Some thermodynamic implications for the thermostability of proteins

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Abstract

An analysis of the thermodynamics of protein stability reveals a general tendency for proteins that denature at higher temperatures to have greater free energies of maximal stability. To a reasonable approximation, the temperature of maximal stability for the set of globular, water-soluble proteins surveyed by Robertson and Murphy occurs at T* ~283K, independent of the heat denaturation temperature, T_m. This observation indicates, at least for these proteins, that thermostability tends to be achieved through elevation of the stability curve rather than by broadening or through a horizontal shift to higher temperatures. The relationship between the free energy of maximal stability and the temperature of heat denaturation is such that an increase in maximal stability of ~0.008 kJ/mole/residue is, on average, associated with a 1°C increase in T_m. An estimate of the energetic consequences of thermal expansion suggests that these effects may contribute significantly to the destabilization of the native state of proteins with increasing temperature.

Keywords: Protein stability; thermal expansion; protein volumes; stability curve

The temperature dependence of the free energy change, ΔG(T), for protein unfolding:

$$N \rightleftharpoons D \tag{1}$$

under a given set of conditions (pH, ionic strength, reduction potential, etc.) may be conveniently represented by the stability curve (Becktel and Schellman 1987) as depicted in Figure 1. When the heat capacity is temperature independent, the stability curve is determined by the values of three parameters, T_m, ΔH_m, and ΔC_p through the relationship (Hawley 1971; Privalov and Gill 1988):

$$\Delta G(T) = \Delta H_m\left[\frac{T_m - T}{T_m}\right] - \Delta C_p\left[T_m - T\left(1 - \ln\left[\frac{T}{T_m}\right]\right)\right] \tag{2}$$

in which T_m is the temperature of heat denaturation with $\Delta G(T_m) = 0$; ΔH_m is the enthalpy of unfolding at T_m; ΔC_p is the heat capacity change on unfolding. The positive ΔC_p of protein denaturation likely reflects the exposure to water of hydrophobic groups that were buried in the native state. One consequence of $\Delta C_p > 0$ is that the stability curve is indeed a curve (Brandts 1964), and it shows a free energy of maximal stability at a temperature T*. In addition to T_m, there is a second point on the stability curve where ΔG is also equal to zero that corresponds to the phenomenon of cold denaturation (Privalov 1990).

The stability curve may be approximated as a quadratic function of the temperature with a free energy of maximal stability occurring at a temperature T* (Zipp and Kauzmann 1973; Stowell and Rees 1995), in which:

$$\Delta G(T^*) \cong \left[\frac{\Delta H_m^2}{2T_m\Delta C_p}\right]$$

$$T^* \cong T_m - \left[\frac{\Delta H_m}{\Delta C_p}\right] \tag{3}$$

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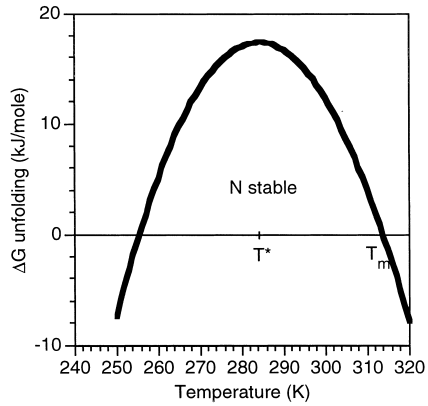

Fig. 1. Schematic representation of the stability curve (Becktel and Schellman 1987) illustrating the temperature dependence of the free energy of unfolding, ΔG. With this convention, the native state of the protein is favored in the temperature range when ΔG > 0. The temperatures of maximal stability, T*, and heat denaturation, T_m, are indicated.

Studies relating the thermodynamics of protein stability to structural features have shown that the enthalpy, entropy, and heat capacity of protein unfolding are, to first approximation, proportional to the size of the protein as described, for example, by the number of residues, N. Using a reference state of 60°C = 333K, Robertson and Murphy (1997) determined the following parameterizations, based largely on data for small globular proteins:

$$\Delta C_p \cong 0.058N \text{ kJ/(mole residue K)}$$

$$\Delta H[\text{T} = 333 \text{ K}] \cong 2.92N \text{ kJ/(mole residue)} \tag{4}$$

$$\Delta S[\text{T} = 333 \text{ K}] \cong 0.088N \text{ kJ/(mole residue K)}$$

which reproduced the experimental data with correlation coefficients of 0.86, 0.77, and 0.74, respectively. Because the thermodynamic parameters are all proportional to N, the stability curves of different proteins should scale according to the number of residues.

The existence of hyperthermophilic organisms illustrates dramatically the ability of proteins to maintain a stable tertiary structure to temperatures > 100°C. This phenomenon naturally raises the question as to how thermostability is achieved (see Szilagyi and Zavodszky [2000] for a recent discussion of the structural basis for thermostability). In terms of the stability curve, there are several thermodynamic mechanisms by which the thermostability of proteins could be increased, such as those depicted in Figure 2. These possibilities can be considered to arise by a combination of raising the stability curve, shifting the curve to high temperatures, and broadening the curve.

The thermodynamics of thermostability can be approached by examining how the stability curve of a protein is coupled to T_m by estimating ΔC_p and ΔH_m from the parameterizations of Robertson and Murphy (1997). The maximal stability can be derived by substituting these parameters into equation 3:

$$\Delta H_m(T_m) = \Delta H(T = 333K) + \Delta C_p(T_m - 333)$$

$$\Delta H_m(T_m) = 2.92N + 0.058N(T_m - 333)$$

$$\Delta G(T^*) = \frac{[\Delta H_m]^2}{2T_m \Delta C_p} \tag{5}$$

$$= \frac{0.0290N}{T_m}(T_m - 282.6)^2 \text{ kJ/mole}$$

This predicted relationship between ΔG(T*)/N and T_m is in general agreement with experimentally observed values (Table 1A,B), as illustrated in Figure 3. Similar trends between maximal stability and T_m are also evident when the stabilities of a single protein under different conditions are measured, as illustrated with wild-type and variant T4 lysozymes studied over a range of pH conditions (Fig. 3; Table 1C).

With this parameterization, the temperature of maximal stability, T*, is predicted to be ~283K, independent of the values of ΔG(T*) and T_m:

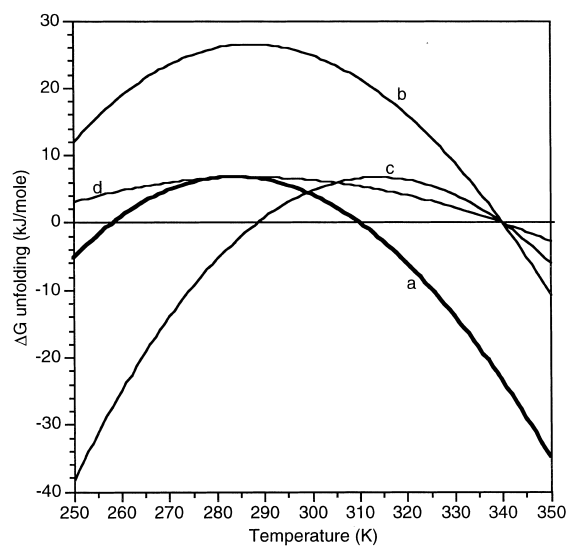

Fig. 2. Stability curves representing different thermodynamic mechanisms for achieving protein hyperthermostability. The thermal stability of a protein can be formally increased relative to the mesophilic homologue (*a*, thick line) by various combinations of raising the stability curve (*b*), shifting the stability curve (*c*), and increasing the breadth of the stability curve (*d*).

$$T^* \cong T_m - \frac{\Delta H_m}{\Delta C_p}$$

$$= T_m - \frac{2.92N + 0.058N(T_m - 333)}{0.058N} \quad (6)$$

$$\sim 283 \text{ K}$$

Observed values of T* for proteins included in the Robertson and Murphy (1997) survey are generally consistent with this expectation (Fig. 4), with average and standard deviations of 285K ± 19K and 280K ± 6K observed for the proteins in Table 1A and the T4 lysozyme in Table 1C. The few hyperthermophilic proteins included in this survey (Table 1B) appear to have systematically higher values for T* (~310K) and values of ΔG(T*) somewhat lower than predicted from equation 5 (Fig. 3), although the generality of this observation is weakened by the small sample size and neglect of the temperature dependence of ΔC_p (Privalov et al. 1989) that will become significant for hyperthermophilic proteins.

There are several implications of these observations. The most significant is that proteins denaturing at higher values of T_m tend to have greater maximal stability (Fig. 3). Although this seems perhaps obvious, there is no a priori reason to expect that this must be true (see Fig. 2). Furthermore, the observation that increased thermostability can be achieved while T* remains approximately constant suggests, as noted by Jaenicke and Böhm (1998), that thermostability is typically achieved by "pulling up" the stability curve (Fig. 2, curve b), at least for nonhyperthermophilic proteins. This observation is clearly a generalization, however, and other thermodynamic mechanisms can be used to generate thermostability; for example, Hollien and Marqusee (1999) have described an analysis of two ribonucleases H, in which the more thermostable protein not only has a greater maximal stability, but also has a small ΔC_p that generates a broader stability curve and hence contributes to a higher T_m. Another example, noted above, may be provided by the shift toward values of T* > 300K for hyperthermophilic proteins, which suggests that these proteins use somewhat different thermodynamic strategies for increased thermostability (see Szilagyi and Zavodszky 2000).

Continuing with this analysis, it is possible to estimate how much T_m will increase with an increase in ΔG(T*) by differentiating equation 5 to give:

$$\frac{1}{N}\frac{d\Delta G(T^*)}{dT_m} = 0.029 - \frac{2316}{T_m^2} \quad (7)$$

This has the value of ~0.0090 kJ/(mole residue K) at 340K, whereas a linear fit to the experimental curve gives a slope of 0.0082 kJ/(mole residue K). Consequently, a ~0.008 kJ/(mole residue) increase in maximal stability corresponds, on average, to an increase in T_m of ~1°C. Three recent examples illustrate that this relationship can capture the behavior of real protein systems:

1. In an analysis of ribonuclease H thermostabilities (Hollien and Marqusee 1999), it was found that ΔG(T* = 293K) = 53.1 kJ/mole and T_m = 359K for the more thermostable protein, and ΔG(T* = 297K) = 31.4 kJ/mole and T_m = 339K for the less thermostable protein. The change in maximal stability associated with these changes in T_m may be calculated (taking 160 as the average number of residues in each protein) to be:

$$\frac{1}{N}\frac{\Delta\Delta G(T^*)}{\Delta T_m} = \frac{1}{160}\frac{21.7}{20} = 0.0068 \text{ kJ/(mole residue K)} \quad (8)$$

2. The thermostabilities of a series of packing mutants of staphylococcal nuclease have been characterized (Chen et al. 2000); the data are described by an approximately linear relationship between the ΔG for unfolding at 293K and T_m (Fig. 5). The value of ΔG(293) should approximate the maximal stability for these variants, because the measurement temperature is close to the generic value for T* (283K). Dividing the slope of this line by the number of residues, N = 149, gives:

$$\frac{1}{N}\frac{d\Delta G(293)}{dT_m} = \frac{1}{149}(1.01) = 0.0068 \text{ kJ/mole/residue} \quad (9)$$

3. Following the introduction of the R3E mutation into the 66 residue *Bacillus caldolyticus* cold shock protein (Pace 2000; Perl et al. 2000), decreases were observed in T_m and ΔG(T*) of 17.8°C and 7.1 kJ/mole, respectively. For this case, the derivative may be estimated:

$$\frac{1}{N}\frac{\Delta\Delta G(T^*)}{\Delta T_m} = \frac{1}{66}\frac{7.1}{17.8} = 0.0060 \text{ kJ/(mole residue K)} \quad (10)$$

In these examples, the relationships between changes in maximal stability and T_m, on a per residue basis, are all close to the value 0.008 kJ/(mole residue K) derived above, illustrating that this general analysis of thermostability provides a useful framework for characterizing the relationship between protein stability and melting temperature.

Temperature dependence of protein structure

A tacit assumption in addressing the structural origins of protein stability is that the native structure is independent of temperature, so that interactions such as hydrogen bonds,

Table 1. *Summary of thermodynamic parameters for protein stability*

Protein	T_m (K)	N_{res}	ΔH_m (kJ/mole)	ΔC_p (kJ/mol K)	$\Delta G(T^*)/N$ (kJ/mole res)	T*(K)
A: Proteins tabulated by Robertson and Murphy (Robertson and Murphy 1997)						
1ALC	298	122	133	7.5	0.03	281
3SIC	323	107	313	8.5	0.16	288
2LZM	324	165	507	10.1	0.23	278
1ACP	326	77	160	3.3	0.15	281
6I1B	326	153	351	8	0.15	285
1BNJ	327	109	546	6.8	0.57	256
1ARR	327	106	297	6.7	0.18	286
1STN	327	136	337	9.3	0.13	293
1BN1	328	108	500	5.8	0.56	252
2STI	332	275	370	20.1	0.04	314
3RN3	332	124	372	6.6	0.24	280
1ABE	332	305	840	13.2	0.25	274
1HRC	333	108	393	5	0.40	263
1HRCB	333	108	307	5.3	0.23	280
1LYS	333	129	427	6.3	0.32	272
5CHA	333	237	710	12.8	0.24	282
2CAB	333	256	725	16	0.18	291
9RNT	334	104	508	4.9	0.69	245
1PMK	335	78	315	5.2	0.34	280
5PEP	336	326	1126	18.8	0.29	281
1LCD	338	51	118	1.3	0.28	258
1SHG	339	57	197	3.3	0.29	284
3PSG	339	365	1134	24.1	0.21	295
1BTA	343	89	292	6.2	0.22	299
2ZTA	343	62	259	3	0.48	267
1CYO	343	88	332	6	0.29	292
9RPR	343	126	580	10.3	0.36	292
2OVO	346	56	207	2.7	0.38	277
2HPR	346	87	248	4.9	0.20	299
1COA	347	64	280	2.5	0.64	251
1PGX	352	56	238	2.9	0.46	279
1LZ1	353	130	579	7.2	0.47	281
9PAP	357	212	904	13.7	0.37	297
4MBN	358	153	837	15.6	0.39	308
2TRX	360	108	411	7	0.29	306
1PBG	361	56	258	2.6	0.58	274
1UBQ	363	76	308	3.3	0.48	281
5CPV	363	108	500	5.6	0.53	284
2WRP	363	105	448	6.1	0.40	297
1CYV	364	98	473	7.4	0.40	305
1SAP	364	66	231	3.6	0.29	305
3AIT	366	74	307	2.9	0.55	274
5BPI	377	58	317	2	1.00	248
B: Extremozymes						
PGK[1] (Grattinger et al. 1998)	358	398	1680	30.9	0.30	308
Sac9d (McCrary et al. 1996)	364	66	242	3.6	0.32	303
COLD (Wassenberg et al. 1999)	367	66	259	4.6	0.29	315
FDX (Pfeil et al. 1997)	398	60	335	3.5	0.62	313
PFRD[1] (Hiller et al. 1997)	450	53	481	3.4	1.42	333
C: T4 lysozyme variants (Kitamura and Sturtevant 1989; Eriksson et al. 1992; Eriksson et al. 1993), as tabulated on pp. 454 and 466 of Pfeil (1998)						
T4	283	164	25	10.5	0.00	281
T4	306	164	328	9.75	0.11	274

Table 1. *Continued*

Protein	T_m (K)	N_{res}	ΔH_m (kJ/mole)	ΔC_p (kJ/mol K)	$\Delta G(T^*)/N$ (kJ/mole res)	T*(K)
T4	309	164	363	9.75	0.13	274
T4	309	164	335	10.5	0.10	279
T4	312	164	406	9.75	0.16	273
T4	313	164	316	10.5	0.09	284
T4	314	164	426	9.75	0.17	273
T4	315	164	234	10.5	0.05	294
T4	316	164	339	10.5	0.10	285
T4	316	164	383	10.5	0.13	282
T4	316	164	466	9.75	0.20	272
T4	316	164	376	10.5	0.13	282
T4	317	164	478	9.75	0.21	271
T4	318	164	479	9.75	0.21	273
T4	321	164	448	10.5	0.17	281
T4	322	164	479	9.75	0.21	276
T4	323	164	485	10.5	0.20	280
T4	325	164	517	9.75	0.24	276
T4	325	164	498	10.5	0.21	281
T4	325	164	481	10.5	0.20	283
T4	327	164	460	10.5	0.18	286
T4	327	164	544	10.5	0.25	279
T4	334	164	531	10.5	0.23	287
T4	336	164	586	10.5	0.28	285
T4	338	164	556	10.5	0.25	289
T4	339	164	565	10.5	0.26	289

Values of T* and $\Delta G(T^*)$ were obtained from the position of the stability curve maximum for each protein, as evaluated from equation 2 with Mathematica.
[1] ΔH_m and ΔC_p were estimated from stability curves presented in the reference.

salt bridges, buried surface area, and others can be enumerated independently of the conditions of the structure determination. Even without consideration of such factors as the possibility of large-scale conformational changes and the influence of crystallization conditions on the structure, the assumption of structural invariance cannot be strictly accurate. Like most materials, proteins expand with increasing temperature. The coefficient of thermal expansion, $\alpha = ((1/V)\ (dV/dT))_P$, has been estimated from thermodynamic measurements and structural studies, and is found to be $\sim 10^{-4}\ K^{-1}$ for many proteins (Frauenfelder et al. 1987; Tilton et al. 1992; Young et al. 1994). This implies, for example, that the volumes of proteins will contract several percent between room temperature and the ~100K typically used for cryocrystallographic data collection.

As indicated by the preceding considerations, this increase in volume with increasing temperature is not energetically benign, but is associated with changes in the thermodynamic parameters of both the N and D states. One example of this is the volume dependence of the internal energy, given by the quantity, $(dE/dV)_T$, which is also known as the internal pressure (Barton 1971). Values of $(dE/dV)_T$ may be obtained from the coefficients of thermal expansion (α) and compressibility $\beta = (-(1/V)\ (dV/dP))_T$, via the thermodynamic relationships:

$$dE = TdS - PdV$$

$$\left(\frac{dE}{dV}\right)_T = T\left(\frac{dS}{dV}\right)_T - P$$

$$= T\left(\frac{dP}{dT}\right)_V - P$$

$$\left(\text{from the Maxwell relationship } \left(\frac{dS}{dV}\right)_T = \left(\frac{dP}{dT}\right)_V\right) \qquad (11)$$

$$= T\frac{\alpha}{\beta} - P\ \left(\text{since } \left(\frac{dP}{dT}\right)_V = \frac{\alpha}{\beta}\right)$$

$$\cong T\frac{\alpha}{\beta}$$

Because $T\alpha/\beta$ tends to be much larger than the external pressure P (1 atm), the latter can be generally neglected. For lysozyme, $\alpha \sim 1 \times 10^{-4}\ K^{-1}$ (Young et al. 1994; Kurinov and Harrison 1995) and $\beta \sim 5 \times 10^{-6}\ atm^{-1}$ (Kundrot and Richards 1987) = 0.08 $Å^3$/(kJ/mole), so that the internal pressure equals ~0.4 kJ/mole/$Å^3$ (= ~6000 atm) at 298K. As a comparison, the volume-dependent term for cavity

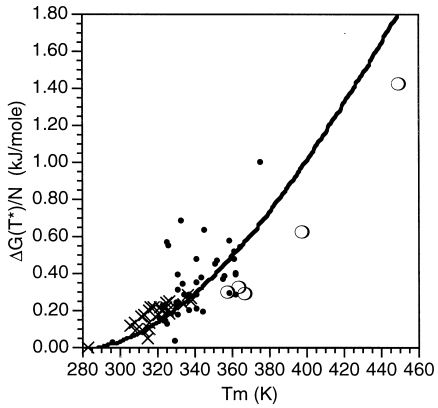

Fig. 3. Relationship between free energies of maximal stability ΔG(T*) and the temperature of heat denaturation T_m for the proteins in Table 1. The filled circles, open circles, and Xs designate water-soluble proteins tabulated in Robertson and Murphy (1997) as shown in Table 1A, proteins from hyperthermophiles in Table 1B, and T4 lysozyme variants in Table 1C, respectively. The black curve represents the predicted dependence from equation 5.

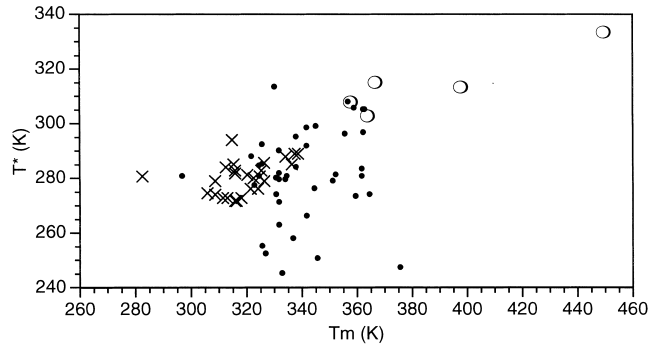

Fig. 4. Relationship between the temperature of maximal stability, T*, and the temperature of heat denaturation, T_m, for the proteins in Table 1. The different symbols are defined in the legend to Figure 3. The expected value for T* from equation 6 is 283K.

mutants engineered into T4 lysozyme is ~0.1 kJ/mole/Å^3 (Eriksson et al. 1992), whereas the intensity of strain associated with small-to-large mutations introduced into the core of T4 lysozyme has been reported in the range of 0.1–0.8 kJ/mole/Å^3 (Liu et al. 2000). The observation that $(dE/dV)_T > 0$ means that the internal energy increases as the volume increases, and is equivalent to the positive correlation between energy and volume fluctuations noted by Cooper (1984).

The increase in energy, ΔE, associated with the expansion of a protein over a temperature range, ΔT, may be estimated as:

$$\Delta E = \frac{dE}{dV}\Delta V = \frac{dE}{dV}(\alpha V \Delta T) \tag{12}$$

For a protein with N residues, an average residue molecular weight of 110, and a density of 1.35 gm/cm^3 (see Quillin and Matthews 2000), V may be approximated as 135 N Å^3, so that an increase in energy associated with a given temperature increase becomes:

$$\frac{1}{N}\frac{\Delta E}{\Delta T} = (0.4)(1 \times 10^{-4})(135)$$

$$\cong 0.005 \text{ kJ/(mole res} \cdot \text{K)} \tag{13}$$

This effect is comparable to the average decrease in ΔG(T)/N with temperature between T* and T_m: (1/N) ΔG(T*)/(T_m–T*) = 0.0049 kJ/(mole residue K) for T_m = 340K (from equation 5), which indicates that these destabilizing effects because of volume expansion are indeed energetically significant. For reference, these volume expansion effects should also be significant relative to the activation volumes measured for protein unfolding (33 Å^3 for staphylococcal nuclease [Vidugiris et al. 1995]), with increases of 40 to 120 Å^3 accompanying temperature increases of 20°C to 60°C for a protein of ~150 residues and $\alpha \sim 10^{-4}$ K^{-1}.

It is possible that the effects of volume changes on the energy of the native state could be offset by comparable effects on the denatured state. A direct analysis of this phenomenon appears problematic, however, because both α and β for the protein-solvent system change on denaturation in a protein-dependent fashion (Brandts et al. 1970; Hawley 1971; Zipp and Kauzmann 1973; Gavish et al. 1983; Prehoda et al. 1998; Panick et al. 1999) that has been difficult to generalize in terms of the relative consequences on $(dE/dV)_T$.

Conclusions

The general behavior of the stability curves for the proteins observed in this study suggests that, on average, increases in

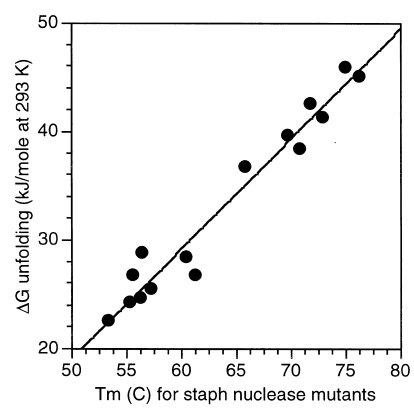

Fig. 5. Relationship between T_m and ΔG for unfolding of staphylococcal nuclease variants at 293K (Chen et al. 2000). A linear fit to these points has a slope of 1.01 kJ/(mole K) and a correlation coefficient of 0.96.

T_m are associated with increases in the free energy of maximal stability $\Delta G(T^*)$. Furthermore, the temperature of maximal stability for these proteins tends to be roughly constant at ~283K. These effects may be summarized in the form of a generic stability curve that gives the dependence of $\Delta G(T)$ on T_m and N:

$$\Delta G(T) = (2.92N + 0.058N(T_m - 333))\left[\frac{T_m - T}{T_m}\right] - 0.058N\left[T_m - T\left(1 - \ln\left[\frac{T}{T_m}\right]\right)\right] \text{kJ/mole} \quad (14)$$

As a useful rule of thumb, increases in $\Delta G(T^*)$ of ~0.008 kJ/(mole residue) are associated with an average increase in T_m of 1° C.

The expansion of proteins with increasing temperature is associated with a destabilizing increase in energy. Estimates of this effect suggest that the destabilization energy increases by about 0.005 kJ/(mole residue) per 1°C, roughly comparable to decreases in ΔG with increasing T above T*. A similar effect has been described by Palma and Curmi (1999) who noticed a correlation between thermal expansion of protein surface area and stability in computational studies. Protein expansion could contribute not only to the thermodynamics of protein stability, but also to the kinetics of this process, because the introduction of defects should permit conformational rearrangements required for unfolding (Vidugiris et al. 1995), much as the volume of solids increases near their melting temperature (Frenkel 1946; Bondi 1968). The combination of thermodynamic measurements and structural studies at different temperatures should help further illuminate the relationships between protein stability and volume, which would be particularly informative for proteins that function under more exotic conditions, such as hyperthermostable proteins and membrane proteins.

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