Hydrogen Bonding Progressively Strengthens upon Transfer of the Protein Urea-Denatured State to Water and Protecting Osmolytes†

Luis Marcelo F. Holthauzen, Jörg Rösgen,*,‡ and D. Wayne Bolen*

Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, Texas 77555-1052
†Present address: Department of Biochemistry and Molecular Biology, Penn State College of Medicine, Hershey, PA 17033-0850

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ABSTRACT: Using osmolyte cosolvents, we show that hydrogen-bonding contributions can be separated from hydrophobic interactions in the denatured state ensemble (DSE). Specifically, the effects of urea and the protecting osmolytes sarcosine and TMAO are reported on the thermally unfolded DSE of Nank4−7*, a truncated notch ankyrin protein. The high thermal energy of this state in the presence and absence of 6 M urea or 1 M sarcosine solution is sufficient to allow large changes in the hydrodynamic radius ($R_h$) and secondary structure accretion without populating the native state. The CD change at 228 nm is proportional to the inverse of the volume of the DSE, giving a compact species equivalent to a premolten globule in 1 M sarcosine. The same general effects portraying hierarchical folding observed in the DSE at 55 °C are also often seen at room temperature. Analysis of Nank4−7* DSE structural energetics at room temperature as a function of solvent provides rationale for understanding the structural and dimensional effects in terms of how modulation of the solvent alters solvent quality for the peptide backbone. Results show that while the strength of hydrophobic interactions changes little on transferring the DSE from 6 M urea to water and then to 1 M TMAO, backbone−backbone (hydrogen-bonding) interactions are greatly enhanced due to progressively poorer solvent quality for the peptide backbone. Thus, increased intrachain hydrogen bonding guides secondary structure accretion and DSE contraction as solvent quality is decreased. This process is accompanied by increasing hydrophobic contacts as chain contraction gathers hydrophobes into proximity and the declining urea−backbone free energy gradient reaches urea concentrations that are energetically insufficient to keep hydrophobes apart in the DSE.

The relative importance and roles of hydrogen bonding and hydrophobic interactions in protein folding and stability is, arguably, one of the most contentious issues in protein folding (1). Because organic osmolytes affect hydrogen-bonding and hydrophobic interactions differentially (1−3), these physiologically important small molecules can be exploited to better understand the thermodynamics of protein folding/unfolding and stability. Here, we illustrate how knowledge of the energetic effects of osmolytes on the structure of the denatured state ensemble (DSE)(1) provides insight into the driving forces for solvent-mediated protein folding.

Numerous organisms use small organic osmolytes to offset protein denaturing conditions brought on by the environment (4, 5). Given their important positions in biology, these molecules add new dimensions and viewpoints to studies of protein folding (1−3, 6−12). The broad scope of osmolyte effects on protein folding ranges from the denaturing action of the physiologically important osmolyte, urea, to the exceptional ability of the protecting osmolytes, sarcosine and trimethylamine N-oxide (TMAO), in forcing proteins to fold (13−16). Understanding the impact of solvent components on proteins involves quantifying the changes they cause in the structure and dynamics of both native and denatured ensembles and identifying the underlying forces responsible. The native state structure is not noticeably perturbed by osmolytes, but the denatured state ensemble (DSE) exhibits plasticity evident in the changes in the DSE hydrodynamic radius ($R_h$) as a function of osmolyte type and concentration (17). In fact, an array of solvent effects on the contraction/expansion of the DSE has been described and sometimes associated with varying degrees of secondary and/or tertiary structure accretion (17−22). Notably, Uversky and Fink find strong correlation between the hydrodynamic volume of precursors of the native state and their degree of secondary structure, a correlation we extend to species that populate cosolvent-induced contraction of the DSE (23).

Owing to its dynamical and transient nature during folding, one of the effects most difficult to study involves contraction of the DSE to the point of collapse. This effect is observed in proteins that exhibit folding kinetics for which an initial rapid collapse of the DSE is followed by a rate-determining step leading to the native protein. Many of the mechanistic issues in folding kinetics involve limited subpopulations within the DSE such as those that promote nucleation and/or a coalescence of nonpolar side chains. The thermodynamics of native to denatured transitions, on the other hand, is inclusive in that it must account for the total population and thermodynamic character of the DSE.
and of the native state. Given that the DSE is the state most affected by osmolytes such as urea, sarcosine, and TMAO, it is desirable to dissect the overall energetics of osmolyte-DSE interaction and accompanying structural changes.

Urea, sarcosine, and TMAO are known to cause substantial changes in RH and secondary structure in the DSE of proteins (17, 20). Here, we take advantage of the fact that the high degree of thermal energy in thermally denatured protein favors the denatured state to the extent that substantial amounts of either the denaturing osmolyte, urea, or the protecting osmolyte, sarcosine, can be present without populating the native state significantly. This increases the experimentally accessible range for observing osmolyte-induced accretion of structure and corresponding contraction of the denatured state RH in the absence of the native state. The protein we use, Nank4–7*, 15 kDa, is a truncated version of the ankyrin domain of the Drosophila notch receptor (16), comprising four tandem ankyrin repeats. Nank4–7* is marginally stable at pH 7 in 200 mM NaCl, with a melting temperature of 34 °C (14, 16), and at 55 °C in the presence and absence of osmolytes, the protein populates the denatured state to >99.5% under experimental conditions adopted in this study.

We use transfer free energies to assess how urea, sarcosine, and TMAO affect protein stability and the denatured ensemble (2, 17, 24). Adopting the basic approach of Nozaki and Tanford, we previously redetermined and extended their measurements by including activity coefficient data for glycine in water and 1 M urea to obtain corrected side chain transfer free energies (3, 25, 26). Contrary to Nozaki and Tanford’s earlier conclusions, our published data show that urea does not denature proteins by favorable interactions with nonpolar side chains; rather it is through its favorable interactions with the peptide backbone that urea is such a good denaturant (3, 8, 27–29). In the present work we show that increased backbone–backbone interactions are coordinated with secondary structure accretion and contraction of the DSE that occurs upon dilution from a comparatively good solvent (urea) to the successively poorer solvents, water, sarcosine, or TMAO solution. That is, backbone–backbone interactions (intrachain hydrogen bonding) progressively increase as solvent quality is shifted from good to poor. In marked contrast, the strength of hydrophobic interactions remains relatively unchanged during this dilution process. These results permit separation of the relative roles of hydrogen-bonding and hydrophobic interactions in the DSE.

MATERIALS AND METHODS

Chemicals. NaCl was from Fisher Scientific; Na2HPO4 and NaH2PO4·H2O were obtained from Mallinckrodt. Ultrapure urea was purchased from USB, sarcosine from Fluka, glycinebetaine from Sigma, and TMAO was synthesized and purified as described previously (30). Prior to use, all sarcosine solutions were treated with activated carbon (Aldrich) and filtered through 0.22 μm sterile filters (Millipore Millex GP), and their molar concentrations were determined refractometrically. The buffer used for all final experiments was 10 mM sodium phosphate, pH 7, and 200 mM NaCl.

Protein Expression and Purification. The C-terminal His6-tagged Nank4–7* construct used in this study contains four tandem ankyrin repeat sequences and was expressed and purified as in refs 31 and 16.

Circular Dichroism. Temperature scans from 5 to 75 °C were performed in a Jasco J-720 spectropolarimeter at 228 nm in two different ways. In one set of experiments continuous temperature scans were performed with a temperature slope of 1 °C/min. In another set, after jumping to a desired temperature (increments of 3–5 °C) the sample was equilibrated for 30 s, and a time course was measured for 4 min at lower temperatures (from 45 to 50 °C depending on the osmolyte concentration) and 90 s at higher temperatures to prevent aggregation, which in the slower, continuous scans was detectable at the highest temperatures in 1 M sarcosine (cf. denatured state baselines in Figure 1). Because of the strong absorbance of concentrated sarcosine and TMAO solutions in the range of ≤220 nm the ellipticity was recorded at 228 nm, where the signal is dominated by the contribution of α-helical and β-structures. Nank4–7* was present at 0.16 mg/mL, and a capped 1 mm cuvette was used.

Dynamic Light Scattering. Dynamic light scattering (DLS) experiments were performed at 55 °C with the protein at 0.32 mg/mL in the presence of 0, 2, and 4 M urea and at 15 °C in the absence of urea. All measurements were done in a DynaPro 99-D-50 MSTC 800 dynamic light scattering instrument from Protein Solutions. The refractive indices for the several samples at the proper temperature were obtained in a Milton Roy tabletop refractometer (Abbé 3L). The viscosities at the various temperatures for the solutions were obtained according to Perl et al. (32), and the hydrodynamic radii (RH) obtained from DLS are all corrected for viscosity at the reported temperature.

Calculation of Transfer Free Energies. ΔGtr,N is calculated from crystallographic coordinates of segments 4–7 of notch ankyrin protein in the manner described previously (3). Briefly, the Nank4–7* surface is interrogated with a probe the size of a water molecule to obtain the total solvent surface areas for each type of side chain and backbone. For the side chains, the areas are divided by the surface area of the fully exposed side chain to determine the number of each type of side chain exposed; the
RESULTS

Reversible Thermal Denaturation of Nank4−7* at Different Urea and Sarcosine Concentrations. For optimal signal-to-noise ratio, CD of Nank4−7* was measured at 228 nm, a wavelength for which β-sheet, β-turns, and α-helices all give negative ellipticity while unordered structures are either positive or near zero (33). Temperature scans monitoring the mean residue ellipticity [θ]228 were performed in two ways, resulting in different effective heating rates (Figure 1) as explained in Materials and Methods. The two sets of experiments show good agreement, reproducibility, and excellent reversibility. Only in the slow scan at 1 M sarcosine is evidence of protein aggregation indicated by the irregular and nonlinear behavior above 65 °C, whereas such irregularities are not seen in the temperature-jump experiments, which successfully minimize the exposure time of denatured protein at high temperatures. Urea concentration ≥2 M denatures this protein at all temperatures shown (blue lines in Figure 1). In contrast, sarcosine stabilizes the protein against temperature denaturation, increasing the transition midpoint temperature Tm (red lines in Figure 1) relative to the protein in buffer alone (black line in Figure 1). The denatured state [θ]228 at temperatures ≥55 °C becomes progressively more negative on successive transfer from high urea concentration to buffer and to 1 M sarcosine. For urea-denatured protein, the decrease in [θ]228 with increasing temperature is presumably due to conversion of polyproline II structure that populates the denatured ensemble at low temperature to β structure that becomes dominant at high temperature (34, 35).

The CD and Volume of Nank4−7* Species Are Inversely Correlated. In considering the specific effects of urea concentration on denatured Nank4−7*, dynamic light scattering (DLS) experiments were performed on thermally denatured Nank4−7* at 55 °C in 0, 2, and 4 M urea solutions, and Rg of the DSE was determined at each condition. Rg for thermally denatured Nank4−7* is 29.5 ± 1.0 Å in dilute buffer and 31.8 ± 1.6 Å in 2 M and 33.4 ± 1.3 Å in 4 M urea solutions, demonstrating expansion of the DSE at 55 °C as a function of urea concentration. Unfortunately, sarcosine and other protecting osmolytes give an excessively noisy DLS signal even in the absence of protein, thus preventing determination of Rg in solutions of protecting osmolytes. Rg of the protein in the presence of protecting osmolytes may be estimated from the relationship between DSE size and its CD signal. Figure 2 illustrates that the inverse of the relative hydrodynamic volume of the thermally denatured Nank4−7* species at 55 °C in the absence and presence of 2 and 4 M urea is proportional to the measured CD at 228 nm. Uversky and Fink reported such proportionality behavior at a different wavelength for intermediate states of a variety of proteins (23). The proportionality is observed to hold for the denatured species and native Nank4−7* as defined by

\[
(R_g^0 / R_g)^3 = (1.298 \pm 0.002)([θ]_{228} / [θ]_{228}^0) - (0.297 \pm 0.003)
\]  

(1)

Here, [θ]_{228}^0 and (R_g^0)^3 represent the ellipticity and hydrodynamic volume of Nank4−7* in buffer at 55 °C as a reference state, and

- Figure 2: Relationship between hydrodynamic volume and CD. The hydrodynamic volume (degree of compactness) of Nank4−7* species thermally denatured at 55 °C ([θ]_{228}^0) relative to the volume of thermally denatured Nank4−7* species in 0, 2, and 4 M urea (R_g^0). The ratio of these two parameters is plotted as a function of the corresponding θ_{228} for the species of interest relative to θ_{228} (the ellipticity of thermally denatured Nank4−7* in 0 M urea). The linear fit is defined by eq 1 in the text. From left to right, symbols are for 4, 2, and 0 M urea for thermally denatured (55 °C) Nank4−7* with native Nank4−7* (θ_{228} and R_g determined at 15 °C) at the top right of the plot. The arrows indicate values of Rg extracted from the graph using the respective θ_{228} values recorded for 0.3, 0.6, and 1.0 M sarcosine.

Sarcosine and TMAO Are More Effective in Inducing Secondary Structure Than Urea Is at Eliminating It. Figure 4 shows how [θ]_{228} for thermally denatured Nank4−7* changes as a function of osmolyte concentration at 61 °C. A temperature...
higher than 55 °C was used so that higher osmolyte concentration could be employed while still not populating the native state. Clearly, urea is not as effective in reducing secondary structure in thermally denatured Nank4−7* as sarcosine and TMAO are at inducing such structure. It is known that TMAO is frequently thermally denatured Nank4−7* that is coordinated in a well-defined manner with the hydrodynamic volume. The experiments explore how the structure of a DSE responds to changes in solvent quality induced by physiologically important osmolytes at a temperature in which the denatured state is >99% populated. The same types of tendencies exhibited at high temperature also occur at temperatures in which folding/unfolding occurs, and this lower temperature range, that is of interest in protein folding, is the focus of the remainder of the Results section.

**Folding Cooperativity of Nank 4−7** in Urea and Sarcosine Solutions Is Dominated by Backbone Contributions. The m-value, an experimental thermodynamic quantity determined by use of the linear extrapolation method, measures the cooperativity of protein folding/unfolding induced by an osmolyte (41). It is evaluated as the slope of the linear plot of protein stability vs osmolyte concentration, and it represents the water to 1 M osomolyte transfer free energy contributed by those side chain and backbone groups that become newly exposed upon denaturation by urea or newly buried on being forced to fold by protecting osmolytes (2, 3, 24). Using transfer free energies and the Tanford transfer model, we recently demonstrated the ability to predict m-values of proteins successfully (2, 3). The method accounts for transfer free energies of the native (ΔGtr,N) and denatured states (ΔGtr,D) by summing individual side chain and backbone contributions (ΔGc) (2, 24, 42). The free energy difference (ΔGtr,D − ΔGtr,N = m-value) is then used to quantify residue-level free energy contributions of those groups that contribute to the m-value (2, 3, 24). For Nank 4−7*, Table 1 lists the collective free energy contributions of side chains and of backbone upon transfer of the native and denatured states from water to 1 M urea or sarcosine. The model used for the denatured state is a self-avoiding random coil (2, 24, 43). Determined in this way, m-values of opposite sign are obtained for denaturants and stabilizers, respectively, and the calculated and experimentally determined m-values are found to be in good agreement (Table 1).

It is clear from Table 1 that the side chains collectively contribute very little (0.18 and 0.32 kcal/mol) to the sarcosine and urea effects on protein stability; the major contribution comes from the backbone (2.62 and −1.96 kcal/mol). With urea, the large favorable backbone transfer promotes denaturation and overcomes a small resistance to unfolding (positive sign) collectively from the side chains; conversely, with sarcosine the large unfavorable interaction with backbone promotes folding while side chains collectively contribute little.

**Urea Is a Good Solvent for the Peptide Backbone but Not for Side Chains.** For transfer of Nank4−7* DSE from water to 1 M urea, Figure 5A gives a residue-level accounting of the
While the $m$-value gives the free energy dependence of folding/ unfolding on osmolyte type and concentration, the transfer free energy of the denatured state ($\Delta G_{\text{tr},\text{D}}$) provides the driving force responsible for the physical and spectral changes the DSE undergoes in response to osmolyte type and concentration as shown in Figures 2–4. The breakdown of $\Delta G_{\text{tr},\text{D}}$ in Table 1 shows that transfer of the DSE from water to 1 M urea is favorable (0.87 $\pm$ 3.05 = $-2.18$ kcal/mol). For the sake of illustration, DSE transfer to 6 M urea, as in the case of Nank4-7*, is highly favorable ($C_{\text{urea}}\Delta G_{\text{tr},\text{D}} = -13.1$ kcal/mol), and this net favorable transfer free energy is composed of a $\sim 18$ kcal/mol contribution from the favorable interaction of 6 M urea with the DSE peptide backbone and a (0.32 $\times$ 6 = 1.92 kcal/mol) unfavorable interaction of the urea with those groups destined to be buried on folding, along with 3.3 kcal/mol unfavorable urea interaction with the solvent-exposed side chains of the native protein. Based on these transfer free energies, it can be concluded that 6 M urea is a good solvent for the peptide backbone but not for the side chains. The magnitude of the favorable interaction of urea with peptide backbone is sufficient to drive DSE structural and spectral changes as shown in Figures 1, 2, and 3B.

**TMAO and Sarcosine Are Poor Solvents for the Peptide Backbone with Side Chains Contributing Differentially but Marginally Overall to the DSE Transfer Free Energy.** Figure 5 shows residue-level free energy contributions upon transfer of the denatured state from water to 1 M sarcosine (Figure 5B) and from water to 1 M TMAO (Figure 5C), and with both protecting osmolytes the unfavorable interaction with peptide backbone dominates the DSE transfer free energy. Clearly, sarcosine and TMAO are poor solvents for the peptide backbone. It is interesting to note that for transfer to sarcosine the side chains collectively contribute very little to the DSE transfer free energy (as reflected in Table 1). This is because the unfavorable interaction with the hydrophobic class of side chains is offset by the favorable interaction between sarcosine and the polar and charged side chain classes of residues. In contrast to sarcosine, the hydrophobic side chains collectively interact favorably with TMAO as do the polar and charged side chains, leading to an overall favorable TMAO interaction with side chains that is opposed by a very large unfavorable and dominant TMAO interaction with backbone. Whereas both TMAO and sarcosine have the same general effects on the dimensions and structural accretion of the Nank4-7* DSE (see ref 17 and Figure 4), the differences in their interactions with side chains (Figure 5 and Table 2) serve to illustrate important distinctions between TMAO and sarcosine.

**DISCUSSION**

Osmolytes Induce Large Changes in the Hydrodynamic Volume and Secondary Structure of Thermally Denatured Nank4-7*. Transfer of (55°C) thermally denatured Nank4-7* in 6 M urea to progressively poorer solvents, water and then sarcosine or TMAO solutions, causes large reduction in $R_b$ of the
DSE with concomitant accretion of secondary structure. Specifically, the DSE contracts (Figures 2 and 3B) such that in 1 M sarcosine its volume ratio is about twice that of native Nank4-7*, a highly compact ensemble approximating the relative size expected of a premolten globule state (44). Moreover, the magnitude of structure accretion (C to B in Figure 1) accompanying this contraction is itself substantial, amounting to ~30% of the total CD change possible on going from the protein denatured in 6 M urea to the fully folded state (C to A in Figure 1) at this temperature.

What kind of structure is accumulated upon Nank4-7* contraction? While the ellipticities of unstructured polypeptide structures at θ_{228} are positive or close to zero, β-sheet, β-turns, and α-helices are all negative at this wavelength (33). Intrachain hydrogen bonding defines backbone–backbone interactions, and the low contact order α-helix and β-hairpin structures are likely to be populated because they are particularly effective at reducing exposure of the peptide backbone to sarcosine or TMAO, thus mitigating the positive free energy of the DSE upon transfer of the peptide backbone to these poor solvents. Relative to water, osmolytes like sarcosine and TMAO increase helical content in peptides that have helix-forming propensity (45). Nank4-7* has ample α-helix and β-hairpin propensities, given that the native protein is 46% helical and contains several β-hairpins (46). Indeed, the type of interactions responsible for decreasing Rθ through intrachain hydrogen bonds is also the one responsible for secondary structure, resulting in the accretion of secondary structure and contraction of the denatured ensemble being interrelated processes (Figure 2).

These results illustrate that under the equilibrium conditions presented the DSE can accommodate dimensional and structure accretion changes from a random coil to a premolten globule without substantively populating the native state. Such behavior provides strong support for the hierarchical model of protein folding under equilibrium conditions, and the high population of the DSE species enables investigations of the nature of accreted structure in the DSE and the extent to which it is native-like.

There is ample evidence that structural and dimensional changes we observed with the DSE at high temperature also occur around room temperature, where most protein folding studies have been performed. The osmolyte urea is known to expand, and osmolytes like TMAO and sarcosine contract the Rθ of the DSE at 25 °C (17) and at 55 °C shown here. Contraction and structure accretion are interrelated by (Rθ)^-3 being directly proportional to [Θ] as shown by Uversky and Fink in the range of room temperature and at 55 °C shown here. What is missing is the relationship between the free energy of the DSE and the DSE’s structural and dimensional changes as solvent quality is changed. Such information is available at 25 °C from transfer free energy measurements (17), thus allowing a structural energetic interpretation of the solvent-induced DSE contraction and structural accretion frequently observed at room temperature.

Structural Energetics of Osmolyte-Mediated Changes in the DSE of Nank4-7*. The sign of the transfer free energy for a denatured protein provides the basis for concepts such as solvophobicity, solvophobicity, and solvent quality (1, 24, 47). The sign and magnitude of the transfer free energy for the entire denatured state (ΔG_τ,D), obtained by summing the transfer free energy contributions of all its constituent groups, determine the overall solvent quality experienced by the polymer chain (1, 17, 47). Key to discussions of hydrophobic interactions and hydrogen bonding are the striking parallels in how these forces are mediated by water and protecting osmolytes and in how they effect protein folding and changes in the DSE. On the basis of the positive free energy of transfer (ΔGw,D) of nonpolar groups from a nonpolar solvent to water, water is said to be a poor solvent for nonpolar groups (48–50). Upon transfer to water the hydrodynamic radius of a DSE that contains nonpolar residues contracts in a process referred to as hydrophobic collapse (51). The DSE collapses because monomer–monomer contacts (nonpolar side chain–nonpolar side chain, i.e., hydrophobic interactions) are more favorable than monomer–solvent contacts (50, 52–54).

As with hydrophobic collapse, protecting osmolytes such as sarcosine and TMAO solutions also are quite effective at causing contraction of the DSE, but a different part of the protein structure is responsible, namely, the peptide backbone (17). ΔGw,D of the peptide backbone from water to either TMAO or sarcosine is positive and depends monotonically on osmolyte concentration, thus classifying sarcosine and TMAO as poor solvents for the peptide backbone (14, 47, 55–57). Upon transfer of the denatured state from water to sarcosine or TMAO solution, monomer–monomer contacts (backbone–backbone, i.e., intrachain hydrogen-bonding interactions) of the DSE are more favorable than backbone–sarcosine/TMAO contacts, thus causing DSE contraction and accretion of secondary structure (17, 45) in what is known as the osmophobic effect (6). In both instances, the impetus for DSE contraction is the respective positive free energy of transfer, with nonpolar interactions favored over the interactions between the nonpolar group and water in the case of hydrophobic collapse and with intrachain hydrogen-bonding interactions favored over the interactions between peptide backbone units and sarcosine or TMAO solution in the case of osmophobic collapse. In essence, both hydrophobic interactions and hydrogen bonding owe their magnitudes to solvent quality.

DSE Contraction and Secondary Structure Accretion Are Driven by the Free Energy Increase on DSE Transfer to Successively Poorer Solvents, Water and Sarcosine. To understand how the driving force for DSE structural and spectral changes arises from denatured state transfer free energies (ΔG_τ,D), it is useful to focus on the details of free energy contributions to the transfer. Table 1 shows that Nank4-7* DSE transfer from 1 M urea to water at 25 °C, ΔG_D is 2.18 kcal mol^{-1} M^{-1}, and the net transfer from 6 M urea to water is 6-fold larger, 13.1 kcal/mol (Figure 6A). The DSE will respond...
to this large unfavorable free energy in ways to reduce contact between the peptide backbone and water. Depending on conditions this may include transition to the native state, DSE aggregation, or DSE contraction (47).

Figure 6B shows how this large net unfavorable free energy upon transfer of the DSE from 6 M urea to water is parsed into backbone and side chain contributions. First, \( (6 \times 3.0 \approx 18.3 \text{ kcal/mol} ) \) in free energy change occurs on transfer to the DSE peptide backbone, showing that in comparison to 6 M water is a poor solvent for the peptide backbone. Thus, a strong relative increase in intrachain hydrogen bonding occurs on DSE transfer as urea—backbone interactions decline to zero, and this drives contraction of \( R_b \) and accretion of structure. At the same time, the side chains collectively favor transfer to water \( (6 \times (-0.875) \approx -5.3 \text{ kcal/mol} ) \), tending to cause some \( R_s \) expansion of the DSE, but this is overridden by the \( +18.3 \text{ kcal/mol} \) effect on the backbone (see Table 2 and Figure 5A). As a class, the DSE’s nonpolar groups favor the transfer to water \( (6 \times (-0.21) \approx -1.26 \text{ kcal/mol} ) \) (Table 2), but as also shown by others the effect is quite small \((59)\), particularly in comparison with transfer of the peptide backbone.

In summary, we emphasize that the strength of hydrophobic interactions is not altered substantively by the presence of urea. Indeed, small clusters of hydrophobic groups have been observed in the DSEs, even in high urea concentration. By contrast, urea significantly affects hydrogen-bonding interactions in the DSE. Urea interacts favorably with the peptide backbone through direct H-bonding \((12)\), which causes disruption of hydrogen-bonded secondary structure and expansion of the polymer chain. Denaturation occurs at urea concentrations in which the favorable urea—backbone interactions that promote the DSE exceed the strength of those intramolecular interactions that maintain the integrity of the native state. Thermodynamically, these processes are reversed on dilution of urea-denatured protein to native conditions.

**Hydrophobic Interactions in Osmolyte Solutions.** For decades hydrophobic interactions have been considered the driving force for DSE contraction on transfer from urea solution to water \((50, 59)\). However, from a polymer science perspective Figures 5 and 6 show that the free energy of intrachain hydrogen-bonding interactions changes considerably more than hydrophobic interactions upon DSE transfer from urea to water. This is not to say that hydrophobic interactions play no role in the DSE contraction and ultimate transition to the native state. Indeed, hydrophobic interactions remain strong even in 8 M urea solution. Our view is that while hydrophobic interaction free energy remains approximately the same in urea and in water, the reduction in DSE \( R_b \) resulting from the increased intrachain hydrogen bonding upon diluting the urea ultimately increases the local concentration of hydrophobic side chains. This leads to increased nonpolar group proximity, and hydrophobic interactions increasingly engage. With further urea dilution the reduced urea—backbone interactions continue to decline until they are unable to rival the strength of all the intramolecular interactions involved in maintaining the integrity of the native state, and at this point the native state populates to a measurable degree.

The free energy effects of sarcosine and TMAO on Nank4−7* DSE structure and dimensions provide interesting contrasts to that of urea. First, the large unfavorable interaction between these osmolytes and the peptide backbone (see Figure 5B,C) is responsible for the large positive \( \Delta G_{tr,b} \) which is ameliorated by reducing contact of the backbone with solvent through contraction of the \( R_b \) concomitant with accretion of secondary structure. Table 2 shows that for both protecting osmolytes the side chains collectively oppose the large unfavorable interaction of osmolyte with backbone. However, a major important difference between the two protecting osmolytes can be found in how the nonpolar groups respond to the transfer. Panels B and C of Figure 5 show that the nonpolar groups interact unfavorably with sarcosine while interacting favorably with TMAO. That is, in TMAO solution secondary structure accretion, DSE contraction, and protein folding are driven by the unfavorable interaction with the peptide backbone, and these processes are opposed to a small extent by hydrophobic interactions. In the presence of sarcosine, DSE contraction, structure accretion, and protein folding again are driven by the unfavorable interaction with the peptide backbone, but in this case hydrophobic interactions favor contraction and folding. These differences between osmolytes can potentially be used in further differentiation of hydrophobic and osmophilic effects within DSEs \((6)\).

Intrinsically disordered proteins provide an opportunity to examine further the roles of hydrophobic groups and backbone in contracting the DSE and folding the protein. These proteins are disordered in aqueous solution and are significantly depleted in bulky nonpolar and aromatic side chains \((60)\), and those whose sequence contains folding information can be forced to fold if solvent quality is made poor enough. Addition of TMAO or sarcosine to examples of such intrinsically disordered proteins causes contraction of the DSE and cooperative folding to species that have structure and function \((15, 61–63)\). For a DSE the size of Nank4−7*, Table 1 provides data from which it can be shown that a 6 M TMAO solution raises the backbone free energy by 42 kcal/mol and that whatever nonpolar groups are present should oppose folding to a modest degree. TMAO solution is clearly a much poorer solvent for the backbone than water, and the large unfavorable free energy of the peptide backbone is reduced through contraction/secondary structure accretion of the DSE and particularly from burial of the backbone through folding. The fact that such proteins have low hydrophobicity indices emphasizes the key role of hydrogen bonding and backbone burial in TMAO-induced folding of IDPs.

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