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Enthalpy-Entropy Balance and Convergence Temperatures in Protein Unfolding*

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We find that isoenthalpic and isoentropic temperatures characterizing the unfolding of small globular proteins are linked by a simple relationship, which takes into account the occurrence of common values of specific unfolding enthalpy and entropy changes. The difference between these temperatures implies that the hydration effect favors protein folding over a quite large range of temperatures.

Following the publication of the paper by Murphy *et al.* (1990), many authors have been concerned with the intriguing existence of isoenthalpic and isoentropic temperatures (T_h* and T_s*, respectively) in protein unfolding. The difference between these temperatures determines the sign of the quantity $\Delta G_{hyd}° = \Delta C_p°[T - T_h* - T\ln(T/T_s*)]$ ($\Delta C_p°$ is the unfolding heat capacity change), which Murphy *et al.* (1990) originally called "hydrophobic hydration" with $T_h* = T_s*$.

This definition has been criticized, since it was proposed originally by Privalov and co-workers (Murphy *et al.*, 1990) on the hypothesis that the hydrophobic free energy goes to 0 at T_s*. Consensus on this hypothesis, as well as about the role played by the hydrophobic free energy in protein folding, is lacking (Muller, 1992), because $\Delta C_p°$ for the transfer of small non-polar molecules into water is far from being 0 at T_s*, as it must be if the water-ordering effect vanishes at that temperature (Gill *et al.*, 1985; Muller, 1990, 1992; Makhatadze and Privalov, 1990; Privalov and Makhatadze, 1990, 1992; Baldwin and Muller, 1992). Privalov and co-workers themselves corrected their original definition introducing the term "hydration effect" to describe the heat capacity temperature-dependent component of the unfolding free energy. Their conclusion was that "the hydration effect, not the hydrophobic effect, leads to destabilization of protein structures" (Privalov *et al.*, 1990). However, this adjusted definition again suffers from the objection that the hydration term vanishes at T_s*, where $\Delta C_p°$ is still substantial (Baldwin and Muller, 1992). The focal point is whether the hydration effect opposes protein folding or not. In this article we use this terminology for indicating $\Delta G_{hyd}°$, but we do not make any limitation about the values of the two convergence temperatures. According to Lee (1991), $\Delta G_{hyd}°$ is the difference between $\Delta C_p°[T - T_{hn} - T\ln(T/T_s*)]$ and $\Delta C_p°(T_h* - T_{hn})$. The first term represents the purely hydrophobic contribution, reflecting the unusual free energy temperature dependence for the transfer of non-polar surface from the liquid organic phase into water (Dill, 1990) as measured on small non-polar molecules. The second term is the polar contribution to the hydration effect, which equals the non-polar contribution at $T = T_h$* (Lee, 1991). T_{hn} is the temperature at which the transfer enthalpy is 0, *i.e.* 295 K (Baldwin, 1986).

Another source of criticism relies on the observation that $\Delta C_p°$ of unfolding is likely to depend on water exposure of both polar and non-polar surface (Makhatadze and Privalov, 1990; Privalov and Makhatadze, 1990, 1992; Murphy and Gill, 1991; Murphy *et al.*, 1992; Fu and Freire, 1992; Spolar *et al.*, 1992; Ragone and Colonna, 1993). According to this finding, $\Delta C_p°$ of unfolding can be expressed as the sum of non-polar, as well as polar, contributions. We will not discuss this point further, since it is beyond the scope of this report.

Since $\Delta C_p°$ is positive at temperatures lower than that hypothetical temperature at which it goes to 0, an additional source of debate is the appraisal of the exact values of T_h* and T_s*. Their difference determines the sign of the term $[T - T_h* - T\ln(T/T_s*)]$. Namely, if T_h* and T_s* are chosen coincident, the hydration effect is never positive, thus opposing folding (Murphy *et al.*, 1990). On the contrary, the non-coincidence determines the existence of a quite large range of temperatures within which the hydration effect is positive. Thus, the need of a firm statement about the exact (within the limits of the experimental uncertainty) value of T_h* and T_s* is not a trivial matter for speculation.

Differential scanning calorimetry of proteins provided a quite large set of data by which hypotheses about protein folding have been developed, with a particular concern to the balance between hydrophobic and polar interactions. Privalov (1979) first observed the convergence of specific unfolding enthalpies and entropies for several proteins toward common temperatures. The conclusion originally drawn by Privalov and co-workers (Murphy *et al.*, 1990) was that these temperatures were coincident to a common value around 385 K. In a subsequent analysis based on the same data, Murphy and Gill (1991) used non-coincident values for T_h* and T_s*. This discrepancy arises from Privalov's original data (Privalov and Gill, 1988), which report an unfolding enthalpy change for pepsinogen at 298 K of −0.24 kJ mol^{-1} residue^{-1}, instead of 0.24 kJ mol^{-1} residue^{-1} (Doig and Williams, 1992). This error brought to the issue that T_h* and T_s* were coincident and was at the origin of various interpretations that supposed the hydrophobic effect to play a role in protein stabilization, in opposition to the common view that it is the driving force of protein folding (Creighton, 1991). After correction and reexamination of experimental data, T_h* and T_s* were found non-coincident, with T_h* ~ 374 K and T_s* ~ 385 K (Murphy and Gill, 1991).

The existence of T_s* encountered a "natural" explanation in Baldwin's liquid hydrocarbon model (Baldwin, 1986), which shows the protein T_s* to be coincident with that temperature at which the entropy change accompanying the dissolution of non-polar solutes goes to 0. The shift of T_h* from the value around room temperature typical of non-polar solutes (Baldwin, 1986)

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to a value around the boiling point of water still is matter of debate.

In a recent paper, Lee (1991) suggested that the enthalpic nature of polar interactions, which counterbalance hydrophobic hydration in proteins, provides a simple explanation of the observed T_h* shift. Lee also argues that the near coincidence of T_h* and T_s* for protein unfolding appears "as a consequence of the fact that the free energy changes upon exposure of the polar and non-polar groups happen to be closely similar on a per area basis at T_s*" (Lee, 1991). Two further papers address the question of whether it is really necessary to invoke the balance between hydrophobic and polar interactions in order to explain the high T_h* value in proteins. Doig and Williams (1992) show that the intersection of specific unfolding enthalpies around 383 K is due to the existence of T_s* assuming the unfolding free energy of proteins at 298 K constrained by the evolution to a nearly constant value of 340 J mol^{-1} residue^{-1}. A quite different approach drove Baldwin and Muller (1992) to the conclusion that the difference between T_h* and T_s* originates from the fact that the melting temperatures of globular proteins have values close to 331 K, given that, at T_s* ~ 385 K, specific unfolding entropies are equal. These authors state that, as a consequence, different proteins should have the same specific unfolding enthalpy at a temperature about 4 K below T_s*, *i.e.* T_h* ~ 381 K.

We present here an equivalent alternative to these observations, which does not require any of the above mentioned assumptions about the stability or the melting temperature of different globular proteins. However, it must be stressed that our argument, as well as those of Baldwin and Muller (1992) and Doig and Williams (1992), merely replace one thermodynamic observation for another (enthalpy convergence), although our demonstration seems numerically more accurate than the others, thus providing a precise theoretical explanation. Lee's argument is different, since it replaces a molecular mechanism for the thermodynamic observation (Lee, 1991).

Our argument lies on the close vicinity of T_h and T_s, the temperatures at which the unfolding enthalpy change and the unfolding entropy change are respectively zero (Becktel and Schellman, 1987). According to the widespread assumption that $\Delta C_p°$ does not depend on temperature, we can write Equations 1 and 2.

$$\Delta H° = \Delta H^* + \Delta C_p°(T - T_h^*) \quad \text{(Eq. 1)}$$

$$\Delta S° = \Delta S^* + \Delta C_p°(\ln T - \ln T_s^*) \quad \text{(Eq. 2)}$$

ΔH^* and ΔS^* are the unfolding enthalpy and entropy changes that different proteins share at T_h* and T_s*, respectively. Evaluation of these equations at $T = T_h$ and $T = T_s$, respectively, gives Equations 3 and 4.

$$\Delta C_p°(T_h - T_h^*) = -\Delta H^* \quad \text{(Eq. 3)}$$

$$\Delta C_p°(\ln T_s - \ln T_s^*) = -\Delta S^* \quad \text{(Eq. 4)}$$

By dividing the last two equations we obtain Equations 5 and 6.

$$\Delta H^*/\Delta S^* = (T_h - T_h^*)/(\ln T_s - \ln T_s^*) \quad \text{(Eq. 5)}$$

$$\ln T_s = \ln T_s^* - (\Delta S^*/\Delta H^*)T_h^* + (\Delta S^*/\Delta H^*)T_h \quad \text{(Eq. 6)}$$

Equation 6 can be fitted to a straight line, plotting $\ln T_s$ against T_h. As a first approximation, we assume $T_s = T_h$ within 273–373 K (with increments of 0.5 K). We have obtained $(\Delta S^*/\Delta H^*) = 3.111 \times 10^{-3} \pm 2 \times 10^{-6}$ and $\ln T_s^* - (\Delta S^*/\Delta H^*)T_h^* = 4.7687 \pm 6 \times 10^{-4}$ ($r = 1.000$). If T_s* is 385 K, it follows that T_h* = 380.8 K, a value very close to that predicted by Baldwin and Muller (1992). As a consequence, the assumptions made by these authors and by Doig and Williams (1992) seem to hold because of the close proximity for each protein of T_h and T_s.

TABLE I
Values of T_h and T_s for globular proteins

These values are calculated using $\Delta H°$, $\Delta S°$, and $\Delta C_p°$ of unfolding at 298 K reported by Privalov and Gill (1988).

Protein	T_h	T_s
	K	
Ribonuclease A	243.5	255.4
Lysozyme	258.9	267.8
Plasm. fragment K4	262.8	270.5
β-Trypsin	275.0	280.8
α-Chymotrypsin	276.2	280.1
Papain	282.5	290.2
Staphylococcus nuclease	284.1	288.0
Carbonic anhydrase	285.4	289.8
Cytochrome *c*	288.3	294.0
Pepsinogen	294.7	299.1
Myoglobin	297.5	301.2

In order to assess the validity of the assumptions made by these authors, we also evaluated T_h and T_s for the protein set given by Privalov and Gill (1988) by using the thermodynamic quantities reported by them. Calculated values are shown in Table I. After least squares fitting to Equation 6, we obtained $(\Delta S^*/\Delta H^*) = 3.09 \times 10^{-3} \pm 9 \times 10^{-5}$ and $\ln T_s^* - (\Delta S^*/\Delta H^*)T_h^* = 4.789 \pm 2.4 \times 10^{-2}$ ($r = 0.997$), from which it follows that T_h* = 376.8 K when T_s* = 385 K. These values are obviously in better agreement with those coming from the experiment (Murphy and Gill, 1991; Fu and Freire, 1992). It must be stressed that this result is only affected by experimental uncertainty. It depends neither on any speculation regarding the role played by evolution in constraining protein stability to a constant value at room temperature (Doig and Williams, 1992) nor on the assumption that all globular proteins share the same melting temperature at 331 K (Baldwin and Muller, 1992).

Even if the difference between T_h* and T_s* is small, nevertheless it has dramatic consequences on the sign of $\Delta G_{hyd}°$. We have pointed out that it no longer seems possible to make a one-to-one correlation between $\Delta G_{hyd}°$, as originally defined, and the hydrophobic hydration. At a glance, the confusion generated about the role played by the hydrophobic hydration in protein folding seems to arise largely from the wrong appraisal of the convergence temperatures. Therefore, it should be interesting to determine the consequences of having T_h* < T_s*, even if $\Delta G_{hyd}°$ is now devoid of the supposed meaning (Murphy *et al.*, 1990). By using T_h* = 377 K and T_s* = 385 K, the hydration effect is positive within 311–466 K, where it favors folding. To the best of our knowledge heat-induced unfolding of proteins in the absence of denaturants does not take place at a temperature lower than 311 K. This means that the hydration effect drives the thermal stabilization of globular proteins above 311 K. Around this temperature, the residual unfolding free energy changes its sign, becoming negative ($\Delta H^* - T\Delta S^* = 0$ at $T \cong$ 324 K) and thus disfavoring folding. The hydration effect favors unfolding below 311 K, where heat denaturation does not occur because ΔH^* (the residual unfolding enthalpy) overwhelms $T\Delta S^*$ (the residual entropic contribution).

The Baldwin and Muller analysis implies that the positive temperature span of the hydration effect must be restrained to 332–442 K. Therefore, it leads to the conclusion that it should favor protein folding only above 331 K, which is assumed to be the melting temperature for each protein (Baldwin and Muller, 1992). At this temperature the stabilizing polar contribution to protein folding must exactly counterbalance the destabilizing conformational entropy change. This seems to contradict a

large body of recent experimental evidence (Murphy and Gill, 1991; Fu and Freire, 1992; Murphy *et al.*, 1992).

In conclusion, for proteins similar to those analyzed by Privalov and Gill (1988), T_h* and T_s* appear to be closely related, T_h* being smaller than T_s* by about 8 K. According to the view of the quoted authors (Baldwin and Muller, 1992; Doig and Williams, 1992), nothing has to be specified about the nature of the forces stabilizing the native state of proteins. No assumption is necessary other than the convergence of specific unfolding entropy changes to 385 K and the constancy of the unfolding heat capacity change with temperature. However, it must be stressed that the substitution of whatever thermodynamic observation for the enthalpy convergence leaves us with the task of explaining that observation and does not provide any insight into the molecular mechanism. From this point of view, the argument presented by Lee (1991) has the merit of evidencing the molecular aspects of the experimental finding.

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