REVIEW

Lessons in stability from thermophilic proteins

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Abstract

Studies that compare proteins from thermophilic and mesophilic organisms can provide insights into ability of thermophiles to function at their high habitat temperatures and may provide clues that enable us to better define the forces that stabilize all proteins. Most of the comparative studies have focused on thermal stability and show, as expected, that thermophilic proteins have higher T_m values than their mesophilic counterparts. Although these comparisons are useful, more detailed thermodynamic analyses are required to reach a more complete understanding of the mechanisms thermophilic protein employ to remain folded over a wider range of temperatures. This complete thermodynamic description allows one to generate a stability curve for a protein that defines how the conformational stability (ΔG) varies with temperature. Here we compare stability curves for many pairs of homologous proteins from thermophilic and mesophilc organisms. Of the basic methods that can be employed to achieve enhanced thermostability, we find that most thermophilic proteins use the simple method that raises the ΔG at all temperatures as the principal way to increase their T_m. We discuss and compare this thermodynamic method with the possible alternatives. In addition we propose ways that structural alterations and changes to the amino acid sequences might give rise to varied methods used to obtain thermostability.

Keywords: protein folding; thermodynamics; protein stability curves; thermophiles; thermostability

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Abbreviations: T_m, melting temperature or temperature at midpoint of transition from native to denatured state in a thermal denaturation; ΔG_T, free energy of stabilization at a temperature T; ΔC_p, change in heat capacity associated with protein unfolding; ΔH, change in enthalpy; ΔS, change in entropy; T_S, temperature of maximal stability or temperature where change in entropy between native and denatured states is zero; ΔG_S, free energy of stabilization at T_S; T_E, environment or habitat temperature of an organism; ΔASA, change in solvent accessible surface area upon protein unfolding; GuHCl, guanidine hydrochloride; CspB, cold shock protein B; CheY, chemotactic protein Y; HPr, histidine containing phospho-carrier protein; *Mf*A, archaeal histone A from hyperthermophile *Methanothermus fervidus*; *Mf*B, archaeal histone B from hyperthermophile *Methanothermus fervidus;* *Py*A1, archaeal histone from hyperthermophile *Pyrococcus* strain GB3a; *Mfo*B; archaeal histone B from mesophile *Methanobacterium formicicum*; IPMD, isopropyl malate dehydrogenase; MGMT, O^6Methyl guanine DNA methyl transferase; DHFR, dihydrofolate reductase.

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Life exists almost everywhere on the earth, from deep-sea hydrothermal vents to the heights of the Himalayas, from boiling waters of hot springs to the cold expanses of Antarctica. The organisms that inhabit and have adapted to these extreme and diverse environments are often classified by their altered habitat, such as temperature adaptations (psychrophiles to hyperthermophiles), high salinity adaptations (halophiles), pH adaptations (acidophiles and alkaliphiles), and pressure adaptation (barophiles), to name a few groups. In general, these organisms are often called extremophiles and have been of interest to many protein chemists over the years, dating back to early studies by Perutz and colleagues (Perutz and Raidt 1975; Perutz 1978). In case of adaptations to extremes of pH, salinity, and pressure, membrane components and protective small molecules often play an important role (Jaenicke 1991) and these have been studied quite extensively (Yancey et al. 1982; van de Vossenberg et al. 1998). For temperature adaptations,

however, environmental stress generally cannot be avoided by compensatory mechanisms, and thus the cellular components themselves, specifically the proteins, have to achieve thermostability (Jaenicke and Zavodszky 1990). For this reason, much interest has been directed to understanding how proteins from thermophilic organisms retain their structure and function at these elevated temperatures (Argos et al. 1979; Rees and Adams 1995; Somero 1995; Vieille and Zeikus 1996; Jaenicke 1998; Sterner and Liebl 2001).

Proteins perform important tasks in all biological systems, and they do so by maintaining a specific globular conformation. This functional state, called the native state, is marginally stabilized in a balancing act of opposing forces. The players in this balancing act have long been identified (Kauzmann 1959), although their relative contributions have been debated (Dill 1990; Creighton 1992; Rose and Wolfenden 1993; Pace et al. 1996; Honig 1999). The major stabilizing forces include the hydrophobic effect and hydrogen bonding while conformational entropy favors the unfolded state. The forces stabilizing the native state outweigh the disruptive forces marginally in a folded protein, in the range of 5–10 kcal mol^{-1} (Pace 1975). This balance of forces is known as the conformational stability of a protein and is defined thermodynamically as the free energy change, ΔG, for the native $\leftrightarrow$ unfolded state transition. Measurements of and studies on protein stability have remained important over several decades owing to the central role these macromolecules play in maintaining life and their involvement in many diseases affecting humans. Studies on protein stability explore the sequence–structure–stability relationship, with stability being the measured thermodynamic quantity, since sequence defines structure, whose interactions afford stability. Sequence is also the variable that organisms alter as they evolve to adapt their proteins to the environments they inhabit. The stability of proteins is usually determined experimentally by perturbing the native state using temperature or denaturing solvent additives (urea, GuHCl) and following this "reaction" by direct (calorimetric) and indirect (spectroscopic) probes (for further details, see Lopez and Makhatadze 2002; Grimsley et al. 2003).

Since this review focuses on thermodynamics of protein stability and protein stability curves in particular, an introduction to these concepts is in order. Becktel and Schellman (1987) introduced protein stability curves, showing plots of free energy of stabilization (ΔG) as a function of temperature (Fig. 1). Such data are described by a modified version of the Gibbs–Helmholtz equation, and important thermodynamic parameters can be determined (Equation 1):

$$\Delta G(T) = \Delta H_m\left(1 - {}^{T}/_{T_m}\right) - \Delta C_p \times \left[(T_m - T) + T\ln\left({}^{T}/_{T_m}\right)\right] \quad (1)$$

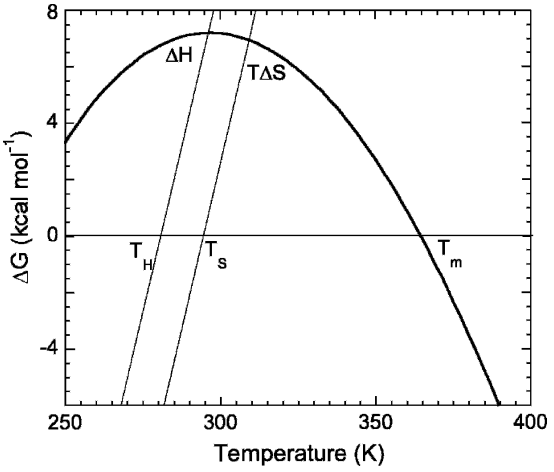

Figure 1. A stability curve for a hypothetical protein (Becktel and Schellman 1987). The stability of a protein is plotted as a function of temperature, and the data can be explained by a modified version of the Gibbs Helmholtz equation (Equation 1). Some key thermodynamic parameters are also marked on the plot. (For an explanation of the terms used, refer to the text.)

where $\Delta G(T)$ is the free energy at a temperature T; ΔH_m is the change in enthalpy at T_m; ΔC_p is the change in heat capacity associated with the unfolding of the protein; and T_m is the melting temperature or the temperature at midpoint of transition from native to denatured state. Other parameters of interest that can be calculated using modifications of Equation 1 include T_S and ΔG_S, where T_S is the temperature of maximum stability or temperature where the change in entropy between native and denatured states is zero and ΔG_S is the conformational stability at this temperature. Protein stability curves also allow the calculation of conformational stability at any temperature, including the habitat temperature of an organism (T_E).

Three-dimensional structures of proteins are often instrumental in our attempts to understand protein stability and the forces involved. Atomic resolution structures are required for enumerating stabilizing interactions like hydrogen bonds and electrostatic interactions; they are also necessary for theoretical studies attempting to correlate features like buried surface areas with magnitude of stabilizing forces like the hydrophobic effect (Pace 1992). Structures are also used to measure ΔASA or change in solvent accessible surface area upon unfolding of a protein. It has been shown that ΔASA correlates with thermodynamic quantities like ΔC_p (Livingstone et al. 1991; Murphy and Freire 1992; Spolar et al. 1992) and the *m*-value (Myers et al. 1995), a parameter that is used to describe the denaturant-induced unfolding of a protein. Protein structures also emphasize the fact that the native state is held together by a large number of weak

noncovalent interactions between constituent amino acids. The fact that proteins are only marginally stable in general makes the ability of thermophilic proteins to function particularly intriguing because, unlike membranes that show heterogeneity in their building block lipids (Russell and Fukunaga 1990), proteins are composed of the same 20 amino acids irrespective of the organism and its habitat.

There have been several studies designed to understand the thermodynamic strategies that proteins from thermophiles use to remain folded at their high habitat temperatures. Proteins from thermophiles alter their sequence such that it optimizes the interactions holding their native conformations together; these optimizations in turn alter key thermodynamic parameters like ΔC_p, ΔG, and ΔH in a way that ''tunes'' the stability characteristics to the habitat of the organism. As protein chemists, we can measure these cardinal parameters, construct stability curves, and possibly learn about the strategies employed in thermostabilization. Nojima et al. (1977) proposed three different methods of modulating the stability curve of a protein to achieve higher thermostability (greater T_m; Fig. 2). Briefly, a hypothetical mesophilic protein can (I) raise the entire stability curve to higher ΔG so it now has a higher T_m, (II) broaden its stability curve so it now intersects the abscissa at a higher temperature, or (III) shift the entire stability curve to the right (to higher temperatures). All three methods of achieving higher thermostability have been observed in nature, some independently and others in combination. These methods have underlying thermodynamic mechanisms, for example, increasing the value of ΔH_S (the change in enthalpy measured at T_S) without compensating changes in ΔS will result in a similar stability curve, but with higher ΔG values at all temperatures (method I). A broadened stability curve (method II) is caused by a reduced ΔC_p. Lowering the ΔS or the change in entropy for the folding transition shifts the T_S to higher temperatures and has the effect of shifting the stability curve to the right (method III).

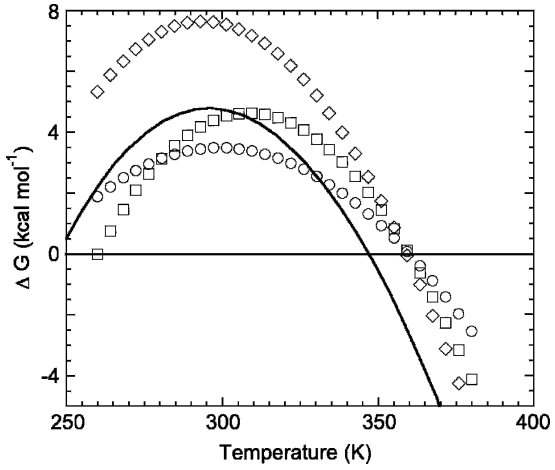

Figure 2. Stability curves showing different methods to achieve a higher T_m. Starting with a stability curve for a hypothetical mesophilic protein (solid line), the protein may increase T_m by shifting the curve up (method I [diamonds]), by making the curve flatter (method II [circles]), or by shifting the curve to the right (method III [squares]). The thermodynamic bases and explanations for all these methods are discussed in the text.

Here we have compiled results from studies reporting thermodynamic characterization of proteins (or domains) from thermophilic species. More specifically, thermodynamic parameters have been compiled so comparisons can be made with values from mesophilic homologs where possible and conclusions drawn on the mode of thermostabilization employed in each case. Our results show which mode of thermostabilization is more commonly employed and we discuss possible reasons for the results.

Construction of the database

A literature search was performed to find experimental thermodynamic characterization of proteins from thermophilic organisms. Our search focused on studies reporting a comparison of thermodynamic data on homologous proteins from thermophiles and mesophiles. The results of the literature search were augmented with data from the Protherm database (Bava et al. 2004). In all, we found 26 sets of proteins for which conclusions concerning the thermodynamic mode of stabilization have been made or can be, based on the information provided (Table 1). Of the 26 sets of proteins, 19 make comparisons with a homologous protein from a different organism, four make comparisons with a collection of similarly sized proteins, and the remaining three do not make comparisons with other proteins. Most of the thermodynamic data are from the analyses of circular dichroism (CD) and differential scanning calorimetry (DSC) unfolding experiments, either in the presence or the absence of denaturants like urea or GuHCl; only in the case of the ferredoxin proteins was HD exchange as monitored by NMR used to estimate stability (Pfeil et al. 1997).

The transitions from native to denatured state exhibited a two-state behavior for most proteins in our compilation. Although nine proteins were dimers in solution, they were shown to follow the two-state model of folding, and the remaining 17 were monomeric and also followed the two-state model. There is one pair of proteins, namely the IPMD enzymes from *Thermus thermophilus* and *Escherichia coli* (Motono et al. 2001), where the former appears to be two-state and the latter follows a three-state unfolding model (entry 13 in Table 1).

The data show that, on average, a thermophilic protein has a T_m 31.5°C higher than its mesophilic homolog, for

Table 1. *Thermodynamic parameters for homologous proteins derived from mesophiles and thermophiles*

	Protein	Source[a]	PDB ID (no. of residues)	T_m (°C)	ΔC_p (kcal mol^{-1} K^{-1})	ΔG (kcal mol^{-1})[b]	T_S (°C)[c]	Mode of stabilization[d]	Reference
1	CspB	*Thermotoga maritima*	1G6P (67)	83.1	0.22			I and II*	Schindler and Schmid 1996; Jacob et al. 1999; Wassenberg et al. 1999
		Bacillus subtilus	1CSP (67)	54	0.88	ΔG_S: *5.5*	*−6*		
2	RNase H	*Thermus thermophilus*	1RIL (166)	86	1.8	ΔG_S: 12.7	20	I and II	Kanaya and Itaya 1992; Hollien and Marqusee 1999
		Escherichia coli	1JXB (155)	66	2.7	ΔG_S: 7.5	24		
3	SSo7D	*Sulfolobus solfataricus*	1SSO (64)	98	0.63	ΔG_S: 7	9	I and II*	Knapp et al. 1996, 1998
		SH3 domain proteins		69–80	0.69–0.81	ΔG_{25}: 3–4	16–23		
4	CheY	*Thermotoga maritima*	1TMY (120)	101	1.17	ΔG_S: 9.54	29	I and II	Deutschman and Dahlquist 2001
		Bacillus subtilus	— (120)	55	2.34	ΔG_S: 3.15	27		
5	Ribosomal protein L30E	*Thermococcus celer*	1H7M (100)	93.8	1.27	ΔG_S: *12*	*17*	I and II	Lee et al. 2005
		Saccharomyces cerevisiae	1CN7 (104)	45.7	2.51	ΔG_S: *3.5*	*17*		
6	Aspartate aminotransferase	*Sulfolobus solfataricus*	(362)		2.87	ΔG_{25}: 16.8		I and II	Arnone et al. 1997
		Cytosolic pig heart	(413)		4.78	ΔG_{25}: 13.8			
7	Phosphoglycerate kinase	*Thermotoga maritima*	1VPE (398)	85		ΔG_{20}: 28.9		I and II*	Hu and Sturtevant 1989; Grattinger et al. 1998; Jaenicke and Bohm 2001
		Saccharomyces cerevisiae	3PGK (416)	60		ΔG_{20}: 6			
8	Phosphoglycerate kinase	*Thermus thermophilus*	1V6S (390)			ΔG: 12		I and II*	Nojima et al. 1977
		Saccharomyces cerevisiae	3PGK (416)	60		ΔG_{20}: 6			
9	Sac7d	*Sulfolobus acidocaldarius*	1SAP (66)	91	0.5–0.86[e]	ΔG_S: *6.5*	*22*	II	McCrary et al. 1996
		Other small proteins		54–90	0.62–1.6	ΔG_{25}: 2.3–8.9			
10	Ssh10B	*Sulfolobus shibattae*	— (97)	125	0.9	ΔG_{25}: 30.1	*15*	II	Milla et al. 1994; Xu et al. 2004
11	HU DNA binding protein	Arc repressor-bacteriophage P22	1ARQ (106)	54	1.53	ΔG_{25}: 11	*19*		
12	Glutamate dehydrogenase domain II	*Thermotoga maritima*	1B8Z (90)	101.9	0.76	ΔG_S: 6.8	20	II*	Ruiz-Sanz et al. 2004
13	Isopropyl malate dehydrogenase	*Thermotoga maritima*	2TMG (149)	69.5	1.4	ΔG_S: 3.7	33	II	Consalvi et al. 2000
		Thermus thermophilus	1OSI (345)	57	1.73	ΔG_S: 15.8	31	II*	Motono et al. 2001
		Escherichia coli	1CM7 (363)	107	20.7	ΔG_S: 32.7	25		
14	Histidine containing protein (HPr)	*Bacillus staerothermophilus*	1Y4Y (88)	88.9	1.37	ΔG_S: 8.2	24.8	I	Sridharan et al. 2005
		Bacillus subtilus	2HPR (88)	74.4	1.33	ΔG_S: 5.2	24.1		
		Streptococcus thermophilus	— (87)	77	1.28	ΔG_S: 6.3	22		
15	Histidine containing protein (HPr)	*Bacillus subtilis*	2HPR (88)	74.4	1.33	ΔG_S: 5.2	24.1	I	Razvi and Scholtz 2006
		Bacillus halodurans	— (87)	82.2	1.3	ΔG_S: 6.7	25		
16	Histidine containing protein (HPr)	*Bacillus subtilus*	2HPR (88)	74.4	1.33	ΔG_S: 5.2	24.1	I	Razvi and Scholtz 2006

(*continued*)

Table 1. *Continued*

	Protein	Source[a]	PDB ID (no. of residues)	T_m (°C)	ΔC_p (kcal mol^{-1} K^{-1})	ΔG (kcal mol^{-1})[b]	T_S (°C)[c]	Mode of stabilization[d]	Reference
17	Histone proteins (*Mf*A)	*Methanothermus fervidus*	1B67 (68)	104	2.16	ΔG_S: 15.5	35	I	Li et al. 1998
		Methanobacterium formicicum (*Mfo*B)	— (67)	74.8	2.55	ΔG_S: 7.2	32		
18	Cellulase	*Themomonospora fusca* $E2_{cd}$	1TF4 (~270)	72.2		ΔG_{30}: 11.2		I*	Beadle et al. 1999
		Cellulomonas fimi $CenA_{P30}$	1GU3 (~270)	56.4		ΔG_{30}: 4.3			
19	Histone (*Mf*B)	*Methanothermus fervidus*	1A7W (69)	113	1.87	ΔG_S: 14.6	40	I, II, and III	Li et al. 1998
		Methanobacterium formicicum (*Mfo*B)	— (67)	74.8	2.55	ΔG_S: 7.2	32		
20	Rubredoxin	*Pyrococcus furiosus*	1BRF (53)	176–195		ΔG_L: 15	*50*	I, II*, and III	Hiller et al. 1997; Hernandez and LeMaster 2001
		GB1, BPTI, and *C. pasteurianum* rubredoxin							
21	O^6Methyl guanine- DNA methyl transferase (MGMT)	*Thermococcus kodakiensis*	1MGT (174)	98.6	1.24	ΔG_S: 10.2	29.5	I, II*, and III	Shiraki et al. 2001
		Escherichia coli C terminal domain of ADA	1SFE (180)	43.8	1.77	ΔG_S: 4	7.4		
22	Ferridoxin	*Thermotoga maritima*	1VJW (60)	125.4	0.86	ΔG_S: 9.32	45	I, II*, and III	Pfeil et al. 1997
		Bovine (fdx), Rabbit ($Cytb_5$), Yeast (isoCyt C)		50–70		ΔG_S: 4.8–6	0–25		
23	Histone protein(*Py*A1)	*Pyrococcus* GB3a	(67)	114	2.39	ΔG_S: 17.2	44	I and III	Li et al. 1998
		Methanobacterium formicicum (*Mfo*B)	(67)	74.8	2.55	ΔG_S: 7.2	32		
24	DHFR	*Thermotoga maritima*	1CZ3 (172)		5.35	ΔG_{25}: 30.11	41	I and III	Ohmae et al. 1996; Dams and Jaenicke 1999; Ionescu et al. 2000
		Escherichia coli	1RX1 (159)			ΔG_{25}: 6.1	15		
25	Cytochrome *c*-552	*Thermotoga maritima*	1C52 (131)		1.2–1.7	ΔG_{25}: 28.5		I* and III	Nojima et al. 1978
		Bovine, horse, and *C. kruseii*			1.5–2.5	ΔG_{25}: 13–15			
26	Farnesyl diphosphate/geranyl-geranyl diphosphate synthase	*Thermococcus kodakiensis*	– (343)	91	2.03	ΔG_S: 3.82	60	II and III*	Fujiwara et al. 2004

Values in italics indicate those that have been calculated using variations of the Gibbs–Helmholtz equation (Equation 1). Ranges of certain values indicate lower and upper bound values in cases where comparisons were made with multiple proteins.

[a] The thermophilic organism is listed first followed by the mesophilic homolog, where available. In some cases the thermophilic protein was compared with a collection of similarly sized proteins; the names of the proteins and their sources are listed.

[b] Free energy of stabilization (ΔG), which is usually ΔG_S or the ΔG at T_S. In other cases, the subscript indicates the temperature at which ΔG was measured. ΔG_E is the ΔG at T_E, the habitat temperature of the organism in question. Values in italics indicate values that have been calculated using variations of the Gibbs–Helmholtz equation (Equation 1).

[c] The temperature of maximum stability or the temperature where change in entropy between native and denatured states is zero.

[d] Method of stabilization as postulated by Nojima et al. (1977). The three methods are I, higher overall ΔG, shifting the curve up; II, reduced ΔC_p, flattening the curve; and III, higher T_S, shifting the curve to the right. Some entries are marked with asterisks to distinguish cases where the conclusions are based on insufficient data or entries where the magnitude of difference between homologs is too small to support the conclusion drawn.

[e] See text for an explanation of ΔC_p values reported for this protein.

the 15 cases where data are available. In the case of ΔG, data were available for 17 of the 26 cases, and the average increase in ΔG of stabilization for the thermophilic homolog was 8.7 kcal mol^{-1} (for the ΔG values, the average reported includes ΔG values listed in column 6 of Table 1 irrespective of the temperature at which they were measured; for both ΔT_m and $\Delta\Delta G$, entry 13 was not included). The sequence identity for the protein homologs was also compared where sequences were available. For this purpose, we used the CLUSTALW (Thompson et al. 1994) program as implemented on the EBI server (http://www.ebi.ac.uk/clustalw/) with the default settings. For the 16 cases where alignments were possible, the average sequence identity was 51%. The alignment scores varied from 11% identity (for aspartate aminotransferases from *Sulfolobus solfataricus* and pig heart cytosol) to 82% (for histone proteins *Mf*A and *Mfo*B). The average value of 51% identity is high considering the diversity of the proteins and the sources from which they are derived.

Classification of data and example cases

The proteins compiled here have been classified based on the methods for thermostabilization first proposed by Nojima et al. (1977). However, classification into just three groups as originally proposed was not possible because we find that most proteins use different combinations of these three general methods. Also, for one of the methods proposed (method III), example cases could be found only where this method was used in combination with other methods of thermostabilization. For these reasons we have grouped proteins based on the methods of stabilization (or combinations) and arranged these groups in descending order of the number of occurrences. This scheme gives six groups of proteins; a brief description of each group with details of an example study for each is provided below.

Stabilization by increased ΔG *and reduced* ΔC_p *(methods I and II)*

The combination of increased ΔG and reduced ΔC_p is the most commonly used way to achieve a higher T_m. Of the 26 sets of proteins, there are eight cases where this combination of stabilizing effects is used to increase the T_m of the thermophilic homolog. The proteins in this group are diverse with a range of sizes from the small (67 residues) nonenzymatic cold shock proteins to the large (398 residues) glycolytic enzyme phosphoglycerate kinase. The range of size and function observed points to broad applicability of this combination of methods to enhance thermostability.

A representative example from this group is the RNase H enzyme (entry 2 in Table 1) from *Thermus thermophilus* (*Tt*) and *Escherichia coli* (*Ec*) (Hollien and Marqusee 1999). RNase H is a small enzyme, which cleaves RNA from RNA–DNA hybrids; the protein from the thermophile (*Tt*RNase H) has 166 residues and shares a 52% sequence identity with its 155-residue mesophilic homolog (*Ec*RNase H). High-resolution structures are available for the two proteins and the structures are very similar (Katayanagi et al. 1992; Ishikawa et al. 1993). To understand the thermodynamic basis of the difference in stability between the two proteins, GuHCl denaturation experiments as a function of temperature were performed to obtain stability curves for these proteins. The data reveal that *Tt*RNase H is indeed more thermostable because of a lowered ΔC_p and a higher ΔG over a broad range of temperatures. The ΔC_p for the thermophilic protein *Tt*RNase H is 0.9 kcal mol K^{-1} lower than that for *Ec*RNase H. The ΔG_S is $\sim$5 kcal mol^{-1} higher than the mesophilic homolog; however, the T_S are very similar for the two proteins.

Stabilization by smaller ΔC_p *(method II)*

For the set of proteins included in this study, stabilization by reduced ΔC_p is the second most common method to attain a higher T_m. Five thermophilic proteins have a smaller ΔC_p compared to their mesophilic homologs, resulting in broader stability curves that allowed them to remain folded over a wider range of temperatures. Proteins in this group show some diversity in terms of their size and function, from the small DNA binding proteins like the 66-residue Sac7d to the large enzyme IPMD, which contains 345 residues. Three of the five proteins in this group are small DNA binding proteins, one is a subdomain of the enzyme phosphoglycerate kinase, and the last protein in this group is IPMD.

As a representative example of this group, consider the Sac7d protein (entry 9 in Table 1) from *Sulfolobus acidocaldarius* (McCrary et al. 1996). Sac7d is a small DNA binding protein that is highly basic and whose structure has been solved by NMR spectroscopy (Edmondson et al. 1995). No mesophilic homolog for Sac7d is known; hence, comparisons have been made with a number of proteins of similar size (McCrary et al. 1996). Sac7d is stable over a broad range of pH (0–10), and DSC experiments have been performed over this pH range to estimate ΔC_p from a Kirchoff analysis. Solvent denaturation experiments with GuHCl were also performed and a global fit to these data provides a ΔC_p. This estimate for ΔC_p was found to be higher than that obtained from the DSC data, and the authors provide an excellent discussion on possible causes for this disparity (McCrary et al. 1996). In any case, either value of ΔC_p produces stability curves that look very similar to those for other mesophilic proteins of similar size, and the use

of either value of ΔC_p does not cause significant differences in stability at 80°C, the habitat temperature of the organism. Surprisingly, estimates of free energy of at this temperature reveal that the protein is only marginally stable (1.6 kcal mol^{-1}).

Stabilization by a higher overall ΔG *(method I)*

Stabilization by higher ΔG is found to be as common a method of stabilization as stabilization by reduced ΔC_p. In five of the 26 cases, proteins from thermophilic organisms show a higher ΔG over a broad range of temperatures compared to their mesophilic homologs, thus shifting the stability curve up and achieving a higher T_m in the process. This group contains the three cases of HPr homologs, a pair of small archaeal histones and the enzyme cellulase subdomains. Most proteins in this group are small in size, like the archaeal histones (67 residues) or HPr homologs (88 residues), except for the cellulase catalytic domains, which have ~270 amino acids. The composition of this group, with three of the six proteins being HPr homologs, precludes much insight into the diversity of this class in terms of both size and function.

As a representative example from this group, consider the HPr proteins from the thermophile *Bacillus staerothermophilus* (*Bst*) and the mesophile *Bacillus subtilus* (*Bs*) (entry 14 in Table 1). HPr or histidine containing protein is involved in the PEP:glycose phosphotransferase system (PTS) in bacteria (Meadow et al. 1990). The *Bst*HPr protein is the same size (88 residues), shows high sequence identity (72%), and has a structure almost identical to that of the mesophilic homolog (Sridharan et al. 2005). The *Bst*HPr protein, however, has a higher T_m (~15°C) and a larger ΔG_S (~3.2 kcal mol^{-1}). The complete analyses of stability curves reveal that the ΔC_p values for the two HPr proteins are very similar at 1.3 kcal $mol^{-1}K^{-1}$ (Razvi and Scholtz 2006). The T_S values for the two proteins also are similar at 24.1° and 24.8°C (*Bs* and *Bst*HPr respectively). Therefore, this pair of proteins is a nearly perfect example for stabilization by method I or by a higher overall ΔG at all temperatures.

The archaeal histone proteins use different methods to gain thermostabilty

We now consider the cases of four archaeal histone homologs: Three of these homologs were derived from hyperthermophilic archaea, and the fourth, from a mesophilic archaeon (Li et al. 1998). The thermophilic histones from *Methanothermus fervidus* (*Mf*A, *Mf*B) and *Pyrococcus* strain GB3a (*Py*A1) (entries 17, 19, and 23, respectively, in Table 1) were compared to the mesophilic homolog from *Methanobacterium formicicum* (*Mfo*B). The curious feature of these proteins is that each thermophilic homolog uses a different thermodynamic approach to achieve a higher T_m. The histone *Mf*A, for example (entry 17 in Table 1), belongs to the group of proteins that utilize a higher ΔG at all temperatures (method I). The other two homologs increase their T_m by a combination of all three methods (*Mf*B) or by combining a higher overall ΔG with a higher T_S (*Py*A1).

Stabilization by increased ΔG*, smaller* ΔC_p*, and higher* T_S *(methods I, II, and III)*

This group of thermophilic proteins achieves higher T_m by a combination of all three methods, and the archaeal histone *Mf*B is representative of this class. This histone attains a higher T_m by combining a reduced ΔC_p with higher T_S and ΔG (methods I, II, and III). Three other thermophilic proteins were found to use this approach to increase their T_m; two of them are metal-cluster-containing proteins ferridoxin and rubredoxin. The third is the MGMT enzyme, which is also the largest protein in this group (174 residues).

*Mf*B (entry 19 in Table 1) shares 80% sequence identity with *Mfo*B, the mesophilic histone homolog, and both proteins, like other histones studied here, are dimers in solution and unfold in a two-state manner to monomers (Li et al. 1998). DSC and thermal denaturation experiments monitored by CD have been used to construct stability curves for these proteins and the ΔC_p estimates are in good agreement. Also, theoretical estimates (Murphy and Freire 1992; Spolar et al. 1992; Myers et al. 1995) for the thermophilic homolog for which a structure is available are in good agreement with experimental values. The ΔC_p for histone *Mf*B, the thermophilic homolog, is 1.9 kcal $mol^{-1}K^{-1}$, which is lower than that for *Mfo*B (2.6 kcal $mol^{-1}K^{-1}$). The ΔG_S for the thermophilic *Mf*B is 14.6 kcal mol^{-1}, which is more than twice that for *Mfo*B. The T_S for *Mf*B (40°C) is significantly greater than that for *Mfo*B (32°C). Together these thermodynamic features of the *Mf*B protein cause the T_m to be 113°C, significantly higher than that for *Mfo*B (74.8°C).

Stabilization by increased ΔG *and higher* T_S *(methods I and III)*

The third thermophilic histone homolog *Py*A1 represents a small group of proteins that achieve high T_m by combining a higher overall ΔG with a higher T_S (methods I and III). Two other thermophilic proteins use this approach to achieve higher T_m: DHFR and cytochrome c-552 from *Thermotoga maritima*. *Py*A1 (entry 23 in Table 1) is the same size as the mesophilic homolog, *Mfo*B (67 residues), and they share 57% sequence identity (Li et al. 1998). Analyses of DSC and CD monitored unfolding experiments provided nearly identical ΔC_p estimates for the two proteins. The ΔC_p for

*Py*A1 is 2.4 kcal $mol^{-1}K^{-1}$ and for *Mfo*B it is 2.6 kcal $mol^{-1}K^{-1}$. The ΔG_S for *Py*A1 is, however, larger than for *Mfo*B (17.2 kcal mol^{-1} vs. 7.2 kcal mol^{-1}). The T_S for *Py*A1 is 44°C, which is significantly higher than that for *Mfo*B (32°C). The high ΔG_S and T_S for *Py*A1, in comparison with the mesophilic homolog *Mfo*B, make this protein a good example for thermostabilization by a combination of methods I and III.

Stabilization by reduced ΔC_p *and higher* T_S *(methods II and III)*

The only case we found of a thermophilic protein using a combination of a reduced ΔC_p and higher T_S to achieve a higher T_m is farnesyl diphosphate/geranylgeranyl diphosphate synthase. This is a dimeric enzyme from *Thermococcus kodakiensis* that has 343 residues (Fujiwara et al. 2004). GuHCl denaturation experiments performed at different temperatures were combined to construct a stability curve for this enzyme. Other thermodynamic parameters, like T_m (91°C), ΔC_p (2 kcal mol^{-1} K^{-1}), ΔG_S (3.8 kcal mol^{-1}), and T_S (60°C), were estimated from the stability curve. From comparisons with other thermostable proteins, the investigators concluded that their enzyme achieved a higher T_m by a combination of lower ΔC_p with higher T_S. No details of this comparison were provided.

General observations on enhanced thermostability

A comprehensive collection of thermodynamic data comparing protein homologs from thermophiles and mesophiles has been compiled. An inspection of this broad compilation lends itself to some conclusions regarding the methods of thermostabilization adopted by proteins from thermophiles and allows us to rank the different modes of thermostabilization originally proposed by Nojima et al. (1977) in terms of their occurrence.

We find that the most common way to attain a higher T_m in proteins from thermophiles is to raise the stability curve to higher values of ΔG (higher intrinsic stability) at all temperatures; 77% of the thermophilic proteins in this study use higher ΔG independently or in combination with other stabilizing effects to increase T_m. The next most popular method used to attain a higher T_m is to lower ΔC_p (70% of thermophilic proteins in this study). Finally, the least number of occurrences (31% of the thermophilic proteins in this study) are reported for cases where the thermophilic protein exhibits a higher T_S compared to the mesophilic homolog. However, it is pertinent to rationalize these observations based on how it might be easier to adopt one strategy rather than the other in terms of sequence changes because changes in ΔG, ΔC_p, or T_S are effects of sequence alterations manifested through changes in structure.

To increase the ΔG of a protein at all temperatures, many options are available at the sequence level since any number of interactions like salt bridges, hydrogen bonds, or hydrophobic interactions may be added by single amino acid changes (for example, Pace 2000; Perl et al. 2000)). Similarly, to attain a lower ΔC_p, the sequence can be altered in many ways to provide for tighter core packing or by simply promoting structured clusters that persist in the denatured state, since ΔC_p is strongly correlated with ΔASA for protein unfolding (Murphy and Freire 1992; Spolar et al. 1992; Myers et al. 1995). For example, it was suggested that a structured cluster in denatured *Tt*RNase H caused the reduced ΔC_p for this protein, and this effect could be reversed by a single-amino acid change (Guzman-Casado et al. 2003; Robic et al. 2003).

Stabilization by shifting the stability curve to higher temperatures (higher T_S) is the least common method in our data set. This might be because very specific changes to the sequence would be required to reduce ΔS or the change in entropy between the folded and the unfolded states. Since this requires that either the entropy of the denatured state be reduced relative to the folded state or the entropy of the folded state be enhanced to more closely match that of the denatured state, it will require rather precise changes in the sequence that affect one of the two states differentially. For example, constraining a certain loop in the denatured state by introduction of proline residues or introduction of glycine residues in structured regions of a protein can reduce ΔS, by decreasing the configurational entropy of the denatured state or increasing it for the native state, respectively. Specialized mutations like these will reduce the ΔS of folding, resulting in higher T_S. A curious feature of proteins in this class is that a majority of them are not enzymes. This could be because nonenzymatic proteins are tolerant of more stable conformations (thus more ''rigid'' conformations as discussed by Jaenicke 2000) afforded by enhanced T_S. Unlike a lowered ΔC_p, which makes for a shallow stability curve, a high T_S (method III) or high ΔG (method I) both provide for higher ΔG at high temperatures.

At the molecular level, however, each of the methods of stabilization are results of features like an increased number of hydrogen bonds, salt bridges, improved core packing, shorter and/or tighter surface loops, enhanced secondary structure propensities, or oligomerization. There have been many studies (Matthews 1993; Vogt and Argos 1997; Vogt et al. 1997; Szilagyi and Zavodszky 2000) that compare homologous proteins with known three-dimensional structures in which the number of stabilizing interactions have been compared with the aim of developing a ''unifying set of

rules" for thermostabilization (Petsko 2001). Although such a set of rules remains elusive, the results have been used to rationally design variants of proteins with desired properties (Bryan 2000; Eijsink et al. 2004). Computational methods have also been used with some success to rationally design proteins with enhanced thermostability (Dahiyat et al. 1997; Malakauskas and Mayo 1998; Korkegian et al. 2005). Directed evolution is another novel technique used to design protein variants with desired properties, which requires no a priori knowledge of stabilizing/destabilizing interactions like the rational design methodology. This technique has been applied to a large number of proteins with a good measure of success (Sieber et al. 1998; Wintrode and Arnold 2000; Arnold et al. 2001; Eijsink et al. 2005). It remains to be determined which thermodynamic methods have been used to achieve higher thermostability in the proteins designed by these methods. It should be especially enlightening to see the results for proteins designed by directed evolution, since this method is least constrained in terms of the sequence space explored and types of interactions that might be altered.

In conclusion, it appears that molecular studies, as well as those that characterize the thermodynamics, both fail to reveal a single cause (molecular) or effect (thermodynamic) that completely explains the ability of thermophilic proteins to survive and function at their high habitat temperatures. Instead, proteins appear to rely on combinations of stabilizing effects that manifest themselves in alterations of different thermodynamic parameters. Thus, as proteins have taught us time and time again, they are extremely adaptable and there is no single mechanism they use to maintain structure and function at high temperatures.

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