referred to as "molecular chaperones” (Ellis et al., 1993). As chaperones, HSPs guard against inappropriate interactions between proteins damaged by heat or chemical stresses, and they also function as midwives in the process of *de novo* protein folding. This hypothesis not only provides a functional link between HSP synthesis and thermotolerance, but it also addresses one of the most fundamental questions in cell biology: How do proteins fold in the highly concentrated and apparently chaotic mixture of macromolecules in the living cell?

While the molecular chaperone hypothesis still has many supporters, there is a growing body of evidence that it is fundamentally wrong. The critical HSPs in cells are not involved in folding or refolding proteins *in vivo*, and the underlying assumption of the hypothesis—that the interior of the cell is “chaotic” and molecules need specific chaperones to function—is misleading.

THE CHAPERONE CONCEPT: VICTORIOUS OR VICTORIAN?

Current investigation at Ames Research Center on HSPs in the hyperthermophilic acidophile *Sulfolobus shibatae* has led to the conclusion that HSP60 is the most critical of the HSPs. In *S. shibatae*, the HSP60s predominate. Other heat-inducible proteins are either absent or present at very low concentrations. For example, HSP70, which is abundant in some organisms, appears to be absent from *S. shibatae*, as well as from other crenarcheaota for which whole genome sequences are now available (Klenk et al.,

1997). In the molecular chaperone model, HSP60 plays a central role by providing the core particle known as the "chaperonin," within which protein folding is believed to occur (Figure 2). The chaperonin system has been best studied in *E. coli*, in which the chaperonin (GroEL) interacts with a co-chaperonin (GroES) and, in an ATP-dependent process, does indeed influence the refolding of a select set of model proteins *in vitro*. It has been observed, however, that many proteins do not interact with chaperonins, which brings into question their universality as "protein-folding machines" (Hartl and Martin, 1995; Horwich et al., 1993). Co-immunoprecipitation experiments indicate that fewer than 15% of *E. coli* proteins bind to GroEL in cell lysates—i.e., 85% of *E. coli* proteins do not bind (Ewait et al., 1997). The rates of protein folding and the abundance of GroEL + GroES in cells indicate that not more than 2% of *E. coli* proteins could be folded by chaperonins *in vivo*—i.e., 98% are folding without using the chaperonin (Lorimer, 1996). In addition, the model for the function of GroEL + GroES indicates that folding occurs within the central cavity of the chaperonin (Weissman et al., 1996). The high-resolution structural information that is now available for GroEL + GroES (Xu et al., 1997) provides precise information about the size of this central cavity. This information can be combined with structural information about *E. coli* proteins to determine the size of proteins that will physically fit within the central cavity of the chaperonin (Figure 3), an exercise demonstrating that only small proteins (<40 kDa) can be accommodated. In summary, the data indicate that only a small fraction of small proteins can be folded by the chaperonin system in *E. coli*. This finding is inconsistent with the underlying rationale for the chaperonin system—i.e., that chaperonins are needed to mediate protein folding within the crowded, chaotic intracellular environment. At this point one may well ask: Why only some proteins? And why, in particular, the smaller ones, when the larger ones may be slower and more problematic to fold?

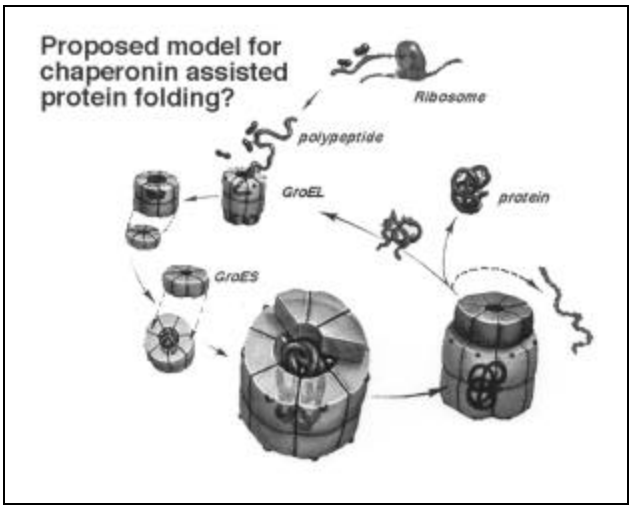

Figure 2. The Putative Chaperonin Cycle, Beginning with the Production of the Polypeptide at the Ribosome and Ending with a Finally Folded Protein. *The polypeptide is thought to be escorted by DnaK (peanut-shaped blobs) as it emerges from the ribosome and is transferred to the cavity in GroEL; folding to take place within the cavity under the cover formed by GroES. The process requires energy from ATP hydrolysis, which causes conformational changes in GroEL to accommodate GroES and ultimately release the protein.*

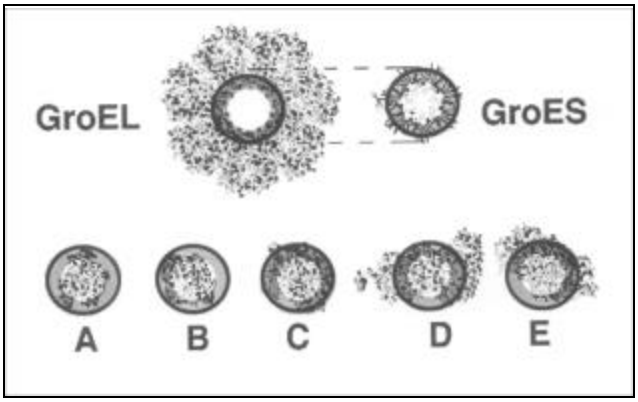

Figure 3. The Crystallographic Structures of the *E. Coli* Chaperonins GroEL and GroES, with Structures of Proteins Placed Inside. *The size of GroES is outlined and superimposed on GroEL to indicate the maximum size to which the cavity of GroEL can be enlarged. The protein structures shown are drawn to scale, indicating the severe space limitations of the putative protein-folding cavity of the chaperonin.*

- *(A) RNase H = 17 kDa*
- *(B) malate dehydrogenase = 32 kDa*
- *(C) isocitrate dehydrogenase = 45 kDa*
- *(D) glycerol kinase = 56 kDa*
- *(E) fragment of topoisomerase = 67 kDa*

THE MEMBRANE IS THE THING

Recent observations reveal that, in some bacteria, the majority of chaperonins are not present in the cell cytoplasm (where most protein folding is believed to occur); rather, they are associated with the plasma membranes (Garduno et al., 1998; Török et al., 1997). Our investigations of chaperonins in hyperthermophilic archaea reveal that most of the chaperonin proteins are associated with the plasma membrane in these organisms, too (Trent, Yaoi, and Kagawa, unpublished). Some years ago it was discovered that the archaeal chaperonin is more closely related to a group of eukaryotic proteins known as TCP1s than to the bacterial chaperonins (Trent et al., 1991). TCP1s are present in the cytoplasm of eukaryotes and are purified from cells as a supramolecular structure similar to the chaperonin. There are claims that TCP1 may be involved in specifically folding actin

and tubulin (Vinh and Drubin, 1994; Yaffe et al., 1992), but genetic manipulations of yeast do not support this interpretation (Ursic and Culbertson, 1991). In addition, the abundance and localization of the eukaryotic cytosolic chaperonins in yeast suggest a role for TCP1 other than protein folding (Ursic et al., 1994). In both yeast and mammalian cells, TCP1-chaperonins are associated with membranes and are involved in cytoskeleton organization (Ursic and Culbertson, 1991; Ursic and Culbertson, 1992; Ursic et al., 1994), cell division (Brown et al., 1996), and exocytosis (Creutz et al., 1994). This apparent structural and membrane-associated role for chaperonins supports the hypothesis that the membrane, not the cytoplasm, is the site of chaperonin activity.

AN ALTERNATE HYPOTHESIS: HSP60-CHAPERONINS MEDIATE MEMBRANE PERMEABILITY

It has been known for many years that bacteria, eukarya, and archaea adapt their membranes to different environmental temperatures by reconstituting their lipids (de Mendoza and Cronan, 1983). Such reconstitution includes increasing the chain length of the lipid acyl chains, the ratio of iso- to anteiso-branching, or the degree of saturation (Prado et al., 1988). It has also been demonstrated that membrane permeability to protons is maintained within a narrow range in bacteria and archaea, and that both natural membranes and liposomes become highly permeable to protons when cells are exposed to high-temperature stress (Peeples and Kelly, 1995). The correlation between the loss of membrane integrity and the synthesis of HSPs has not gone unnoticed (Mejia et al., 1995). However, under the influence of the prevailing chaperonin theory, the increased membrane permeability has been interpreted as a signal to the cell to initiate HSP synthesis in order to prepare itself for damaged proteins, not so that the HSPs will influence the membrane itself (Török et al., 1997).

We suggest that chaperonin interactions with membranes allow cells to rapidly adjust membrane permeability and respond to short-term fluctuations in their environment. The reconstitution of membrane lipids is a secondary, more permanent adjustment the cells make in response to long-term environmental changes. Thus, chaperonin-mediated membrane stabilization allows cells to survive rapid changes in their environments and gives them time to "decide" if they should modify their lipids to accommodate the environmental change.

This hypothesis explains the localization of chaperonins at the cell membrane and is consistent with observations that most of the stresses that induce cells to produce HSPs are suspected or known to damage membranes. HSP-inducing stresses, such as alcohol, peroxide, and heavy metals, clearly impact membranes; but even unfolded proteins, with their exposed hydrophobic and hydrophilic peptide chains, may impact membranes (this remains to be demonstrated). It has long been known, however, that proteins are surface-active, with the ability to form monolayers at air-water interfaces (Deamer, 1992). More recently, it has become clear that nascent membrane proteins penetrate lipid bilayers and that the lipid hydrophobic phase is essential to the folding process. The mechanism of penetration and folding, not yet completely understood, is currently the subject of intense interest.

Surprisingly, the presence of a folded protein in a lipid bilayer ordinarily does not lead to an increased ionic conductance across the bilayer, unless the protein happens to produce an ion-conducting channel by a specific folding of transmembrane alpha helices. On the other hand, non-specific penetration of a lipid bilayer by hydrophobic peptides can markedly affect bilayer permeability. Oliver and Deamer (1994) demonstrated that alpha helical strands of polyalanine and polyleucine incorporate themselves into lipid bilayers, both in the form of liposomes and planar membranes. It was discovered that, under these conditions, liposomes and planar membranes became leaky to ionic flux. Significantly, the leaks showed channel-like behavior in planar bilayers, with a remarkable specificity for protons. This finding confirmed earlier studies indicating that synthetic hydrophobic peptides produced proton-specific ion-conducting channels under certain conditions (Deamer, 1992). If heat shock can produce even a small number of denatured proteins with exposed hydrophobic residues that penetrate lipid bilayers and thereby introduce ion leaks, it follows that HSP-binding to these peptides may protect the membrane from proton leaks.

Since the proton-permeability of membranes increases with temperature, the problem of maintaining an effective proton gradient is exaggerated for hyperthermophiles (Driessen et al., 1996). If this hypothesis is correct, increases in membrane proton permeability can be compensated for by increases in the level of HSP60—a possible explanation for the extraordinary abundance of HSP60 in the hyperthermophilic archaea. HSP60 reaches 12% of total protein in the *S. shibatae* at its upper growth temperature; and, in one of the most extreme hyperthermophiles, *Pyrodictium occultum*, HSP60 reportedly reaches 76% of total protein near the organism's upper growth temperature (Phipps et al., 1991).

CONCLUSION

As indicated above, the HSP60s are currently under intense investigation to determine their role in protein folding. Our research seeks to clarify their potential role in membrane stabilization. Our hypothesis, based on empirical data showing that chaperonins are associated with membranes *in vivo*, suggests that this membrane interaction is the primary function of chaperonins *in vivo*, but it does not exclude a secondary or indirect role for chaperonins in protein folding and assembly. The chaperonin-membrane interaction may influence membrane integrity and thereby affect membrane permeability, which may influence not only the conditions in the cytoplasm but also protein folding indirectly. We are not questioning observations that chaperonins influence protein folding *in vitro*. It has been firmly established that chaperonins (like BSA, PEG, urea, detergents, and a variety of other reagents) influence the folding of a limited number of proteins *in vitro*, which

is a capability that may be of significant value in biotechnology.

Still unanswered are questions about how cells live at hyperthermophilic temperatures and, more generally, how all cells cope with the fluctuating temperatures in their natural habitats. It may be time to abandon the prevailing protein-folding hypothesis in favor of a membrane stabilization hypothesis if we are to understand the role of HSP60 *in vivo*. Fundamental questions arise about the assumptions inherent in the molecular chaperone model. For example:

- Are the protein-protein, protein-substrate, or, more generally, protein-macromolecule interactions that inevitably occur in the crowded confines of the cell leading to non-productive aggregations?
- Are macromolecular interactions part of the natural intracellular dynamics and could they even be productive?
- Are chaperones necessary?

Besides, if a protein is forced into a non-productive interaction under what may be rare circumstances, cells seem well equipped with proteases for dealing with this mistake.

The alternative hypothesis—that the weak link in the cell is the plasma membrane, not proteins, and that HSP60s effectively stabilize the cell membrane—provides a new perspective for understanding HSP60 function *in vivo*. Our research on the extremophile *S. shibatae* certainly supports this model. Perhaps it is serendipitous that we chose that organism, living as it does at low pH and high temperatures. With neutral pH inside a cell that is under constant threat of membrane heat damage, it may be no surprise that 12% of total cellular protein is HSP60, a protein that limits the permeability of the membrane.

Extremophiles are revealing a diversity of subtle biochemical adaptations that allow them to thrive under extreme conditions. On the molecular level, these adaptations include specific modifications of their essential biomolecules, such as lipids, nucleic acids, and proteins. On the cellular, they include production or regulation of compounds such as organic solutes, salts, and proteins that interact with essential biomolecules to help stabilize specific macromolecules and/or the organization of the organism as a whole. This may be one of the advantages of studying extremophiles: the opportunity to look at the biochemical and molecular adaptations that allow them to live under extreme conditions, thereby expanding our perspective on the physical and chemical limits for life on Earth and enhancing our ability to rationally explore for signs of life on planets beyond Earth.

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