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Thermodynamics of the temperature-induced unfolding of globular proteins

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Abstract

The heat capacity, enthalpy, entropy, and Gibbs energy changes for the temperature-induced unfolding of 11 globular proteins of known three-dimensional structure have been obtained by microcalorimetric measurements. Their experimental values are compared to those we calculate from the change in solvent-accessible surface area between the native proteins and the extended polypeptide chain. We use proportionality coefficients for the transfer (hydration) of aliphatic, aromatic, and polar groups from gas phase to aqueous solution, we estimate vibrational effects, and we discuss the temperature dependence of each constituent of the thermodynamic functions. At 25 °C, stabilization of the native state of a globular protein is largely due to two favorable terms: the entropy of nonpolar group hydration and the enthalpy of interactions within the protein. They compensate the unfavorable entropy change associated with these interactions (conformational entropy) and with vibrational effects. Due to the large heat capacity of nonpolar group hydration, its stabilizing contribution decreases quickly at higher temperatures, and the two unfavorable entropy terms take over, leading to temperature-induced unfolding.

Keywords: Gibbs energy; heat capacity; hydration; protein folding

The stability of globular proteins is a delicate balance between enthalpic and entropic terms derived from the physical-chemical difference between the native and unfolded polypeptide chain and the surrounding solvent. Differential-scanning calorimetry (DSC) measures thermodynamical parameters for temperature-induced unfolding: heat capacity, enthalpy, entropy, and Gibbs energy (Privalov, 1979; Privalov & Gill, 1988). These experimental values, which are relevant to the overall process of unfolding, should be interpreted as sums of a number of different contributions. The most significant are from changes in interactions between atoms within the polypeptide chain, in conformational degrees of freedom of the chain, in vibrational modes, and in the hydration of chemical groups. In cases where the three-dimensional structure of the native protein is known, the contribution of these changes to the thermodynamic parameters can in principle be calculated (see Creighton, 1991, for a review). To a good approximation, the contribution of hydration is additive (Murphy & Gill, 1990) and linearly related to the change in solvent-accessible surface area (Ooi et al., 1987). It can be derived with the help of proportionality coefficients derived from small molecule studies (Makhatadze & Privalov, 1988, 1990, 1993, 1994; Ooi & Oobatake, 1988; Spolar et al., 1989, 1992; Khechinashvili, 1990; Privalov & Makhatadze, 1990, 1992, 1993; Livingstone et al., 1991; Makhatadze et al., 1993). The heat capacity change determines the evolution of other parameters with temperature, as opposed to their value at a given temperature. We present here estimates of its value for unfolding 11 monomeric globular proteins of known three-dimensional structures, based on semi-empirical calculations of the hydration and vibrational components. We then derive the temperature dependence of the enthalpy, entropy, and Gibbs energy of protein unfolding, and compare their values with data from DSC experiments. By difference, we obtain an estimate of the enthalpy change due to novel interactions being made between protein atoms upon folding, and of the entropy change due to degrees of freedom lost by the polypeptide chain. The comparison with experimental data confirms that temperature-induced unfolding can be qualitatively accounted for in this simple way, even though the accuracy and reliability of some parameters may still be insufficient for the analysis to be quantitative and exact.

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Methods and results

Atomic coordinates and ASA calculations

Atomic coordinates for 11 monomeric globular proteins (Table 1) were taken from the Brookhaven Protein Data Bank (Bernstein et al., 1977). They represent well-refined high-resolution (2 Å or better) X-ray structures of proteins for which calorimetric data are available. Solvent molecules and ions were deleted and only the first position was retained when alterna-

Table 1. *The protein sample*

Protein	MW	File	Reference
Pancreatic trypsin inhibitor, bovine	6,550	4PTI	Wlodawer et al. (1987)
Erabutoxin B, sea snake	6,860	3EBX	Smith et al. (1988)
Parvalbumin, carp	11,500	5CPV	Swain et al. (1989)
Cytochrome *c*, tuna (oxidized)	12,400	3CYT	Takano and Dickerson (1981)
Ribonuclease A, bovine	13,600	3RN3	Borkakoti et al. (1982)
Lysozyme, hen	14,300	2LZT	Ramanadham et al. (1989)
Myoglobin, sperm whale (met)	17,800	4MBN	Takano (1977)
Lysozyme, phage T4	18,600	3LZM	Weaver et al. (1989)
Papain	23,400	9PAP	Kamphuis et al. (1984)
β-Trypsin, bovine	23,500	1TLD	Bartunik et al. (1989)
α-Chymotrypsin, bovine	25,200	2CHA	Birktoft and Blow (1972)

tive atomic positions were listed in the files. Erabutoxin B (Smith et al., 1988) was used as a model of neurotoxin II from cobra venom for which thermodynamic parameters of denaturation are known (Khechinashvili & Tsetlin, 1984). Both are short neurotoxins, comprising 61 and 62 residues, respectively, with a high degree of sequence homology (Karlsson, 1979).

Solvent-accessible surface areas (ASA; Lee & Richards, 1971) were calculated with a program written by Prof. A. Lesk (Cambridge, UK) implementing the Shrake and Rupley (1973) algorithm. The probe radius (1.4 Å) and the group radii were consistent with those of Ooi et al. (1987). Seven types of chemical groups were considered: aliphatic carbon (-CH_3, -CH_2-, >CH-), aromatic carbon, hydroxyl, amide and amine, carbonyl and carboxyl carbon, carbonyl and carboxyl oxygen, and sulfur. Aliphatic and aromatic carbons are considered below as nonpolar; all other atoms, including carbonyl and carboxyl carbons, are considered as polar.

A model of the unfolded proteins was derived from the Protein Data Bank coordinates by systematically setting the main-chain ϕ angles to $-140°$ and the ψ angles to 140°, giving the polypeptide chain an extended conformation as in a β-strand. The native side-chain conformations were retained. Whereas individual side-chain conformations are fixed by the native structure, their overall distribution is the same in protein structures and in small peptides (Janin et al., 1978). It is therefore representative of a random polypeptide chain.

ASA(N) values obtained with native proteins were consistent with previous estimates (Miller et al., 1987), with minor differences (1–2%) resulting from the different set of group radii. ASA(N) is not proportional to the molecular weight M; it varies as M^{α} where $\alpha \approx 0.73$ (Miller et al., 1987). Because ASA(U), the accessible surface area of the unfolded proteins, is a linear function of M, ΔASA, the ASA change occurring upon unfolding, increases with M faster than linearly.

ASA(U) has often been derived by summing the ASA of residues in tripeptides. Like Livingstone et al. (1991), we find that ASA(U) values measured on the extended chains are systematically smaller. The difference amounts to 17–30% depending on the protein, with an average of 25%, and it can be attributed mostly to $(i, i \pm 2)$ side-chain contacts. The tripeptide model has been developed to find the maximum ASA an amino acid residue can have, not its average accessibility in an unfolded and fluctuating polypeptide chain, where contacts take place between second and other neighbors. $(i, i \pm 2)$ side-chain contacts dominate in β-strands. Most other main-chain conformations have more $(i, i \pm 1)$ and $(i, i \pm n)$ contacts for $n > 2$. For instance, when the (ϕ, ψ) angles were set to $(-57°, -47°)$, values typical of an α-helix, ASA(U) was 40% smaller on average than with $(-140°, 140°)$. The average ASA of a denatured protein with a fluctuating random conformation is certainly larger than in an α-helix and closer on average to that of a β-strand. Still, approximating a thermally unfolded protein with an extended chain is likely to overestimate ASA(U) and ΔASA, especially in proteins with disulfide bonds.

ΔASA was derived separately for each type of chemical group. Proportionality coefficients were applied to calculate values of thermodynamic parameter changes associated with hydration upon denaturation: the heat capacity at constant pressure, enthalpy, and Gibbs energy changes. The entropy change at $T = 298$ K was derived from the enthalpy and Gibbs energy changes. The proportionality coefficients, taken from Ooi et al. (1987) and Ooi and Oobatake (1988), have been derived from experimental data on transfer of low molecular weight model compounds from gas phase to water at 25 °C (Cabani et al., 1981). Aspartate and glutamate side chains were assumed to be ionized. Carbon or nitrogen atoms in the heme group of myoglobin and cytochrome *c* were treated like their counterparts in aromatic side chains. The results are quoted in Table 2, separating contributions from aliphatic, aromatic, and polar groups.

The heat capacity of protein unfolding

Heat denaturation of globular proteins is invariably accompanied with a positive change in heat capacity, ΔC_p. This is largely due to the exposure of nonpolar groups to water when the polypeptide chain unfolds (Brandts & Hunt, 1967; Privalov & Khechinashvili, 1974). Exposure of aliphatic groups, and to a lesser extent of aromatic groups, makes a large positive contribution that we call ΔC_p^{NP}. Exposure of polar groups has the opposite effect, but its contribution ΔC_p^{P} is 5–7 times smaller at 25 °C in proteins studied here.

Sturtevant (1977) and Kanehisa and Ikegami (1977) pointed out that changes in the frequency of internal vibrational modes also contribute to the heat capacity change. The unfolded chain has more soft (low frequency) modes than the native protein,

Table 2. *Thermodynamic parameters for hydration of protein groups upon unfolding at 25 °C*[a]

Protein	Hydration	ΔASA (Å^2)	ΔC_p (kJ·mol^{-1}·K^{-1})	ΔH (kJ·mol^{-1})	ΔS (kJ·mol^{-1}·K^{-1})	ΔG (kJ·mol^{-1})
PTI	Al	1,300	2.02	−142	−0.624	44
	Ar	750	0.93	−120	−0.319	−25
	P	1,480	−0.57	−401	−0.362	−293
Erabutoxin B	Al	1,700	2.63	−185	−0.812	57
	Ar	400	0.50	−64	−0.168	−14
	P	2,310	−1.00	−636	−0.517	−482
Parvalbumin	Al	3,220	4.98	−350	−1.537	108
	Ar	975	1.21	−155	−0.409	−33
	P	2,510	−1.36	−677	−0.513	−524
Cytochrome *c*	Al	3,650	5.66	−397	−1.742	122
	Ar	1,110	1.37	−176	−0.466	−37
	P	3,170	−1.06	−883	−0.956	−598
Ribonuclease A	Al	3,980	6.17	−433	−1.899	133
	Ar	850	1.05	−135	−0.359	−28
	P	3,880	−1.79	−1,146	−0.913	−874
Lysozyme, hen	Al	4,190	6.48	−456	−2.000	140
	Ar	820	1.01	−130	−0.346	−27
	P	3,500	−1.63	−1,037	−0.832	−789
Myoglobin	Al	5,820	9.00	−633	−2.778	195
	Ar	1,235	1.53	−196	−0.520	−41
	P	2,980	−1.37	−780	−0.708	−569
Lysozyme, T4	Al	6,080	9.41	−661	−2.903	204
	Ar	1,130	1.41	−180	−0.480	−38
	P	3,430	−1.40	−1,165	−1.057	−850
Papain	Al	8,940	13.84	−973	−4.268	299
	Ar	1,970	2.44	−313	−0.829	−66
	P	6,030	−2.62	−1,746	−1.554	−1,283
β-Trypsin	Al	9,810	15.18	−1,067	−4.681	328
	Ar	1,510	1.87	−240	−0.638	−50
	P	6,770	−2.93	−1,459	−1.453	−1,026
α-Chymotrypsin	Al	10,270	15.89	−1,117	−4.903	344
	Ar	1,660	2.06	−264	−0.698	−56
	P	6,870	−2.71	−1,155	−1.359	−750

[a] ΔASA is the accessible surface change upon protein unfolding; ΔC_p, ΔH, ΔS, and ΔG are the heat capacity, enthalpy, entropy, and Gibbs energy for hydration of: Al, aliphatic; Ar, aromatic; and P, polar groups upon unfolding.

and a larger vibrational heat capacity. Kanehisa and Ikegami (1977) calculated a value of 0.105 J·g^{-1}·K^{-1} from the infrared and Raman spectra of the 20 amino acids. More recently, Sochava and Smirnova (1993) obtained 0.190 J·g^{-1}·K^{-1}, probably an overestimate, from calorimetric measurements done on several globular proteins at 4–7% moisture content. This value was virtually the same for the five proteins studied: ribonuclease, lysozyme, α-chymotrypsin, cytochrome *c*, and myoglobin. All these studies suggest that vibrational modes contribute significantly to the heat capacity of unfolding. For all proteins considered here, we shall use the value 0.105 J·g^{-1}·K^{-1} in calculating the vibrational contribution ΔC_p^{vib}.

The heat capacity of unfolding derived by considering the hydration and vibrational terms is then:

$$\Delta C_p^{calc} = \Delta C_p^{vib} + \Delta C_p^{NP} + \Delta C_p^{P}. \quad (1)$$

In Figure 1, its value is compared to the experimental value derived for the 11 proteins at 25 °C from the temperature dependence of the denaturation enthalpy in DSC measurements. The two sets are linearly correlated. The calculated values are within 10% of the experimental ones for the pancreatic trypsin inhibitor, cytochrome *c*, and myoglobin. In other proteins, the calculation overestimates ΔC_p by 22% on average, probably because we overestimate ASA(U) and ΔASA.

Temperature dependence of the heat capacity change

Microcalorimetric studies performed on many different globular proteins indicate that the enthalpy of thermal denaturation is a linear function of temperature to within the experimental error (less than 5%). This implies that, to a good approximation, the heat capacity of thermal denaturation does not depend

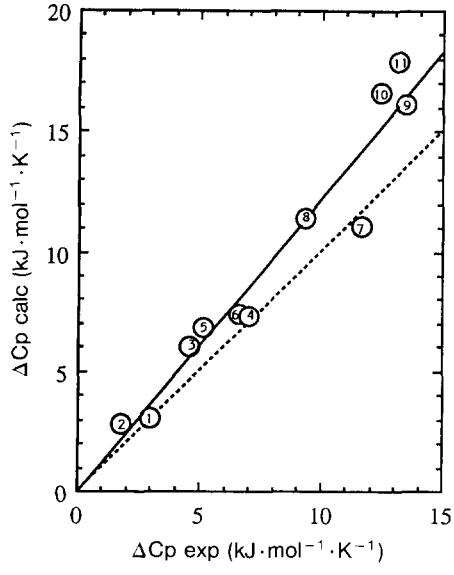

Fig. 1. Experimental and calculated heat capacities for protein unfolding. ΔC_p^{calc}, calculated from the vibrational and hydration contributions is plotted against ΔC_p^{exp}, an experimental value derived from the temperature dependence of the heat capacity of thermal unfolding in DSC experiments. The dotted line has a slope of 1, the regression line ($R^2 = 0.94$), a slope equal to 1.22; 1, pancreatic trypsin inhibitor; 2, erabutoxin; 3, parvalbumin; 4, cytochrome *c*; 5, ribonuclease A; 6, hen lysozyme; 7, myoglobin; 8, phage T4 lysozyme; 9, papain; 10, trypsin; 11, chymotrypsin. 1, 3, 8, 10, Experimental data from Privalov (1979); 2, from Khechinashvili and Tsetlin (1984); 4, 5, 6, 7, 11, from Privalov and Khechinashvili (1974); 9, from Tiktopulo and Privalov (1978).

on temperature (Privalov & Khechinashvili, 1974; Privalov, 1979). Nevertheless, each of the three components of ΔC_p^{calc} in Equation 1 is temperature dependent, and we want now to examine this dependence.

Kanehisa and Ikegami (1977) found that, whereas the vibrational heat capacity is similar in native and unfolded proteins at 25 °C (1.046 ± 0.013 and 1.151 ± 0.013 $J \cdot g^{-1} \cdot K^{-1}$, respectively), it increases faster with temperature in the native (4.184 versus $3.766 \cdot 10^{-3}$ $J \cdot g^{-1} \cdot K^{-2}$). The difference between the two is ΔC_p^{vib}. It must decrease linearly with increasing temperature with a slope $-0.418 \cdot 10^{-3}$ $J \cdot g^{-1} \cdot K^{-2}$ that we call α^{vib}. Taking $T_0 = 298$ K, we write:

$$\Delta C_p^{vib}(T) = \Delta C_p^{vib}(T_0) + \alpha^{vib}(T - T_0). \quad (2)$$

Naghibi et al. (1986, 1987a, 1987b) have measured the hydration heat capacity of aliphatic hydrocarbons: methane, propane, butane, and isobutane. It is positive and decreases linearly with increasing temperature in the range 0–50 °C. A study by Makhatadze and Privalov (1988) of the hydration heat capacity of aromatic compounds leads to the same conclusion over a wide temperature range. In the region 25–140 °C, where heat capacity measurements are most reliable for benzene, we calculate a correlation coefficient $R^2 = 0.999$ from their data. Thus, we can write an equation like Equation 2 for the hydration of the nonpolar surface, with a slope α^{NP}. The data from Naghibi et al. (1986, 1987a, 1987b) and Makhatadze and Privalov (1988) yield values of α^{NP} that are almost identical for aliphatic and aromatic groups: $-3.28 \cdot 10^{-3}$ $J \cdot g^{-1} \cdot K^{-2}$ and $-3.24 \cdot 10^{-3}$ $J \cdot g^{-1} \cdot K^{-2}$, respectively.

The first two components of the heat capacity of unfolding are linear decreasing functions of temperature. To get a temperature-independent value of ΔC_p^{calc} requires that ΔC_p^{P}, the hydration heat capacity of the polar surface, increases linearly with temperature. This is at variance with Privalov and Makhatadze (1990, 1992), who consider its temperature dependence to be nonlinear. In data from Table 7 of Makhatadze and Privalov (1990) and Table 1 of Privalov and Makhatadze (1990), the source of nonlinearity is the hydration heat capacity of the peptide unit. Its contribution was derived from measurements of the partial molar heat capacity of glycine homopeptides $(Gly)_n$ by taking the difference between $(Gly)_{n+1}$ and $(Gly)_n$. The temperature dependence is nonlinear for $n = 3$, but it becomes linear for $n = 4$ and above. We took the value derived for $n = 4$ to be representative of peptide units in proteins, in agreement with microcalorimetric results of Jolicoeur and Boileau (1978).

Components of the heat capacity of unfolding ΔC_p and of its temperature derivative α are represented in Figure 2 for the 11 proteins. The nonpolar surface hydration and vibrational components of ΔC_p are positive in the range of temperatures that is accessible to experiment, and they become smaller at higher temperatures. The nonpolar component is almost equal to ΔC_p^{calc} at 25 °C. This results from ΔC_p^{vib} and ΔC_p^{P} having opposite signs and approximately the same absolute value at this temperature. At 25 °C, the polar surface hydration component is negative; above, it increases to reach a value of zero somewhere between 70 and 120 °C.

Temperature dependence of thermodynamic parameters for hydration

In the case where ΔC_p is a linear function of temperature, the classical relationships:

$$\Delta C_p = \frac{d(\Delta H)}{dT} = T\frac{d(\Delta S)}{dT} \quad (3)$$

integrate to:

$$\Delta H(T) = \Delta H(T_0) + \Delta C_p(T_0)(T - T_0) + \frac{\alpha}{2}(T - T_0)^2 \quad (4)$$

and

$$\Delta S(T) = \Delta S(T_0) + \Delta C_p(T_0)\ln\frac{T}{T_0} + \alpha\left(T - T_0 - T_0\ln\frac{T}{T_0}\right). \quad (5)$$

Here, $\Delta H(T_0)$, $\Delta S(T_0)$, and $\Delta C_p(T_0)$ are the enthalpy, entropy, and heat capacity changes at the reference temperature of 25 °C ($T_0 = 298$ K); α is the derivative of ΔC_p versus temperature as in Equation 2.

Hydration of nonpolar groups is accompanied with an enthalpy change ΔH^{NP} and an entropy change ΔS^{NP}. It can be seen in Figure 3 that the two quantities are negative at 25 °C, but because the associated heat capacity change is positive and

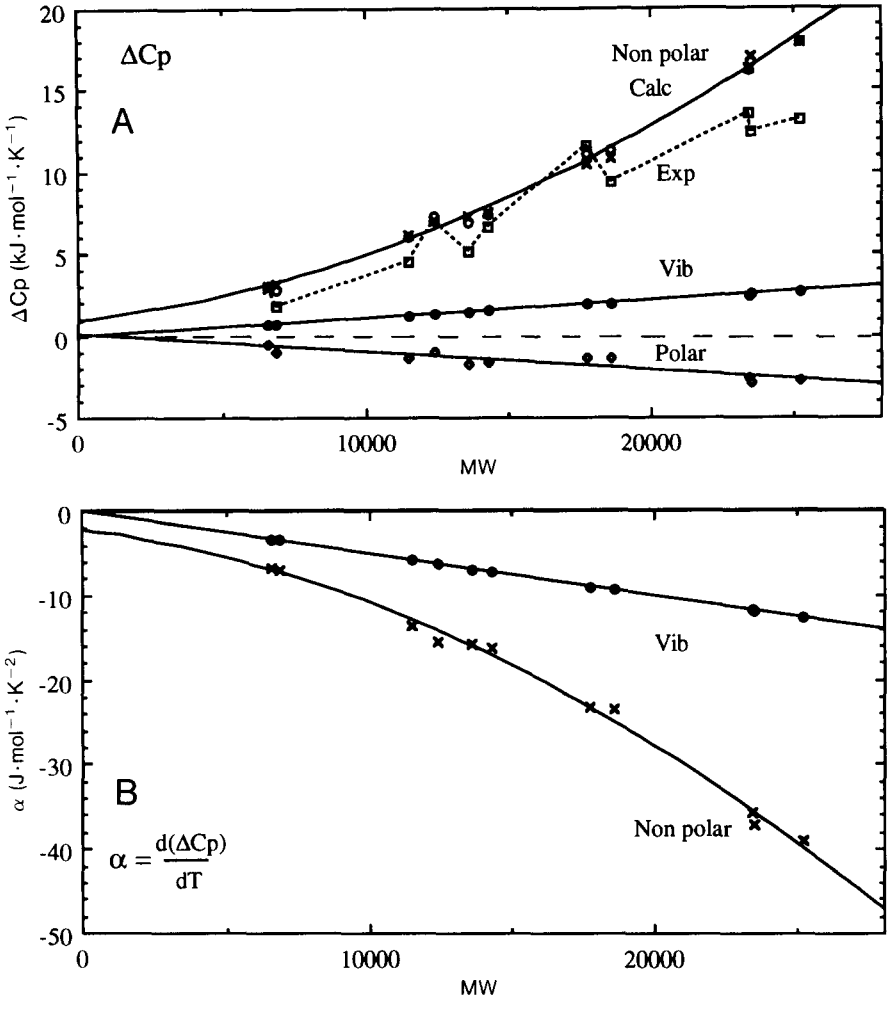

Fig. 2. Contributions to the heat capacity for protein unfolding, ΔC_p, and its temperature derivative, α. **A:** Contributions of X, nonpolar surface hydration; ◇, polar surface hydration; and ●, vibrations to ○, the heat capacity change ΔC_p^{calc} at 25 °C, plotted against the molecular weight M of the 11 proteins in Table 1. □, Experimental values ΔC_p^{exp} as in Figure 1. The polar contribution is fitted to a regression line of slope $-1.1 \cdot 10^{-4}$ ($R^2 = 0.81$); the nonpolar contribution, to a second-order polynomial $\Delta C_p^{NP} = 1 + 2.1 \cdot 10^{-4} M + 1.9 \cdot 10^{-8} M^2$. **B:** Plot of ●, vibrational and X, nonpolar surface hydration contributions to the temperature derivative α of ΔC_p^{calc}.

large, they both increase with temperature and must change sign at some point. The temperature where $\Delta H^{NP} = 0$ is in the range 110–125 °C for the 11 proteins studied here. The temperature where $\Delta S^{NP} = 0$ is higher, near 150 °C, but it is also approximately the same for all 11 proteins.

In Table 2, we quoted values for the hydration enthalpy and heat capacity of two kinds of nonpolar surface, aliphatic and aromatic. They show the following trends: at 25 °C, the hydration enthalpy is less negative and the hydration heat capacity is more positive for the aliphatic than for the aromatic surface. Thus, the aliphatic surface has zero hydration enthalpy at a lower temperature than that for the aromatic surface (Makhatadze & Privalov, 1993). Most proteins in our sample bury a similar ratio of aliphatic to aromatic surface area, and therefore, the temperature where the overall contribution of nonpolar hydration is zero is also similar. Conversely, pancreatic trypsin inhibitor has relatively more buried aromatic groups than others, and it has zero ΔH^{NP} at a higher temperature (125 °C).

We derive ΔG^{NP}, the hydration Gibbs energy for nonpolar surface hydration, from the corresponding enthalpy and entropy. It is positive around 25 °C for all proteins. It should be noted that this is entirely due to buried aliphatic groups, the contribution of aromatic surfaces to ΔG^{NP} being negative but small. ΔG^{NP} increases with temperature and should reach a maximum near 150 °C, where $-\Delta S^{NP}$, its derivative, is zero. At the other extreme of the temperature range, ΔG^{NP} is also zero somewhere between −5 and −18 °C, depending on the protein, and it is negative below. The pancreatic trypsin inhibitor has zero ΔG^{NP} at 10 °C, well above other proteins, again due to a relatively high content of buried aromatic groups.

Hydration of more polar groups upon protein unfolding is accompanied by a very large negative enthalpy ΔH^P (Table 2). The entropy component $-T\Delta S^P$ is positive, but it compensates ΔH^P only in part, so the Gibbs energy ΔG^P is also negative and large. The heat capacity of polar surface hydration ΔC^P is negative at 25 °C. We took the value of α^P, its temperature derivative, to be equal to $-(\alpha^{NP} + \alpha^{vib})$, so as to make ΔC_p^{calc} temperature invariant as discussed above, and applied Equations 4 and 5. The temperature dependences of ΔH^P and ΔS^P were derived for the 11 proteins. They do not display the regularity that we observed for their nonpolar counterparts, i.e., a tendency for the curves to intersect at a single point. In Figure 4A, we show the temperature dependence of thermodynamic parameters for polar surface hydration in myoglobin. It is typical of other proteins studied here. ΔH^P and ΔS^P decrease as the temperature increases from 25 to 70 °C, they reach a minimum near 70 °C, then start increasing. At 70 °C, the hydration heat capacity is zero for the polar surface. In other proteins, ΔH^P and

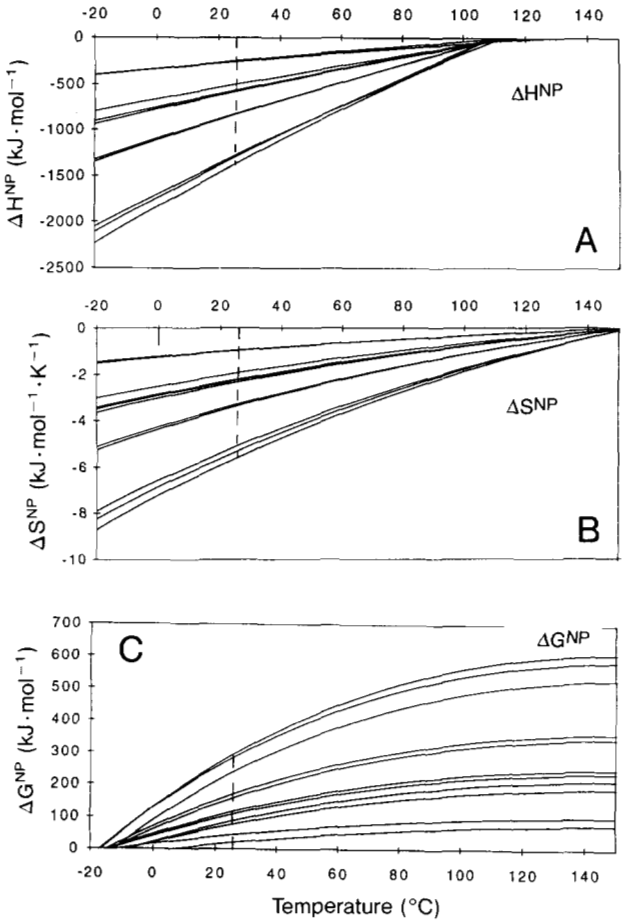

Fig. 3. Temperature dependence of the contribution of nonpolar surface hydration to thermodynamic parameters for protein unfolding. Enthalpy ΔH^{NP} and entropy ΔS^{NP} changes were derived by applying Equations 4 and 5 to values calculated at 25 °C and quoted in Table 2; the Gibbs energy change is $\Delta G^{NP} = \Delta H^{NP} - T\Delta S^{NP}$. From top to bottom in the first two panels and in reverse order in the bottom panel: pancreatic trypsin inhibitor, erabutoxin, parvalbumin, cytochrome *c*, ribonuclease A, hen lysozyme, myoglobin, phage T4 lysozyme, papain, trypsin, chymotrypsin.

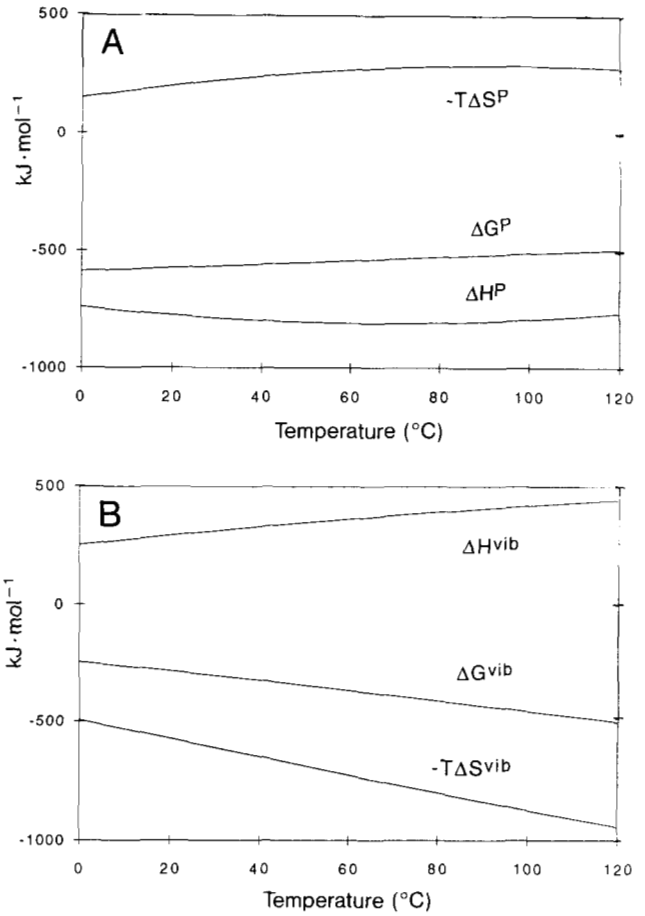

Fig. 4. Temperature dependence of the contributions of **(A)** polar surface hydration and **(B)** vibrations to thermodynamic parameters for myoglobin unfolding. Enthalpy, ΔH, and entropy, ΔS, changes are derived by applying Equations 4 and 5 to the values calculated at 25 °C and quoted in Table 2. The Gibbs energy change is $\Delta G = \Delta H - T\Delta S$.

ΔS^P also go through a minimum at temperatures that range from 70 to 120 °C.

The vibrational contribution

To derive the vibrational contributions to the enthalpy, entropy, and Gibbs energy changes at 25 °C, we used empirical relationships proposed by Sturtevant (1977):

$$\Delta H^{vib} = 0.53T\Delta C_p^{vib}$$

$$\Delta S^{vib} = 1.05\Delta C_p^{vib}$$

$$\Delta G^{vib} = -0.52T\Delta C_p^{vib}.$$

The vibrational heat capacity change per unit mass was assumed to be the same (0.105 $J \cdot g^{-1} \cdot K^{-1}$) for all proteins. Therefore, ΔH^{vib}, ΔS^{vib}, and ΔG^{vib} are simply proportional to molecular weight. At 25 °C, their value per unit mass are 16.6 $J \cdot g^{-1}$, 0.110 $J \cdot g^{-1} \cdot K^{-1}$, and −16.3 $J \cdot g^{-1}$, respectively.

The temperature dependence of ΔC_p^{vib} being given by Equation 2, that of ΔH^{vib} and ΔS^{vib} was obtained by applying Equations 4 and 5. The result is shown for myoglobin in Figure 4B. The vibrational enthalpy and entropy changes are positive and increase with temperature. The entropy contribution prevails, so the Gibbs energy change is negative over the whole range of temperature and more so at higher temperatures.

The role of intramolecular bonds and conformational degrees of freedom

Values of the enthalpy and Gibbs energy changes associated with the hydration and vibrational components may now be compared to results of DSC studies of heat denaturation. Experimental values were obtained for each protein under conditions corresponding to their maximal thermostability (Privalov & Khechinashvili, 1974; Tiktopulo & Privalov, 1978; Khechinashvili & Tsetlin, 1984; Kitamura & Sturtevant, 1989; Connelly et al., 1991; Hu et al., 1992). They have been extrapolated to 25 °C to yield numbers that we call ΔH^{exp}, ΔS^{exp}, and ΔG^{exp}.

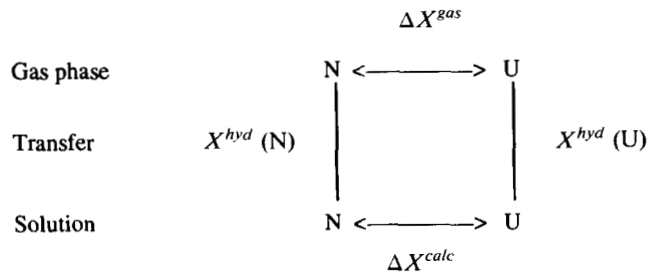

Fig. 5. Thermodynamic cycle for protein unfolding. The change of any thermodynamic parameter X (enthalpy, entropy, Gibbs energy, or heat capacity) when the protein unfolds can be calculated first in the gas phase, then in solution as:

$$\begin{aligned}\Delta X^{calc} &= \Delta X^{gas} - X^{hyd}(\mathrm{N}) + X^{hyd}(\mathrm{U}) \\ &= \Delta X^{gas} + \Delta X^{hyd} \\ &= \Delta X^{int} + \Delta X^{vib} + \Delta X^{NP} + \Delta X^{P},\end{aligned}$$

where $X^{hyd}(\mathrm{N})$ and $X^{hyd}(\mathrm{U})$ are the values of the hydration parameter for the native and unfolded protein; ΔX^{hyd} is their difference; ΔX^{gas} includes a vibrational term, ΔX^{vib}, and a term ΔX^{int} related to breaking interactions within the protein in gas phase. ΔX^{hyd} has a nonpolar ΔX^{NP} and a polar ΔX^{P} component. ΔX^{calc} may be compared to the experimental value ΔX^{exp}.

As shown by the thermodynamic cycle of Figure 5, each of the experimental numbers should be equal to the sum of a hydration term and a term for unfolding in the gas phase. At this point, we have determined values for the hydration terms and for the vibrational component of the gas phase unfolding terms. We are still missing the gas phase contribution of intramolecular bonds, hydrogen bonds, and van der Waals interactions that break when the protein unfolds. Their energy contributes to the enthalpy change, and the concomitant loss of conformational degrees of freedom, to the entropy change. Up to now, we have ignored all these internal contributions. Let us assume that they are the only source of the difference between the experimental values and the sum of the calculated hydration and vibrational contributions. We label the residual enthalpy, entropy, and Gibbs energy, ΔH^{int}, ΔS^{int}, and ΔG^{int}, respectively.

Figure 6 shows how the four terms, hydration of polar and nonpolar surface, vibrations and intramolecular bonds, contribute to the thermodynamic parameters of unfolding in the 11 proteins. The vibrational contributions are proportional to the molecular weight M. Those for nonpolar surface hydration are nonlinear functions of M due to the nonlinear variation of ΔASA. In Figure 6, ΔH^{NP}, ΔS^{NP}, and ΔG^{NP} were fitted to second-order polynomials that yield very good approximations of their values. Data points representing the polar surface hydration and internal contributions show more dispersion than for the nonpolar surface. Figure 6 suggests that these two contributions increase linearly with M. ΔH^{int}, the interaction enthalpy change, is within 30% of 0.10 kJ·g^{-1}·mol^{-1}, equivalent to 11 kJ·mol^{-1} per residue on average. ΔS^{int} is about 20 J·mol^{-1}·K^{-1} per residue. It includes all main-chain and side conformational entropy changes upon protein unfolding and happens to be equal to the entropy for a helix–coil transition calculated by Schellman (1955). The large negative value of $-T\Delta S^{int}$ partly compensates the positive ΔH^{int}, so that ΔG^{int} is positive like ΔH^{int}, but smaller, about 6 kJ·mol^{-1} per residue. Overall, intramolecular bonds contribute positively to the Gibbs energy of unfolding at 25 °C.

The thermodynamics of thermal unfolding

At 25 °C, the Gibbs energy of unfolding is positive but small, in the range 20–80 kJ·mol^{-1} for the proteins of our sample: the native state is only marginally stable. This is true of globular proteins in general and results from compensating terms. Components of ΔG that have a positive sign at 25 °C (ΔG^{int} and ΔG^{NP}) favor the native state; components that have a negative sign (ΔG^{vib} and ΔG^{P}) favor denaturation.

Each contribution to ΔG can be further broken into an enthalpic term ΔH and an entropic term $-T\Delta S$, making it a total of eight terms, which we now take into consideration separately. In addition to their sign at 25 °C, we shall consider how each term varies with increasing temperature. In Table 3, an arrow pointing up (↑) indicates that a given term increases and becomes more stabilizing (if positive) or less destabilizing (if negative) at high temperatures; an arrow pointing down (↓) indicates that it decreases and becomes more destabilizing or less stabilizing.

Intramolecular bonds have an enthalpy change ΔH^{int} and an entropy change ΔS^{int} that are both positive and independent of temperature in first approximation. ΔH^{int} is large: as expected, intramolecular bonds stabilize the native state. The entropic term $-T\Delta S^{int}$ is also large. It favors the denatured state, which has many more conformational degrees of freedom. The value of ΔG^{int} is positive nevertheless: internal interactions promote folding in spite of the conformational entropy loss. Vibrational modes also have a positive enthalpy change ΔH^{vib} that increases with temperature, but it remains small compared to the associated entropic term $-T\Delta S^{vib}$, which is negative and also increases in absolute value with temperature. Thus, the vibrational contribution always favors the unfolded state, especially so at high temperatures.

Hydration of the nonpolar surface has a large entropy change ΔS^{NP} that makes a major contribution in favor of the native state. Water becomes ordered and loses entropy upon protein unfolding as it solvates more nonpolar groups and especially more aliphatic groups. With Kauzmann (1959), Tanford (1970, 1979) and many others, we maintain that the hydrophobic effect stabilizes globular proteins. However, this is not the whole

Table 3. *Sign and temperature dependence of thermodynamic parameters for protein unfolding*[a]

Contribution	ΔG		ΔH		$-T\Delta S$	
Interactions	+	↓	++	−	−−	↓
Vibrations	−	↓	+	↑	−−	↓
Nonpolar hydration	+	↑↓	−	↑	++	↓
Polar hydration	−	↑	−−	↓	+	↑
Overall	+	↑↓	+	↑	−	↓

Arrows mark quantities that are increasing (↑) or decreasing (↓) functions of temperature in the 0–120 °C range. ↑↓ indicates a quantity that goes through a maximum in this range. Signs are for 25 °C. In addition to the sign of the quantity, −− and ++ indicate terms that have a large absolute value relative to others at 25 °C.

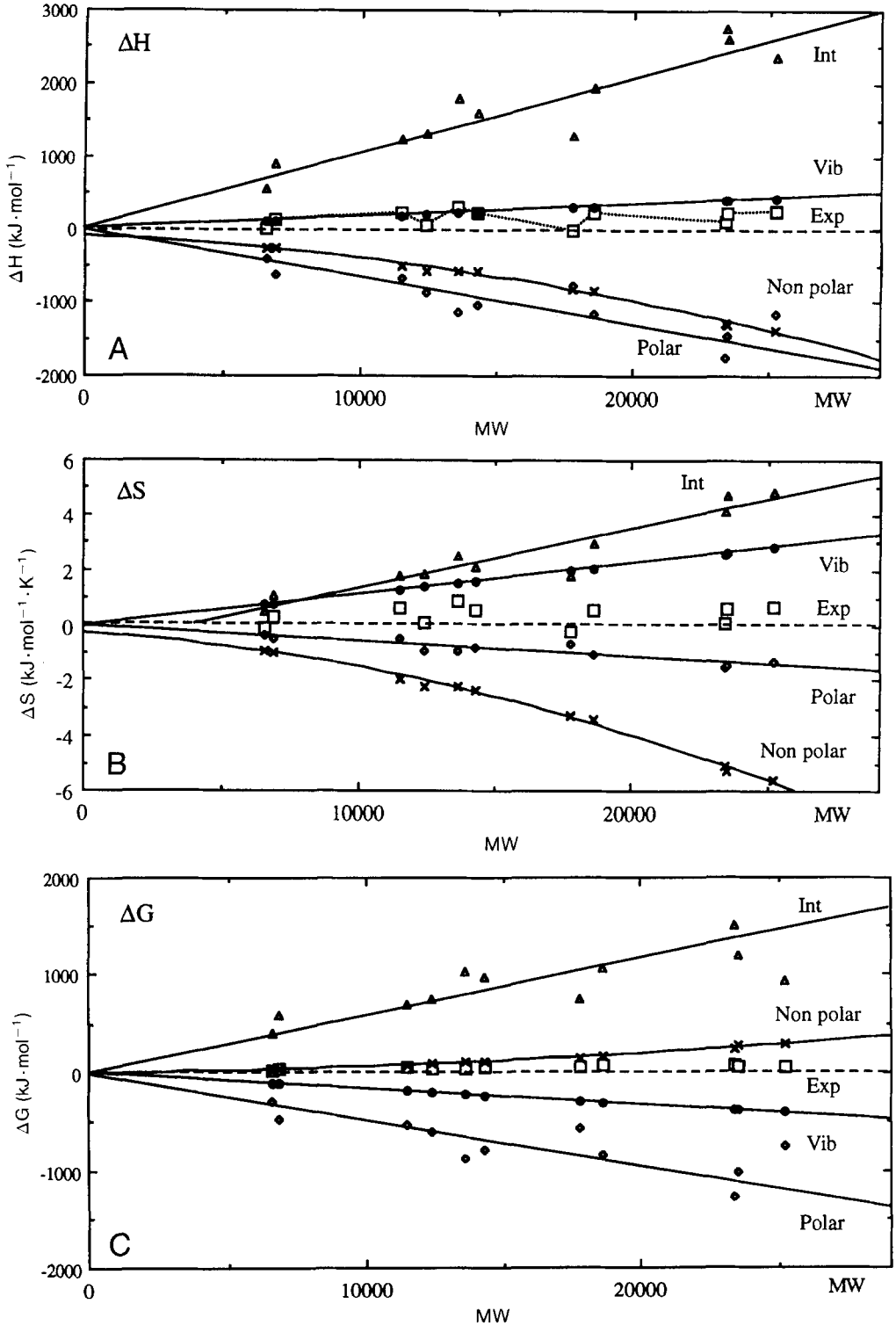

Fig. 6. Contributions to the enthalpy, entropy, and Gibbs energy for protein unfolding at 25 °C. Contributions of: X, nonpolar surface hydration; ◇, polar surface hydration; ●, vibrations; and △, intramolecular bonds, are plotted against the molecular weight of 11 proteins. □, Experimental values from references given in the legend of Figure 1. **A:** Enthalpy changes. Contribution of intramolecular bonds is fitted to a regression line of slope 0.10 kJ·mol^{-1}·g^{-1} (R^2 = 0.86); polar contribution, to a line of slope $-4\cdot10^{-3}$ kJ·mol^{-1}·g^{-1}; nonpolar contribution, to a second-order polynomial $\Delta H^{NP} = -90 + 1.6\cdot10^{-2}\,M + 1.4\cdot10^{-6}\,M^2$. **B:** Entropy changes. Contribution of conformational degrees of freedom is fitted to a regression line $\Delta S^{int} = -0.75 + 2.1\cdot10^{-4}\,M$ (R^2 = 0.90); polar contribution is fitted to a line of slope $-5.6\cdot10^{-5}$ kJ·mol^{-1}·K^{-1}·g^{-1}, nonpolar contribution, to a second-order polynomial $\Delta S^{NP} = -0.3 + 6.6\cdot10^{-5}\,M + 5.9\cdot10^{-9}\,M^2$. **C:** Gibbs energy changes. Contribution of intramolecular bonds is fitted to a line of slope 0.06 J·mol^{-1}·g^{-1}; polar contribution, to a line of slope $-4.6\cdot10^{-2}$ J·mol^{-1}·K^{-1}·g^{-1}; nonpolar contribution, to a second-order polynomial $\Delta G^{NP} = 3.2\cdot10^{-3}\,M + 3.4\cdot10^{-7}\,M^2$.

story (Murphy et al., 1990). The entropic nature of the hydrophobic effect has often been stressed. Yet, ΔS^{NP}, the entropy of nonpolar surface hydration, decreases in absolute value at higher temperatures. Moreover, nonpolar surface hydration also has an enthalpic component, ΔH^{NP}. It is negative at 25 °C, and more so at lower temperatures due to the large heat capacity of nonpolar surface hydration. The rapid evolution of ΔH^{NP} and ΔS^{NP} with temperature illustrated in Figure 3 implies that the contribution of nonpolar surface hydration cannot be described as purely entropic and stabilizing near 25 °C or below. As temperature goes down, ΔH^{NP} becomes more unfavorable, especially when buried aromatic groups are abundant. It may overcome the favorable entropic term, and then nonpolar surface hydration becomes a destabilizing factor for the native state, leading to cold denaturation (Privalov et al., 1986; Griko et al., 1988; Chen et al., 1989; Tamura et al., 1991; Griko & Privalov, 1992).

Last, hydration of the polar surface has a large negative enthalpy change ΔH^{P} favoring the denatured state. The entropic term $-T\Delta S^{P}$ favors the native state just as it does for the nonpolar surface, but it is smaller than ΔH^{P}. Both ΔH^{P} and $-T\Delta S^{P}$ depend on temperature. As they do so in opposite direction, they more or less compensate. Thus, polar surface hydration favors the unfolded state at all temperatures in the range we consider. Polar group hydration is the only component in ΔG^{calc} where the entropic term favors the native state and increases with temperature. The temperature dependence of ΔH^{vib} and ΔH^{NP} would also favor the native state at high temperature, but these terms are small. At higher temperatures, the stabilizing entropic term due to nonpolar hydration decreases fast, whereas the vibrational and conformational entropic terms become even more destabilizing. Thermal unfolding results from the evolution of these three large entropic terms.

Discussion

Our analysis is similar in its principle to the work of Spolar et al. (1992) and of Privalov and collaborators. Spolar et al. (1992) analyzed thermodynamic data on 14 proteins, Makhatadze and Privalov (1994) on 9 proteins. Five (pancreatic inhibitor, cytochrome *c*, ribonuclease A, hen lysozyme, and myoglobin) are common to all three studies, which makes a comparison possible.

Spolar et al. (1992) used parameters for transfer from organic solvent rather than from the gas phase to water as we do. Nevertheless, their results are consistent with ours. Buried surface areas, also evaluated with an extended chain model, are systematically larger by about 15%. This is due in part to the choice of group radii, but, as these are consistent with the proportionality coefficients, the calculated hydration heat capacities of unfolding are within 10% of ours at 25 °C. Hydration enthalpy changes, evaluated in a rather different manner, represent the difference between interactions made by protein groups with water and with the reference organic solvent. This difference is expected to be small for nonpolar groups and large for polar ones. Accordingly, the values of ΔH^{NP} quoted by Spolar et al. (1992) are much smaller than ours and those of ΔH^{P} differ by only 10–30%. Because the temperature dependence of these terms is not explicitly considered, the comparison can be made only at 25 °C, but the agreement is satisfactory at this temperature.

Because our approach is essentially the same as that of Makhatadze and Privalov (1994), very similar results could be expected. Yet, there are large discrepancies that we attribute primarily to the coefficients used to calculate thermodynamical parameters from ΔASA values. The set of coefficients derived by Privalov and collaborators (Makhatadze & Privalov, 1990, 1993; Privalov & Makhatadze, 1993) is quite different from the one we used (Ooi et al., 1987), for the polar surface at least. Makhatadze and Privalov (1994) obtain excessively high estimates of the enthalpy and entropy of polar group hydration (see the Discussion attached to their paper). At 25 °C, ΔH^{P} comes out 4–8 times larger and ΔS^{P} is 3–6 times larger than here in Table 2 for the same proteins. The contribution of internal interactions, derived by difference between calculated and observed values, is also much larger: ΔH^{int} is about 50 kJ·mol^{-1} and ΔS^{int} 50 J·mol^{-1}·K^{-1} per residue, well above our estimates of 11 kJ·mol^{-1} and 20 J·mol^{-1}·K^{-1}. The omission of vibrational effects and discrepancies concerning nonpolar surface hydration are minor differences in comparison.

Thus, there is no quantitative agreement between these two analyses of the thermodynamics of protein unfolding. Still, we draw the same qualitative conclusions concerning the role of each component in stabilizing globular proteins and in promoting their temperature-induced unfolding. The vibrational and hydration terms dominate the large heat capacity change associated with unfolding. We show that, like hydration, the vibrational component can be derived from small molecule studies. The role of intramolecular bonds formed and of conformational degrees of freedom lost as the polypeptide chain folds is not accessible in this way. It was inferred by difference and confirmed that at 25 °C, intramolecular bonds are the dominant stabilizing term in the enthalpy change and conformational degrees of freedom are the dominant destabilizing term in the entropy change. These conclusions are in agreement with Makhatadze and Privalov (1994) and they must therefore be largely independent of the details of the method and of the set of coefficients used in deriving thermodynamic parameters for thermal unfolding.

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