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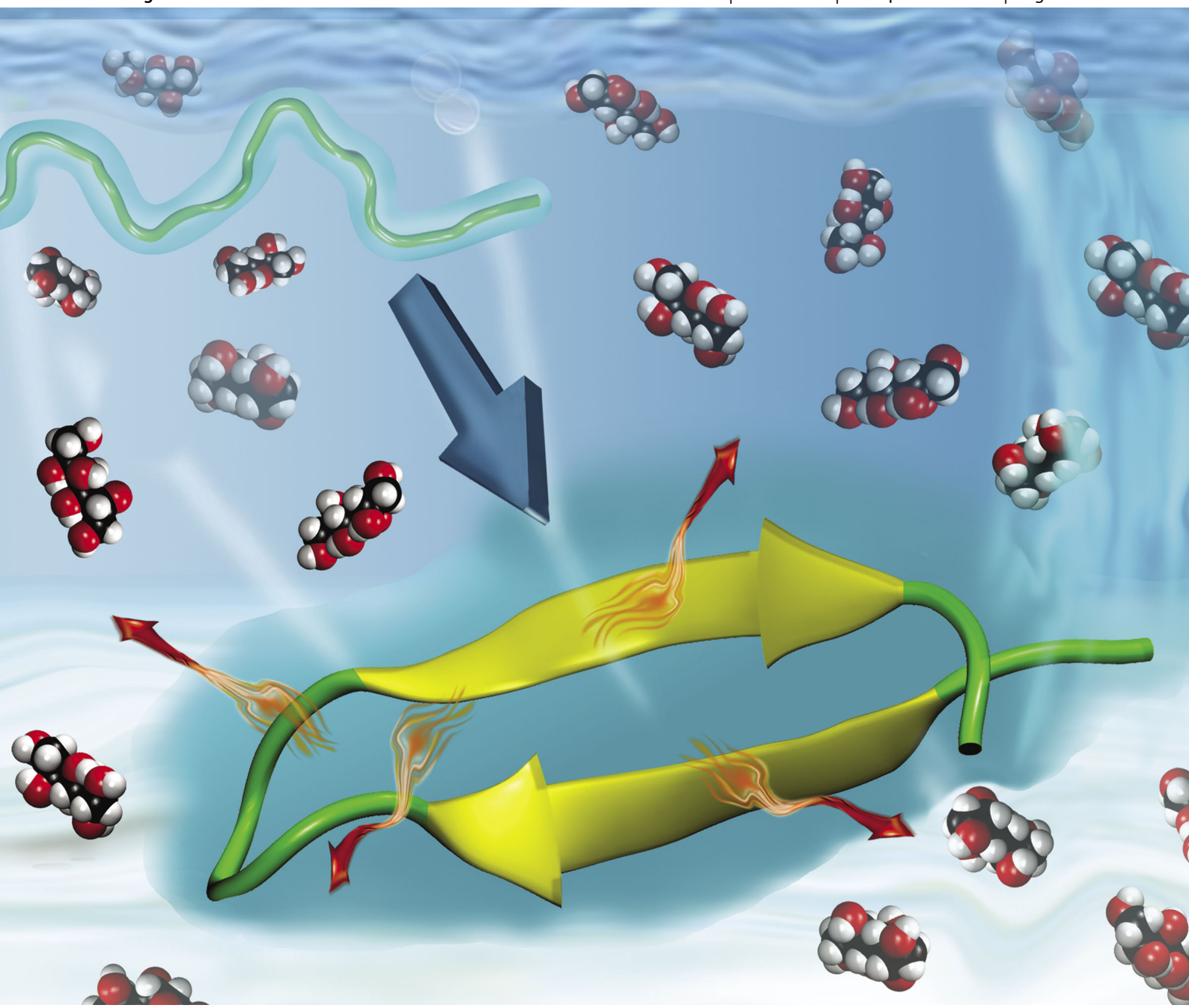

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Enthalpically driven peptide stabilization by protective osmolytes†

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We determined the added enthalpic and entropic contributions of protective osmolytes to the folding of a model peptide into its native β-hairpin state. In contrast to entropically driven steric "crowding", this study shows that sugars and polyols can act as protective osmolytes by primarily diminishing the unfavourable enthalpic contribution to folding.

Living cells have developed multiple strategies to ensure that correct protein folding occurs even under substantial environmental stresses. Among the most widely employed methods to counteract external osmotic pressure is the use of small cosolutes called osmolytes.[1] The exact mechanism of macromolecular stabilization by osmolytes is still largely unknown, but it has been recognized that solutes stabilizing the native state of proteins (protective osmolytes) tend to be preferentially excluded from protein–water interfaces.[2]

Molecular crowding due to excluded volume interactions has been widely invoked to explain how osmolytes can drive protein stability. For example, it was shown that high fractional volume occupancy of crowding agents, such as soluble polymers, significantly shifts the non-native to folded thermodynamic equilibrium (D $\rightleftharpoons$ N) toward the more compact native states.[3] This effect was shown to be related to the restriction of protein conformations to allow larger free volume for osmolytes, thereby destabilizing the unfolded state with respect to the native conformation.[4] However, such molecular crowding mechanisms that are based on steric interactions and are entropic in nature do not usually consider the possibility of enthalpic contributions that may be mediated by the aqueous solution.

In this communication we focus on the effects of various molecularly small osmolytes on the stability of a model 16-residue peptide‡ that can fold to a β-hairpin structure. In aqueous solutions and at neutral pH, this peptide folds in an endothermic, entropically driven process.[5] Surprisingly, in contrast to the crowding mechanism, polyol and sugar osmolytes act to drive further folding primarily through diminishing the enthalpic loss with concomitant reduction in the favourable entropic gain for folding.

Although the osmolyte effect is strongly enthalpic, the action of different osmolytes correlates with their size, as also expected for crowding. Our results further suggest that osmolytes act indirectly by altering the solvating environment, rather than by interacting directly with the peptide. While the osmotic effect we find for this peptide is quite small (*ca.* −1.5 kJ mol^{-1} change in folding free energy at 1 M osmolyte), the effect is known to translate into substantial added stabilization for proteins, and expected to roughly scale with peptide length.

This previously unreported mechanism for osmolyte action on peptide folding highlights the possible importance of water structuring forces, in addition to the entropic crowding effects, in determining protein stabilization by osmolytes. Such insights should provide a better understanding of the variety of physical forces by which protective osmolytes stabilize proteins in biologically realistic solutions.

We used circular dichroism (CD) spectroscopy to determine the free energy of folding, $\Delta G^0_{D\rightarrow N}$, of the model peptide in the presence and absence of different concentrations of polyols and carbohydrates, acting as small molecular osmolytes. The uniqueness of this model peptide, first designed and studied by Searle and co-workers,[5] is its marginal stability in aqueous solutions ($\Delta G^0_{D\rightarrow N} \approx 0$ at T = 298 K, and pH 7), so that even small shifts in its thermodynamic equilibrium due to osmolyte addition can be sensitively gauged. We studied a range of osmolytes that differ not only in the number of hydroxyl groups (varying from 3 for glycerol to 8 for trehalose) but also in structure (isomers).

To determine $\Delta G^0_{D\rightarrow N}$ we follow the method developed and extensively validated by Searle *et al.*[5] We further extended and verified the procedure's applicability in determining the peptide folding free energy also in the presence of added sugars and polyols (see ESI† for experimental details). The isodichroic point at wavelength 209 nm, seen for all osmolytes tested, supports a two-state model for folding (see Fig. S1a, ESI† for spectra taken at several trehalose concentrations). Using this model, we can determine $\Delta G^0_{D\rightarrow N} = -RT\ln([N]/[D])$, where [N] and [D] represent the concentrations of native and non-native structures, T is absolute temperature, and R the ideal gas constant, as described in the ESI† and in ref. 5.

To further quantify the effect of osmolytes on peptide stability, we follow $\Delta\Delta G$, defined as the difference between $\Delta G^0_{D\rightarrow N}$ measured in water and its value obtained in the presence of osmolytes. Fig. 1a shows that for all osmolytes tested, $\Delta\Delta G$ varies linearly with Osmolal concentration. Furthermore, while all osmolytes tested enhance β-hairpin stability, the variation in $\Delta\Delta G$ generally grows with osmolyte size. Previous studies that determined the effect of cosolutes on several proteins found a similar dependence of $\Delta\Delta G$ on osmolyte concentration and size.[6] The linearity of folding free energy with osmolyte concentration implies a constant change in the number of solute-excluding water molecules

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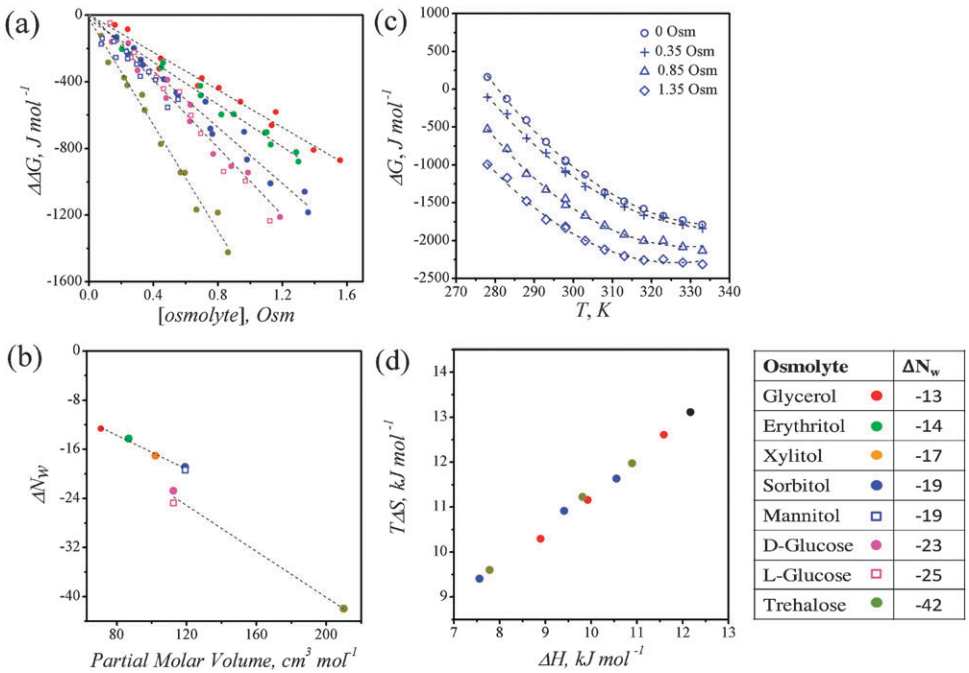

Fig. 1 Effect of osmolytes on β-hairpin stability. (a) Folding free energy, $\Delta\Delta G_{D\rightarrow N}$, *versus* osmolyte concentration at T = 298 K for different polyols and sugars. (b) Preferential hydration upon folding, ΔN_W, *versus* osmolyte partial molar volume. Dashed lines are guides to the eye. (c) Peptide folding free energy, ΔG, *versus* temperature, T, at different sorbitol concentrations (see Fig. S4, ESI† for similar plots for glycerol and trehalose). (d) Entropy–enthalpy plot for folding in the presence of glycerol, sorbitol and trehalose at different concentrations of each. Values of ΔH and ΔS are derived from the free energy dependence on temperature. Data plotted for water (in black); glycerol concentrations of 0.49, 0.94 and 1.44 Osm; sorbitol: 0.38, 0.85, 1.35 Osm; trehalose: 0.25, 0.54 and 0.87 Osm. For all panels errors in ΔG are expected to be on the order of 10% of the measured value (see ESI† for details).

(preferential hydration) upon folding, ΔN_W. This link can be expressed as

$$\Delta N_W = -\frac{\partial\Delta\Delta G}{\partial\mu_W} = \frac{55.6}{RT}\frac{\partial\Delta\Delta G}{\partial[\mathrm{Osm}]} \qquad (1)$$

where μ_W is the water chemical potential, 55.6 is the number of moles of water in 1 kg, and [Osm] represents the solute osmolal concentration, a measure of water chemical potential.[7] Note that we can equally speak about ΔN_W or $\Delta\Gamma_S$, the difference in the osmolyte preferential interaction coefficient between the D and N states, because the two differences are necessarily related.[2] We focus our discussion on ΔN_W, which remains constant for each osmolyte over the range of concentrations studied. We evaluate ΔN_W for each osmolyte (table in Fig. 1) from the slope in Fig. 1a, according to eqn (1). Fig. 1b shows that ΔN_W grows approximately linearly with partial molar volume inside of each group, polyols and carbohydrates. This observation is consistent with the fact that larger osmolytes are more strongly excluded from the peptide surface. Moreover, the two stereoisomers, sorbitol and manitol, demonstrate no significant difference in their exerted effect, further supporting the trend of size dependent exclusion (as determined, *e.g.*, by molar volumes or number of hydroxyl groups).

In addition, we tested the effect of the two enantiomers L- and D-glucose on the change in $\Delta\Delta G$. Because water is achiral, any difference in the action of these osmolytes should necessarily be attributed to solute–peptide interactions. Fig. 1a shows, however, that the two isomers have no detectable difference in their effect on $\Delta\Delta G$. This insensitivity of peptide folding to the enantiomer used is consistent with the finding that osmolytes are excluded from the peptide interfaces, and that osmolyte–peptide interactions are probably rare. Osmolytes, therefore, seem to act from within the aqueous environment and not through direct (binding) interactions with the peptide.

To further dissect $\Delta\Delta G$ into enthalpic and entropic contributions, we used the ellipticity at 215 nm to follow the temperature dependence of hairpin stability in the presence of glycerol, sorbitol, and trehalose at various concentrations of each (see ESI† and ref. 5 for details). Fig. 1c shows the variation of folding free energy ΔG with temperature, in the presence of different sorbitol concentrations. We determined ΔH, ΔS and ΔC_P for folding at T = 298 K by fitting experimental ΔG values to $\Delta G = \Delta H + \Delta C_P(T - 298) - T[\Delta S + \Delta C_P \ln(T/298)]$. Although this expression implies ΔC_P is temperature independent, the same results (within experimental errors) were also found when this assumption was relaxed.

Nonlinear least square fits, summarized in Table 1, verify that in the absence of osmolytes the β-hairpin conformation is entropically stabilized (ΔS = 44.0 J mol^{-1} K^{-1}) and that folding is endothermic (ΔH = 12.2 J mol^{-1}), as previously found.[5] Positive ΔS and ΔH, as well as negative ΔC_P, are often interpreted as signatures of hydrophobic interactions that drive burial of nonpolar residues by increasing the accessible

Table 1 Osmolyte effect on the thermodynamic parameters of peptide folding[a,c]

Solvent	ΔH/kJ mol^{-1}	ΔS/J K^{-1} mol^{-1}	ΔC_P/J K^{-1} mol^{-1}
Aqueous solution	12.1	44.0	−354
Glycerol[b]	9.9	37.4	−330
Sorbitol[b]	8.8	35	−340
Trehalose[b]	7.4	31	−340

[a] At pH 7 and 298 K. [b] Values evaluated for 1 Osm of osmolyte. [c] Errors are estimated to be within 10% of the measured value (see ESI†).

states of water, and by contributing to weakened hydrogen bonding near the folded *versus* unfolded states.[8] The small differences between the values determined using solution NMR at pH 5.5[5] and this study can be attributed to the differences in pH, as well as to the CD *versus* NMR-based methods used to follow the population of the folded state.

For water–osmolyte mixtures, Table 1 shows that β-hairpin stability increased in the presence of osmolytes, primarily due to a decreased enthalpy loss, so that folding is less endothermic than in water. Remarkably, the favourable entropy for folding in water is *diminished* by added solutes, a mechanism that cannot be explained by steric crowding. Plotting the entropy *versus* enthalpy changes (Fig. 1d) well fits linear "entropy–enthalpy" compensation with a slope of 0.79, reflecting the strong osmolyte-induced enthalpic driving force to folding. Other studies have shown that alcohols induce similarly strong enthalpic driving forces in DNA condensation,[9] and that the transfer enthalpy of an amide unit from water to solutions of another protective osmolyte, TMAO, is strongly unfavourable.[10] Finally, simulations of short hydrophobic polymers in solution have shown a reduction in the positive enthalpic contribution to the polymer's folding transition due to TMAO.[11]

Interestingly, we further found that the folding enthalpy loss decreases with osmolyte size, with a corresponding loss in folding entropy. We suggest that an important contribution to these changes could be water restructuring in the presence of osmolytes that has also been shown to be related to increases in solute size.[8]

The decrease in enthalpy loss could be a result of an increase in the number of hydrogen bonds or to an increase in their strength for the N *vs.* D states in the presence of osmolytes compared to their value in water. An additional enthalpic contribution could be due to variations in van der Waals forces acting in modified N and D states, or to the lowered dielectric constant (ε) of osmolyte–water solutions that may strengthen attractive electrostatic interactions between the C-terminal carboxylate group and N-terminal lysines. However, taking the experimentally estimated energetic contribution of ion pairing interactions within this peptide,[5] and the small decrease in ε typically caused by the addition of polyols,[12] we believe that the effect of these osmolytes on electrostatic interactions is only secondary.

In summary, our study shows that osmolytes exert stabilizing forces on peptides that go beyond what simple steric exclusion would predict. These effects should become significantly stronger for protein folding, where the chain length is much longer, and hence the exposure to osmolytes is larger. This force must be considered together with excluded volume type interactions that also necessarily exist. It is interesting to determine how these additional enthalpic contributions we find are related to protein and osmolyte properties and structure. Further work is required to link these to the way such cosolutes affect water structuring in solution and near macromolecules.

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‡ The peptide sequence is AcKKYTVSINGKKITVSI.

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