Role of Hydration Water in Protein Unfolding

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ABSTRACT In this paper, following our work on the two-state outer neighbor mixed bonding model of water, it is proposed that polar groups promote the formation of the low density ice Ih-type bonding in their neighborhood, whereas nonpolar groups tend to promote the higher density ice II-type structure. In a protein, because of the large numbers of exposed polar and nonpolar groups, large changes in the neighboring water structure can occur. These changes, of course, depend on whether the protein is in its native or its unfolded state and will be shown here to have a direct impact on the thermodynamics of protein unfolding at both high and low temperatures. For example, it is known that the polar hydration entropies become rapidly more negative with increasing temperature. This very unusual behavior can be directly related to the promotion in the outer bulk liquid of the more stable Ih-type bonding at the expense of II-type bonding by polar groups of the protein. In contrast, nonpolar groups have an opposite effect on the thermodynamics. It is the delicate balance created by these outer hydration contributions, mixed with ordinary thermodynamic contributions from the inner hydration shell and those from hydrogen-bond and van der Waals forces within the protein molecule itself that is responsible for both heat and cold denaturation of proteins.

INTRODUCTION

A number of recent papers (Livingstone et al., 1991; Spolar et al., 1992; Matouschek et al., 1994; Abseher et al., 1996; Bryant, 1996; Covell and Wallqvist, 1997; Giorgione and Epand, 1997; Parker and Clarke, 1997; Schwabe, 1997; Wiggins, 1997; Makarov et al., 1998; Shaltiel et al., 1998; Fitter, 1999) have stressed the importance of water on protein structural changes. References to the large amount of older work on this topic can be found in the above papers.

From a series of studies on the properties of pure water as a function of temperature and pressure (Bassez et al., 1987; Vedamuthu et al., 1994; Cho et al., 1997; Robinson et al., 1998, 1999), it is becoming evident that this liquid, on the average, is composed of dynamically transforming microdomains of two very different bonding types. One type is the regular tetrahedral water–water bonding similar to that in ordinary ice Ih, whereas the other is a more dense nonregular tetrahedral bonding similar to that in ice II. Crucially, the transformations between these two structural forms occur not at the nearest neighbor level, but in the first nonhydrogen-bonded outer region, through a bending of O···O···O intermolecular bonds (Kamb, 1968). Unlike all other ideas, both mixture components in Kamb's model have hydrogen-bonded inner tetrahedral structure with the four neighbors to the central molecule all having roughly 2.8 Å nearest neighbor O···O distances. However, the O···O···O angle can be either the regular tetrahedral 109.5° angle as in ice Ih, or strongly bent to angles of ~80° as they are in the dense ice polymorphs. Because of this bending, the outer next-nearest neighbor O···O distances cluster around roughly two values, 4.5 Å ($2 \times 2.8 \sin ½ 109.5°$) as in ordinary ice and the shorter O···O distance of 3.5 Å ($2 \times 2.8 \sin ½ 80°$) as in the dense ice forms, ice II in particular. The stability of ice Ih is dominated by the entropy, not present in ice II, associated with disordered hydrogen bonds in this polymorph (Nagle, 1966). This entropy difference between ice Ih and ice II would be expected to be diminished or absent in the liquid. Thus, ignoring it and realizing that ice II is nearly equienergetic with ice Ih, it might be expected that this dense structural characteristic would be prominent in the liquid. This is the basis of Kamb's and our own two-state model for the liquid.

The transformation between these two bonding forms is evidenced experimentally by the increase of outer second-neighbor O···O structure near 3.5 Å and the concurrent decrease of the ordinary 4.5 Å outer O···O regular tetrahedral structure in the pure liquid through structural studies as a function of both temperature (Bosio et al., 1983) and pressure (Okhulkov et al., 1994). In fact, the good structural isosbestic characteristics of the radial distribution function with changing temperature and pressure (Robinson et al., 1999) have now fully confirmed a precise two-state description of this liquid at the second-neighbor level. Changes with temperature in the fractional compositions, f_I and f_{II}, of Ih-type and II-type bonding in pure liquid water can, in fact, be obtained from a density analysis (Vedamuthu et al., 1994; Urquidi, et al., 1999). The results are depicted in Fig. 1, and numerical values of f_I and the specific volumes of Ih-type and II-type structure in the liquid as a function of temperature are summarized in Table 1. These changes in water structure must, of course, be included in a full thermodynamic description of any hydration problem, including protein hydration.

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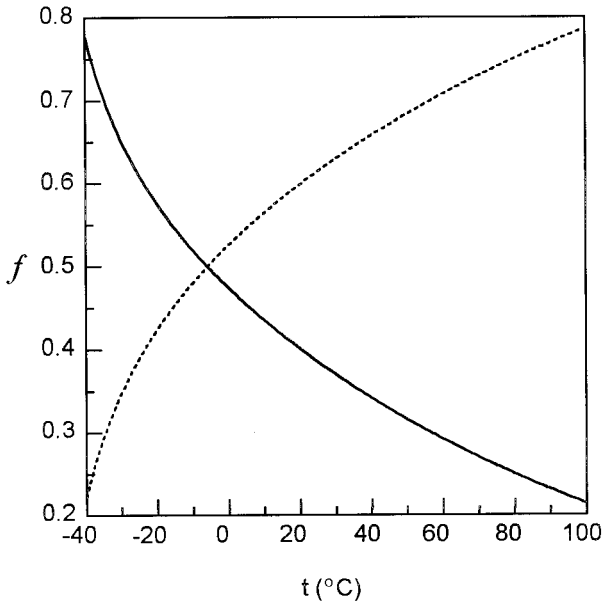

FIGURE 1 Graphical representation of the change in fractional composition, f_I (*solid line*) and $f_{II} = 1 - f_I$ (*dashed line*), of Ih-type and II-type bonding in the liquid (from Vedamuthu et al. 1994).

How does this picture help in the understanding of interfacial effects near surfaces and solutes? A central issue is that the above structural transformations involve a huge volume change. For example, from Table 1, it is seen that the volume of the liquid at 25°C is proportionately composed of 38.55% of Ih-type bonding with a volume of 36.399 Å^3 per molecule mixed with 61.45% of II-type bonding having a volume of 25.992 Å^3 per molecule. Any solute or surface perturbation that disturbs these proportions will have an immense effect on the volume of the solution, and on other properties, such as the thermodynamics of the liquid near the interface.

SIMPLE SOLUTES IN WATER

The molecular level description of the effect of a strongly polar solute, such as an ion, on the volume of the nearby water structure depends on the expected locally strong binding of the ion to water. Taking the sodium ion as an example, one notes that nearly the entire enthalpy of hydration of Na^+ comes from the inner hydration shell of about six water molecules (Caldwell et al., 1990). How this strong bonding in the inner shell affects the volume change is evidenced by the short $Na^+ \cdots O$ distances in solution, about 2.3 Å, compared with 2.8 Å $O \cdots O$ distances in the normal liquid (Robinson et al., 1996). Considering the rather modest overall reduction of volume for Na^+ in water, it would appear that what is happening for a polar solute is a contraction in the strongly bound first water shell, counterbalanced by an expansion, "iceberg formation" (Frank and Evans, 1945), farther out. Using the two-state bonding model of water, this would involve a transformation to an increased preponderance of low-density Ih-type bonding in the surrounding liquid.

In contrast, evidence exists that a nonpolar molecule such as CH_4 has an opposite effect on the structure. The overall decrease of 22.7 ml/mol transfer volume of this solute from hexane to water (Masterton, 1954; Kauzmann, 1959) implies an overall shrinkage of the water solvent to the more dense II-type configuration in the surroundings. In fact, in agreement with this view, the last paragraph of the Masterton (1954) paper states that there is "apparently a breaking down of the cage-like structure (Ih-type) of water molecules around the aliphatic hydrocarbon molecules." "This produces a decrease in the partial molal volume analogous to that observed when ice melts" (Ih→II transformation).

PROTEIN HYDRATION

According to the Concluding Remarks in the review of Makhatadze and Privalov (1995), it is stated that polar and nonpolar groups comprising the surface of a protein contribute approximately additively to the overall thermodynamic effects of unfolding. In what specific way does the neighboring water structure, and its possible dependence on the polarity of the protein groups, contribute to this thermodynamics? From what has been said in the previous section about simple solutes, one can conjecture what might happen to the water structure near a protein. The polar groups of the protein would be expected to bind water molecules fairly strongly in their first shell, then, outside this shell, promote a greater preponderance of open low-density Ih-type structure than is present in the pure liquid. The extent of these polar perturbations is probably not as strong nor as long range as it is for Na^+. Nonpolar groups, outside their own inner hydration shell, could promote a transformation in the other direction to give more II-type bonding in the surrounding liquid, or, because of adverse thermodynamics (Wiggins, 1997), they may merely prevent the Ih-type structure caused by adjacent polar groups from forming near them. Thus, the polar groups would give rise to a lower entropy, but a greater enthalpic stability (more negative ΔH) in the outlying water regions, whereas nonpolar groups would do just the opposite. Because the water-accessible surface areas (ASA) in unfolded proteins range from ~10 000 to 60 000 Å^2 (Makhatadze and Privalov, 1995), and, because a water molecule occupies an area of ~9–10 Å^2, about 1000 to 6000 water molecules per unfolded protein could occupy the first layer, with as many as two to three times this number in the outlying regions being involved in the overall protein hydration process. Even a small effect on the water structure could give an effect on the total hydration thermodynamics of protein unfolding that is very large indeed.

A well studied, relatively simple protein is ubiquitin (Wintrode et al., 1994; Ibarra-Molero et al., 1999). It will be used here to illustrate the important hydration thermodynamics of its reversible native to unfolded transition N⇄U

TABLE 1 Temperature-dependent component fractions and volumes in bulk water

t°C	f_I	V_I	V_{II}	t°C	f_I	V_I	V_{II}
−40	.7787	1.09694	.83608	45	.3290	1.26750	.88359
−35	.7049	1.10371	.83778	50	.3163	1.28120	.88761
−30	.6517	1.11089	.83961	55	.3042	1.29531	.89178
−25	.6101	1.11847	.84159	60	.2925	1.30983	.89608
−20	.5757	1.12647	.84370	65	.2813	1.32475	.90052
−15	.5461	1.13487	.84594	70	.2705	1.34008	.90510
−10	.5199	1.14368	.84833	75	.2601	1.35582	.90981
−5	.4963	1.15290	.85085	80	.2501	1.37197	.91466
0	.4746	1.16252	.85350	85	.2404	1.38853	.91964
5	.4545	1.17255	.85630	90	.2312	1.40549	.92477
10	.4357	1.18300	.85923	95	.2222	1.42287	.93003
15	.4181	1.19384	.85230	100	.2137	1.44064	.93543
20	.4014	1.20510	.86550	105	.2054	1.45883	.94096
25	.3855	1.21676	.86885	110	.1974	1.47743	.94663
30	.3704	1.22883	.87233	115	.1898	1.49643	.95244
35	.3560	1.24131	.87594	120	.1824	1.51584	.95839
40	.3422	1.25420	.87970	125	.1753	1.53566	.96447

Data compiled from an algebraic fit of H_2O density data (Vedamuthu et al., 1994). For the two-state model, f_I is the fraction of the Ih-type component, whereas $1 - f_I$ is the fraction of the II-type component (see Fig. 1). The units of V_I and V_{II} are ml/g.

presumed to take place at both high and low temperatures as a general property of proteins (Privalov, 1990; Ibarra-Molero et al., 1999). Ubiquitin contains 76 amino-acid residues and has a molecular mass of 8433 daltons. Most importantly, for our interests here, are the ASAs in Å^2 for polar *(p)* and aliphatic/aromatic nonpolar *(n)* groups comprising the native (N) and unfolded (U) forms of ubiquitin: $ASA^N = 2320n + 2430p$; and $ASA^U = 6200n + 4340p$. It is seen that, on unfolding, the increases in ASA, Δ^U_NASA, are 3880 Å^2 for the nonpolar groups, and roughly half this, 1910 Å^2, for the polar groups. At 9.5 Å^2 per water molecule, these ASA changes correspond to an increase on unfolding of about 408 and 201 first-shell water molecules, respectively, for nonpolar and polar groups in ubiquitin. The idea to be considered here is that each polar group, in addition to the closely hydrated inner water molecules, promotes Ih-type structure in the water outside this first hydration shell, whereas each nonpolar group outside its first shell promotes transformations in the opposite direction.

In Table 2, the thermodynamics of the ubiquitin unfolding reaction (Makhatadze and Privalov, 1995) is broken down into polar and nonpolar hydration contributions and internal contributions. These values have been smoothed by a procedure to be described later and may differ slightly from those in the above article. It is to be kept in mind that the hydration contributions consist of two parts, an inner shell of strongly hydrated water molecules and perturbed water molecules outside of this inner region. The specific separation of the thermodynamics of these two types of hydration contributions will be made in the Results section,

TABLE 2 Thermodynamics of ubiquitin unfolding

	polar hydration			nonpolar hydration			internal			total		
t°C	$\Delta^U_N H_p$	$\Delta^U_N S_p$	$\Delta^U_N G_p$	$\Delta^U_N H_n$	$\Delta^U_N S_n$	$\Delta^U_N G_n$	$\Delta^U_N H_i$	$\Delta^U_N S_i$	$\Delta^U_N G_i$	$\Delta^U_N H_t$	$\Delta^U_N S_t$	$\Delta^U_N G_t$
−40	−2238	−775	−2057	−1035	−4289	−35	+2921	+3568	+2089	−352	−1496	−3.3
−30	−2276	−930	−2050	−946	−3914	+6	+2928	+3596	+2053	−295	−1248	+8.8
−20	−2313	−1073	−2041	−858	−3557	+42	+2935	+3622	+2018	−236	−1008	+19.1
−10	−2347	−1203	−2031	−772	−3219	+75	+2943	+3647	+1983	−177	−775	+27.5
0	−2380	−1323	−2018	−687	−2897	+105	+2949	+3668	+1947	−117	−552	+33.9
5	−2395	−1379	−2012	−645	−2743	+118	+2952	+3677	+1930	−87	−445	+36.3
10	−2410	−1432	−2005	−603	−2593	+131	+2955	+3685	+1912	−58	−340	+38.1
15	−2425	−1483	−1998	−561	−2447	+144	+2958	+3693	+1894	−29	−238	+39.5
20	−2439	−1532	−1990	−520	−2304	+155	+2960	+3698	+1876	0	−138	+40.4
25	−2453	−1579	−1983	−480	−2166	+166	+2961	+3703	+1857	+28	−42	+40.7
30	−2467	−1624	−1975	−439	−2031	+176	+2962	+3706	+1839	+56	+51	+40.5
40	−2493	−1707	−1958	−360	−1772	+195	+2963	+3706	+1802	+110	+227	+38.7
50	−2518	−1783	−1941	−282	−1527	+211	+2961	+3700	+1765	+161	+389	+35.1
60	−2541	−1853	−1924	−206	−1296	+225	+2956	+3685	+1728	+208	+537	+29.7
75	−2574	−1948	−1896	−95	−971	+243	+2941	+3646	+1672	+272	+727	+18.9
100	−2625	−2085	−1847	+80	−486	+262	+2897	+3528	+1580	+352	+956	−4.5
125	−2671	−2207	−1792	+243	−62	+268	+2820	+3331	+1494	+393	+1063	−30.3

Units of G and H are kJ/mol and of S are J/mol-K.

where it will be seen that, although the inner hydration shell makes large contributions to the hydration thermodynamics, the dominant temperature-dependent terms come from the outer transformations. This results in a nonlinear temperature dependence of the thermodynamics, in agreement with the direct enthalpy measurements of Wintrode et al. (1994) for ubiquitin.

The internal contributions comprise those interactions among amino acid residues that totally exclude hydration effects and can be broken down farther into van der Waals and hydrogen bond contributions. Interestingly, as illustrated in Table 2, temperature effects on the internal $\Delta_N^U S_i$ and $\Delta_N^U H_i$ are fairly small. The resulting large positive internal $\Delta_N^U G_i$ values strongly favor the native protein conformation. Because of the increased ASA in the unfolded protein for both polar and nonpolar groups, their combined hydration effects, which together give rise to large negative polar + nonpolar $\Delta_N^U G_h$ hydration contributions to the overall transformation, are responsible for the unfolding. In fact, as seen in Table 2 and further illustrated in Fig. 2, the subtle small differences between the large thermodynamic quantities for internal interactions and hydration create the delicate balance that exists for the total free energy values. These values cross the $\Delta_N^U G_t = 0$ line near +90°C and −40°C, giving rise to both heat and the so far experimentally inaccessible (except probably under pressure) cold denaturation of ubiquitin dissolved in pure water (Ibarra-Molero et al., 1999).

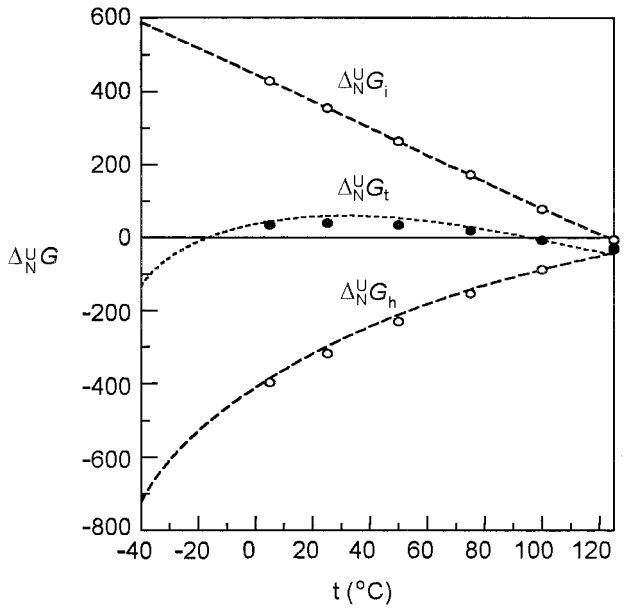

FIGURE 2 Free energy changes on unfolding of ubiquitin in a pure water solution. The upper curve is ($\Delta_N^U G_i$ −1500) using the values in Table 2, the lower curve is the polar + nonpolar total hydration contribution ($\Delta_N^U G_h$ +1500), and the middle curve is their sum. The circles represent experimental data from Table 2, and the lines for the middle and lower curves are from the two-state water theory of Eqs. 2–5, using parameters given in Table 3. The 1500 kJ/mol added and subtracted from $\Delta_N^U G_h$ and $\Delta_N^U G_i$ are merely to bring the curves together and improve the vertical scale so that the behavior of their sum can be more clearly comprehended.

THERMODYNAMICS

In the two-state bonding representation of water, at any given temperature and pressure, bulk water consists of a fraction f_I of Ih-type bonding and a fraction f_{II} of II-type bonding (Vedamuthu et al., 1994). As the temperature (or pressure) is raised, more of the Ih-type bonding in bulk water is replaced by II-type bonding. Table 1 has given this temperature dependence at atmospheric pressure, whereas for the two-state representation, $f_{II} = 1 - f_I$ (see also Fig. 1).

From what has been said so far, W water molecules outside the strongly adsorbed inner hydration layer perturbed by the polar groups of the protein should give rise to the transformation of $f_I W$ Ih-type plus $(1 - f_I)W$ II-type molecules making up the bulk liquid over to purely Ih-type bonding near the protein. This II→Ih transformation would then correspond to a net loss of $(1 - f_I)W$ II-type molecules with a gain of this same number of the stiffer (lower entropy) but more stable (more negative enthalpy) Ih-type molecules. As the temperature rises, and there are more II-type molecules in the bulk, the factor $(1 - f_I)$ becomes larger, so a greater number of the II-type molecules are available for transforming to Ih-type molecules. This directly accounts for the highly unusual decrease of entropy but increasingly negative ΔH of polar hydration with increasing temperature. It is further supposed that a nonpolar group on the protein tends to drive this water transformation in the reverse direction. Each nonpolar group would therefore cause the entropy to increase and the enthalpy to become less negative with increasing temperature. These predicted polar and nonpolar trends are evident from Table 2 and Figs. 3 and 4.

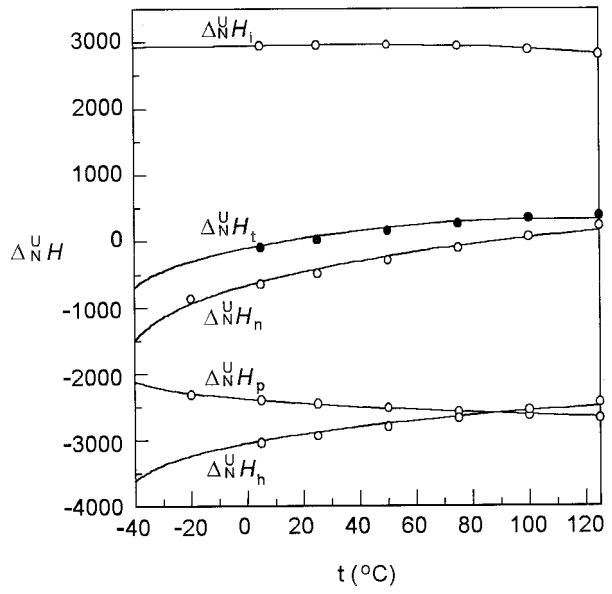

FIGURE 3 Enthalpy changes in kJ/mol on unfolding of ubiquitin in pure water solution. *Symbols*, data from Table 2; *solid lines*, theoretical curves as described in Fig. 2 caption.

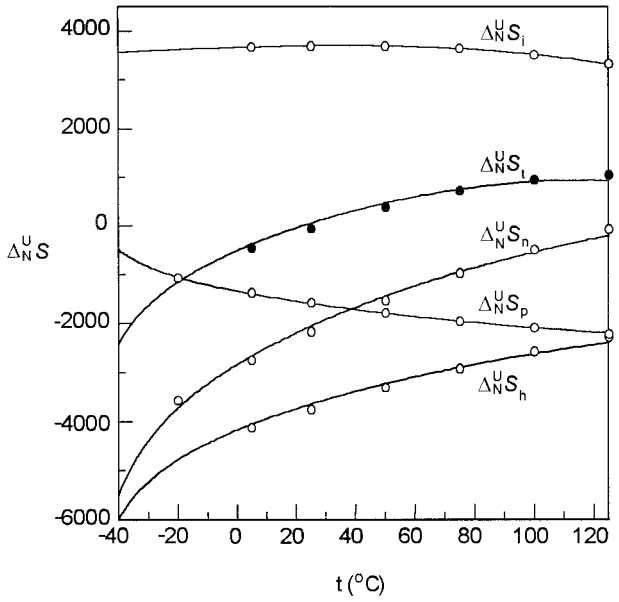

FIGURE 4 Entropy changes in J/mol · K on unfolding ubiquitin in pure water solution. Same notation as in Figs. 2 and 3.

RESULTS

At the end of the previous section, we remarked that the trends in the hydration thermodynamics for polar and nonpolar protein groups are what would be expected from transformations promoted by these groups between Ih- and II-type bonding arrangements in the nearby water structure. If plots of f_I and f_{II} as functions of temperature (Fig. 1) are compared with plots of the individual experimental $\Delta_N^U H$ and $\Delta_N^U S$ values for hydration, a similarity in the curvatures is noticed. Compare, for example, $\Delta_N^U S_n$ and $\Delta_N^U S_p$ in Fig. 4 with f_{II} and f_I, respectively, in Fig. 1. The ΔH curves, as plotted in Fig. 3, are similar but, because they are flatter, do not compare as well graphically with the f curves. This similarity between the curvatures could mean that the primary source of temperature variations of the hydration thermodynamics of protein unfolding is caused by these outer-region water transformations.

One way of checking this is to try to bring the temperature-dependent thermodynamic plots into coincidence with the f_I and f_{II} plots by applying a shift, $\Delta_N^U X^0$, and a scale factor, Y,

$$(\Delta_N^U X - \Delta_N^U X^0) Y = f, \tag{1}$$

where X refers to polar or nonpolar H or S. Because of the connection between f_I and f_{II} and the form of Eq. 1, which will absorb any constant term on the right-hand side, this equation will apply equally accurately for either f-value, giving rise to a number of possible relationships. The choice of acceptable relationships depends on the signs and magnitudes of the resulting parameters obtained from this analysis.

Rewriting Eq. 1 in more understandable thermodynamic language gives,

$$\Delta_N^U H_p = \Delta_N^U H_p^0 + f\Delta_{II}^I H_p \tag{2}$$

$$\Delta_N^U S_p = \Delta_N^U S_p^0 + f\Delta_{II}^I S_p \tag{3}$$

$$\Delta_N^U H_n = \Delta_N^U H_n^0 + f\Delta_I^{II} H_n \tag{4}$$

$$\Delta_N^U S_n = \Delta_N^U S_n^0 + f\Delta_I^{II} S_n, \tag{5}$$

where $\Delta_{II}^I X$ and $\Delta_I^{II} X$ ($1/Y$ in Eq. 1) are the thermodynamic changes in the surrounding water caused by the presence of the polar and nonpolar groups, and the subscripts p and n again refer to these groups. The $\Delta_N^U X^0$ quantities concern thermodynamics that has nothing to do with structural changes in the outlying water, but rather with the direct surface adsorption of water molecules to the protein groups, with possible secondary effects on the internal interactions.

To simplify the forthcoming analysis, we have made some approximations. One not very serious approximation is that the aliphatic and aromatic nonpolar contributions can be combined, because the two contributions vary in the same direction and the aromatic contributions in ubiquitin, in any case, are fairly small. Naturally, the $\Delta_N^U X^0$, $\Delta_{II}^I X$, and $\Delta_I^{II} X$ terms would be expected to have a temperature dependence. However, considering the weak temperature dependence of the thermodynamics of the internal interactions, we will make a perhaps more serious approximation, and ignore these dependencies here. The reason for doing this, at the cost of obtaining less good agreement with the experimental data, is so as not to confuse what we believe to be the main temperature dependence of the overall hydration thermodynamics caused by the strong temperature dependence of the f-terms.

Table 3 presents the parameters obtained by fitting Eqs. 2–5 to the four sets of data points in the Makhatadze/Privalov review (1995), where the temperatures reported there are +5, +25, +50, +75, +100, and +125°C. A seventh experimental point at −20°C for each data set has been added to bridge more satisfactorily the very low temperature data and the higher temperature data points starting

TABLE 3 Fitting parameters for hydration of ubiquitin

	$\Delta_N^U H_p$	$\Delta_N^U S_p$
$\Delta_N^U ASA$	1910	1910
N	201.1	201.1
$\Delta_N^U X^0$	−1914	+157
$\Delta_{II}^I X$	−898	−2848
	$\Delta_N^U H_n$	$\Delta_N^U S_n$
$\Delta_N^U ASA$	3880	3880
N	408.4	408.4
$\Delta_N^U X^0$	−2113	−7456
$\Delta_I^{II} X$	+2761	+8808

The units of $\Delta_N^U ASA$ are Å^2, whereas those of H and S are kJ/mol and J/mol-K, respectively. N is the change in the number of water molecules in the strongly adsorbed first layer on the protein, assuming 9.5 Å^2 per water molecule.

at +5°C. These −20°C data points, as well as the other values of $\Delta_N^U X$ listed in Table 2, are derived from a polynomial fit of degree $n = 3$,

$$\Delta_N^U X = \sum_{i=0}^{n} A_i T^i,$$

using the above six temperature points, T being the absolute temperature. In the analysis of these experimental data, f must be taken to be f_{II} for the polar groups. As mentioned earlier, both the $f = f_{II}$ and $f = f_I$ fits give identical final results because of the relationship $f_{II} = 1 - f_I$. For the polar groups, however, in the case where $f = f_I$, the resulting parameters are unacceptable, because $\Delta_{II}^I X$ would have to be positive for both $X = H$ and $X = S$. For the same reasons, $f = f_I$ is required for the nonpolar groups.

Table 4 shows the numerical agreement obtained between the seven experimental points and data derived from Eqs. 2–5 using the parameters in Table 3. Considering uncertainties in the experimental values, combined with the wide range of temperatures covered, and our omission of any temperature dependence of the parameters in Table 3, the agreement is certainly very suggestive. The plots shown in Figs. 2–4 emphasize this perhaps more clearly.

DISCUSSION

It is seen that the presence of both heat and cold denaturation arises naturally from the ideas presented here. According to the extended experimental data given in Table 2, the total $\Delta_N^U G_t$ passes through zero, where the equilibrium favors neither the unfolded nor native form, at both a high and a low temperature point. The direct cause is the near balance at all temperatures between the negative hydration free energies and the positive internal free energies, combined with a severe change of slope of the hydration free energy curve (see Fig. 2). At low temperatures, the positive slope of the hydration curve is greater than the negative slope of the internal free energy data, whereas, at high temperatures, the situation is reversed. This allows the $\Delta_N^U G_t$ values to be negative at low temperatures, slightly positive at intermediate temperatures, but negative again at higher temperatures. The change of slope of the hydration data is a direct consequence of the behavior of f_I and f_{II} with temperature. Ordinary contributions to $\Delta_N^U G_t$ do not possess this strongly changing slope, and could not give rise to denaturation at two different temperatures. Figures 3 and 4 show that $\Delta_N^U X_h$ almost exactly parallels the total $\Delta_N^U X_t$ for both $X = S$ and $X = H$ because of the small slopes of the internal values.

According to Fig. 3 of Ibarra-Molero et al. (1999), the $\Delta_N^U G_t = 0$ equilibrium points for ubiquitin in the absence of an added denaturant, such as guanidinium hydrochloride or urea, occur near −40°C and +90°C. Our theoretical values are close to −20°C and +90°C. Because of the slightly incorrect curvatures of these theoretical curves caused by the omission of the temperature dependence of $\Delta_N^U X^0$, $\Delta_{II}^I X$, and $\Delta_I^{II} X$, together with the fragile balance between positive and negative contributions to $\Delta_N^U G_t$, it is really surprising that even this good a theoretical description of heat and cold denaturation was achieved. With slightly different curvatures of the theoretical data coming from the missing temperature dependence, the low temperature crossing point of $\Delta_N^U G_t$ will move from its present location near −20°C downward toward −40°C, in agreement with Ibarra-Molera et al. (1999). These more refined results will be reported in a later paper for 18 different proteins.

CONCLUSIONS

Though this paper was originally meant only as a suggestion of how the thermodynamics of hydration waters, through changes in the outer water structure itself, can influence the protein unfolding reaction, some rather remarkable findings have emerged. First of all, it is easy to confirm, as we have done for a number of other proteins, that not only ubiquitin, but all proteins fit this same pattern simply for the reason that "protein groups contribute additively to the overall thermodynamic effects" (Makhatadze and Privalov, 1995). Therefore, fits of the quality seen in Figs. 2–4 for all proteins can be achieved even with the broad approximations used here. For example, considering myoglobin and RNase A, each of which has a very different thermodynamic profile than ubiquitin, the simplified analysis based on outer-water structural transformations gives a very low temperature (−30°C) cold denaturation for RNase A but one near −5°C for myoglobin. These results are consistent

TABLE 4 Experimental compared with calculated hydration using the parameters in Table 3

	$\Delta_N^U H_p$		$\Delta_N^U S_p$		$\Delta_N^U H_n$		$\Delta_N^U S_n$	
t°C	exp	calc	exp	calc	exp	calc	exp	calc
−20	−2313	−2295	−1073	−1051	−858	−941	−3557	−3720
5	−2395	−2404	−1378	−1396	−645	−607	−2743	−2652
25	−2454	−2466	−1581	−1593	−479	−416	−2166	−2044
50	−2517	−2528	−1783	−1790	−282	−225	−1526	−1435
75	−2574	−2579	−1945	−1950	−97	−70	−973	−939
100	−2625	−2621	−2088	−2082	+82	+58	−485	−530
125	−2671	−2655	−2206	−2192	+243	+164	−62	−192

The units of H and S are kJ/mol and J/mol-K, respectively.
The experimental data 5–125°C are from Makhatadze and Privalov (1995).

with the experimental temperatures (Makhatadze and Privalov, 1995), but, once again, are perhaps 10–20°C too high because of the approximations used in this paper.

The theoretical thermodynamic changes, with increasing temperature, of the protein-perturbed water itself have been found to be in opposite directions for the polar and nonpolar groups. This agrees with the experimental findings for the protein hydration thermodynamics and further emphasizes that the temperature dependence of the hydration thermodynamics for proteins closely follows changes expected from changes with temperature in the water structure contributions, f_I and f_{II}. This and the interesting point about the curvature of the *f*-functions (Fig. 1) being the direct cause of cold denaturation further confirm the presence of important thermodynamic contributions from outer-water structural transformations to the protein unfolding problem. Participation of the surrounding water itself is a completely separate contribution from the internal interactions within the protein molecule or the thermodynamics of the surface water bound to the protein, the two areas where all past emphasis in the protein unfolding problem has rested.

Another important conclusion of this paper is that cold denaturation can occur only when there is sufficient Ih-type structure in the bulk liquid, which the protein nonpolar groups can transform to II-type structure. In this respect, it is noteworthy (Vedamuthu et al., 1995) that increased pressure, just as increased temperature, diminishes Ih-type structure in the bulk liquid, transforming it to II-type structure. Because of this, the slope of the hydration free energy curve becomes flatter at high pressures, just as it does at high temperatures. As known experimentally (Privalov, 1990), this would cause the total $\Delta_N^U G_t$ to cross zero at a higher temperature, moving cold denaturation to higher temperatures with increasing pressure. Eventually, at very high pressures, there would be no more Ih-type bonding in the bulk and only heat denaturation can occur. In other words, at such pressures, water behaves as an ordinary liquid and the cold denaturation anomaly disappears. This type of result should provide predictive information that may be valuable in future research on protein design.

Note added in proof: A recent paper by I. M. Klotz (1999. Parallel change with temperature of water structure and protein behavior. *J. Phys. Chem.* 103:5910–5916) has also pointed out similarities between the "dome-shaped" curvatures of the protein unfolding thermodynamics and temperature variations of certain water properties.

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