Journal of Thermal Analysis and Calorimetry, Vol. 93 (2008) 2, 429–438

IS THERE A RELATIONSHIP BETWEEN PROTEIN THERMAL STABILITY AND THE DENATURATION HEAT CAPACITY CHANGE?

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It is shown the non-occurrence of a correlation between the values of the denaturation temperature and those of the denaturation heat capacity change for a set of 13 proteins possessing the 'SH3-type' fold from both mesophilic and thermophilic microorganisms. This seems to be a rather general result, because, fixed the size and the folding pattern, the denaturation heat capacity change is a nearly constant quantity, within the uncertainty limits of experimental determinations, regardless of the thermal stability of the protein. A precise definition of the thermodynamics of the hydrophobic effect is presented to clarify that the above finding does not imply that the hydrophobic effect does not play a role in the extra-thermal stability of thermophilic and hyperthermophilic proteins.

Keywords: *denaturation heat capacity change, enthalpy–entropy compensation, hydrophobic effect, thermal stability, work of cavity creation*

Introduction

It is widely recognized that the large and positive heat capacity change associated with the thermal denaturation of globular proteins, $\Delta_d C_p$, is the thermodynamic signature of the role played by the hydrophobic effect for the stability of the native conformation of globular proteins [1–4]. On this basis it would be predictable the existence of a relationship between the values of the denaturation temperature, T_d, of a family of globular proteins and the corresponding values of $\Delta_d C_p$. This relationship has also been invoked in order to try to explain the origin of the extra-stability against temperature of proteins from thermophilic and hyperthermophilic microorganisms [5]. Specifically, it has been suggested that a lowering of $\Delta_d C_p$ passing from a mesophilic protein to a thermophilic analogue may lead to a broader stability curve and so to a larger T_d value [6].

Detailed analyses of available sequences and structures of mesophilic and thermophilic pairs have not succeeded in identifying a unique mechanism selected by nature to enhance the thermal stability of globular proteins [7–15]. It could be a better choice to analyze experimental data for a family of globular proteins possessing the same folding pattern but spanning a large range of thermal stability. Baker found that topology determines the folding mechanism: pairs of structurally related proteins with little or no sequence similarity have very similar folding transition-state ensembles [16]. The major features of the folding Gibbs energy landscape appear to be dictated by the native structure topology (i.e., the folding pattern).

To this aim I have selected a set of 13 small globular proteins, including seven SH3 domains, four cold shock proteins and the thermophilic Sac7d and Sso7d, because they all possess a similar structure with 60–70 residues and no disulfide bridges. The common fold consists of 5 anti-parallel β-strands, twisted into a barrel comprising two β-sheets that pack against each other in a nearly orthogonal manner [17–20]. It appears that this fold 'has survived in all kingdoms due to its (thermal) stability and because it forms a suitably small and stable platform for different functions in various organisms' [19]. In addition, there is a lot of reliable experimental data on the thermodynamic stability of both mesophilic and thermophilic representatives of this set. The analysis shows that there is no correlation between the $\Delta_d C_p$ values and the T_d ones for the selected set of globular proteins. This finding, however, does not imply that the hydrophobic effect is not a fundamental factor of protein stability. The matter is analyzed in detail: (a) by assigning a precise meaning to the thermodynamics of the hydrophobic effect; (b) by testing the thermodynamics associated with point mutations in some of the selected proteins. It emerges that the hydrophobic effect is always a stabilizing factor, but it is often hidden in the very intricate mix of enthalpic and entropic contributions determining the marginal thermodynamic stability of the folded conformation of globular proteins.

Selected literature data

For Btk-SH3, Itk-SH3 and Tec-SH3, experimental data come from the calorimetric and spectroscopic

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1388–6150/$20.00

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study of Ladenstein *et al*. [21]; for Abl-SH3, Fyn-SH3 and Spc-SH3, experimental data come from the calorimetric investigation of Mateo *et al.* [22]. For Sem5-SH3 experimental data come from the spectroscopic study of Richards *et al*. [23], but the Δ_dC_p was not determined and it has been assigned on the basis of the values of the other SH3 domains. For Sso7d, extracted from the hyperthermophilic archaeon *Sulfolobus solfataricus*, I have used data from both the study of Ladenstein *et al.* [21, 24], and the study of Shriver et al. [25]. For Sac7d, extracted from the hyperthermophilic archaeon *Sulfolobus acidocaldarius*, I have selected two sets of data both coming from the calorimetric and spectroscopic studies of Shriver et al. [25, 26]. For Ec-CspA data come from the study of Petrosian and Makhatadze [27]; for Bs-CspB and Bc-CspB data are from the spectroscopic study of Schmid et al. [28]. For Tm-Csp, extracted from the hyperthermophilic microorganism *Thermotoga maritima*, I have used data from the calorimetric study of Jaenicke *et al.* [29]; in particular, I have selected both the values at pH 4.8 and 6.0 because there is a large difference in the $\Delta_dH(T_d)$ numbers, and I have considered two Δ_dC_p values: the experimental one for Tm-Csp, 4.6 kJ K^{-1} mol^{-1} [29], and that determined for several variants of Bs-CspB and Bc-CspB, 4.0 kJ K^{-1} mol^{-1} [30]. The set contains only wild-type proteins, to avoid the occurrence of artifacts due to point mutations, in the conditions of maximal thermal stability measured by the T_d value.

The temperature-induced denaturation of all of these proteins is a reversible process well represented by the two-state N⇔D transition model [31, 32]. In the assumption that Δ_dC_p is a constant quantity, the temperature dependence of Δ_dG is given by [1, 33]:

$$\Delta_dG(T)=\Delta_dH(T_d)[1-(T/T_d)]+\Delta_dC_p[T-T_d-T\ln(T/T_d)] \quad (1)$$

The plot of Δ_dG *vs.* T has a parabola-like shape and is called the stability curve of the protein [34]. Since Δ_dC_p is a large and positive quantity, the denaturation enthalpy and entropy changes vanish at two temperatures, T_H and T_S, respectively [34]. If Δ_dC_p is a constant quantity, it results:

$$T_H=T_d-[\Delta_dH(T_d)/\Delta_dC_p] \quad (2)$$

$$\ln T_S=\ln T_d-[\Delta_dH(T_d)/T\Delta_dC_p] \quad (3)$$

Experimental data, T_d, $\Delta_dH(T_d)$ and Δ_dC_p, together with the calculated values of T_H, $T_S\equiv T_{max}$ (i.e., the temperature at which Δ_dS is zero corresponds to the temperature where Δ_dG achieves the maximum), and $\Delta_dG(T_S)$ are collected in Table 1.

Table 1 Parameters characterizing the maximal thermal stability, at pH around neutrality, of a family of small globular proteins, all possessing the 'SH3-type' fold. Values of T_d, $\Delta_dH(T_d)$ and Δ_dC_p are directly obtained from DSC or CD measurements; those of T_H, T_S and $\Delta_dG(T_S)$ are calculated by means of Eqs (1)–(3)

Protein	N_{res}	T_d/K	$\Delta_dH(T_d)$/ kJ mol^{-1}	Δ_dC_p/ kJ K^{-1} mol^{-1}	T_H/K	T_S/K	$\Delta_dG(T_S)$/ kJ mol^{-1}
Btk-SH3 [21]	64	353	196	3.1	289.8	295.1	16.4
Itk-SH3 [21]	57	342	178	3.4	289.6	293.5	13.3
Tec-SH3 [21]	63	344	169	2.9	285.7	290.4	13.6
Abl-SH3 [22]	63	342	194	3.3	282.7	288.0	17.5
Fyn-SH3 [22]	64	344	233	3.3	273.0	280.2	23.8
Spc-SH3 [22]	62	339	197	3.4	281.0	285.7	16.0
Sem5-SH3 [23]	60	352	271	3.3	270.2	278.8	28.4
Sso7d [21, 24]	62	372	274	2.7	270.5	283.2	34.3
Sso7d [25]	62	372	272	3.1	284.3	293.8	29.5
Sac7d [26]	66	364	275	3.6	287.5	295.1	27.4
Sac7d [25]	66	363	245	3.0	282.3	290.8	25.5
Ec-CspA [27]	70	330	181	3.3	275.4	279.5	13.5
Bs-CspB [28]	67	327	193	4.0	278.4	282.1	14.8
Bc-Csp [28]	66	350	245	4.0	288.8	293.8	20.0
Tm-Csp[a] [29]	66	367	259	4.6 4.0	310.6 302.2	314.8 307.6	19.3 21.6
Tm-Csp[b] [29]	66	365	312	4.6 4.0	297.4 287.2	303.1 294.8	26.2 30.8

[a]pH 4.8; [b]pH 6.0

Analysis of literature data

The existence of a correlation between Δ_dC_p and T_d can be tested by constructing a plot of these two quantities. The plot of the Δ_dC_p values *vs.* the T_d values for the 13 selected proteins is shown in Fig. 1 (i.e., 16 entries because I considered two Δ_dC_p values for the three hyperthermophilic proteins; values at pH 6.0 for Tm-Csp have not been taken into account because they would practically duplicate those at pH 4.8). Data do not show any correlation: the linear correlation coefficient r=–0.035 for 16 points. The latter number indicates that there is no relationship between Δ_dC_p and T_d for the present set of globular proteins possessing the 'SH3-type' fold and spanning a large range of T_d values (i.e., from 327 up to 372 K). Also considering solely the three proteins from hyperthermophilic sources, Sso7d, Sac7d and Tm-Csp, the Δ_dC_p values of 2.7, 3.6 and 4.6 kJ K^{-1} mol^{-1} (or 3.1, 3.0 and 4.0 kJ K^{-1} mol^{-1} using the second set of values reported in Table 1 for these three proteins) do not correlate with the T_d values of 372, 364 and 367 K, respectively. This suggests that, fixed the size and the folding pattern of a set of globular proteins, the Δ_dC_p value remains practically constant within the uncertainty limits of experimental determinations. In fact, the experimental Δ_dC_p values of the 13 selected proteins are in line with those expected on the basis of the proposed empirical relationships between the buried accessible surface area, *ASA* (that is determined by the size and the folding pattern) and Δ_dC_p [3, 4, 35, 36]. For instance, according to the simple relationship provided by Robertson and Murphy [35], $\Delta_dC_p \approx 0.058N_{res}$, so that Δ_dC_p would amount to 3.5 and 4.1 kJ K^{-1} mol^{-1} for proteins of 60 and 70 residues, respectively. Another simple relationship is the following [36]:

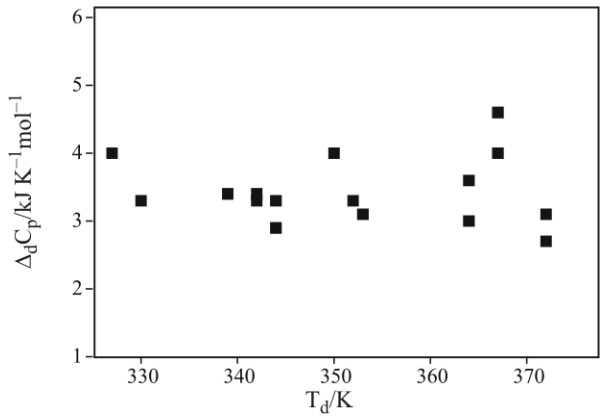

Fig. 1 Plot of Δ_dC_p *vs.* T_d for the 13 proteins collected in Table 1; two different values of Δ_dC_p have been considered for Sso7d, Sac7d and Tm-Csp (i.e., 16 points). There is no correlation between these two quantities

$$\Delta_dC_p=[\Delta C_{p,CONH}f_{b,pol}+\Delta C_{p,CH}f_{b,np}<N_{CH}>]N_{res} \quad (4)$$

where one uses information derived from small molecule studies for the heat capacity contribution of a CONH group and a CH group (i.e., a hydrogen atom bonded to a carbon atom, regardless of whether it is aliphatic or aromatic, and considering that, for instance, a methyl group corresponds to 3CH groups), together with the average value of the buried fraction of polar and non-polar *ASA* of the protein, $f_{b,pol}$ and $f_{b,np}$, respectively, and the mean number of CH groups in the protein residues, $<N_{CH}>$. By using Eq. (4) with $<N_{CH}>$=6, that is an average value for globular proteins, one obtains Δ_dC_p=2.5 and 3.2 kJ K^{-1} mol^{-1} for N_{res}=60 and 70, respectively [36]. It is worth noting that quantitative failure of empirical relations between Δ_dC_p and buried *ASA* has been reported [37].

Davidson *et al.* [38] found that for Fyn-SH3 domain and 48 hydrophobic core single point mutants the $\Delta_dH(T_d)$ and T_d values correlate well and the slope of the linear fit gives Δ_dC_p=2.8 kJ K^{-1} mol^{-1}. These 49 proteins, even though they show T_d values ranging from 36 up to 82°C, are characterized by the same Δ_dC_p value. This robust datum confirms the non-occurrence of a correlation between T_d and Δ_dC_p values for a family of structurally similar globular proteins. Forman-Kay *et al.* [39] found that for drkN-SH3 and 9 point mutants a Δ_dC_p value of 3.0 kJ K^{-1} mol^{-1} is able to fit all the thermal unfolding curves characterized by T_d values ranging from 58 up to 89°C. Makhatadze *et al.* found that Δ_dC_p=3.5 kJ K^{-1} mol^{-1} for both Bs-CspB having T_d=52.9°C, and its thermostable variant form having T_d=71.6°C [40]. These data suggest that: (a) the observed lack of correlation between Δ_dC_p and T_d should be biologically significant; (b) the relationship between the hydrophobic effect and Δ_dC_p should be more subtle than it is usually believed. It is worth noting that a lack of correlation between Δ_dC_p and T_d was originally pointed out by Nussinov *et al.* [41], even though partially retracted in subsequent analyses [42–44] and by Ragone [45].

These findings indicate that the flattening of the Δ_dG *vs.* T parabola-like curve due to a Δ_dC_p decrease passing from a mesophilic protein to the thermophilic counterpart is not an operative mechanism to increase the thermal stability of the 'SH3-type' fold. The other two limiting thermodynamic mechanisms to increase the thermal stability of globular proteins are [8, 46]: (a) lifting up of the Δ_dG *vs.* T parabola-like curve; (b) shifting toward higher temperature of the Δ_dG *vs.* T parabola-like curve due to an increase of the temperature corresponding to the maximum of the parabola, $T_{max} \equiv T_S$. The T_S values of the 13 selected proteins are reported in the seventh column of Table 1, and fall within the range determined by Rees and Robertson [47], over

a large set of mesophilic proteins, T_S=285±19 K (note that the values of T_H, listed in the sixth column of Table 1, are close to those of T_S, indicating the occurrence of a large enthalpy–entropy compensation [48]). Even though the latter range is wide, there is no clustering of T_S values for thermophilic proteins in the high temperature side of the range. Therefore, also the shifting toward higher temperature of the $\Delta_d G$ *vs.* T parabola-like curve does not seem to be operative for the selected proteins. This implies that the lifting up of the $\Delta_d G$ *vs.* T parabola-like curve is the preferred thermodynamic mechanism to increase the thermal stability of the 'SH3-type' fold. A linear regression of $\Delta_d G(T_S)$ *vs.* T_d, reported in Fig. 2, (18 entries, since all the values listed in the eighth column of Table 1 have been considered), gives r=0.7782, confirming the existence of a correlation between these two thermodynamic quantities. The sensitivity of $\Delta_d G(T_S)$ estimates to differences in the values of T_d, $\Delta_d H(T_d)$ and $\Delta_d C_p$ can be appreciated by looking at the four entries reported for Tm-Csp.

The lifting up of the $\Delta_d G$ *vs.* T parabola-like curve has also been found by Razvi and Scholtz in an experimental study of the conformational stability of five histidine-containing phosphocarrier protein homologues derived from microorganisms living in very different environments [49] and in a general survey of available data [50].

This finding does appear to be in line with the principle of 'corresponding states' advanced by Somero [51] and Jaenicke [52]: the folded state of thermophilic proteins should be characterized by larger $\Delta_d G$ values around room temperature (i.e., around $T_{max} \equiv T_S$) than the folded state of mesophilic counterparts in order to have a similar flexibility at their respective physiological temperatures to perform the biological function [53, 54]. It would be a simple consequence of the basic fact that the random thermal energy RT increases with temperature, amounting to 2.3 kJ mol^{-1} at 0°C, 2.5 kJ mol^{-1} at 25°C and 3.1 kJ mol^{-1} at 100°C. Note that water molecules bombarding the protein surface possess an average translational kinetic energy amounting to $3RT/2$ per mole and that folded proteins should resist to this bombardment.

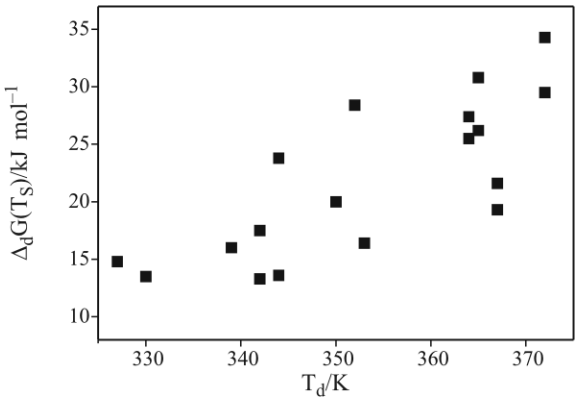

Fig. 2 Plot of $\Delta_d G(T_S)$ *vs.* T_d for the 13 proteins collected in Table 1; all the entries have been considered (i.e., 18 points). A linear least squares regression gives a correlation coefficient r=0.7782

Relationship with the hydrophobic effect

The absence of a relationship between T_d and $\Delta_d C_p$ may appear strange because the large and positive heat capacity change is considered to be the distinctive signature of the fundamental role played by the hydrophobic effect for the conformational stability of globular proteins [1–4, 55]. However, the relationship between the Gibbs energy change associated with the hydrophobic effect, that, since the famous Kauzmann's analysis [56], should be the main stabilizing factor of the native conformation, and $\Delta_d C_p$ needs a closer scrutiny.

The hydrophobic effect may be quantified by the changes in thermodynamic functions associated with the transfer of nonpolar molecules from a nonpolar organic liquid phase to water over a large temperature range. This choice contrasts with the widespread use of values at a single temperature, usually 25°C [57–60], but is the right one because temperature is a fundamental variable in this matter [55, 61]. In order to provide specific numerical values, I use the transfer from neat liquid phase to water of neopentane, cyclohexane and benzene over the 5–100°C temperature range [61–63]. The experimental values of $\Delta C_p^\bullet$, $\Delta H^\bullet$, $\Delta S^\bullet$ and $\Delta G^\bullet$, where the superscript filled circle indicates that such quantities are referred to the so-called Ben–Naim standard (i.e., transfer from a fixed position in the non-polar liquid phase to a fixed position in water [64]), are collected in Table 2. In addition, the plots of $\Delta H^\bullet$, $-T\Delta S^\bullet$ and $\Delta G^\bullet$ for the neat liquid-to-water transfer of neopentane (panel a), cyclohexane (panel b) and benzene (panel c) are shown in Fig. 3. It is evident that $\Delta C_p^\bullet$ for all the three hydrocarbons is a large and positive quantity that decreases by about 30% over the 5–100°C temperature range. The functions $\Delta H^\bullet$ and $\Delta S^\bullet$ increase strongly with temperature, whereas $\Delta G^\bullet$ is always large and positive, and shows a little temperature dependence due to the occurrence of enthalpy–entropy compensation. Thus, $\Delta G^\bullet$ slightly increases with temperature while $\Delta C_p^\bullet$ decreases: the two functions are anti-correlated over a large temperature range (Table 2).

Even though the large and positive $\Delta C_p^\bullet$ is a signature of the hydrophobic effect, the molecular mechanism determining its magnitude is different from that producing the large and positive $\Delta G^\bullet$ values [61, 65].

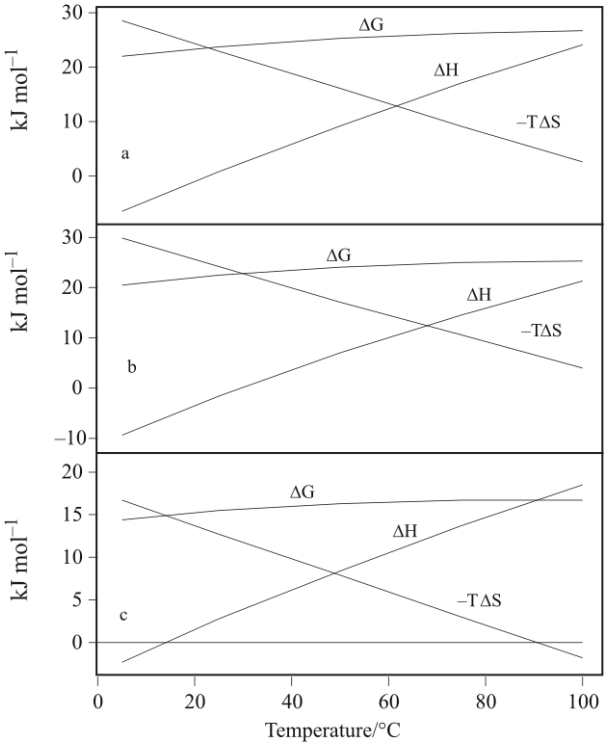

Fig. 3 Temperature dependence of the Ben-Naim standard thermodynamic functions $\Delta G^{\bullet}$, $\Delta H^{\bullet}$ and $-T\Delta S^{\bullet}$ associated with the transfer from neat liquid phase to water of neopentane (panel a), c-hexane (panel b), and benzene (panel c)

The large and positive $\Delta C_p^{\bullet}$ is mainly associated with the reorganization energetics of water molecules around the inserted non-polar molecule, as convincingly demonstrated by the analysis of a lot of experimental data [66–68], and computer simulations in water models [69–71]. The reorganization of water molecules in the hydration shell of the nonpolar solute molecule is characterized by enthalpy–entropy compensation and does not affect the overall Gibbs energy change [72–75]. In particular, a large and positive $\Delta C_p^{\bullet}$ seems to be a distinctive feature of the reorganization of H-bonded systems. Cooper underscored that the transition ice⇔water is characterized by a large and positive heat capacity change associated with the disordering of the 3D H-bonded network of ice [76, 77]. In fact, the $\Delta C_p^{\bullet}$ values associated with the hydration of nonpolar solutes and their temperature dependence can be reproduced by means of the Muller's model [78–80], which is an extension of a two-state lattice model originally developed by Angell [81]. The Muller's model accounts for the reorganization of H-bonds upon non-polar solute insertion by distinguishing the hydration shell region from the bulk water [78, 79]. In addition, it has been shown that the H-bonds in the hydration shell should be more broken than those in bulk water in order to qualitatively reproduce the decrease of $\Delta C_p^{\bullet}$ with temperature [82–85].

In contrast, the large and positive $\Delta G^{\bullet}$ values, measuring the poor solubility of hydrocarbons in water, are determined by the balance between [61–63]: (a) the work spent to create a suitable cavity in the two liquid phases, $\Delta\Delta G_c$, and (b) the work gained to turn on attractive interactions between the inserted solute and the surrounding molecules in the two liquid phases, $\Delta\Delta G_a$. The temperature dependence of $\Delta G^{\bullet}$ for the neat liquid-to-water transfer mainly reflects the different temperature dependence of the density of water and nonpolar organic liquids. Over the 5–100°C temperature range the density of water decreases slightly, whereas that of hydrocarbons decreases more (about 4% *vs.* about 10%, respectively [86]). The work of cavity creation is always larger in water than in nonpolar liquids, and the difference increases with temperature due to the density effect [87–91]. See the $\Delta\Delta G_c$ values listed in the last column of Table 2, calculated by means of scaled particle theory, SPT [92–95], using (a) the experimental liquid densities over the 5–100°C temperature range; (b) the following, temperature-independent, hard-sphere diameters: 2.80 Å for water, 5.80 Å for neopentane, 5.63 Å for cyclohexane and 5.26 Å for benzene. The difference $\Delta G^{\bullet}-\Delta\Delta G_c$, that is a reliable estimate of $\Delta\Delta G_a$, is positive at room temperature, and decreases with temperature due to the density effect. Non-polar solute–solvent attractive interactions appear to be stronger in non-polar liquids than in water around room temperature, but the difference tends to vanish with temperature and also to reverse its sign [61–63]. It is worth noting that the density effect reflects the temperature dependence of the structural features of the different liquids and is absolutely unrelated to solvent reorganization upon the insertion of a nonpolar solute molecule (i.e., in water it does not depend on the reorganization of water–water H-bonds around the inserted nonpolar solute).

The validity of empirical relations such as Eq. (4) implies that $\Delta_d C_p$ is mainly determined by the reorganization of water–water H-bonds around nonpolar moieties exposed upon unfolding. Since this water–water H-bond reorganization is a compensating process that does not affect the overall Gibbs energy change, the non-occurrence of a correlation between $\Delta_d C_p$ and T_d should not be surprising, and should not be interpreted as an indication that the hydrophobic effect does not work. The strength of the hydrophobic effect is measured by $\Delta G^{\bullet}$ values which are always large and positive. Thus the hydrophobic effect should be considered the non-specific glue that drives the collapse of the polypeptide chain towards the folded compact conformation. Moreover, since the $\Delta G^{\bullet}$ magnitude increases over the 5–100°C temperature range, the hydrophobic effect should provide

Table 2 Ben-Naim standard thermodynamic quantities for the transfer of neopentane (a), cyclohexane (b) and benzene (c) from pure liquid phase at equilibrium vapour pressure to water at 1 atm over the 5–100°C temperature range [61–63]. In the last column are reported the values of the difference in the work of cavity creation to insert a molecule of neopentane (a), cyclohexane (b), and benzene (c) in water and in the neat liquid phase, calculated by means of SPT, $\Delta\Delta G_c=\Delta G_c$(water)$-\Delta G_c$ (neat liquid); see text for more details

	T/ °C	$\Delta C_p^\bullet$/ J K^{-1} mol^{-1}	$\Delta H^\bullet$/ kJ mol^{-1}	$\Delta S^\bullet$/ J K^{-1} mol^{-1}	$\Delta G^\bullet$/ kJ mol^{-1}	$\Delta\Delta G_c$/ kJ mol^{-1}
a	5	394	–9.4	–107.5	20.5	17.6
	25	372	–1.6	–80.8	22.5	21.5
	50	315	7.0	–52.9	24.1	26.2
	75	280	14.5	–30.4	25.0	30.3
	100	264	21.3	–10.7	25.3	35.0
b	5	380	–6.5	–102.5	22.0	13.1
	25	360	0.8	–76.8	23.7	16.0
	50	330	9.2	–49.8	25.3	19.2
	75	295	17.1	–26.1	26.2	21.8
	100	255	24.1	–7.0	26.7	24.3
c	5	270	–2.3	–60.1	14.4	8.9
	25	243	2.8	–42.7	15.5	11.5
	50	219	8.4	–24.4	16.3	14.2
	75	199	13.7	–8.6	16.7	16.6
	100	182	18.5	4.8	16.7	18.7

a positive and important contribution for the stability of globular proteins from thermophilic and hyperthermophilic sources [96]. It is important to note that the T_S values of the hydrophobic effect occur above 100°C (Fig. 3), and so differ strongly from those determined for globular proteins that are clustered around 20°C [42, 47]. This large difference demonstrates the existence of a strongly destabilizing entropic factor in the case of globular proteins, that can reliably be identified with the loss of conformational entropy upon folding of the polypeptide chain, both backbone and side-chain degrees of freedom [2, 56, 97].

Effect of some point mutations

It may be of interest to try to verify if the thermodynamic consequences of some point mutations in the selected proteins qualitatively resemble the thermodynamic features of the hydrophobic effect, considering that the destabilizing conformational entropy contribution should be only marginally affected by a point mutation.

The side-chain of Ile30 is buried in the hydrophobic core of Sso7d (PDB entries 1SSO or 1JIC), and the I30V mutation should lead to the creation of a cavity in the core, causing a destabilization of the folded conformation. Shriver and colleagues [25] found that the I30V mutant has T_d=95.1°C with re-

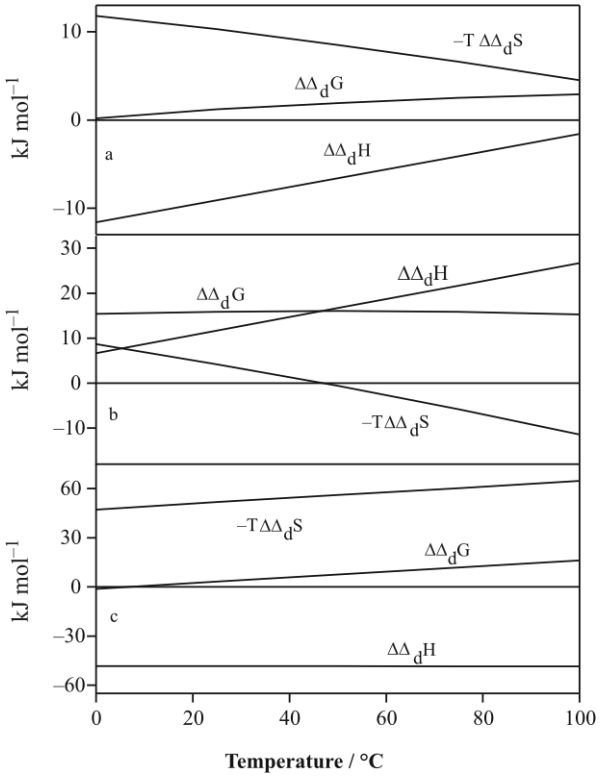

Fig. 4 Temperature dependence of $\Delta\Delta_d G$, $\Delta\Delta_d H$ and $-T\Delta\Delta_d S$ obtained by comparing the denaturation thermodynamics of Sso7d and its mutant I30V (panel a), the same wild-type protein and its mutant F31A (panel b), Bs-CspB and its variant form TB-CspB (panel c)

spect to T_d=99°C of wild-type Sso7d (Table 3). By considering the reverse process, one gains information on the thermodynamic consequences of burying Ile side-chain in substitution of Val side-chain at position 30. The functions $\Delta\Delta_dX=\Delta_dX$(Sso7d)–$\Delta_dX$(I30V), where $X=H$, S and G, are shown in panel *a* of Fig. 4 over the 0–100°C temperature range. It is evident that, even though the stabilization is entirely entropic, as expected for a process ruled by the hydrophobic effect, and enthalpy–entropy compensation is operative, the $\Delta\Delta_dH$ values are not qualitatively consistent with those characterizing the liquid-to-water transfer of neopentane, cyclohexane and benzene. From the enthalpic point of view the V30I mutation is destabilizing, but it is not possible to distinguish between strain introduced in the folded conformation or energy gain in interacting with water.

Also the side-chain of Phe31 is buried in the hydrophobic core of Sso7d participating in an aromatic cluster (PDB entries 1SSO or 1JIC); the F31A mutation causes a marked destabilization of the folded conformation [98], with T_d=74.4°C with respect to T_d=99°C of wild-type Sso7d (Table 3). By considering the reverse process to gain information on the stabilization caused by the burial of Phe aromatic side-chain in the hydrophobic core, one obtains the functions shown in panel *b* of Fig. 4. The stabilization is both enthalpic and entropic up to temperatures just below 50°C, and then becomes solely enthalpic, at odds with dominance of the hydrophobic effect. However, it has been shown, by means of NMR structure determination [99] (PDB entry 1B4O), that the F31A mutation causes a marked modification of the folded conformation with the burial of Trp23 side-chain, solvent exposed in wild-type Sso7d.

Charge-charge interactions seem to play the major role in the extra-thermal stability of cold shock proteins from thermophilic microorganisms [28]. On this basis Makhatadze *et al.* [40] constructed a hybrid protein, TB-CspB, having the same charge distribution of Csp from *Thermotoga maritima* (PDB entry 1G6P), and the rest of the sequence identical to that of CspB from *Bacillus subtilis* (PDB entry 1CSP). TB-CspB proved to be significantly more resistant to temperature with T_d=71.6°C to be compared to T_d=52.9°C of Bs-CspB (Table 3). This marked stabilization is entirely entropic since the $\Delta\Delta_dH$ values are always large and negative, as shown in panel *c* of Fig. 4. Clearly, this entropy dominance cannot be attributed to the hydrophobic effect since only charge-charge interactions have been modified.

As a final system, I considered Fyn-SH3 domain and three point mutants, namely F20A, I28A and W37A [38]. The three residues Phe20, Ile28 and Trp37 are buried in the hydrophobic core of Fyn-SH3 (PDB entry 1SHF), and their mutation to Ala causes a marked destabilization of the folded conformation, as indicated by the values reported in Table 3. By considering the stabilization gained in the reverse process, one obtains that the stabilization is dominated by enthalpic factors, at odds with expectation based on the thermodynamic features of the hydrophobic effect. This result seems to be in line with the proposal by Burley and Petsko [100] on the energetic stabilization provided by aromatic/hydrophobic clusters in the protein core, and to contrast a recent claim on the entropic stabilization provided by aromatic clusters [101].

These examples clarify that, even though the hydrophobic effect is operative in stabilizing the folded conformation, its role may be masked by other factors, such as the solid-like packing of the protein core [102–104], i.e., the crystal molecule model *vs.* the oil drop model [56, 105, 106], the gain/loss of H-bonds and charge-charge interactions on the protein surface

Table 3 Parameters characterizing the maximal thermal stability of three proteins of the selected set and some mutant forms. Values of T_d, $\Delta_dH(T_d)$ and Δ_dC_p are directly obtained from DSC or CD measurements; those of T_S and $\Delta_dG(T_S)$ are calculated by means of Eqs (1) and (3). See text for further details

Protein	T_d/°C	$\Delta_dH(T_d)$/kJ mol^{-1}	Δ_dC_p/kJ K^{-1} mol^{-1}	T_S/K	$\Delta_dG(T_S)$/kJ mol^{-1}
Sso7d [25]	99.0	272	3.1	293.8	29.5
I30V [25]	95.1	262	3.0	290.6	28.7
Sso7d [24]	99.0	274	2.7	283.2	34.3
F31A [98]	74.4	186	2.5	280.6	18.6
Bs-CspB [40]	52.9	165	3.5	282.2	11.4
TB-CspB [40]	71.6	182	3.5	296.4	13.1
Fyn-SH3 [38]	80.1	215	2.8	284.2	21.8
F20A [38]	57.8	128	2.8	288.2	8.4
I28A [38]	53.0	106	2.8	290.4	5.9
W37A [38]	58.5	105	2.8	296.2	5.7

[11–14]. Such a complexity is because globular proteins are heteropolymers, the twenty building blocks have different chemical features [107], and so the core is a heterogeneous matrix and the effects of a point mutation are context-dependent [108]. The general occurrence of enthalpy-entropy compensation as a consequence of point mutations reflects the basic fact that any weakening (strengthening) of intramolecular interactions tends to be counterbalanced by a gain (loss) of degrees of freedom [48, 109].

Discussion

It is shown the non-occurrence of a correlation between the T_d values and the $\Delta_d C_p$ ones for a set of proteins possessing the 'SH3-type' fold and spanning a large range of thermal stability. This seems to be a rather general result, obtained also by others [41, 45], related to the fact that, fixed the size and the folding pattern, $\Delta_d C_p$ proves to be a nearly constant quantity, within the uncertainty limits of experimental determinations, regardless of the thermal stability of the protein. Moreover, a precise definition of hydrophobic effect is presented to clarify that the lack of correlation between T_d and $\Delta_d C_p$ does not imply that the hydrophobic effect does not play a role in the extra-thermal stability of thermophilic and hyperthermophilic proteins. The large and positive heat capacity change associated with the hydrophobic effect is mainly caused by the reorganization of water–water H-bonds around the nonpolar molecule, a process that is characterized by an almost complete enthalpy–entropy compensation and cannot affect the Gibbs energy change. The latter is always a large and positive quantity increasing in magnitude with temperature. It is determined by a balance between the work spent to create a cavity in water and organic liquid, respectively, suitable to host the nonpolar solute, and the work gained to switch on attractive interactions between the nonpolar solute and the surrounding solvent molecules in the two liquids.

Kumar and Nussinov [44], performing calculations by means of Eq. (1) with the constraint that T_S has to be around room temperature (see above), found evidence of an anti-correlation between T_d and $\Delta_d C_p$. In other words, $\Delta_d C_p$ should be lower for thermophilic and especially hyperthermophilic proteins in comparison to homologous mesophilic ones. Even though such an anti-correlation would be expected if electrostatic interactions play a key role for the stability of the folded structure at high temperature [5], one has to remember that the calculations by Kumar and Nussinov cannot be considered a demonstration. They are phenomenological in nature and cannot provide any information at molecular level.

It should be important to try to shed light on the relation between the solid-like nature of protein interior and the hydrophobic effect defined on the basis of the non-polar liquid-to-water transfer thermodynamics. Honig *et al.* emphasized that the enthalpic contribution of van der Waals forces due to the solid-like nature of the protein core should be substantial, but, since it is counterbalanced to a large extent by the freezing of side-chain degrees of freedom, the consequences of the solid-like packing are expected to be small for the stabilization Gibbs energy [110]. Due to enthalpy-entropy compensation the solid-like packing should provide a second-order contribution with respect to the hydrophobic effect.

On the other hand, one can assume that the fundamental step of folding is the collapse in water of the polypeptide chain, i.e., the drastic change from a random coil to a compact globule. Collapse thermodynamics would be ruled by the difference in the work of cavity creation in water associated with the conformational transition. It is worth noting that, even though, as a first approximation, one could consider that the volume of the polypeptide chain does not change upon collapse, the magnitude of the cavity creation work in a liquid depends not only on the cavity volume but also on the cavity shape. SPT-based calculations indicate that the work to create a rod-like cavity is larger than that to create a spherical cavity, the two volumes being equal [111, 112]. The collapse in water of the polypeptide chain would be guided by a substantial reduction in the cavity creation work associated with the conformational transition from a random coil to a compact globule. This approach would be in line with the analysis by Chandler [113], and would allow the explanation of some unexpected results [114, 115]: the burial of polar side-chains, involved in intramolecular H-bonds, stabilizes the folded conformation more than the burial of nonpolar ones. Simple additivity does not hold for protein folding thermodynamics (i.e., attractive interactions among residues are not independent of polypeptide collapse), and we have still to learn how to single out the quantitative role of the different contributions.

Acknowledgements

The article is dedicated to professor Guido Barone in the occasion of his 70th birthday. I would like to thank professor Guido Barone for the complete trust he placed in me since the starting days of my PhD thesis in his laboratory. A trust, I hope, he still places in me.

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Received: September 6, 2007
Accepted: January 29, 2008

DOI: 10.1007/s10973-007-8698-z