amount of data. Obviously, each protein shows individual characteristics. Tentative generalizations refer to (i) the principle of corresponding states reflected by the low structural flexibility around 20°, (ii) the types of weak interactions involved mainly in ion pairs or ion-pair clusters, hydrogen-bond networks, and enhanced hydrophobic packing through van der Waals forces, (iii) an increase in the hydrophobic surface area of folded monomers buried on assembly, and (iv) the distinction between functional and structural amino acids optimized for *flexibility* (catalytic function), on the one hand, and *stability,* on the other. A broader database (now accessible from the complete genome sequence,[117] as well as directed evolution experiments[118]) may provide more general conclusions and finally help in elucidating general strategies of protein stabilization.

Acknowledgments

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[117] K. E. Nelson, R. A. Clayton, S. R. Gill, M. L. Gwinn, R. J. Dodson, D. A. Haft, E. K. Hickey, J. D. Peterson, *et al., Nature* **399,** 323 (1999).
[118] W. P. Stemmer, *Nature* **370,** 389 (1994).

[34] Structural Basis of Thermostability in Hyperthermophilic Proteins, or "There's More Than One Way to Skin a Cat"

By GREGORY A. PETSKO

The great American songwriter Cole Porter was once asked if he wrote the words first or the music first. He said, "Yes." Consider the question of what factor is responsible for the extreme thermostability of proteins isolated from microorganisms whose optimum growth temperature is above 80°. Is it an increase in the number of hydrophobic interactions? An increase in the number of ionic interactions? Shorter surface loops? *Longer* surface loops? Improved packing of the protein core? Oligomerization? More secondary structure? Disulfide formation? Tying down the chain termini? In this paper I shall endeavor to show that the answer here, too, is "Yes."

That we are still even asking the question says more about human nature than it does about what the data have told us. It has been fairly obvious for several years

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that many different factors contribute to the extreme thermostability of any given protein, yet studies continue whose stated goal is to discover a unifying set of rules. It seems hard for some to accept that there is no single factor that dominates. Whether that is because scientists always hope to "discover" something universal, or because it is somehow unsatisfying to conclude that a single great property like extreme thermostability arises from a combination of many different small contributions, is best left for psychologists to ponder; the effect is that studies continue and we have a plethora of new data to examine. It is not the first time this sort of thing has happened in science: two recent examples are the "code" for protein recognition of DNA and the origin of diversity in the immune system. In both cases the search for a single unifying principle led instead to the discovery that several different contributors play a role. The protein–DNA example is particularly relevant to the thermostability issue, because it is now clear that almost every individual protein–DNA complex is *sui generis*. Recognition of, say, an A-T base pair can be done in many different ways and each protein adopts its own strategy. Similarly, we shall see that there are many different ways to make a protein relatively stable to temperatures near the boiling point of water, and although some of them occur frequently, every hyperthermophilic protein employs different mixes. In stabilizing a protein there appears to be, as the saying goes, more than one way to skin a cat. (The origin of this colorful expression, incidentally, is not certain. I'm aware of two possibilities. One is the old British expression "There are more ways of killing a cat than choking it with cream," which implies that whatever was being discussed is foolish, since cats like cream and probably wouldn't choke to death on it. It is conceivable that this expression could have metamorphosed into one indicating that there are more ways than one of accomplishing something. But my preference is for the second possibility, that the original saying was: "More than one way to skin a *catfish*." The meat inside a catfish is tender but the skin is very tough, so over the years many ways have been developed to remove the skin without destroying the meat inside. Why "fish" got dropped I don't know for sure, but because I have a cat I can imagine.... By the way, to a friend who was trying to figure out a way to get rid of his cat, Dorothy Parker suggested, "Have you tried curiosity?")

Pace Gertrude Stein

On her deathbed, Gertrude Stein was asked, "What is the answer?" Exasperating to the last, she replied, "What is the question?" Part of the problem with any discussion of thermostability is that there is more than one way to pose the question. For some investigators thermostability means how long a protein survives at some elevated temperature, usually close to 100°, before it is inactivated. For others, it means whether thermal denaturation (usually measured by the loss of catalytic activity or the disappearance of structure as determined spectroscopically) occurs at relatively high temperature. These do not necessarily reflect the

same underlying molecular events.

Thermal *denaturation* usually occurs when all or a large fraction of the tertiary structural interactions are disrupted, so an increase in denaturation temperature reflects increased stabilization of the structure. We do not yet understand the onset of thermal denaturation well enough to know if merely "tying down" one or two "hot spots" in a protein is always sufficient to stabilize the overall structure, in which case the increased structural stability may be local, or whether many sites distributed throughout the protein must always be stabilized in order for the protein to survive very high temperatures. Like everything else connected with thermostability, it probably varies from protein to protein, but we can't even be sure of that at present. For the purpose of this discussion, we shall assume that stabilizing interactions can, and do, occur pretty much anywhere in the structure.

Thermal *inactivation* can reflect something very different. For one thing, the loss of activity when a protein is held at elevated temperatures for extended periods of time is usually irreversible; thermal denaturation usually is a reversible process. The reason for this difference is that, as first detailed by Klibanov and associates,[1] there are a number of covalent chemical changes in protein structure that occur on prolonged exposure to high temperatures, in addition to any reversible or irreversible unfolding that takes place. Often these covalent changes prevent the reforming of the active, native structure when the temperature is reduced. Which specific changes are found depends, once again, on the protein being studied, but typical examples include polypeptide chain cleavage,[2] side chain isomerization,[3] and deamidation of asparagine residues.[4] It's easy to see how these chemical processes can often be prevented by simple amino acid replacement: if, for example, the half-life of protein X at 90° is determined by the deamidation of two critical asparagines at a subunit interface, then simply replacing those residues by, say, valine or leucine may greatly increase the stability of X to thermal inactivation. Yet the same sort of substitution may have no effect on the stability of protein Y, whose half-life at elevated temperature is limited by the rate of chain cleavage. Neither will the replacement of asparagine residues with other side chains be likely to have any effect on the denaturation temperature of this protein, for that temperature, at which simple thermal-driven unfolding occurs, should be unrelated to the rate of covalent changes in the molecule. A striking example of this uncoupling is provided by the work of Lebbink *et al.*,[5] who, by combining mutations, engineered a version of glutamate dehydrogenase from *Thermotoga maritima* that had

[1] T. J. Ahern and A. M. Klibanov, *Science* **228,** 1280 (1985).

[2] T. J. Ahern and A. M. Klibanov, *Methods Biochem. Anal.* **33,** 91 (1988).

[3] S. J. Tomazic and A. M. Klibanov, *J. Biol. Chem.* **263,** 3086 (1988).

[4] T. J. Ahern, J. I. Casal, G. A. Petsko, and A. M. Klibanov, *Proc. Natl. Acad. Sci. U.S.A* **84,** 675 (1987).

[5] J. H. Lebbink, S. Knapp, J. van der Oost, D. Rice, R. Ladenstein, and W. M. de Vos, *J. Mol. Biol.* **289,** 357 (1999).

30 minutes longer half-life for inactivation at 85° but only a 0.5° higher apparent melting temperature.

Hence, in the search for general or at least frequently employed factors that contribute to protein thermostability, we must first agree on the type of stability we are talking about. For the purpose of this discussion, we shall confine ourselves to thermal unfolding as measured either by loss of structure or loss of activity as the temperature is raised. Since a catalog of interactions in proteins can have all the excitement of the Catalog of Ships in the second half of Book Two of *The Iliad* (that's the part that literature courses all *skip,* remember), I have taken the liberty of trying to enliven the discussion by constructing an imaginary dialog between myself and a skeptic desperately trying to find a single, predominant stabilizing effect.

Is It Increased Hydrogen Bonding? I'll Bet It's Increased Hydrogen Bonding.

Sometimes it is. Comparison of the crystal structure of methionine aminopeptidase from the hyperthermophile *Pyrococcus furiosus* with that of the same enzyme from the mesophile *Escherichia coli* led Tahirov *et al.* to conclude that a major stabilizing factor was an increase in the number of hydrogen bonds between positively charged side chains and neutral oxygens.[6] A similar conclusion was reached by Pfeil *et al.* in their study of ferredoxin from *T. maritima,*[7] and by Macedo-Riberio *et al.* [8,9] Tanner, Hecht, and Krause observe the same thing when they compare their structure of *Thermus aquaticus* glyceraldehyde-3-phosphate dehydrogenase with those from mesophiles.[10] They speculate that the reason such hydrogen bonds, where only one of the participants is charged, are so effective in stabilizing proteins is that they provide electrostatic stabilization without the heavy penalty of increased salt bridges (but see below).

But sometimes it isn't. In contrast to these data, a number of other thermophile/mesophile structure comparisons have found no significant increase in the number of hydrogen bonds in the thermophilic protein. One example is the threefold comparison of *Escherichia coli, Salmonella typhimurium,* and *Thermus thermophilus* 3-isopropylmalate dehydrogenase structures[11] where the number of non-salt-bridge hydrogen bonds didn't change much. The authors of that study make

[6] T. H. Tahirov, H. Oki, T. Tsukihara, K. Ogasahara, K. Yitani, K. Ogata, Y. Izu, S. Tsunasawa, and I. Kato, *J. Mol. Biol.* **284,** 101 (1998).

[7] W. Pfeil, U. Gesierich, G. R. Kleemann, and R. J. Sterner, *J. Mol. Biol.* **272,** 591 (1997).

[8] S. Macedo-Ribeiro, B. Darimont, and R. Sterner, *Biol. Chem.* **378,** 331 (1997).

[9] S. Macedo-Ribeiro, B. Darimont, R. Sterner, and R. Huber, *Structure* **4,** 1291 (1996).

[10] J. J. Tanner, R. M. Hecht, and K. L. Krause, *Biochemistry* **35,** 2597 (1996).

[11] G. Wallon, G. Kryger, S. T. Lovett, T. Oshima, D. Ringe, and G. A. Petsko, *J. Mol. Biol.* **266,** 1016 (1997).

the very important point that it is not appropriate to compare numbers of hydrogen bonds between structures that were determined at very different resolutions, because the errors in the precision with which interatomic distances can be determined vary greatly with resolution.

Is It Increased Secondary Structure Formation and Stability?

Sometimes yes. Warren and Petsko examined the amino acid composition of alpha helices in thermophilic proteins and found an increase in the numbers of those amino acids whose presence would be expected to increase helical stability.[12] Of particular interest was an increase in the number of glycine residues. In addition to having a positive delta-s value (Zimm–Bragg helix propagation value) with temperature, glycine is also the most favorable amino acid to form a cap at either the N or C terminus of a helix.[13] Improved capping has been shown by Fersht and co-workers to contribute greatly to the stability of an α helix.[13] And indeed, some thermophilic proteins show more extensive secondary structure, and better capped helices, than their mesophilic counterparts. Examples include the archaeal *O*(6)-methylguanine-DNA methyltransferase[14] and *P. furiosus* methionine aminopeptidase,[6] in both of which the helices are stabilized by interhelical side-chain interactions of the kind predicted earlier[12]; glyceraldehyde-3-phosphate dehydrogenase from *Sulfolobus solfataricus,*[15] phosphoribosylanthranilate isomerase from *T. maritima,*[16] and lactate dehydrogenase from *T. maritima,*[17] in which the number of alpha helices actually increases over that found in their mesophilic counterparts; and improved stabilization by capping such as found in *T. maritima* ferredoxin[9] and indole-3-glycerol phosphate synthase from *S. solfataricus*.[18] Interestingly, much less attention has been paid to β sheets; it is at this time unclear if any changes in sheet number or increased hydrogen bonding in sheets are important for increased thermostability, although one study, of rubredoxin from *P. furiosus,* suggests they may be.[19]

Sometimes no. A number of other studies of specific thermophile/mesophile pairs have found no obvious increase in helix or sheet number, no additional

[12] G. L. Warren and G. A. Petsko, *Protein Eng.* **8,** 905 (1995).

[13] Y. Harpaz, N. Elmasry, A. R. Fersht, and K. Henrick, *Proc. Natl. Acad. Sci. U.S.A.* **91,** 311 (1994).

[14] H. Hashimoto, T. Inoue, M. Nishioka, S. Fujiwara, M. Takagi, T. Imanaka, and Y. Kai, *J. Mol. Biol.* **292,** 707 (1999).

[15] M. N. Isupov, T. M. Fleming, A. R. Dalby, G. S. Crowhurst, P. C. Bourne, and J. A. Littlechild, *J. Mol. Biol.* **291,** 651 (1999).

[16] M. Hennig, R. Sterner, K. Kirschner, and J. N. Jansonius, *Biochemistry* **36,** 6009 (1997).

[17] G. Auerbach, R. Ostendorp, L. Prade, I. Korndorfer, T. Dams, R. Huber, and R. Jaenicke, *Structure* **6,** 769 (1998).

[18] M. Hennig, B. Darimont, R. Sterner, K. Kirschner, and J. N. Jansonius, *Structure* **3,** 1295 (1995).

[19] M. W. Day, B. T. Hsu, L. Joshua-Tor, J. B. Park, Z. H. Zhou, M. W. Adams, and D. C. Rees, *Protein Sci.* **1,** 1494 (1992).

stabilizing hydrogen bonds or side-chain interactions in helices or sheets, or better helix capping.[11,20]

It's Better Packing, Isn't It?

Maybe. A decrease in the number of internal cavities has been observed in some cases, such as lactate dehydrogenase[16] and glutamate dehydrogenase[21] from *T. maritima* and *Thermococcus litoralis*.[22]

Maybe not. No such decrease has been found in many other comparisons, including the isopropylmalate dehydrogenase case[11] and the studies of *S. solfataricus* glyceraldehyde-3-phosphate dehydrogenase[15] and the superstable superoxide dismutase (melting temperature 125°!) from *Sulfolobus acidocaldarius*.[23] In fact, a test of this idea was actually carried out on *T. thermophilus* isopropylmalate dehydrogenase: a mutation was made that created a new cavity of 32Å^3 in volume in the interior of the protein, but no decrease in thermostability was observed.[11]

OK, It's Not Exactly Better Packing. It's Really Decreased Surface to Volume Ratio.

It may well be, in some cases. Surface loops are often drastically shortened in hyperthermophilic enzymes; examples include *T. thermophilus* isopropylmalate dehydrogenase,[11] *T. maritima* ferredoxin,[8] and *T. aquaticus* glyceraldehyde-3-phosphate dehydrogenase.[10]

But in other cases, it may well not be. The most dramatic exception seems to be two subtilisin-like proteases from *P. furiosus* and *T. stetteri*.[24] These highly thermostable proteases actually have several extra surface loops compared with their mesophilic counterparts!

Still, there seems to be something to this surface-to-volume ratio idea, because a number of normally monomeric enzymes oligomerize when they are found in hyperthermophilic organisms. Adenylate kinase from *S. acidocaldarius* is a trimer, for example, whereas nearly all other adenylate kinases are monomeric.[25] Perhaps the best evidence that this effect may be important comes from an engineered protein, the repressor of primer (ROP). ROP is normally an all helical homodimeric protein that denatures at 71°. Removal of five amino acids from a surface

[20] G. H. Silva, J. Z. Dalgaard, M. Belfort, and P. Van Roey, *J. Mol. Biol.* **286,** 1123 (1999).
[21] S. Knapp, W. M. de Vos, D. Rice, and R. Ladenstein, *J. Mol. Biol.* **267,** 916 (1997).
[22] K. L. Britton, K. S. Yip, S. E. Sedelnikova, T. J. Stillman, M. W. Adams, K. Ma, D. L. Maeder, F. T. Robb, N. Tolliday, C. Vetriani, D. W. Rice, and P. J. Baker, *J. Mol. Biol.* **293,** 1121 (1999).
[23] S. Knapp, S. Kardinahl, N. Hellgren, G. Tibbelin, G. Schafer, and R. Ladenstein, *J. Mol. Biol.* **285,** 689 (1999).
[24] W. G. Voorhorst, A. Warner, W. M. de Vos, and R. J. Siezen, *Protein Eng.* **10,** 905 (1997).
[25] C. Vonrhein, H. Bonisch, G. Schafer, and G. E. Schulz, *J. Mol. Biol.* **282,** 167 (1998).

loop converts the protein to a homotetramer and increases T_m to101°.[26] Finally, in contrast to what is usually observed for mesophilic proteins, a number of hyperthermophilic proteins have their chain termini tucked back into the body of the protein, which not only would decrease the surface to volume ratio but also would prevent these ends of the chain from serving, as loops might also serve, as "fraying points" where the structure might begin to unravel at high temperatures.[27] Examples include phosphoribosylanthranilate isomerase[16] and ferredoxin[8] from *T. maritima,* and rubredoxin[19] from *P. furiosus.*

Wait, I've Got It; It's More Hydrophobic Residues, Right? Because the Hydrophobic Effect Increases with Increasing Temperature, so....

It makes sense. And, yes, a number of hyperthermostable proteins do show a significant increase in the number of hydrophobic residues, especially in the core of the structure or at subunit interfaces: *T. maritima* lactate dehydrogenase,[16] the hypertherophilic subtilisin-like proteases,[24] *Aquifex pyrophilus* superoxide dismutase,[28] and *S. acidocaldarius* superoxide dismutase[23] are just a few of many examples. However, some proteins show no such increase, and a few actually have more polar water molecules in the core instead (e.g., β-glycosidase from *Thermosphaera aggregans,* ref. 29). And then there's this funny business about aromatic residues. If increased content of hydrophobic residues was a major factor in thermostability, one might expect to see a significant increase in the number of aromatic residues in the core, because they bury more hydrophobic surface area than aliphatic residues do, and also have the opportunity for additional stabilization through aromatic–aromatic interactions. Well, it's like everything else: some hyperthermophilic proteins do have more such interactions,[24] but others do not.[11]

Don't Tell Me It's Increased Rigidity.

OK, I won't tell you. Besides, it's awfully hard to figure out if increased rigidity is a cause of hyperthermostability or an effect. It is certainly true that *most* hyperthermostable proteins are more rigid, at ordinary temperatures, than their mesophilic counterparts,[30] but not *all* of them are, and it's unclear whether this

[26] M. W. Lassalle, H. J. Hinz, H. Wenzel, M. Vlassi, M. Kokkinidis, and G. Cesareni, *J. Mol. Biol.* **279,** 987 (1998).

[27] T. Lazaridis, I. Lee, and M. Karplus, *Protein Sci.* **6,** 2589 (1997).

[28] J. H. Lim, Y. G. Tu, Y. S. Han, S. Cho, B. Y. Ahn, S. H. Kim, and Y. Cho, *J. Mol. Biol.* **270,** 259 (1997).

[29] Y. I. Chi, L. A. Martinez-Cruz, J. Jancarik, R. V. Swanson, D. E. Robertson, and S. H. Kim, *FEBS Lett.* **445,** 375 (1999).

[30] P. Zavodszky, J. Kardos, R. Svingor, and G. A. Petsko, *Proc. Natl. Acad. Sci. U.S.A.* **95,** 7406 (1998).

contributes to a high melting temperature or just arises as a consequence of the increased number of different kinds of stabilizing interactions that these proteins all seem to have. The one fact that suggests there may be a causal relationship is the increased number of proline residues in many hyperthermophilic protein sequences.[6,11] Proline reduces the flexibility of the polypeptide chain.

Tang and Dill[31] have attempted a theoretical investigation of this question. Using an HP lattice model, they find a low-temperature point below which large fluctuations are frozen out. They also conclude that proteins having greater stability tend to have fewer large fluctuations, and hence lower overall flexibilities.

Isn't There ANYTHING That's at Least *Common* to All These Proteins?

As a matter of fact, there is. It can't be the *only* stabilizing factor, and may not even be the *most important* stabilizing factor in many cases because of all the other ones already mentioned, but it appears to be observed nearly all the time. It's an increase in the number of ion pairs (salt bridges), especially in networks. Essentially every one of the thermophilic protein structures cited thus far contains an increase in the number of ionic interactions relative to its closest mesophilic homolog. These include intrahelix ion pairs.[10,14] interhelix ion pairs,[14,16] surface ion pairs,[7,29] intersubunit ion pairs,[23,32] and intrasubunit ion pairs,[17,28,33]; any kind of ion pair seems to help, especially when these pairs form networks (e.g., refs. 5, 15, 19). Ogasahara *et al.*,[34] in a very important study with the kind of detail that is sorely needed, used calorimetry to examine the effect of salt on thermal stability of *P. furiosus* methionine aminopeptidase. From this they could establish directly the contribution that the large number of salt bridges in this protein make to its thermostability. It all makes perfect sense.

And yet, even here, there are subtleties. Although it would seem from the above that ion pairs can be put almost anywhere to increase stability, that may not be the case. Lebbink *et al.* tried to enlarge the existing networks in *T. maritima* glutamate dehydrogenase and found that resistance to both thermal denaturation and irreversible thermal inactivation *decreased*. However, combination of destabilizing single mutations often restored stability.[5] From this they conclude that there is a need for a balance of charges at subunit interfaces and high cooperativity between different members of the network. Russell *et al.* found that thermal denaturation in citrate synthase appears to be resisted by intersubunit ion pair networks,

[31] K. E. Tang and K. A. Dill, *J. Biomol. Struct. Dyn.* **16,** 397 (1998).

[32] R. J. Russell, U. Gerike, M. J. Danson, D. W. Hough, and G. L. Taylor, *Structure* **6,** 351 (1998).

[33] G. Auerbach, R. Huber, M. Grattinger, K. Zaiss, H. Schurig, R. Jaenicke, and U. Jacob, *Structure* **5,** 1475 (1997).

[34] K. Ogasahara, E. A. Lapshina, M. Sakai, Y. Izu, S. Tsunasawa, I. Kato, and K. Yutani, *Biochemistry* **37,** 5939 (1998).

whereas cold denaturation appears to be resisted by an increase in intramolecular ion pairs.[32] Tanner, Hecht, and Krause concluded that charged residues play a dual role in stabilization by participating not only in salt bridges, but also in charged-neutral hydrogen bonds.[10] And Knapp *et al.* point out that in *T. maritima* glutamate dehydrogenase the number of intersubunit ion pairs is actually *reduced* vis-à-vis the mesophilic enzyme, whereas in *P. furiosus* glutamate dehydrogenase there is a big increase in the size of the intersubunit ion pair network. In both cases the number of intrasubunit ion pairs is increased.[21] We clearly don't understand yet just where some interaction has to be placed in a structure to guarantee that it will increase stability. And as for how all the different kinds of interactions balance out, well....

So is EVERYTHING Important EVERYWHERE?

Potentially, yes. What all of these studies seem to show is that protein stability at hyperthermophilic temperatures arises from a combination of many factors, each of which contributes to a different extent in different proteins, and not all of which need be present. As to where the increased interactions need to be placed in the structure to contribute to stability, the answer seems to be all over. The structural distribution of stability in a thermophilic enzyme has been examined in detail in a paper by Hollien and Marqusee that has just appeared.[35] Using NMR, they determined the native state hydrogen exchange rates for each residue in RNase H from *T. thermophilus*. They found that the general distribution of stability in the thermophilic protein is similar to that of its mesophilic homolog from *E. coli*, with a proportional increase in stability for almost all residues. Consequently, the residue-specific stabilities of the two proteins are remarkably similar under conditions where their global stabilities are the same. From these data they conclude that this enzyme is stabilized in a delocalized fashion, with the stabilizing interactions—and presumably (although their H/D data measure this only indirectly) any decrease in flexibility—being distributed throughout the structure.

Extreme thermostability thus seems to be achieved in nature by distributing many different kinds of additional intramolecular interactions throughout the protein rather than by concentrating just one kind in one or a few places (but there is no indication yet that it would be impossible to achieve it in that simpler way artificially). Although increased ionic interactions and greater compactness seem to be the most frequently observed strategies based on comparison of thermophilic and mesophilic protein structures, in only a few instances have the contributions of these factors been tested experimentally, for example by site-directed mutagenesis of hyperthermophilic proteins. There is still considerable room for further work along those lines.

[35] J. Hollien and S. Marqusee, *Proc. Natl. Acad. Sci. U.S.A.* **96,** 13674 (1999).

As is so often the case in biology, searches for an overarching principle frequently are doomed to failure from the beginning. Evolution can, and does, make use of anything that works. As the somewhat whimsical title of this piece is meant to remind us: there's more than one way to skin a cat (and I have a cat, so believe me, I've thought about this).

This is All Just Your Opinion, Though, Isn't It?

Well, it's my *conclusion* based on the available data. And it's not uniquely my conclusion, either. A number of other scientists have already made the point that the only general conclusion that can be drawn is that there are many different ways to stabilize a protein. Two papers of note that emphasize this are by Daniel, Dines, and Petach,[36] who state that "there is currently no strong evidence that any particular interaction ... plays a more important role in proteins that are stable at 100°C than in those stable at 50°C," and by Jaenicke and Bohm,[37] who conclude that "proteins are individuals that accumulate increments of stabilization; in thermophiles these come from charge clusters, networks of hydrogen bonds, optimization of packing and hydrophobic interactions, each in its own way."

I'm particularly fond of that last quotation. Anyone who has a cat knows all about individuals that go their own way.

[36] R. M. Daniel, M. Dines, and H. H. Petach, *Biochem. J.* **317,** 1 (1996).
[37] R. Jaenicke and G. Bohm, *Curr. Opin. Struct. Biol.* **8,** 738 (1998).