Why Water-Soluble, Compact, Globular Proteins Have Similar Specific Enthalpies of Unfolding at 110 °C†

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ABSTRACT: The changes in free energy, enthalpy, and entropy of unfolding have been measured for many water-soluble, compact, globular proteins by a number of workers. In principle, a wide range in stability could be achieved by proteins, as measured by the free energy of unfolding; in practice, evolution only allows a narrow range in this quantity. Proteins are only marginally stable at room temperature for many possible reasons, including ensuring that folding is reversible and polypeptide chains are not trapped in incorrectly folded structures. Many of these proteins have approximately the same values of enthalpy of unfolding around 110 °C. We show here that this arises because the change in entropy of unfolding at room temperature and the change in heat capacity on unfolding, which governs the temperature variation of the enthalpy and entropy, both vary with the magnitude of the hydrophobic effect in the protein. As all these proteins have evolved to achieve similar stabilities at room temperature, the enthalpy of unfolding will also vary with the size of the hydrophobic effect in the protein. A consequence of this is that curves of the specific unfolding enthalpy against temperature for different proteins intersect around 110 °C. A similar conclusion, on the basis of similar melting points rather than similar free energies of unfolding, has been reached independently by Baldwin and Muller (R. L. Baldwin, personal communication).

Many folded, functional proteins are approximately globular in shape. If, however, the environment of such proteins is perturbed by heat, cold, acid, alkali, or organic denaturants, they may unfold to a random-coil state. Scanning microcalorimetry has provided us with a considerable degree of thermodynamic data regarding the thermal unfolding transition in proteins. The changes in enthalpy (ΔH_u),[1] entropy (ΔS_u), Gibbs free energy (ΔG_u), and heat capacity at constant pressure (ΔC_{p_u}) have been measured for a large number of proteins by a number of groups, and a number of unifying features have emerged. The thermodynamics of thermal unfolding of water-soluble, compact, globular proteins are considered in this paper.

The stability of a protein to thermal denaturation, measured as a free energy change, is very small, being only 20–80 kJ mol^{-1} at room temperature (Pace, 1975; Privalov, 1979). This is a remarkably small value, considering the size of a protein molecule. The results obtained for 12 typical proteins [Table I in Privalov and Gill (1988)] are summarized in Table I. These data are somewhat unsatisfactory as they were collected at different pH values. Nevertheless, it does give a general indication of the range of thermodynamic quantities found during thermal protein denaturation.

In this paper, the temperature dependence of ΔH_u and ΔS_u is considered. At ≈110 °C, many proteins show common values of ΔH_u and ΔS_u (Privalov, 1979). The reason why ΔS_u is constant at 110 °C was given by Baldwin (1986); however, he did not explain why ΔH_u shows similar behavior. Here, it is shown that the fact that many proteins show the same value of ΔH_u per residue around 110 °C is a consequence of the thermodynamic properties of the hydrophobic effect and the fact that proteins have evolved to have similar stabilities around room temperature.

EQUATIONS FOR PROTEIN UNFOLDING

For any physical process, such as protein unfolding, the variation of the change of enthalpy with temperature is given by eq 1, assuming that ΔC_{p_u} is invariant with temperature:

$$\Delta H_u(T) = \Delta H^* + \Delta C_{p_u}(T - T_H^*) \quad (1)$$

Similarly, the variation in the change in entropy of unfolding with temperature can be given by eq 2, if we make the same assumption:

$$\Delta S_u(T) = \Delta S^* + \Delta C_{p_u} \ln (T/T_S^*) \quad (2)$$

In eqs 1 and 2, T_H^* and T_S^* are hypothetical reference temperatures; ΔH^* is the value of ΔH_u at temperature T_H^*; ΔS^* is the value of ΔS_u at temperature T_S^*. Thus, at temperatures T_H^* and T_S^*, eqs 1 and 2 respectively simplify so that the term containing ΔC_{p_u} disappears. Equations 1 and 2 can also be written as

$$\Delta H_u(T) = \Delta C_{p_u}(T - T_h) \quad (3)$$

$$\Delta S_u(T) = \Delta C_{p_u} \ln (T/T_S) \quad (4)$$

In eq 3, T_h is the temperature at which the enthalpy of protein unfolding is zero; in eq 4, T_S is the temperature at

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[1] Abbreviations: ΔG_u, change in Gibbs free energy on protein unfolding; ΔH_u, change in enthalpy on protein unfolding; ΔS_u, change in entropy on protein unfolding; ΔC_{p_u}, change in heat capacity at constant pressure on protein unfolding; n_r, number of residues in a protein; T, temperature; T_S^*, temperature at which the entropy of protein unfolding is the same for the investigated water-soluble, compact, globular proteins; T_H^*, temperature at which the enthalpy of protein unfolding is the same for the investigated water-soluble, compact, globular proteins; ΔH^*, enthalpy change of protein unfolding at temperature T_H^*; ΔS^*, entropy change of protein unfolding at temperature T_S^*, T_h, temperature at which the enthalpy of protein unfolding is zero; T_S, temperature at which the entropy of protein unfolding is zero; ΔS_{hyd}, contribution of the hydrophobic effect to the entropy of protein unfolding; ΔS_{res}, entropy of protein unfolding with the hydrophobic effect subtracted; ΔH_{hyd}, contribution of the hydrophobic effect to the enthalpy of protein unfolding; ΔH_{res}, enthalpy of protein unfolding with the hydrophobic effect subtracted.

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Table I: Thermodynamic Parameters per Residue (±1 Standard Deviation) for 12 Globular Proteins at 298 K[a]

	ΔH_u, kJ (mol·res)$^{-1}$	ΔS_m, J K^{-1} (mol·res)$^{-1}$	ΔG_u, kJ (mol·res)$^{-1}$	ΔC_{p_u}, J K^{-1} (mol·res)$^{-1}$
minimum	0.04	−0.80	0.20	43.5
maximum	2.37	6.70	0.53	74.5
mean	1.14 ± 0.70[b]	2.69 ± 2.28	0.34 ± 0.09	59.0 ± 9.8

[a] Data are taken from Table I of Privalov and Gill (1988). The proteins used were ribonuclease A, parvalbumin, lysozyme, fragment K4 of plasminogen, β-trypsin, α-chymotrypsin, papain, *Staphylococcus* nuclease, carbonic anhydrase, cytochrome *c*, pepsinogen, and myoglobin. [b] $\Delta H_u(298)$ for pepsinogen is incorrectly reported as −0.24 kJ (mol·res)$^{-1}$ in Privalov and Gill (1988); the correct value is 0.24 kJ (mol·res)$^{-1}$ (Mateo & Privalov, 1981). We thank an anonymous referee for pointing this out.

which the entropy of protein unfolding is zero. Equations 3 and 4 can be combined with the Gibbs equation ($\Delta G_u = \Delta H_u - T\Delta S_u$) to give the free energy of protein unfolding as a function of temperature:

$$\Delta G_u(T) = \Delta C_{p_u}\{T[1 - \ln(T/T_S)] - T_h\} \quad (5)$$

BOUNDS ON POSSIBLE VALUES OF ΔG_U

A typical protein has a remarkably small stability at room temperature. However, it is possible to conceive of proteins with different sequences yet with much greater stabilities. A range of experimental observations supports the idea that proteins could be more stable. First, the structures of ferredoxin and glyceraldehyde-phosphate dehydrogenase from thermophilic bacteria have been characterized. Increased stabilities (at the temperatures at which these organisms function) are achieved in these proteins by modifying the structures seen from mesophilic bacteria to include additional salt bridges or van der Waals contacts (Perutz & Raidt, 1975; Walker et al., 1980). Second, a novel four α-helical bundle protein has been synthesized and was found to be 4 times more stable than a typical natural protein (Regan & DeGrado, 1988), despite any unavoidable design imperfections in this protein due to our imperfect understanding of protein structure and stability. Third, single-site mutations are known which increase the stability of a protein [for example, see Hecht et al. (1985) and Alber (1989)]. In other words, evolution could have generated more stable proteins if it were advantageous (DeGrado, 1988; Richardson & Richardson, 1988; Sauer et al., 1990).

These observations strongly suggest that proteins have not evolved to make ΔG_u as large as possible; rather, natural protein sequences have evolved to make ΔG_u small. A possible reason for this is that a low stability is necessary to make protein degradation feasible (Pace, 1975), as proteolytic enzymes may have difficulty in degrading proteins which are held together too strongly. Additionally, a low ΔG_u is likely to lead to a high degree of protein flexibility which may be essential for function (Becktel & Schellman, 1987; Fischer & Schmid, 1990; Creighton, 1990). However, flexibility relies on there being a number of low-energy conformations close to the folded structure and is not directly related to the difference in energy between the folded and the unfolded states. A small stability may also be necessary for transient unfolding for transport or assembly purposes (Fischer & Schmid, 1990). Folding may also proceed too slowly if the final conformation is too stable (Creighton, 1990) as folding intermediates may then also be too stable.

A low value of ΔG_u may help to ensure that polypeptides are not trapped in incorrectly folded structures. To illustrate this idea, Figure 1 shows a free energy profile for the folding

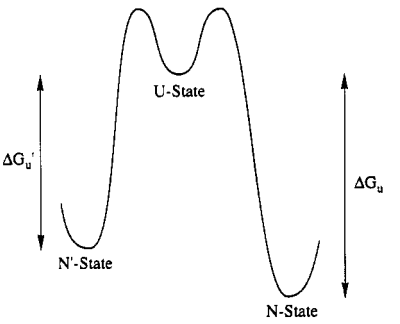

FIGURE 1: Free energy profile for an unfolded protein (U-state) folding into two alternative folded structures (N- and N′-states). The difference in free energy between the U- and N-states is ΔG_u; the difference in free energy between the U- and N′-states is $\Delta G_u'$.

of a polypeptide from the unfolded (U-state) to the native, folded state (N-state) under folding conditions, where the difference in free energy between the U- and N-states is ΔG_u. There is an alternative folding pathway from the U-state to a different folded structure, named the N′-state, which is of slightly higher free energy than the N-state. The lowest energy pathway for the N′- to N-state is via the U-state. The kinetic barriers in this figure are not important; they merely show that both the N- and N′-states are kinetically accessible. Clearly, the N-state is the structure of lowest free energy which is kinetically accessible. Hypothetical structures of lower free energy are kinetically inaccessible and hence are irrelevant to this analysis. As ΔG_u is so small, the protein can readily escape from the N′-state back to the U-state, if the chain has folded in this incorrect structure. It then has another chance to fall into the correctly folded N-state. After a short time, the majority of the polypeptide chains will have achieved the required N-state. If the interactions which develop during folding are very strong, ΔG_u and $\Delta G_u'$ may both be very large. If the protein then folds into the incorrect N′-state, it may be unable to achieve the correctly folded N-state as the U-state, which is an intermediate between the N′- and N-states, is of too high an energy to be reached in a reasonable time. A small value of ΔG_u therefore ensures reversible folding, and most protein molecules synthesized will end up in the same lowest energy structure which is kinetically accessible (the functional N-state). Additionally, misfolded proteins in vivo may be easily degraded by proteolysis as they will be very close to their denaturation transition (Jaenicke, 1991).

However, despite the above arguments, ΔG_u cannot be too small. If ΔG_u was very small, the protein would be highly susceptible to mutations which would lead to the protein unfolding at room temperature, a situation which would make many single-site mutations lethal. For example, even the smallest of mutations, namely, the deletion of a single methylene group in the mutation Ile→Val, can decrease protein stability by as much as 7 kJ mol^{-1} (Shortle et al., 1990), which is a significant fraction of the total protein stability. In general, single-site mutations lead to decreased enzymic efficiency and frequently to reduced stability, but not unfolding. If ΔG_u was too small, protein unfolding could be induced by a small change in external conditions, such as pH, ionic strength, or temperature, which could again be lethal. Finally, if ΔG_u is very small, the fraction of protein which is unfolded will become significant, since the folded and unfolded forms are in equilibrium. Unfolded proteins not only are nonfunctional but also are likely to be highly susceptible to degradation.

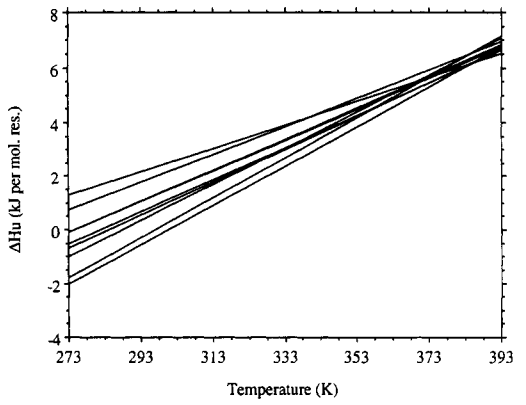

FIGURE 2: Variation of enthalpy of protein unfolding with temperature, assuming a constant ΔC_{p_u}. Data are taken for nine typical proteins with a range of thermodynamic data from Table I of Privalov and Gill (1988).

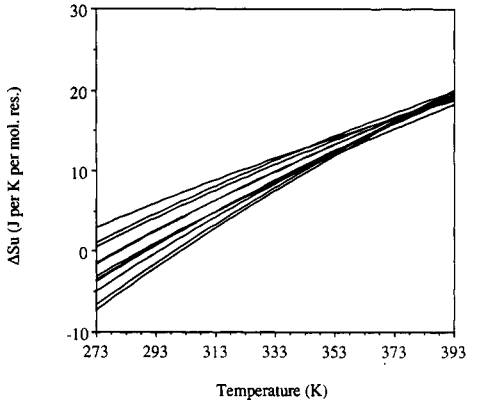

FIGURE 3: Variation of entropy of protein unfolding with temperature, assuming a constant ΔC_{p_u}. Data are taken for 11 typical proteins with a range of thermodynamic data from Table I of Privalov and Gill (1988).

Thus, evolution has ensured that all of these proteins have achieved similar stabilities at room temperature.

TEMPERATURE VARIATION OF THE ENTHALPY OF UNFOLDING

The thermodynamic parameters of unfolding (ΔG_u, ΔH_u, ΔS_u, and ΔC_{p_u}) have been measured for a number of water-soluble medium-sized compact, globular proteins by scanning microcalorimetry. If ΔH_u is plotted against temperature, it is found that they each have a common value of ΔH_u at approximately 383 K (Figure 2). The reason for this intersection is a long-standing puzzle, considered, but not solved, by Baldwin (1986), who did successfully explain the temperature dependence of the entropy of unfolding (see later). In this section, it is shown that it arises from the properties of the hydrophobic effect and the fact that all of these proteins have similar stabilities at the same temperature.

Similarly, if ΔS_u is plotted against temperature, it is found that they each have a common value of ΔS_u at approximately 383 K (Figure 3). The proteins which do not fit these graphs all show values of $\Delta H_u(383)$ and $\Delta S_u(383)$ which differ by large amounts from 6.25 J (mol·res)$^{-1}$ to 17.6 J K^{-1} (mol.res)$^{-1}$, respectively. However, only small variations from these values are found for many water-soluble, medium-sized, compact globular proteins (Privalov, 1979; Privalov & Gill, 1988). Examples of the kinds of proteins which show different values of $\Delta H_u(383)$ and $\Delta S_u(383)$ and which are not relevant to this analysis include histones, which have noncompact folded structures (Tiktopulo et al., 1982), collagen (Privalov, 1982), which is not globular, and small proteins, such as some neurotoxins (Khechinashvili & Tsetlin, 1984), BPTI (Privalov, 1979), and ribonuclease T1 (Pace & Laurents, 1989), which appear to have a smaller number of hydrogen bonds and/or nonpolar contacts in the folded protein (Privalov & Gill, 1988).

The assumption that ΔC_{p_u} is invariant with temperature has been criticized by Franks et al. (1988), who argue that ΔC_{p_u} decreases in magnitude at lower temperatures and becomes negative at some point before the temperature of cold denaturation. However, measurements of ΔC_{p_u} have shown that it is constant, within experimental error, in the temperature range 0–80 °C (Privalov, 1979), and Franks et al. were mostly concerned with temperatures below this. Above 80 °C, ΔC_{p_u} becomes smaller. The graphs in Figures 2 and 3 were calculated assuming ΔC_{p_u} to be invariant with temperature and taking values of ΔC_{p_u} measured at the melting points of the proteins. Whether these graphs become more and more inaccurate as we move away from the melting points of the proteins is irrelevant to the arguments presented in this paper. However, rather than stating that the proteins have the same enthalpy of unfolding at 110 °C, it is more accurate to state that water-soluble, compact, globular proteins have the same enthalpy of unfolding if their enthalpy of unfolding is extrapolated to 110 °C, assuming the change in heat capacity of unfolding is invariant with temperature, as measured at the upper denaturation temperature of the protein.

The reason why these proteins have a common value of $\Delta S_u(383)$ was rationalized by Baldwin (1986). He broke down ΔS_u into two components—the entropic part of the hydrophobic effect (ΔS_{hyd}) and everything else (ΔS_{res}). This is a useful division because it appears that ΔC_{p_u} is largely associated with the hydrophobic effect; i.e., ΔS_{res} does not vary with temperature. Thus, ΔS_u as a function of temperature can be written as

$$\Delta S_u = \Delta S_{hyd} + \Delta S_{res} \quad (6)$$

$$\Delta S_u(T) = \Delta C_{p_u} \ln (T/T_s^*) + \Delta S_{res} \quad (7)$$

In eq 7, T_S^* is the temperature at which the entropic component of the hydrophobic effect (ΔS_{hyd}) is zero, at which $\Delta S_u = \Delta S_{res}$, for all the proteins considered here. Figure 3 shows that for the proteins considered T_S^* is about 383 K (Privalov, 1979). Consideration of the temperature variation of the hydrophobic effect (here defined as that measured by hydrocarbon solvent-transfer experiments from nonpolar solvent to water) shows that at 383 K the entropic part of the hydrophobic effect is zero (Baldwin, 1986). Thus, in these proteins at 383 K, $\Delta S_{hyd} = 0$ and $\Delta S_u = \Delta S_{res}$. Figure 3 shows that, remarkably, the sums of the factors which contribute to ΔS_{res} (which may include conformational freedom and solvation of polar groups) are approximately equal for all the proteins considered. This implies that, apart from the hydrophobic effect, all proteins have similar thermodynamic properties, at least in terms of entropy. The mean value of $\Delta S_u(383)$ ($=\Delta S_{res}$) is 17.6 J K^{-1} (mol.res)$^{-1}$. From eq 7 and the values ΔS_{res} = 17.6 J K^{-1} (mol.res)$^{-1}$, T = 298 K, and T_S^* = 383 K, we can thus obtain $\Delta S_u(298)$ as a function of ΔC_{p_u}:

$$\Delta S_u(298) = 17.6 - 0.251 \Delta C_{p_u} \text{ J K}^{-1} \text{ (mol·res)}^{-1} \quad (8)$$

The entropy of unfolding at 298 K can therefore be estimated solely from ΔC_{p_u}. Equation 8 is physical reasonable because ΔC_{p_u} reflects the size of the hydrophobic effect, which entropically stabilizes a protein at 298 K. If ΔC_{p_u} is large, there is a large hydrophobic effect, and the protein will be stabilized entropically. Thus, $\Delta S_u(298)$ will become more negative, as shown in eq 8.

Observed values of the free energy of protein unfolding at 298 K fall in the narrow range of 20–80 kJ mol^{-1} (Privalov, 1979; Pace, 1975). For the 12 proteins listed by Privalov and Gill, the mean value of $\Delta G_u(298)$ is 340 J (mol·res)$^{-1}$. We can use this mean value for protein stability, which is likely to be constrained by evolution (see above), and the equation for the entropy of unfolding at 298 K [$\Delta S_u(298)$; eq 8] to derive an equation for the enthalpy of unfolding at 298 K [$\Delta H_u(298)$; eq 11] from the Gibbs equation (eq 9):

$$\Delta G_u(298) = \Delta H_u(298) - 298\Delta S_u(298) \quad (9)$$

$$340 = \Delta H_u(298) - 298(17.6 - 0.251\Delta C_{p_u})\ \text{J (mol·res)}^{-1} \quad (10)$$

$$\Delta H_u(298) = 5580 - 75\Delta C_{p_u}\ \text{J (mol·res)}^{-1} \quad (11)$$

To summarize, the properties of the hydrophobic effect and the similar values of ΔS_{res} for all proteins dictate that $\Delta S_u(298)$ is of the form of eq 8 (i.e., a function of ΔC_{p_u} only). Because of the evolutionary requirement that $\Delta G_u(298)$ is about the same for all these proteins, $\Delta H_u(298)$ is also a function of ΔC_{p_u}. Having obtained a equation for ΔH_u at one particular temperature (eq 11), it is possible to derive an equation for ΔH_u at any temperature from eq 12:

$$\Delta H_u(T) = \Delta H_u(298) + \Delta C_{p_u}(T - 298) \quad (12)$$

$$\Delta H_u(T) = (5580 - 75\Delta C_{p_u}) + \Delta C_{p_u}(T - 298)\ \text{J (mol·res)}^{-1} \quad (13)$$

$$\Delta H_u(T) = 5580 + \Delta C_{p_u}(T - 373)\ \text{J (mol·res)}^{-1} \quad (14)$$

Equation 14 thus gives ΔH_u as a function of temperature and ΔC_{p_u}. The values of ΔC_{p_u} which are found in proteins vary by a factor of ≈2 for the 12 proteins in the data set of Privalov and Gill (1988). In general, therefore, different proteins will have different values of ΔH_u at a particular temperature. However, when T = 373 K, eq 14 reduces to $\Delta H_u(373)$ = 5.58 kJ (mol·res)$^{-1}$. In other words, at ≈100 °C, all the proteins considered have approximately the same value of ΔH_u, as seen in Figure 2 (i.e., T_H^* = 373 K). The only two pieces of information we have used in deriving eq 14 are that $\Delta S_u(383)$ = 17.6 J K^{-1} (mol·res)$^{-1}$, a value which can be found by extrapolating experimental data, and that $\Delta G_u(298)$ = 340 J (mol·res)$^{-1}$, which is also obtained from experiment. The assumptions used in deriving T_H^* = 373 K are that T_S^* = 383 K and that $\Delta G_u(298)$ is constant.

This suggests that these proteins show a common value of ΔH_u around 373 K as a result of the properties of the hydrophobic effect and constraints on protein stability (i.e., the free energy of unfolding). $\Delta S_u(298)$ is given by eq 8 and arises from the fact that T_S^* = 383 K (Baldwin, 1986). Given that $\Delta S_u(298)$ is equal to $17.6 - 0.251\Delta C_{p_u}$ (eq 8), the protein makes $\Delta H_u(298)$ equal to $5580 - 75\Delta C_{p_u}$ (eq 11) so that $\Delta G_u(298)$ is always close to 340 J (mol·res)$^{-1}$. In this analysis, no assumptions have been made from about the forces which contribute to ΔH_u so the fact that $\Delta H_u(373)$ is the same for each of these proteins tells us nothing about the enthalpic part of any of the forces which affect protein stability, such as the hydrophobic effect.

In the analysis of Baldwin (1986), ΔH_u is divided (eq 15) into the enthalpic part of the hydrophobic effect (ΔH_{hyd}) and everything else (ΔH_{res}), as with the entropy (eq 6):

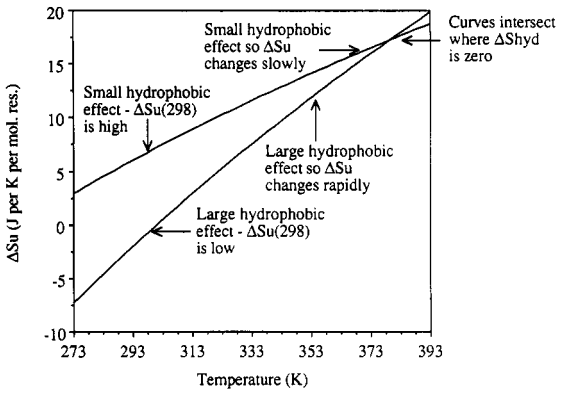

FIGURE 4: Why proteins show common values of ΔS_u around 383 K.

$$\Delta H_u(T) = \Delta H_{hyd} + \Delta H_{res} \quad (15)$$

Again, it is assumed that the change in heat capacity on unfolding (ΔC_{p_u}) arises solely from the hydrophobic effect. From solvent-transfer experiments, ΔH_{hyd} is known to be zero at 295 K (Baldwin, 1986). Thus, ΔH_u as a function of temperature can be written as in eq 16:

$$\Delta H_u(T) = \Delta C_{p_u}(T - 295) + \Delta H_{res} \quad (16)$$

When eqs 14 and 16 are compared, an expression for ΔH_{res} as a function of ΔC_{p_u} can be derived:

$$\Delta H_{res} = 5580 - 78\Delta C_{p_u} \quad (17)$$

The sequence and structure of the protein will ensure that eq 17 is satisfied. The size of the hydrophobic effect (characterized by ΔC_{p_u}) thus affects $\Delta H_u(T)$ directly via the equation $\Delta H_{hyd} = \Delta C_{p_u}(T - 295)$ and indirectly via the equation $\Delta H_{res} = 5580 - 78\Delta C_{p_u}$. There is no evidence that the direct effect of the hydrophobic effect is any different in solvent-transfer experiments and in protein unfolding. Baldwin (1986) has shown that ΔS_{res} has approximately the same value for each protein considered here. However, ΔH_{res} does vary from protein to protein, as shown in eq 17.

An argument similar to that presented above has been independently derived by Baldwin and Muller (R. L. Baldwin, personal communication). However, their explanation of why various proteins converge to similar enthalpies of unfolding at ≈110 °C was based upon the experimental observation that these proteins have similar melting points, rather than similar free energies of unfolding at room temperature.

The arguments presented in this paper are summarized in Figures 4 and 5. Figure 4 shows the variation of the specific entropy of unfolding for two proteins which differ in the size of their specific hydrophobic effects and hence in ΔC_{p_u} and $\Delta S_u(298)$. The entropic component of the hydrophobic effect is zero at 383 K (Baldwin, 1986), so the curves intersect at this temperature. Figure 5 shows the variation of the specific enthalpy of unfolding for the same two proteins. $\Delta H_u(298)$ varies to keep $\Delta G_u(298)$ the same for both proteins. This leads to a correlation between ΔC_{p_u} and $\Delta H_u(298)$ and hence a similar interaction at a higher temperature.

CONCLUSION

The hydrocarbon model of Baldwin has been extended to explain the experimental observation that many water-soluble, compact, globular proteins have the same enthalpy of unfolding, if their enthalpy of unfolding measured at the

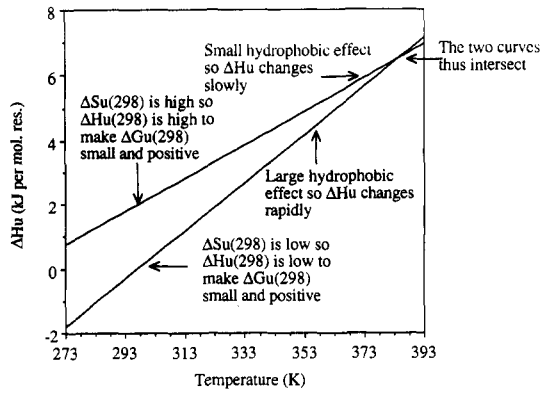

FIGURE 5: Why proteins show common values of ΔH_u around 383 K.

melting point of the protein is extrapolated to ≈110 °C, assuming the change in the heat capacity of unfolding is invariant with temperature. The addition to Baldwin's model of the experimentally verifiable fact that proteins in this class [most succinctly defined as those with $\Delta S_u \approx 17.6$ J K^{-1} (mol·res)$^{-1}$] have similar, small stabilities close to room temperature leads to the conclusion that these proteins will have similar specific enthalpies of unfolding at the higher temperature.

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