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Thermodynamics of Ubiquitin Unfolding

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ABSTRACT **The energetics of ubiquitin unfolding have been studied using differential scanning microcalorimetry. For the first time it has been shown directly that the enthalpy of protein unfolding is a nonlinear function of temperature. Thermodynamic parameters of ubiquitin unfolding were correlated with the structure of the protein. The enthalpy of hydrogen bonding in ubiquitin was calculated and compared to that obtained for other proteins. It appears that the energy of hydrogen bonding correlates with the average length of the hydrogen bond in a given protein structure.**

Key words: microcalorimetry, heat capacity, enthalpy, hydrogen bonding

INTRODUCTION

Studies of correlations between thermodynamic and structural features of proteins provide insight into the principals of protein energetics. Success in this study greatly depends on the choice of the protein—its size, reversibility of unfolding, number of covalent crosslinks, etc.

Ubiquitin is one of the best objects for such studies. It is a small globular protein consisting of 76 amino acid residues with a molecular weight of 8433 Da. The structure of ubiquitin has been determined with high resolution by X-ray crystallography[1] and NMR.[2] It has been shown that crystal and solution structures of ubiquitin are virtually identical.[3]

Ubiquitin was the subject of extensive study by NMR spectroscopy[4] at low pH in the presence of high concentrations of methanol, where it is in a so called A-state.[5–7] A recent calorimetric study of ubiquitin[8] was mainly concerned with compariing the thermodynamics of ubiquitin unfolding in the presence and absence of methanol. These results, unfortunately, are not sufficient for the analysis of the correlation between thermodynamic parameters of ubiquitin unfolding and its structural features. This requires knowledge of not only the enthalpy and entropy of protein unfolding, but also of the absolute value of the partial molar heat capacities of the protein in the native and denatured states in a wide temperature range.[9,10]

In this paper we report the results of calorimetric measurements of the thermodynamic parameters of ubiquitin unfolding in aqueous solution in a wide temperature range, 5–125°C, and analyze the correlation of these parameters with the structural features of the protein.

MATERIALS AND METHODS

Ubiquitin from bovine red blood cells was purchased from Sigma Chemical Co. (cat.#U 6253) and used without further purification.

Concentration of ubiquitin in solution was measured spectrophotometrically using an extinction coefficient of $E^{1\%,1cm}_{280nm} = 1.49$, calculated according to Gill and von Hippel.[11] Correction on light scattering was taken into account as suggested by Winder and Gent.[12]

For calorimetric experiments the protein solution was extensively dialyzed at 4°C against corresponding buffer followed by centrifugation for 10–15 min at 5000*g*. The Spectrapor 2 membranes with the molecular weight cutoff 3,000 Da were used. Protein scans were performed in 10 mM glycine/HCl (for pH 2.0–3.5) or 10 mM sodium acetate/acetic acid (for pH 3.5–4.0) buffer systems.

Heat capacity measurements were performed using the differential scanning microcalorimeter DASM-4M (NPO "Biopribor," Pushchino, Russia) with the temperature operation range 0–130°C at heating rate 1°C/min. The heat capacity difference between the sample cell, containing protein solution, and the reference cell, containing corresponding solvent, $\Delta C_p^{app}(T)$, is related to the partial heat capacity of protein, $C_{p,2}(T)$, as follows:[13]

$$C_{p,2}(T) = C_{p,1}(T)\frac{V_2(T)}{V_1(T)} - \frac{\Delta C_p^{app}(T)}{m(T)} \qquad (1)$$

where $V_1(T)$ and $V_2(T)$ are partial volumes of solvent and protein, respectively, $C_{p,1}(T)$ is partial heat capacity of solvent, and $m(T)$ is amount of protein in the calorimetric cell, at a given temperature, T. The protein concentration in the solution for the experiments varied from 2 to 4 mg ml^{-1}.

The partial specific volume of ubiquitin was estimated from its amino acid composition, according to

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TABLE I. Water Accessible Surface Area Change Upon Unfolding of Ubiquitin

Type of the surface	Surface area (ΔASA) (Å^2)
Aliphatic	4,820
Aromatic	449
Polar parts of	
Arg	105
Asn	28
Asp	89
Cys	0
Gln	242
Glu	90
His	14
Lys	118
Met	36
Ser	66
Thr	124
Trp	0
Tyr	36
–CONH–	1,819

Makhatadze et al.,[14] and was found to be 0.743 cm^3 g^{-1} at 25°C.

The surface areas of different groups of amino acid residues in ubiquitin were calculated from the three-dimensional structure of ubiquitin.[1] Atomic coordinates of ubiquitin (1 ubq) were obtained from the Brookhaven Protein Data Bank.[15] The surface area for the native molecule was computed using the program CAVT66[16] based on the algorithm developed by Shrake and Rupley.[17] The van der Waals radii used were the same as those of Chothia[18] and the solvent probe radius was 1.4 Å^2. The water-accessible surface area of protein in the unfolded molecule was obtained assuming that all protein groups are accessible to the solvent in the same way as in the tripeptide Gly-X-Gly, where X is the residue of interest.[19] The areas of different types of surfaces in ubiquitin that are exposed to solvent upon unfolding are presented in Table I.

The parameters of hydrogen bonding in the ubiquitin molecule were obtained using the computer program HBOND,[20] kindly provided by Dr. George Rose.

RESULTS AND DISCUSSION

Effect of pH on the Thermostability of Ubiquitin

Figure 1 presents the partial molar heat capacity of ubiquitin in solutions of different pH. A remarkable feature of ubiquitin is that it is a highly thermostable protein and its stability significantly depends on pH. With the pH of solution decreasing from 4.0 to 2.0 the temperature of the denaturational transition decreases from 91 to 58°C. Experiments were performed in the pH range below 4.0. At higher pH values the protein tends to aggregate after denaturation. The dependence of the transition temperature on pH is presented in Figure 2 and in Table II. This dependence can be used for the calculation of the number of protons released upon protein unfolding, $\Delta_t v$:[21]

$$\Delta_t v = -\frac{\Delta H_{cal}(T_t)}{2.303RT_t^2}\frac{dT_t}{d\mathrm{pH}}. \quad (2)$$

It follows that $\Delta_t v$ for ubiquitin is equal to 1.9 ± 0.2. According to Lenkinski et al.[22] the pK_a of the only histidine in the ubiquitin molecule is about 6.9. This means that only terminal carboxyl group and side chains of aspartic or glutamic acids can be responsible for the release of these protons in the pH range 2.0–4.0. The calorimetric experiments were performed in glycine and acetate buffer. The heats of ionization of these buffers are very close to that of the carboxylic groups in the protein. Thus, they practically cancel each other.[13]

Mechanism of the Heat Induced Transition of Ubiquitin

In Table II the calorimetrically measured enthalpies of ubiquitin unfolding, ΔH_{cal}, are listed. The obtained enthalpies of unfolding of ubiquitin are close to those reported recently[8] at identical pH values. Table II also presents the corresponding van't Hoff enthalpies, ΔH_{vH}, calculated from the shape of the transition profile as[13]

$$\Delta H_{vH} = \frac{4RT_t^2 C_{p,max}}{\Delta H_{cal}} \quad (3)$$

where T_t is the temperature of the denaturational transition, $C_{p,max}$ is the heat capacity at this temperature, and R is the gas constant. As one can see, the ratio $\Delta H_{cal}/\Delta H_{vH}$ is close to 1, which means that the unfolding of ubiquitin can be closely approximated by a "two state" mechanism.[23,24]

Reversibility of Denaturational Transition

The denaturation of ubiquitin is highly reversible under the conditions used if the heating is stopped just after completion of the transition. Heating of ubiquitin above 120°C produces an irreversibly denatured polypeptide (Fig. 1b), which does not show excess heat absorption upon reheating. Its heat capacity is a smooth curved function of temperature, and is significantly higher than the heat capacity of the native protein. The question is, however, how close is it to the heat capacity of the fully unfolded polypeptide chain?

Heat Capacity of Ubiquitin in the Unfolded State

The heat capacity of the unfolded state can be obtained from the amino acid composition of the protein as[25]

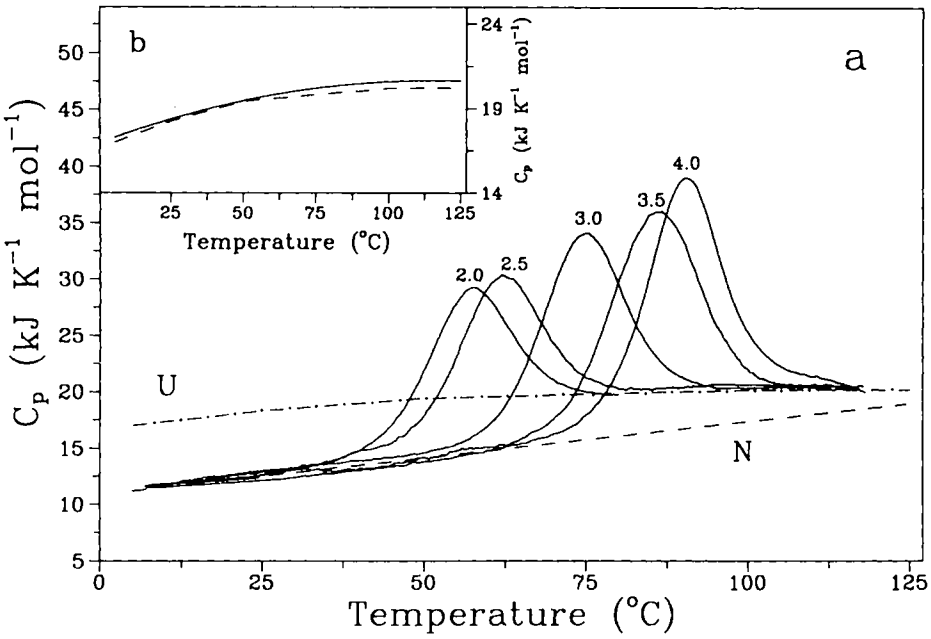

Fig. 1. (a) Temperature dependencies of the partial molar heat capacity of ubiquitin in solutions with different pH values. The dashed-and-dotted line represents the partial molar heat capacity of unfolded ubiquitin calculated according to Eq. (4). The dashed line represents the partial molar heat capacity of native ubiquitin. (b) Comparison of the temperature dependencies of the heat capacity of ubiquitin, obtained for the irreversible denatured protein by heating it up to 120°C (dashed line), with that calculated according to Eq. (4) (solid line).

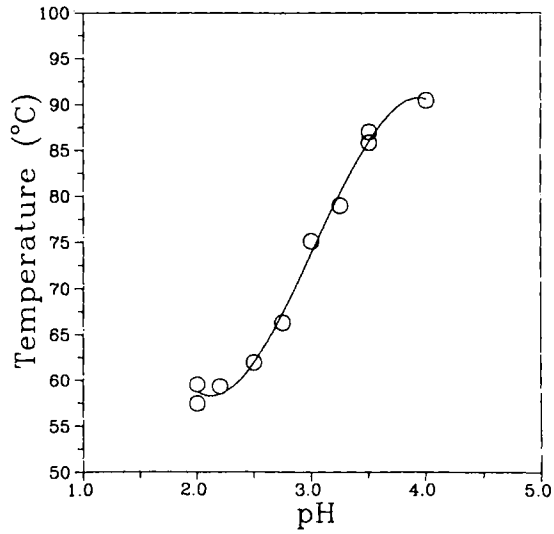

Fig. 2. The dependence of the temperature of protein unfolding on the pH of solution.

$$C^{U}_{p,2} = (N - 1)\, C_{p,2}(\text{–CHCONH–}) + \sum_{i=1}^{N} C_{p,2}(-R_i) \quad (4)$$

where $C_{p,2}$(–CHCONH–) is the heat capacity of the peptide unit, $C_{p,2}(-R_i)$ is the heat capacity contribution of the side chain of the ith amino acid residue, and N is the number of amino acid residues in the polypeptide chain. All of these contributions were reported earlier[26] for the temperature range 5–125°C. Calculated values of the heat capacity of ubiquitin in the unfolded state are listed in Table III and are compared with the calorimetrically measured values for the irreversibly denatured protein in Figure 1b. As one can see the experimentally determined heat capacities of denatured ubiquitin correspond well with the calculated ones. This supports the earlier findings[8] that the thermally denatured state of ubiqutin in aqueous solution is close to the unfolded state and justifies consideration of the thermodynamic characteristics of ubiquitin thermal denaturation as identical to those of its unfolding.

Heat Capacity of Ubiquitin in the Native State

The heat capacity of the native state of ubiquitin appears to be a linear function of temperature (Fig. 1) in the temperature range up to 65°C, where the heat capacity of the native state can be experimentally observed. Above this temperature the heat capacity of the native state can be obtained by linear extrapolation. The linear extrapolation of the heat capacity of the native state was justified from studies on many proteins with different thermostabilities.[25,27–29] The obtained heat capacities of the native state of ubiquitin are listed in Table III.

TABLE II. Thermodynamic Characteristics of Ubiquitin Heat Denaturation

pH	T_t (°C)	ΔH_{cal} (kJ/mol)	ΔH_{vH} (kJ/mol)	$\Delta H_{cal}/\Delta H_{vH}$
2.0	57.0	196	208	0.95
2.2	58.1	185	200	0.93
2.5	61.6	219	231	0.95
2.75	66.3	243	234	1.04
3.0	74.1	265	245	1.08
3.25	79.0	273	250	1.09
3.5	85.1	288	264	1.09
4.0	90.0	302	287	1.05

TABLE III. Partial Molar Heat Capacities of Ubiquitin in the Unfolded, $C^U_{p,2}$, and Native, $C^N_{p,2}$, States and the Heat Capacity Change Upon Unfolding, $\Delta^U_N C_p$, at Different Temperatures

	Temperature (°C)					
	5	25	50	75	100	125
$C^N_{p,2}$	11.2	12.6	14.3	16.0*	17.7*	19.4*
$C^U_{p,2}$	17.0	18.3	19.4	19.8	20.2	20.2
$\Delta^U_N C_p$	5.8	5.7	5.1	3.8	2.5	0.8

*Extrapolated values.

Heat Capacity Change Upon the Unfolding of Ubiquitin

The difference between the heat capacities of the native and the unfolded states is the heat capacity change upon unfolding. The heat capacity increment is presented in Table III as a function of temperature. One can see that the dependence of the ΔC_p on temperature is not linear. A nonlinear temperature dependence of the heat capacity changes upon unfolding has been found in other globular proteins: cytochrome *c*, ribonuclease A, lysozyme, myoglobin,[25] tendamistat,[28] BPTI,[29] and ribonuclease T1.[30]

The heat capacity change can also be obtained from the temperature dependence of the enthalpy. Figure 3 presents the enthalpies of ubiquitin unfolding obtained in experiments covering an extended temperature range from 57 to 90°C. The dependence is not a linear function of temperature, but is curved. The slope of this dependence at a given temperature corresponds to the heat capacity. These mean that the heat capacity change upon protein unfolding is also a temperature-dependent function which decreases with temperature increasing. The temperature dependence of the heat capacity change upon protein unfolding was demonstrated before only by the measurements of the heat capacities of proteins in the native and denatured states.[25,31] The observation of a nonlinear dependence of the enthalpy dependence on temperature was prevented by the fact that experimentally the enthalpies for all proteins studied before were obtained in a rather narrow temperature range. The extreme thermostability of ubiquitin and its broad variation with changing solvent conditions provides a unique opportunity to observe, by direct calorimetric measurements, a nonlinear dependence of the unfolding enthalpy on temperature.

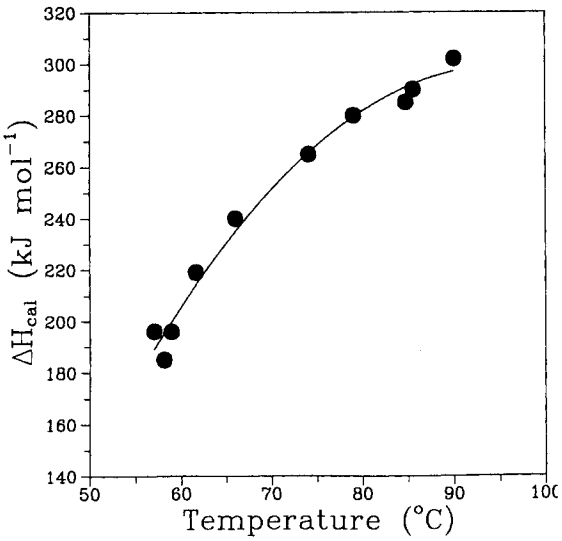

Fig. 3. The dependence of the enthalpy of ubiquitin unfolding on temperature.

Enthalpy and Entropy Functions of Ubiquitin Unfolding in the Temperature Range 5–125°C

The obtained function of the heat capacity change upon ubiquitin unfolding allows us to calculate the enthalpy and entropy of unfolding as functions of temperature in the temperature range 5–125°C:

$$\Delta^U_N H(T) = \Delta^U_N H(T_o) + \int_{T_o}^{T} \Delta^U_N C_p(T)dT \qquad (5)$$

$$\Delta^U_N S(T) = \Delta^U_N S(T_o) + \int_{T_o}^{T} \Delta^U_N C_p(T)d\ln T. \qquad (6)$$

The dependence of the specific enthalpy and specific entropy of unfolding of ubiquitin on temperature is presented in Figure 4 together with similar functions obtained for other small globular proteins.[9,10] It is notable that the specific enthalpy and entropy of unfolding of ubiquitin converge to a values similar to the $\Delta^U_N H$ and $\Delta^U_N S$ of all studied small globular proteins at approximetely 120°C.

Contributions of Hydration, van der Waals Interactions, and Hydrogen Bonding to the Enthalpy of Ubiquitin Unfolding

Recently we have shown[9,10] that the contributions of different interactions (hydration, van der Waals

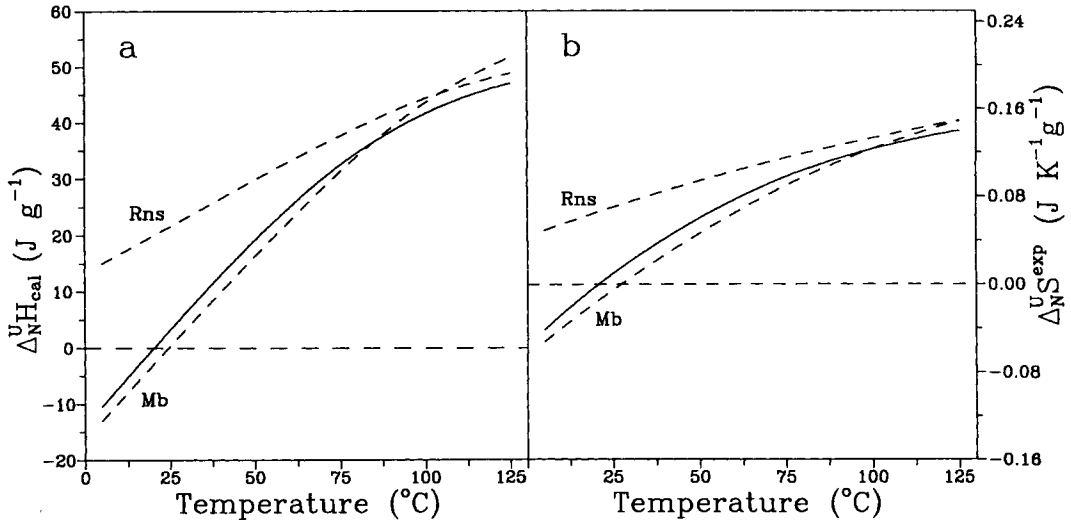

Fig. 4. (**a**) Temperature dependence of the specific enthalpy of unfolding of ubiquitin calculated according to Eq. (5) (solid line). The dashed lines represent the temperature dependencies of the specific enthalpy of unfolding for ribonuclease A (Rns) and myoglobin (Mb).[9] (**b**) Temperature dependence of the specific entropy of unfolding of ubiquitin at pH 2.0, calculated according to Eq. (6) (solid line). The dashed lines represent the temperature dependencies of the specific enthalpy of unfolding for ribonuclease A (Rns) and myoglobin (Mb).[10]

interactions, hydrogen bonding) to the stability of proteins can be correlated with the structural information obtained from their three-dimensional structures.

The experimentally measured integral enthalpy of protein unfolding, ΔH_{cal}, can be presented as a sum of contributions:

$$\Delta H_{cal} = \Delta_N^U H_{pol}^{hyd} + \Delta_N^U H_{npl}^{hyd} + \Delta_N^U H^{vdW} + \Delta_N^U H^{HB} \quad (7)$$

where the right side terms of this equation are the enthalpy the hydration of polar groups

$$\Delta_N^U H_{pol}^{hyd} = \sum_i \Delta \hat{H}_i^{hyd} \Delta_N^U ASA_i \quad (8)$$

the enthalpy of hydration of nonpolar groups

$$\Delta_N^U H_{npl}^{hyd} = \Delta \hat{H}_{arm}^{hyd} \Delta_N^U ASA_{arm} + \Delta \hat{H}_{alp}^{hyd} \Delta_N^U ASA_{alp} \quad (9)$$

the enthalpy of van der Waals interactions

$$\Delta_N^U H^{vdW} = \Delta \hat{H}_{arm}^{int} \Delta_N^U ASA_{arm} + \Delta \hat{H}_{alp}^{int} \Delta_N^U ASA_{alp} \quad (10)$$

and the enthalpy of hydrogen bonding

$$\Delta_N^U H^{HB} = N_{HB} \Delta h^{HB}. \quad (11)$$

In Eqs. (8)–(11) $\Delta \hat{H}_i^{hyd}$ is the reduced enthalpy of hydration of a given type of surface area, listed in Table 5 of Makhatadze and Privalov,[9] $\Delta \hat{H}^{int}$ is the reduced enthalpy of van der Waals interaction listed in Table 7 of Makhatadze and Privalov;[9] $\Delta_N^U ASA_i$ is the change of a given type of surface area upon unfolding listed in Table I of this paper; Δh^{HB} is the enthalpy of hydrogen bonds inside the protein and N_{HB} is the number of hydrogen bonds in the ubiquitin molecule.

All terms in the Eq. (7), except the enthalpy of hydrogen bonding $\Delta_N^U H^{HB}$, are measurable or calculable, and are listed in Table IV. These permit us to estimate the enthalpy of intramolecular hydrogen bonding. The obtained values of the enthalpy of hydrogen bonding in the ubiquitin molecule are listed in Table IV. According to Stickle et al.,[20] there are 76 internal hydrogen bonds in the ubiquitin molecule. Dividing the enthalpy of hydrogen bonding by the number of hydrogen bonds in the molecule we find that one hydrogen bond contributes about 50 kJ/mol to the enthalpy of stabilization of ubiquitin and this contribution does not depend noticeably on temperature in the considered temperature range.

Length Dependence of the Enthalpy of Hydrogen Bonding

It has been shown earlier[9] that the enthalpy of hydrogen bonding correlates with the fraction of protein in the helical conformation. The probable reason for this is the length of hydrogen bonds, which is larger in helices than in β-sheets.[32] Having in mind that the energy of hydrogen bonding decreases with increasing interatomic distance, the energy of interactions in α-helical structures will be smaller than in β-sheets. Analysis of hydrogen bonding patterns in proteins shows[20] that hydrogen bonds are mostly involved in the elements of regular secondary structure and that few hydrogen bonds participate in tertiary structure formation. This means that if we calculate the average length of

TABLE IV. Temperature Dependence of the Enthalpy and Entropy Change Upon Unfolding of Ubiquitin

	Temperature (°C)					
	5	25	50	75	100	125
	Enthalpy (kJ mol^{-1})					
ΔH_{cal}	−88	27	162	273	351	393
$\Delta_N^U H_{npl}^{hyd}$	−881	−654	−387	−136	105	324
$\Delta_N^U H_{pol}^{hyd}$	−3776	−3869	−3969	−4059	−4142	−4220
$\Delta_N^U H^{vdW}$	697	686	671	645	604	543
$\Delta_N^U H^{HB}$	3872	3864	3847	3823	3784	3746
Δh^{HB}	51	51	51	50	50	49
	Entropy (J mol^{-1} K^{-1})					
$\Delta_N^U S^{exp}$	−350	49	488	823	1044	1153
$\Delta_N^U S_{npl}^{hyd}$	−3712	−2923	−2059	−1313	−638	−70
$\Delta_N^U S_{pol}^{hyd}$	−2133	−2456	−2771	−3034	−3265	−3462
$\Delta_N^U S^{conf}$	5498	5431	5319	5174	4944	4683

hydrogen bond in a given protein, it will be longer in proteins with more helical structure. The results of these calculations are presented on Figure 5. As one can see, the greater the amount of helical structure, the longer the average length of hydrogen bonds, and the smaller the energy of hydrogen bonding.

The Entropy of Ubiquitin Hydration Upon Unfolding

The entropy of protein unfolding, can be represented as[10]

$$\Delta_N^U S^{exp} = \Delta_N^U S_{npl}^{hyd} + \Delta_N^U S_{pol}^{hyd} + \Delta_N^U S^{conf} \quad (12)$$

where $\Delta_N^U S_{npl}^{hyd}$ and $\Delta_N^U S_{pol}^{hyd}$ are the entropies of hydration of nonpolar and polar groups exposed to water upon unfolding, respectively, and $\Delta_N^U S^{conf}$ is the configurational entropy of unfreezing the backbone chain, including the entropy of disruption of hydrogen bonds, and unpacking of the side chain groups. The hydration terms can be calculated as

$$\Delta_N^U S_{pol}^{hyd} = \sum_i \Delta \hat{S}_i^{hyd} \Delta_N^U ASA_i \quad (13)$$

$$\Delta_N^U S_{npl}^{hyd} = \Delta \hat{S}_{arm}^{hyd} \Delta_N^U ASA_{arm} + \Delta \hat{S}_{alp}^{hyd} \Delta_N^U ASA_{alp} \quad (14)$$

using the hydration entropies, reduced per square angstrom of a given type of surface area, $\Delta \hat{S}_i^{hyd}$ (Table 4 of Privalov and Makhatadze[10]). The hydration entropies of ubiquitin are listed in Table IV. Knowing the entropies of hydration and experimental entropies of ubiquitin unfolding, one can get the conformational entropy change, $\Delta_N^U S^{conf}$, using Eq. (12). The obtained values of $\Delta_N^U S^{conf}$, are listed in Table IV.

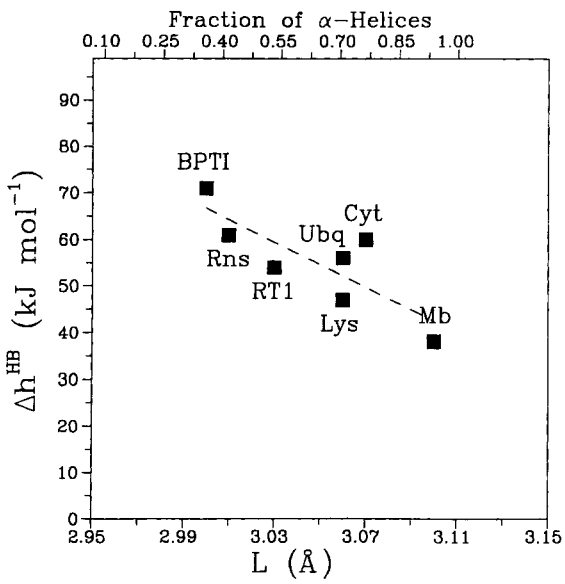

Fig. 5. The dependence of the enthalpy of hydrogen bonding on the average length of hydrogen bond in the structure of different proteins (Rns, ribonuclease A; Lys, lysozyme; Cyt, cytochrome *c*; Mb, myoglobin; RT1, ribonuclease T1; Ubq, ubiquitin). The data are taken from Privalov and co-workers.[9,29,30] The correlation coefficient between the enthalpy of hydrogen bonding and the length of hydrogen bond is 0.82 through whole set or 0.93 if cytochrome *c* will be excluded.

Mechanism of Stabilization of Ubiquitin

The enthalpies and entropies given in Table IV allow us some insight into the mechanism of stabilization of the ubiquitin molecule. Figure 6 presents Gibbs energies of different interactions contributing to the stability of the ubiquitin molecule: Gibbs energy of hydration of nonpolar groups exposed to water upon unfolding, ΔG_{npl}^{hyd}; Gibbs energy of hydrogen

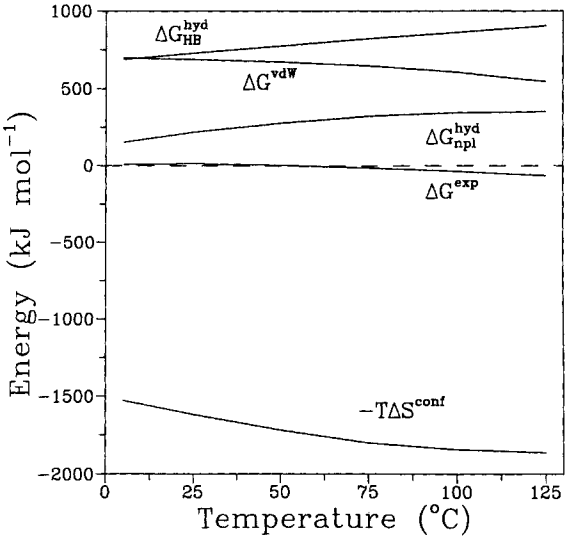

Fig. 6. The relative contribution of various forces to the stability of ubiquitin. The Gibbs energy of van der Waals interactions, ΔG^{vdW}, hydrogen bonding, ΔG_{HB}, and hydration of nonpolar groups, ΔG^{hyd}_{npl}, dissipative forces of thermal motion, $-T\Delta S^{conf}$, and the total Gibbs energy of ubiquitin unfolding, ΔG^{exp}, determined from calorimetric studies of its denaturation.

bonding, which includes the enthalpy of disruption of hydrogen bonds and the Gibbs energy of hydration of polar groups exposed to water upon unfolding, $\Delta^U_N H^{HB} + \Delta^U_N G^{hyd}_{pol}$; van der Waals interactions, which has only enthalpic term, $\Delta^U_N H^{vdW}$; and the effect of the increase of the configurational entropy, $-T\ \Delta^U_N S^{conf}$. The main sources of stabilization are internal hydrogen bonding and van der Waals interactions, while the stabilizing contribution from the hydration of nonpolar groups is considerably smaller. All these positive contributions to the stability of the protein are opposed by the configurational entropy.

The role of solvent ordering effects on the stability of proteins was recently analyzed by Woolfson et al.[8] In an elegant analysis of the influence of methanol on stability, they showed that solvent ordering upon exposure of buried protein groups to water is not obligatory for the folding of a protein. Our analysis supports this conclusion. The addition of methanol affects the solvent ordering and thus decreases the absolute value of the Gibbs energy of hydration. However, addition of methanol should not significantly affect van der Waals interactions. Since the contribution of hydration of nonpolar groups to the stability of proteins is not large, the protein will still be stable even when solvent ordering effects are significantly reduced.

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