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Effect of osmolytes on the thermal stability of proteins: replica exchange simulations of Trp-cage in urea and betaine solutions.

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Abstract

Although osmolytes are known to modulate the folding equilibrium, the molecular mechanism of their effect on thermal denaturation of proteins is still poorly understood. Here, we simulated the thermal denaturation of a small model protein (Trp-cage) in the presence of denaturing (urea) and stabilizing (betaine) osmolytes, using the all-atom Replica Exchange molecular dynamics simulations. We found that urea destabilizes Trp-cage by enthalpically-driven association with the protein, acting synergistically with temperature to induce unfolding. In contrast, betaine is sterically excluded from the protein surface thereby exerting entropic depletion forces that contribute to the stabilization of the native state. In fact, we find that while at low temperatures betaine slightly increases the folding free energy of Trp-cage by promoting another near-native conformation, it protects the protein against temperature-induced denaturation. This, in turn, can be attributed to enhanced exclusion of betaine at higher temperatures that arises from less attractive interactions with the protein surface.

1 Introduction

Betaine and urea are examples of osmolytes, small organic co-solvents, which are generally known to shift the folding equilibrium toward the folded or unfolded states, respectively(56; 14; 48; 39).

It is commonly believed that the destabilizing effect of urea on proteins results mostly from its favorable interactions with the protein surface (8; 58; 16; 7). These favorable interactions are usually explained by the propensity of urea to form hydrogen bonds, especially with the backbone amide and carbonyl groups(8; 50; 1) or by favorable dispersion interactions, particularly with the protein side chains(36; 59; 29; 66; 1). Since the solvent-exposed surface is typically greater in unfolded compared to folded proteins, preferential interactions with the protein surface lead to stabilization of the unfolded state. In addition, urea was found to diminish the hydrophobic effect and thereby to promote solvation of non-polar residues(8).

In contrast, if osmolyte molecules interact unfavorably with the protein surface, the folding equilibrium is shifted toward the folded state(37; 63; 64; 10; 65; 34; 13). Such unfavorable interactions, resulting in effective exclusion from the protein surface, were shown for betaine and similar osmolytes, using transfer free energy measurements(21; 20; 6; 7; 24). Although extensively studied, it is less clear why stabilizing osmolytes (also termed osmoprotectants) are excluded from the protein surface. When modeled by hard-core steric interactions, the osmolyte exclusion appears to be purely entropic in nature(5). Recently, however, this excluded-volume model was extended by including into the effective protein-osmolyte interaction potential the enthalpic component that might also contribute to the observed exclusion(52; 53). Additionally, stabilizers such as TMAO and betaine can also have an increased ability to bind water, which reduces its availability to solvate the protein.(9; 38; 2).

It has been long known that osmolytes alter the folding equilibrium of proteins at room temperatures, however, the temperature dependence of this effect is less understood(60; 47; 35; 4). While it has been found that denaturing action of urea is generally enhanced by temperature(45; 3), the effect of temperature on protein stabilization by osmoprotectants is more complex (47; 4; 49). In particular, it has been observed that at room temperatures betaine and similar protectants (sarcosine, glycine and proline) usually act as relatively weak stabilizers and may even slightly destabilize some proteins. However, as temperature increases, their stabilizing effect is also markedly enhanced (e.g., the effect of betaine on the unfolding free energy of cytochrome c changes from $\approx$0 kcal/mol at 293 K to 0.6 kcal/mol at 353 K)(47; 4). From a biological perspective, this temperature dependence may allow for additional protection against the thermal stress without strongly perturbing the folding equilibrium at normal

temperatures (4; 19). Contrary to that, other studies found that stabilizing effect of β-hydroxyectoine, sorbitol and betaine on RNase A increases only slightly (22; 35) while that of arginine in fact decreases with increasing temperature(60).

The molecular mechanism behind the observed temperature dependence of the osmolyte effect on protein stability is not fully understood(55; 16). Molecular dynamics simulations suggested that at low temperatures the urea-induced denaturation is dominated by Lennard-Jones interactions between the osmolyte and protein surface(16). However, with increasing temperature, electrostatic urea-protein interactions become effectively more attractive, thereby leading to enhanced protein destabilization at higher temperatures. Electrostatic interactions between protein and water apparently also become more favorable which further stabilizes the unfolded state and contributes to protein destabilization (16). Even less is known on how temperature affects protein stabilization by protecting osmolytes, such as betaine and TMAO. The mechanistic studies on these stabilizers are challenging also because their effect on folding equilibrium is known to be protein-specific and has been shown to depend on multiple factors, including osmolyte concentration and pH(35; 4; 54). For example, 1 M betaine has been found to stabilize lysozyme and myoglobin, whereas the opposite is the case for cytochrome c(4). According to the study by Singh et al.(54), betaine molecules stabilize RNase A at neutral pH (6–8) while having destabilizing effect on α-lactalbumin(54) at a pH range of 4–4.5.

In this work, we investigate the molecular mechanism underlying the effect of urea and betaine on thermal denaturation of a small model protein (Trp-cage) using the all-atom Temperature Replica Exchange Molecular Dynamics (T-REMD) simulations. We find that while urea acts synergistically with temperature to promote protein unfolding, betaine protects the protein against temperature-induced denaturation. Our data clearly show that destabilization by urea arises from its enthalpy-driven accumulation at the protein surface, whereas protective effect of betaine is due to its entropically favored exclusion from the surface. In fact, betaine slightly increases the folding free energy at low temperatures by promoting an additional near-native structure, however, as temperature increases, it begins to markedly shift the equilibrium towards the folded state. This, in turn, results from the enhanced exclusion of betaine from the protein surface that can be attributed to weaker osmolyte-protein interactions at higher temperatures.

Methods

Molecular systems and simulation procedure

Initial coordinates of the 20 residue Trp-cage protein (the TC5b variant, NLYIQ WLKDG GPSSG RPPPS) were taken from the NMR structure (PDB code 1L2Y) (inset in Fig. 1A). Significant stability of the TC5b variant of Trp-cage(43), its small size, and fast folding kinetics make it an attractive model for protein folding studies (17; 33; 16; 18; 46). Ionizable side chains of Trp-cage were kept at standard protonation states at pH 7 and both termini were left uncapped (charged). In each system, the protein was solvated with sTIP3P water molecules(32) and a proper number of osmolyte molecules in a dodecahedron box with at least 1.2 nm from the solute to the nearest box edge. Five different solutions were simulated: 3.5 and 5.0 M urea, 1.5 and 3.5 M betaine and the osmolyte-free system (see Table S1 in Supporting Information for detailed compositions of the individual systems and comparison with experimental densities, and Fig. S1 for chemical structures of osmolytes). The above molar concentrations refer to the solution only excluding the volume occupied by the protein (the corresponding mole fractions are given in Table S1). Cl^- and Na^+ ions were added to neutralize the system and achieve physiological ionic strength (150 mM NaCl). The CHARMM36 force field with the backbone CMAP correction was used for the protein and ions (12). This choice was dictated by the fact that CHARMM36 allows for better reproduction of the experimental temperature dependence of secondary structure formation compared to other recent force fields (11). The force field parameters for urea were taken from the CHARMM General Force Field (CGenFF) (62) and for betaine we used the previously validated parameter set obtained by analogy from the CHARMM parameters for phospholipid headgroups (trimethylammonium group from phosphatidylcholine; carboxylic group from phosphatidylserine)(1).

Each system was equilibrated using a 10 ns NPT MD simulation at 300 K and 1 atm, which resulted in an initial set of folded configurations of Trp-cage for subsequent T-REMD simulations. For each REMD simulation, 50 replicas ranging from 300 to 510 K were used, and the intermediate temperatures

were chosen such as to ensure uniform exchange rates (∼10%) with exchange attempts every 2 ps. Each replica was simulated for 600 ns, yielding a total time of 30 μs for each system. All MD simulations were run using Gromacs 4.6.5(27) in the NPT ensemble. Periodic boundary conditions were applied, and electrostatic interactions were calculated using the particle mesh Ewald (PME) method with a real-space cutoff of 1 nm and a Fourier grid spacing of 0.12 nm. A cut-off of 1 nm was used for Lennard-Jones interactions. Bond lengths were constrained using P-LINCS(26) for the protein and SETTLE(41) for water. The equations of motion were integrated using the leap-frog algorithm with a 2 fs time step. All molecular images were created using VMD(30).

Thermodynamics of protein folding

To examine the thermodynamic stability of the Trp-cage protein, we used replica exchange molecular dynamics (REMD) method, which allows for better sampling of the folded and unfolded states and thus for examining the temperature-dependence of the folding equilibrium. To determine the folding free energy, for each system, the melting curve $f(T)$, representing the folded population as the function of temperature, was calculated using trajectories from the last 500 ns of REMD simulations (based on the convergence analysis in the Fig. S3). All structures with the root-mean-square deviation (RMSD) below 0.25 nm calculated for all C_α atoms with respect to the NMR structure were counted as the folded configurations. The obtained $f(T)$ curves were then converted to the folding free energies according to the equation:

$$\Delta G(T) = -RT \ln\left(\frac{f(T)}{1-f(T)}\right) \tag{1}$$

Additionally, by assuming that heat capacity change upon folding, ΔCp, is independent of temperature, we fit the computed $\Delta G(T)$ to the analytical expression

$$\Delta G(T) = \frac{T_m - T}{T_m}\Delta H(T_m) + \Delta C_p(T - T_m) + T\Delta C_p \ln\left(\frac{T_m}{T}\right) \tag{2}$$

for a two-state folding process. From this expression, ΔC_p, transition temperature, T_m, and the folding enthalpy at T_m, $\Delta H(T_m)$, were determined as fitting parameters and compared with experimental data. The melting point, T_m, is defined here as the temperature for which $\Delta G(T) = 0$, i.e., the folded and unfolded states are equally populated.

Alternatively, thermodynamic parameters for the Trp-cage folding transition were obtained from the simulated $\Delta G(T)$ data in a model-free fashion by numerically computing the following derivatives at the melting point T_m:

$$\Delta H(T_m) = -T_m \left.\frac{d\Delta G(T)}{dT}\right|_{T=T_m} \tag{3}$$

$$\Delta C_p(T_m) = \left.\frac{d\Delta H(T)}{dT}\right|_{T=T_m} = -T_m \left.\frac{d}{dT}\left(\frac{d\Delta G(T)}{dT}\right)\right|_{T=T_m} \tag{4}$$

$$\Delta S(T_m) = \frac{\Delta H(T_m)}{T_m} \tag{5}$$

Thermodynamics of protein-osmolyte interaction

To investigate the effect of temperature on preferential interaction between osmolytes and Trp-cage, we calculated the free energy profiles as a function of temperature $\Delta G(r,T) = -kT \ln g(r,T)$, where $g(r,T)$ is the minimum-distance distribution function between the osmolyte and Trp-cage, computed at temperature T. The enthalpic (ΔH) and entropic ($-T\Delta S$) contributions to the association free energy (ΔG) were obtained by fitting the simulation free energy data with the formula

$$\begin{aligned}\Delta G(r,T) = \Delta H(r,T_0) + \Delta C_p(r)(T - T_0)\\ -T\left[\Delta S(r,T_0) + \Delta C_p(r)\ln\left(\frac{T}{T_0}\right)\right]\end{aligned} \tag{6}$$

where the two fitting parameters, $\Delta H(r, T_0)$ and $\Delta S(r, T_0)$, are the enthalpic and entropic components of the free energy profile at the reference temperature T_0, and the third fitting parameter, ΔC_p, is the corresponding heat capacity change, assumed to be independent of the temperature. Accordingly, the temperature dependence of enthalpic and entropic contributions was obtained as follows:

$$\Delta H(r, T) = \Delta H(r, T_0) + \Delta C_p(r)\,(T - T_0) \tag{7}$$

$$\Delta S(r, T) = \Delta S(r, T_0) + \Delta C_p(r) \ln\left(\frac{T}{T_0}\right) \tag{8}$$

Results and Discussion

Betaine- and urea-induced shift of the Trp-cage folding equilibrium is non-uniform in temperature

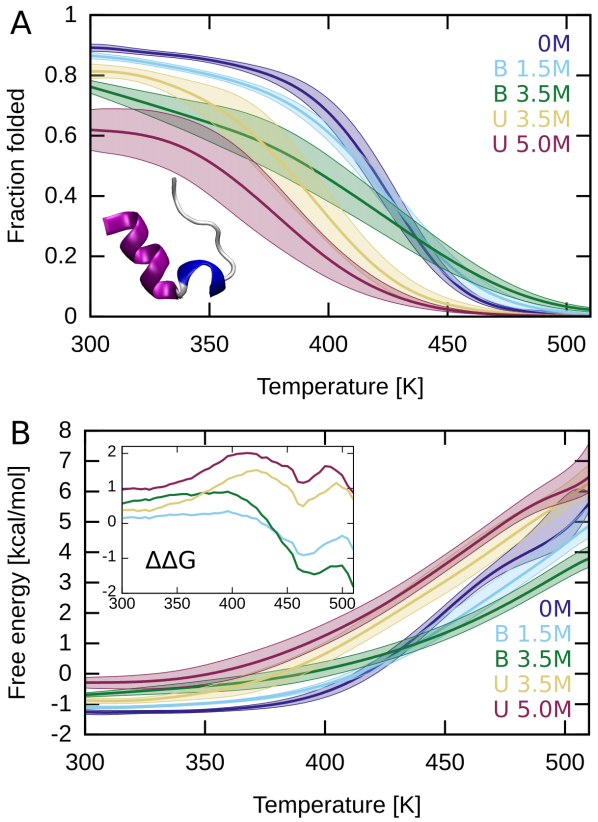

Figure 1: A, Temperature dependence of the fraction of folded Trp-cage in the urea (U) and betaine (B) solutions, as well as in pure water (0 M). Statistical uncertainties were estimated by block-averaging with the autocorrelation times of the fraction folded found to be 10–30 ps and 2–80 ns using a double-exponential fit (25). It is seen that urea destabilizes Trp-cage in the entire temperature range, while betaine enhances stability at higher temperatures. B, Temperature dependence of the folding free energy of Trp-cage calculated from the folded fraction shown in panel A. Inset shows the difference in the folding free energies between a given osmolyte solution and pure water.

To investigate the effect of osmolytes on the thermal stability of Trp-cage, we first calculated how the fraction of folded protein depends on temperature in two urea (3.5 M and 5.0 M) and two betaine (1.5 M and 3.5 M) solutions, as well as in the absence of osmolytes (0 M). To this end, all conformations of Trp-cage in the generated REMD ensemble, for which C_α-RMSD to the NMR structure was below 0.25 nm, were assigned to the folded state (probability distribution of the RMSD and convergence of the conformational sampling in simulations are shown in Fig. S2 and Fig. S3, respectively).

Fig. 1A shows that for the pure water and urea-containing systems the folded fraction depends on temperature in a sigmoidal manner indicating two-state folding equilibrium (see also Fig. S4), similarly to what was found previously for Trp-cage and other two-state folders (57; 31). At physiological temperature (300 K), Trp-cage is predominantly folded in pure water, with the equilibrium folded population of 89 %, in a good agreement with the experimental data(44). Urea, a potent denaturing agent, causes partial protein unfolding, by decreasing the fraction of the folded state at 300 K to 81 and 62 % for 3.5 and 5.0 M solution, respectively. Interestingly, at this temperature, betaine also partially destabilizes Trp-cage, reducing the fraction folded to 87 and 76 % for 1.5 and 3.5 M solution, respectively. However, betaine present as co-solvent reduces the extent of unfolding upon temperature increase which, beyond ca. 425 K, leads to higher fractions of the folded state compared to the osmolyte-free system. In contrast, urea acts synergistically with thermal stress, especially at intermediate temperatures (370–450 K).

Consistently, Figure 1B shows that 5.0 M urea destabilizes Trp-cage by at least 1 kcal/mol for all temperatures, with the largest change of the folding free energy ($\Delta\Delta G$) occurring in the 370–450 K range (inset in Fig.1B). On the other hand, 3.5 M betaine decreases the stability of Trp-cage by up to 0.6 kcal/mol at lower temperatures, but above 425 K begins to stabilize the folded state relative to pure water, with $\Delta\Delta G$ increasing to -1.6 kcal/mol.

To more quantitatively characterize the effect of osmolytes on the thermal denaturation of Trp-cage, we determined thermodynamic parameters of this process, by fitting the calculated free energy profiles in Fig.1 to the equation 2 (see Methods).

These parameters, shown in Table S2, in particular folding enthalpy $\Delta H(T_m)$ and heat capacity change upon folding ΔC_p, are in a fairly good agreement with the experimental values. However, the comparison of the fraction folded obtained directly from the simulation data (Fig. 1A) with the analytical curves (eq. 2) in Fig. S4, indicates some deviations from the applied two-state model that are particularly pronounced for both betaine solutions. Therefore, to obtain more accurate estimates of the thermodynamic folding parameters, we also employed a more direct approach by numerically evaluating derivatives eq. 3 and eq. 4 at the melting point T_m inferred from Fig. 1A. As expected, relative differences between the parameters obtained in this model-free approach and experimental values are smaller than those resulting from the two-state model fitting, which additionally justifies the applied force field model and strengthens our predictions.

Betaine, in contrast to urea, stabilizes the Trp-cage structure against thermal denaturation.

To check how osmolytes modulate the effect of temperature on the Trp-cage structure, we computed the equilibrium distributions for a number of geometric descriptors, including secondary structures (Fig. 2 and Fig. S5), radius of gyration (Fig. S6), intramolecular contacts (Fig. S7) and hydrogen bonds (Fig. S8), in the entire temperature range considered.

Comparison of the secondary structure probabilities between the osmolyte solutions and pure water (Fig. 2) clearly shows that urea disrupts the local structure of the protein causing its almost complete loss at higher temperatures, as can also be seen from the most populated structural clusters of Trp-cage in Fig. S9. The only region of the protein that is resistant to urea- and temperature-induced denaturation is the 3–9 portion of the α-helix, in agreement with the previous computational studies on the denaturation of Trp-cage(23; 51; 40). Consistently with its disruptive effect on secondary structure, urea also increases the radius of gyration of the protein (Fig. S6) and reduces the number of intramolecular contacts and hydrogen bonds (Fig. S7 and Fig. S8), compared to these observed in pure water. Thus we conclude that at all temperatures addition of urea shifts the equilibrium further towards the unfolded conformational ensemble (see also Fig. S9).

Fig. 2 also clearly indicates that, regardless of the temperature, the secondary structure of Trp-cage in the presence of 3.5 M betaine is largely similar to this observed in pure water. The distribution of the C_α-RMSD to the NMR native state (Fig. S2) further indicates that the apparent increase in the folding free energy of Trp-cage caused by betaine at low temperatures stems from the osmolyte-induced stabilization of an additional near-native structural state with RMSD peak around 0.28 nm. Indeed, cluster analysis reveals the existence of the other populated structural state similar to the dominant native structure, with probabilities 18 and 20 % in the 1.5 and 3.5 M betaine solutions, respectively (see gray boxes in Fig. S9 and comparison with the native state in the Fig. S10). The appearance of

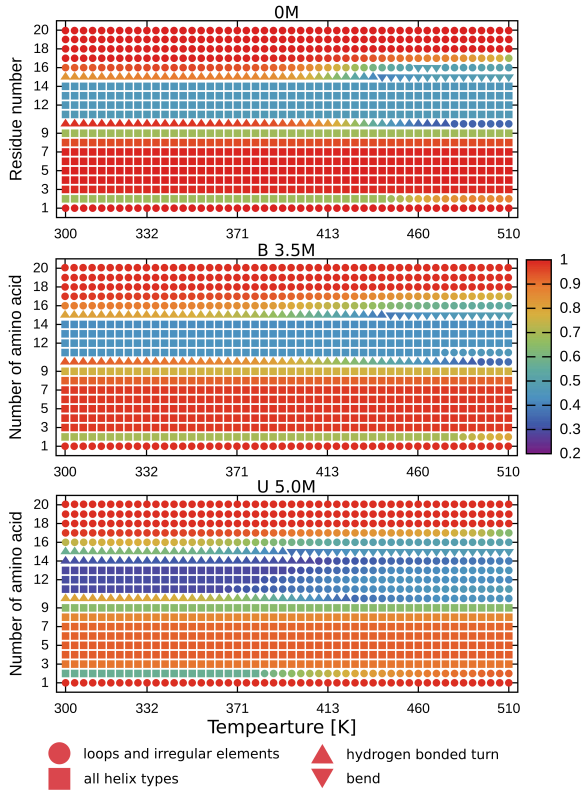

Figure 2: Probability of the most likely secondary structure adopted by two consecutive Trp-cage residues in pure water (0M), 3.5 M betaine (B 3.5M) and 5.0 M urea (U 5.0M), as a function of temperature. Shapes indicate the most likely secondary structure at a given temperature calculated by DSSP program(61). The analysis reveals urea-induced disruption of the secondary structure over the entire temperature range. In contrast, betaine does not largely affect the secondary structure at low temperatures and stabilizes it slightly against temperature denaturation (see, e.g., residues 2 and 9–10).

additional stable states might account for the above-mentioned inadequacy of a two-state model for the folding transition in the presence of betaine. Our predictions coincide with previously reports that betaine, present at low concentrations, causes misfolding of GST-GFP fluorescent protein(42). Further, from probability distributions of geometric descriptors (Fig. S6–S8), it can be seen that while at low temperatures betaine does not significantly affect the overall structure of Trp-cage, it begins to protect the native state when temperature increases, by promoting more compact structures containing more native contacts than found in pure water.

Exclusion of betaine is enhanced and accumulation of urea suppressed by temperature

Next, we sought to identify the molecular determinants of the observed effect of osmolytes on the thermal denaturation of Trp-cage. Since, it is commonly believed that the stabilizing or destabilizing effect of osmolytes on the protein structure results from their attractive (favorable) or repulsive (unfavorable) interactions with the protein, we examined whether urea and betaine are preferentially excluded from or accumulated at the Trp-cage surface, and how these preferential interactions vary with temperature.

For this purpose, we calculated the preferential interaction coefficient Γ_c, defined as follows:

$$\Gamma_c(r,T) = \langle N_o(r)f(T) - \frac{N_o^b}{N_w^b} N_w(r)f(T)\rangle, \tag{9}$$

where $N_o(r)$ and $N_w(r)$ are the number of osmolyte and water molecules within a distance r from

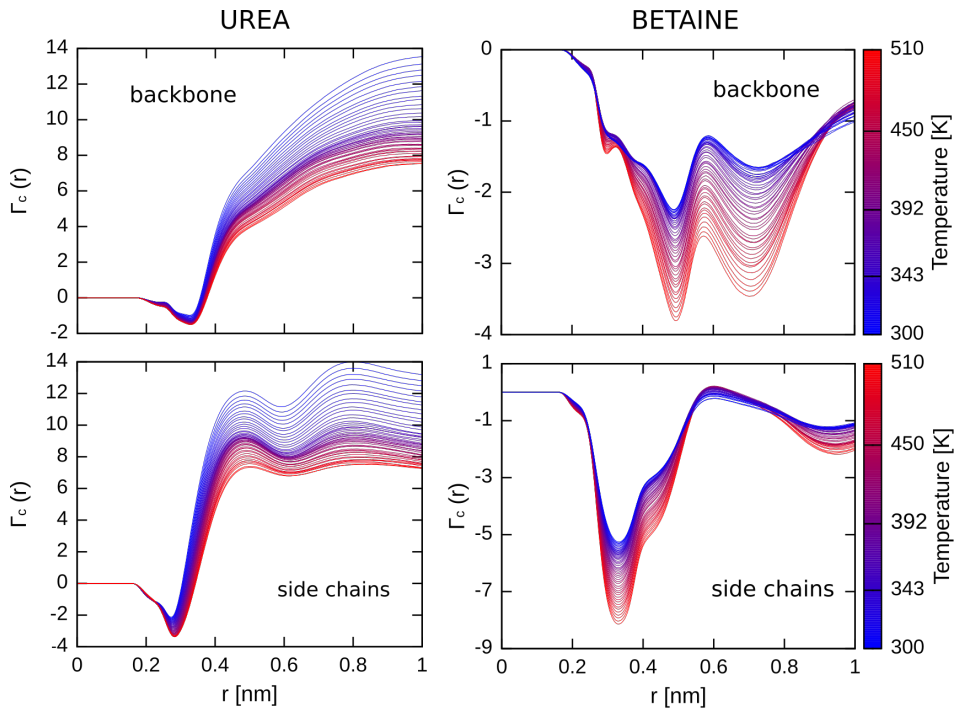

Figure 3: Preferential interaction coefficient (Γ_c) between the osmolyte and Trp-cage, calculated for 5 M urea and 3.5 M betaine solutions, as a function of temperature and the separation distance from the protein surface. The protein backbone and side chains were treated separately. Decreasing values of Γ_c show that betaine is more strongly excluded from and urea less accumulated at the protein surface with increasing temperature.

the protein, respectively, and N_o^b and N_w^b are the number of osmolyte and water molecules in bulk solvent. Since the volume of the simulated systems increases markedly with the temperature (see Fig. S11 for the $V(T)$ curves), affecting the observed Γ values, to disentangle the volume effect from the actual temperature-induced changes in preferential interaction, a correction factor $f(T) = \frac{V(T)}{V_0}$ has been introduced to the standard expression for $\Gamma(r)$, where V_0 is the volume of the simulation box at the lowest temperature. $\langle\rangle$ denotes averaging over an MD trajectory at a given temperature. By construction, positive values of Γ_c indicate preferential accumulation of osmolyte or exclusion of water from the protein surface, whereas negative values represent the exclusion of osmolyte or accumulation of water.

Fig. 3 shows the temperature dependence of the preferential interaction coefficients computed in 3.5 M betaine and 5.0 M urea as a function of the distance from the protein, separately for the backbone and side chains. For the urea solution, it can be seen that Γ_c is positive in almost the entire distance range considered (except for very short distances from the protein where water molecules are preferred due to their smaller size), indicating accumulation of the osmolyte at the protein surface, which is typical for denaturants.(15; 1) Moreover, accumulation of urea around side chains is slightly greater than around the backbone, which is also consistent with some previous reports (29; 66; 15; 1). Importantly, the amount of urea accumulated at the surface of Trp-cage is markedly decreased with increasing temperature, suggesting the enthalpic nature of the observed accumulation.

In contrast, negative values of Γ_c obtained for betaine indicate that the osmolyte molecules are effectively repulsed by the Trp-cage surface, with the exclusion being most pronounced in the immediate vicinity of the protein side-chains ($\Gamma_c = -8.2$ at T = 510 K for r = 0.36 nm). These findings are in general agreement with the transfer free energies and local bulk partition coefficients obtained from osmometric measurements (6; 28) that showed exclusion of stabilizing osmolytes, and betaine in particular, from the protein surface. It can be also seen that the number of betaine molecules present at the protein surface decreases with temperature, with the exclusion from the protein backbone increasing more rapidly. Therefore, our results imply that the observed enhancement in betaine-induced stabilization in response to high temperature is related to its stronger exclusion from the

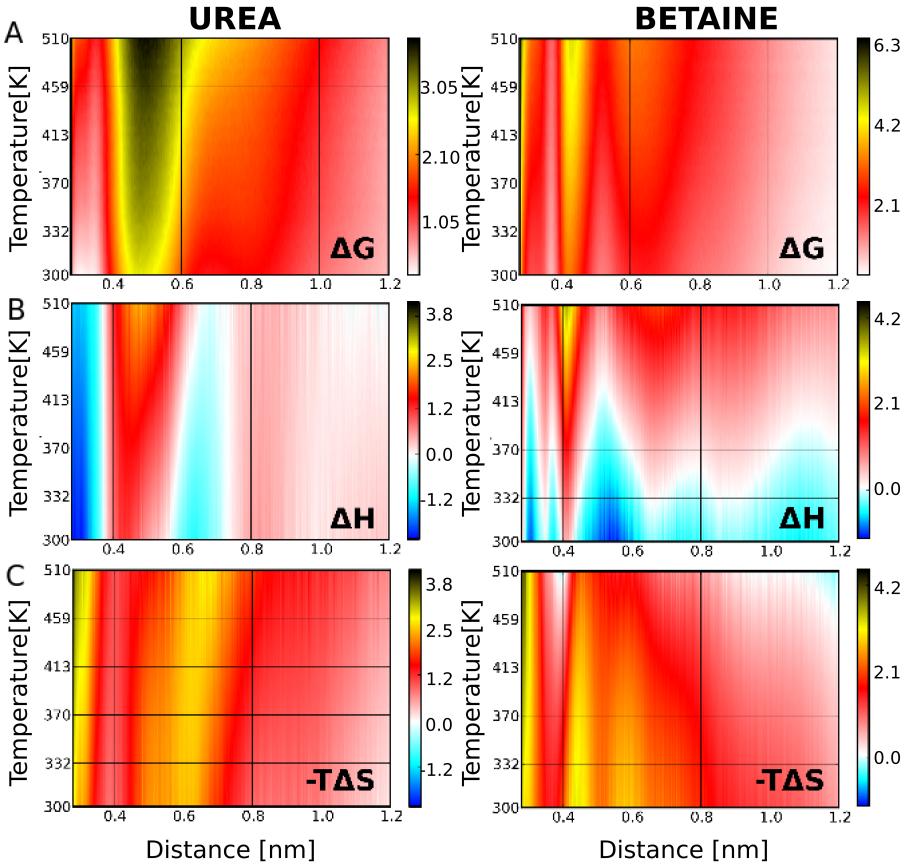

Figure 4: Thermodynamics of protein-osmolyte interaction. A, Temperature dependence of the free energy profile (in kJ/mol) for the association of the osmolyte with Trp-cage in 5 M urea and 3.5 M betaine systems BC, Enthalpic (ΔH) and entropic ($-T\Delta S$) contributions to the free energy profiles shown in panel A. Free energy decomposition shows that main enthalpic driving force for the association of osmolytes with Trp-cage diminishes with temperature.

surface of Trp-cage, according to the widely accepted mechanism of action of protein stabilizers(47; 22; 35; 4; 19).

Enthalpic driving force for the association of urea and betaine with Trp-cage decreases with temperature

To understand why temperature increases exclusion of betaine and reduces accumulation of urea at the protein surface, we first calculated how the association free energy of both osmolytes with Trp-cage vary with temperature. Fig. 4A shows the relevant free energy profiles, calculated as $\Delta G(r,T) = -kT \ln g(r,T)$, where $g(r,T)$ is the minimum-distance distribution function between the central atoms of the osmolyte molecules (methylene carbon atom of betaine and carbon atom of urea) and Trp-cage at temperature T. Next, in Fig. 4BC, the free energy profiles were decomposed into the enthalpic (ΔH) and entropic ($-T\Delta S$) contributions, as described in Methods.

The free energy minimum in the range of 0.2–0.4 nm seen for urea in Fig. 4A is indicative of the osmolyte accumulation at the protein surface. Clearly, the depth of this minimum decreases with temperature, confirming that association of urea with Trp-cage is less favorable at higher temperatures. Similarly, for betaine, the association free energy, that is unfavorable already at 300 K, becomes even more positive with increasing temperature, indicating once again that temperature enhances exclusion of the osmolyte from the protein surface.

As can be seen from Fig. 4B, the association enthalpy of urea with the protein surface (distance up to 0.4 nm) is negative for the entire range of the studied temperatures, varying from -2.1 kJ/mol at

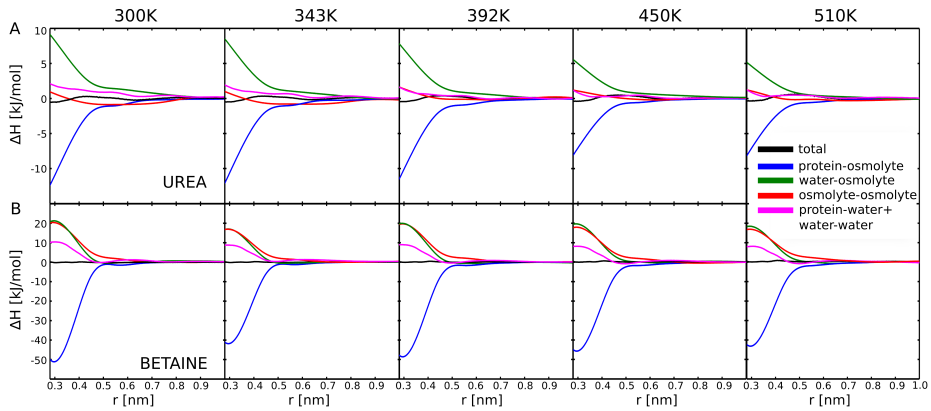

Figure 5: Decomposition of the association enthalpy between urea (A) or betaine (B) and Trp-cage (ΔH) into interaction energy changes between individual components of the systems. It can be seen that with increasing temperature, the protein-osmolyte term become less favorable, causing depletion of osmolytes at the protein surface.

$T = 300\,\mathrm{K}$ to $-0.4\,\mathrm{kJ/mol}$ at $T = 510\,\mathrm{K}$. Because the corresponding entropy contribution (Fig. 4B) is unfavorable, we find that the observed accumulation of urea at the surface of Trp-cage is enthalpically driven. Importantly, this enthalpic driving force decreases with temperature, accounting for most (ca. 70%) of the temperature-induced change in the association free energy. Consequently, it can be concluded that the reduced accumulation of urea at higher temperatures results from its weaker interactions with the protein. Indeed, Fig. S12 shows that this reduced accumulation is accompanied by a substantial decrease in the average number of hydrogen bonds between protein-associated urea molecules and the protein surface. In fact, 13 and 18% of hydrogen bonds between urea and the protein backbone and side chains, respectively are lost on average in response to the temperature increase from 300 to 510 K. Smaller loss observed in the former case is due to the marked preference of urea to form hydrogen bonds with the backbone amide groups that become exposed upon thermal unfolding. Concomitantly, van der Waals interactions between urea and the backbone and side chains also become weaker (by 10 and 13%, respectively), additionally contributing to the decreased association driving forces (Fig. S13).

Fig. 4B reveals that enthalpically-driven attraction between the protein surface and betaine is noticeably less pronounced than for urea, however, at low temperatures, the association of betaine with Trp-cage is also marginally enthalpically favorable. Two distinct enthalpy minima, a narrower at 0.3 nm and a broader one at 0.5–0.6 nm, can be attributed to two preferred orientations of betaine, with the carboxylic or trimethylammonium group, respectively, oriented towards the protein surface. Because even at low temperatures, the enthalpic gain due to betaine-protein association is minor (> -0.4 kJ/mol), we find that the observed effective repulsion of betaine from the protein surface is dominated by the unfavorable entropic contribution (up to 2.5 kJ/mol), consistently with the steric model of osmolyte exclusion.(53)

However, since at higher temperatures the association of betaine with Trp-cage becomes entropically less costly (Fig. 4C), it is clear that the temperature-enhanced exclusion of betaine is due to relatively quickly increasing enthalpic term that eventually becomes unfavorable (Fig. 4B). Similarly as for urea, the observed increase in ΔH can be at least partly attributed to a reduced hydrogen bonding interaction between betaine and Trp-cage. Indeed, Fig. S12 shows that the total number of hydrogen bonds decreases by 6%, as a result of lost interactions between betaine and the protein side chains (a decrease from 0.60 to 0.52 bonds per molecule upon temperature change from 300 to 510 K). Regardless of this general tendency, a slight increase in the number of hydrogen bonds with the protein backbone (from 0.09 to 0.12 bonds) is due to exposure of the backbone amide sites during thermal unfolding. At the same time, both electrostatic (Fig. S14) and van der Waals (Fig. S13) interactions between betaine and Trp-cage also become significantly weaker, contributing to the unfavorable change in ΔH.

Weaker protein-osmolyte interactions are responsible for temperature-induced depletion of osmolytes at the protein surface

To investigate in more detail the molecular determinants of decreased accumulation of urea and enhanced exclusion of betaine at higher temperatures, we decomposed the enthalpy for association of both osmolytes with Trp-cage into individual energetic contributions, by averaging the appropriate potential energy terms over the trajectories at various temperatures. In Fig. 5, these contributions are shown as a function of the separation distance between the osmolyte molecules and the protein surface for 5 different temperatures.

As can be seen from Fig. 5, the overall association enthalpies (ΔH, in black) are the result of the compensation of large contributions of opposite signs. Notably, in both solutions, the association of the osmolyte is enthalpically favorable due to the attractive interaction between the protein and osmolyte. This favorable interaction, in turn, is almost completely compensated by the loss of interactions between the osmolyte and water as well as between the protein and water (note that the water-water contribution is expected to be favorable, as the association of an osmolyte molecule with the surface releases a number of water molecules to the bulk solvent). Furthermore, changes in the average number of hydrogen bonds formed by an osmolyte molecule with the distance from the protein surfaces (Fig. S15) confirm the above compensation, showing a partial replacement of h-bonds to water with h-bonds to the protein. In fact, bringing a betaine molecule from the bulk to the position near the protein surface is associated with only a slight dehydration (20% decrease in the number of h-bonds with water) that is fully compensated by new h-bonds with hydrogen-donating groups of Tpr-cage. As it has been argued previously, this strong preference of betaine to interact with water may be the main reason for its exclusion from the immediate vicinity of a protein (1; 13). In turn, for urea, which can act as both a hydrogen-bond acceptor and a donor, Fig. S15 reveals a much more pronounced replacement of water (up to 50%). Therefore, the ability to exchange water for the polar groups of a protein appears to be crucial for the accumulation at the protein surface.

Fig. 5 and Table S3 also reveal that with increasing temperature the osmolyte-protein contribution to ΔH becomes markedly less favorable (by 4.0 and 8.0 kJ/mol for urea and betaine solutions, respectively). Since, at the same time, other enthalpic contributions are clearly decreasing (by -4.6 and -7.2 kJ/mol, respectively), we conclude that reduced accumulation of urea and enhanced exclusion of betaine at higher temperatures result from weaker interactions between osmolytes and the protein.

Conclusions

In this work, we studied the effect of two osmolytes, urea and betaine, on temperature denaturation of Trp-cage, using replica-exchange molecular dynamics simulations. We found that urea, a well known denaturant, shifts the conformational equilibrium of Trp-cage towards the unfolded state in the entire range of temperature studied. In contrast, betaine slightly increases the folding free energy of Trp-cage at low temperatures, by promoting another near-native conformation, but at the same time stabilizes the protein against temperature-induced unfolding. Due to this temperature dependence, betaine may possibly provide protection against thermal stress without strongly affecting the folding equilibrium at physiological temperatures.

Our data indicate that the destabilizing effect of urea results from its enthalpy-driven accumulation at the protein surface, in agreement with the most widely accepted mechanism of urea-dependent denaturation. Additionally, we found that accumulation of urea at the protein surfaces decrease with increasing temperature due to its weaker interactions with the protein. These weaker interactions stem from the reduced van der Waals energy and the average number of hydrogen bonds between protein-associated urea molecules and the protein surface with increasing temperature.

We also found that the protective effect of betaine against temperature denaturation results from its exclusion from the protein surface that is even more pronounced at higher temperatures. Our thermodynamic analysis suggests that while the exclusion itself is entropic in nature, its increase with temperature can be attributed to less favorable hydrogen-bonding and van der Waals interactions with the protein surface.

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