Assessing the Interaction of Urea and Protein-Stabilizing Osmolytes with the Nonpolar Surface of Hydroxypropylcellulose[†]

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ABSTRACT: The interaction of urea and several naturally occurring protein-stabilizing osmolytes, glycerol, sorbitol, glycine betaine, trimethylamine oxide (TMAO), and proline, with condensed arrays of a hydrophobically modified polysaccharide, hydroxypropylcellulose (HPC), has been inferred from the effect of these solutes on the forces acting between HPC polymers. Urea interacts only very weakly. The protein-stabilizing osmolytes are strongly excluded. The observed energies indicate that the exclusion of the protein-stabilizing osmolytes from protein hydrophobic side chains would add significantly to protein stability. The temperature dependence of exclusion indicates a significant contribution of enthalpy to the interaction energy in contrast to expectations from "molecular crowding" theories based on steric repulsion. The dependence of exclusion on the distance between HPC polymers rather indicates that perturbations of water structuring or hydration forces underlie exclusion.

Solutes are widely used to modulate the stability of native or folded conformations of proteins and nucleic acids (*1–7*). There are several naturally occurring osmolytes that cells synthesize to protect proteins in response to denaturing environmental conditions such as heat shock. Stabilization of compact structures typically results from an increased level of exclusion of solutes from the unfolded or more open conformations. There is an unfavorable interaction of solutes with exposed surfaces. The exclusion of osmolytes from surfaces necessarily means the inclusion of water and has quite naturally been termed a preferential hydration (*6*). Excluded, stabilizing osmolytes that are naturally occurring include glycerol, sorbitol, glycine betaine, proline, and trimethylamine oxide (TMAO)[1] (*8*). Denaturation results when there are favorable interactions of solutes with exposed surface; more solutes are "bound" or included with unfolded structures. Urea is probably the best known denaturant. The nature of the interaction between the solute and the macromolecule that results in exclusion or inclusion has not been satisfactorily characterized. Crowding theories that have been successful for the interaction of macromolecules (*9*) have been reformulated for small solute−macromolecule forces (*10*). This, however, does not explain the chemical specificity of the interaction. Bolen and co-workers, for example, have concluded (*8, 11, 12*) that the inclusion of urea and the exclusion of stabilizing osmolytes from proteins are dominated by the interaction of these small molecules with the peptide backbone with little contribution from the exclusion of these polar solutes from hydrophobic side chains. One method for elucidating the physics of the interaction is to measure the distance dependence of the force through either a radial distribution function of solutes surrounding a macromolecule or a change in solute concentration between two macromolecular surfaces as they approach each other.

For some time now, we have been measuring intermolecular forces through the dependence of the distance between macromolecules in an ordered array measured by X-ray scattering on the osmotic pressure of a polymer that is excluded from the macromolecular phase and applies a force on it (*13–21*). The effect of solute exclusion on forces can be used to infer changes in solute concentration in the space between macromolecules as the distance between them changes. We have previously used this approach to measure the exclusion of nonpolar alcohols from ordered arrays of DNA (*22, 23*) and of salts and some polar solutes from hydroxypropylcellulose (HPC) (*24*). Here we use the osmotic stress technique coupled with X-ray scattering to investigate the inclusion or exclusion of urea and of several common protein stabilizers with the modified polysaccharide HPC as a model for the interaction with hydrophobic amino acid side chains. We find that urea interacts only weakly with HPC but that the polar protein stabilizers are all significantly excluded from this hydrophobic polymer. The dependence of exclusion on the distance between HPC chains in the condensed phase is approximately exponential with a decay length of ∼3 Å. The same functional form has been observed for the intermolecular force between many biomacromolecules (charged, polar, and nonpolar) and for the exclusion of nonpolar osmolytes from the highly charged DNA

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[1] Abbreviations: HPC, hydroxypropylcellulose; PEG, poly(ethylene glycol); TMAO, trimethylamine oxide.

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surface and of salts from HPC. We have interpreted this distance dependence as being due to a water structuring force. If the solute is within one or two hydration layers of the HPC polymer, the intervening water structuring is perturbed, resulting in a repulsive force. Hydration energies become more unfavorable since water must accommodate both molecules simultaneously.

We also report that the exclusion of the polar solutes from HPC is significantly temperature-dependent, unlike our previous observations for the exclusion of salt from HPC or of alcohols from highly charged DNA. Exclusion results from an enthalpy of interaction that is more unfavorable than the entropic contribution to the free energy, which is favorable. For both glycerol and sorbitol, in particular, ΔH and $T\Delta S$ are much larger than the free energy of exclusion. This enthalpy−entropy compensation has often been attributed to hydration.

We test the estimate of the number of excess water molecules for each osmolyte determined by the osmotic stress−X-ray scattering method by measuring the dependence of the precipitation temperature of dilute HPC on solute concentration. Not surprisingly, hydrophobically modified HPC precipitates from dilute aqueous solution as the temperature is increased. We compare the observed dependence of the transition temperature on osmolyte concentration with the value calculated from our measured solute exclusion from condensed HPC arrays. The measured values of excess water are in reasonably good agreement with measurements of the change in the transition temperature. The observed discrepancies are consistent with the temperature dependence of the exclusion.

The insensitivity of HPC forces and precipitation temperature to urea is consistent with other measurements indicating that urea preferentially solvates the peptide bond and interacts very little with hydrophobic side chains (*11, 25*). The exclusion energies of the polar protein-stabilizing osmolytes from the hydrophobic HPC chain, however, are comparable to the energies that have been ascribed to exclusion from the peptide backbone (*8, 11, 26*). This indicates that the exclusion of these osmolytes from nonpolar peptide side chains should significantly contribute to the stabilization of native protein structure. This is contrary to the current assignment of exclusion energies of these osmolytes.

MATERIALS AND METHODS

Hydroxypropylcellulose was purchased from Polysciences, Inc., and used without further purification. The average degree of hydroxypropyl substitution was three per glucose unit. Trimethylamine oxide (purum, >99%), sorbitol (ultra, >99.5%), L-proline (>99%), glycine betaine monohydrate (>99%), and 8000 MW poly(ethylene glycol) (PEG) (Biochemika grade) were all purchased from Fluka Chemical Corp. Glycerol (ultrapure) was purchased from Gibco-BRL Life Technologies. All solutes were used without further purification.

Ordered HPC arrays were prepared by dialyzing HPC solutions against a solution of 30% PEG (20K MW) as described by Bonnet-Gonnet et al. (*13*). Small pieces (1 mm × 1 mm × 0.5 mm) were cut from the solid film and equilibrated against PEG or PEG/solute solutions. HPC pellets remain phase-separated from PEG solutions for weight fractions of >0.2. HPC samples were transferred to fresh PEG/solute solutions after an initial equilibration for ~5 days. Osmotic pressures of PEG solutions and of PEG/solute mixtures were measured using a Wescor Vapro vapor pressure osmometer (model 5520XL). Osmotic pressures were additive to within 10% for urea and glycerol. Osmotic pressures were additive to only within 35% at the highest PEG concentration used for glycine betaine, TMAO, proline, and sorbitol. We assume that to within 10% this excess pressure results entirely from an increase in solute activity, due to an exclusion from PEG, as we observed for salt/PEG mixtures (*24*). Solute osmotic pressures were taken as the difference of the PEG/solute and PEG alone osmotic pressures. At a fixed PEG concentration, the apparent osmotic coefficients of the solutes were insensitive to solute concentration over the range examined, further indicating that the nonideality of the mixtures is due to exclusion of solute from PEG.

X-ray Scattering. An Enraf-Nonius Service Corp. (Bohemia, NY) fixed copper anode Diffractis 601 X-ray generator equipped with double-focusing mirrors (Charles Supper Co.) was used for X-ray scattering measurements. HPC samples were sealed with a small amount of equilibrating solution in the sample cell and then mounted into a temperature-controlled holder. A helium-filled Plexiglas cylinder with Mylar windows was between the sample cell and image plate, a distance of ~16 cm. Diffraction patterns were recorded by direct exposure of Fujifilm BAS image plates and digitized with a Fujifilm BAS 2500 scanner set for a 50 μm pixel size and 16 bit intensity. The images were analyzed using FIT2D (copyright A. P. Hammersley, ESRF) and SigmaPlot 9.01 (SPSS Inc.). The sample−image plate distance was calibrated using powdered *p*-bromobenzoic acid. Mean pixel intensities between scattering radii $r - 0.05$ mm and $r + 0.05$ mm averaged over all angles of the powder pattern diffraction, $\langle I(r)\rangle$, were used to calculate integrated radial intensity profiles, $2\pi r\langle I(r)\rangle$. The scattering peaks correspond to interaxial Bragg diffraction from HPC polymers that we correct for packing in a hexagonal array. X-ray scattering patterns were reproducible over at least several months of storage. No sample degradation was apparent. Duplicate samples were prepared for 20% of the samples and showed that measured interaxial spacings were reproducible within 0.2 Å.

Critical Temperature of HPC Precipitation in Dilute Solution. The transition temperature of HPC was measured from the intensity of 90°-scattered light at 500 nm using a Jobin-Yvon-Horiba Fluoromax-3 fluorospectrophotometer equipped with a Wavelength Electronics model LFI-3751 Peltier temperature controller. The HPC concentration was 100 μg/mL. The temperature was increased in steps of 0.25 °C and the sample allowed to equilibrate for 3 min at each step. Twofold changes in concentration or temperature ramp rate did not change the transition temperature. Precipitation was reversible.

Thermodynamic Analysis. The thermodynamic analysis of the effects of solutes on the forces between macromolecules has been developed in more detail elsewhere (*22–24*). We only briefly outline the results here. A macroscopic phase of ordered HPC polymers is in equilibrium with the bulk solution of osmolyte and a polymer such as PEG that is excluded from the HPC phase. The osmolyte is free to

equilibrate between the HPC and bulk solution phases. We consider that PEG simply applies an osmotic pressure, Π_{PEG}, on the HPC phase. A difference in solute concentration between the bulk solution and HPC phase can be equivalently analyzed as the solute contribution to the osmotic pressure, Π_{solute}, acting on an excess or deficit number of water molecules in the HPC phase per saccharide, Γ_w, or the solute chemical potential acting on an excess or deficit number of solute molecules, Γ_s, in the HPC phase. Since we observe that Γ_w is constant with a changing solute concentration, we focus on the contribution of solute to osmotic pressure. The Gibbs−Duhem equation becomes

$$d\mu_{HPC} = V_w\, d\Pi_{PEG} + \bar{\nu}_w \Gamma_w\, d\Pi_{solute} \quad (1)$$

where μ_{HPC} is the chemical potential of HPC per glucose monomer, V_w is the volume of water per glucose unit in the condensed phase, and $\bar{\nu}_w$ is the molecular volume of water (assumed to be 30 Å^3). The number of excess water molecules is given by the difference in solute concentration between the bulk solution and HPC phase. If the HPC phase contains N_s solute and N_w water molecules per glucose unit and the bulk solution contains an n_s/n_w ratio of solute to water molecules, then Γ_w per glucose unit is defined as

$$\Gamma_w = N_w\left(1 - \frac{N_s/N_w}{n_s/n_w}\right) \quad (2)$$

This is the number of water molecules that would have to be removed or added to the HPC phase to result in the same solute concentration as in the bulk solution. The ratio $(N_s/N_w)/(n_s/n_w)$ is the same as the solute partition coefficient defined in ref *27*. If the solute is completely excluded, then $\bar{\nu}_w\Gamma_w = V_w$.

Rearrangement of the Maxwell relation of eq 1 gives the change in the number of excess waters as helices move closer as a function of the change in PEG osmotic pressure needed to maintain a constant V_w as the solute osmotic pressure is varied

$$\frac{\partial \Gamma_w}{\partial V_w} = -\frac{1}{\bar{\nu}_w}\frac{\partial \Pi_{PEG}}{\partial \Pi_{solute}}\bigg|_{V_w} \quad (3)$$

For hexagonal packing of polymers with spacing D_{int}, $dV_w = \sqrt{3}LD_{int}\, dD_{int}$, where L is the length of a glucose monomer (assumed to be 5 Å). We have previously observed that Π_{PEG} and Π_{solute} are linearly interdependent at a constant V_w for the exclusion of salts, glycerol, glycine betaine, and α-methyl glucoside from HPC arrays. For a linear interdependence, the slope $\partial\Pi_{PEG}/\partial\Pi_{solute}$ is simply given by the solute osmotic pressure, Π_0, and the difference in PEG osmotic pressures at a constant interaxial spacing with and without added osmolyte, the apparent Π_{excess}. For a concentration m of solute

$$\Pi_{excess}(D_{int},m) = \Pi_{PEG}(D_{int},m=0) - \Pi_{PEG}(D_{int},m) \quad (4)$$

and

$$\frac{\partial \Pi_{PEG}}{\partial \Pi_{solute}}_{V_w} = -\frac{\Pi_{excess}}{\Pi_0} \quad (5)$$

An overlap of Π_{excess}/Π_0 for different solute concentrations means a linear interdependence of Π_{PEG} and Π_{solute} at a constant D_{int}. The number of excess waters, Γ_w, can be calculated by integrating eq 3.

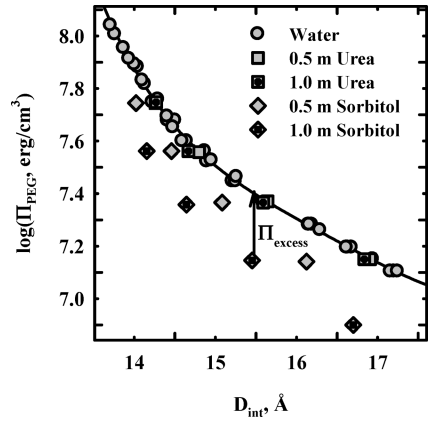

FIGURE 1: Effect of urea and sorbitol on HPC osmotic stress force curves at 20 °C. The PEG osmotic pressure dependence of the spacing between HPC chains determined from the Bragg scattering peak is shown for HPC in water, in 0.5 and 1 *m* urea, and in 0.5 and 1 *m* sorbitol. Urea has no effect on HPC forces, while intermolecular spacings are significantly closer in sorbitol at the same PEG osmotic pressure. The arrow illustrates the apparent excess osmotic pressure, Π_{excess}, exerted by 1 *m* sorbitol at ~15.5 Å.

HPC precipitates from dilute solution as the temperature is increased to 42 °C in water. Since there is an energy associated with solute exclusion and HPC precipitation releases excess waters, the presence of excluded osmolytes will lower the transition temperature. As also developed previously (*24*), the dependence of the precipitation temperature, T_t, of HPC in dilute solution on the osmotic pressure of the excluded solute, Π_s, is given by an analog of the Clapeyron equation

$$\frac{dT_t}{d\Pi_s} = -\frac{\bar{\nu}_w \Delta\Gamma_w}{\Delta S} \quad (6)$$

$\Delta\Gamma_w$ and ΔS are the differences in the number of excess water molecules and in the entropy, respectively, between the condensed and extended states per glucose monomer. We have previously determined the transition entropy from the temperature dependence of forces between HPC polymers (*13*). $\Delta\Gamma_w$ can be calculated by integrating eq 3.

RESULTS

Figure 1 shows a force curve for HPC at 20 °C measured by the osmotic stress technique. The osmotic pressure of poly(ethylene glycol) (PEG) in the bulk solution that is excluded from a condensed macroscopic HPC phase is used to compact the HPC phase. The spacing between HPC chains is determined from the Bragg reflection peak of scattered X-rays. The spacing between HPC polymers dried at a very low relative humidity (<10%) is 12.6 Å. Also shown in the figure is the effect of adding sorbitol and urea to the bulk solution on the spacing between HPC chains as a function of the PEG osmotic pressure. No change is seen with urea, whereas the spacing decreases with an increase in sorbitol concentration. The partitioning of solute between the condensed HPC phase and the bulk solution can be inferred from the change in spacing between HPC macromolecules as the solute concentration is varied at a constant PEG osmotic pressure as derived in eqs 1–3. Essentially, the exclusion of solutes results in an extra pressure exerted on the HPC phase by the solutes as represented by the Π_{excess} arrow in the figure.

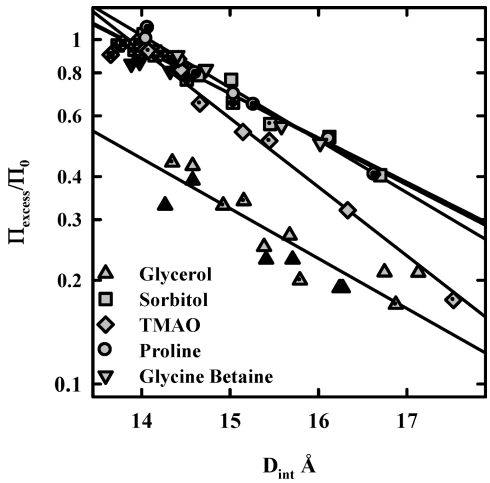

FIGURE 2: Dependence of osmolyte exclusion on the distance between HPC chains. The excess osmotic pressure as indicated in Figure 1 normalized by the osmolyte contribution to the solution osmotic pressure is shown as a function of the interaxial spacing for several protein-stabilizing solutes at two or three concentrations. $\Pi_{excess}/\Pi_0 = 1$ corresponds to complete exclusion, while $\Pi_{excess}/\Pi_0 = 0$ indicates no preferential inclusion or exclusion. The exclusion curves for sorbitol, glycine betaine, and proline are virtually identical. Glycerol is excluded more weakly. TMAO has a somewhat different exponential decay length compared with the others. The following solute concentrations were examined: 0.4 and 0.8 *m* glycine betaine; 0.4, 0.8, and 1.2 *m* glycerol; 0.25, 0.05, and 1 *m* sorbitol; 0.25, 0.5, and 1 *m* TMAO; and 0.25 and 0.5 *m* proline. Plain symbols are used for the lowest solute concentration, followed by dotted and crossed symbols. The different concentrations for each osmolyte overlap within experimental error, indicating an osmotic effect; i.e., Γ_w is constant at a fixed HPC interaxial spacing that is insensitive to osmolyte concentration.

Figure 2 shows the variation of Π_{excess} at 20 °C with the spacing between HPC polymers for several osmolytes commonly used to stabilize native protein structures: sorbitol, proline, glycine betaine, TMAO, and glycerol. The data for glycerol and glycine betaine have been reported previously but are included here for completeness. At least two concentrations are shown for each solute. Π_{excess} has been normalized by the solute contribution to the bulk solution osmotic pressure, Π_0, i.e., by the excess pressure if no solute was present in the HPC phase. Thus, $\Pi_{excess}/\Pi_0 = 1$ means complete exclusion, while $\Pi_{excess}/\Pi_0 = 0$ indicates no preferential interactions resulting in exclusion or inclusion. Π_{excess}/Π_0 can be related to changes in preferential hydration of the HPC phase through eqs 3 and 5. The exclusion of solute can be characterized by a number of water molecules, Γ_w, in excess of that expected if the concentration of solute in the bulk solution and HPC phase was the same. The overlap of the data for the different concentrations of each solute indicates that the number of waters associated with HPC that exclude solute at a fixed spacing is constant, independent of solute concentration. The curves for proline, sorbitol, and glycine betaine are virtually indistinguishable. Complete exclusion is attained at a D_{int} of ~14 Å. The exclusion of glycerol is only approximately half of that of those three osmolytes. Π_{excess}/Π_0 ratios can be adequately described by an exponential function with a decay length λ; $\Pi_{excess}/\Pi_0 \sim A \exp(-D_{int}/\lambda)$. The decay lengths for glycerol, sorbitol, proline, and glycine betaine are all 3 Å. This decay length is also characteristic of the exclusion of salts from HPC (*24*) and of nonpolar solutes from charged DNA (*22, 23*).

Table 1: Exclusion of Osmolytes from HPC at 5 and 20 °C[a]

osmolyte	$\Delta N_{w,total}$ at 20 °C	λ at 20 °C (Å)	$\Delta N_{w,total}$ at 5 °C	λ at 5 °C (Å)
urea	0	–	0	–
glycerol	9.7	3.25	20.3	3.45
sorbitol	19.8	2.9	32.7	3.5
TMAO	14.7	2.2	17.5	1.95
proline	19.9	2.9	27.1	3.3
glycine betaine	21.0	3.1	26.9	3.2

[a] The total of excess water per glucose monomer is calculated by integrating the curves shown in Figure 2 from 12.6 Å to ∞ as prescribed in eq 3, assuming that $\Pi_{exces}/\Pi_0 = 1$ is the maximum excess pressure. The error is ~10% for decay length λ and ~15% for ΔN_w.

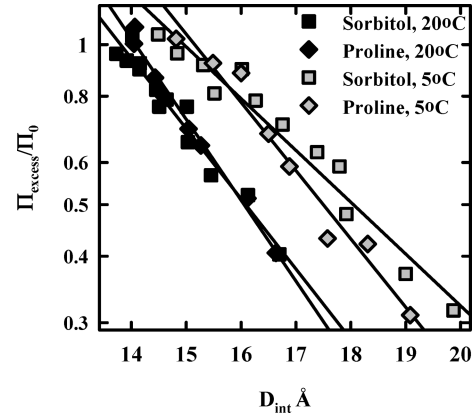

FIGURE 3: Temperature dependence of sorbitol and proline exclusion. The dependence of apparent excess osmotic pressure normalized by the osmolyte contribution to the solution osmotic pressure, Π_{excess}/Π_0, on interaxial HPC spacing is shown for sorbitol and proline at 5 and 20 °C. The exclusion of both is significantly higher at 5 °C than at 20 °C.

TMAO exhibits a somewhat different behavior. As with the other osmolytes, the Π_{excess}/Π_0 ratio is insensitive to TMAO concentration between 0.25 and 1.0 *m*. The exponential decay length λ, however, is only ~2.2 Å. The exclusion of TMAO is complete at ~14 Å.

A total number of excess waters that can be released in pushing HPC polymers to touching can be estimated from integrating Π_{excess}/Π_0 from ∞ to 12.6 Å (eqs 3 and 5). Table 1 gives this total number of excess water molecules per glucose unit and the observed decay length for the six osmolytes that were examined. Urea had no observable effect on the force curves and so is assigned a ΔN_w of 0.

Temperature Dependence of Exclusion. The free energy of solute exclusion can depend significantly on temperature (*28, 29*). This would indicate that the solute−surface interaction is more than a simple steric exclusion but that actual physical forces underlie exclusion. Figure 3 shows Π_{excess}/Π_0 values for sorbitol and proline at 5 and 20 °C. In both cases, there is more exclusion at 5 °C. There are 33 excess waters per glucose monomer at 5 °C compared to ~20 at 20 °C for sorbitol. The exponential decay length λ, however, does not depend significantly on temperature. Decay lengths and total numbers of excess waters calculated by integrating Π_{excess}/Π_0 from ∞ to 12.6 Å for 5 °C are also given in Table 1 for the osmolytes that were examined.

Effect of Osmolytes on HPC Precipitation. Hydroxypropylcellulose will spontaneously precipitate from dilute solution as the temperature is increased due to favorable hydrophobic interactions. The exclusion of solutes from HPC

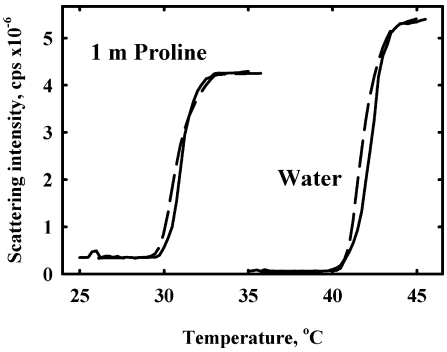

FIGURE 4: Effect of proline on the temperature-favored precipitation of HPC. The temperature dependence of the 90° scattering intensity is shown for HPC in water and in 1 *m* proline. The HPC concentration is 100 μg/mL. Heating curves are shown with solid lines and cooling curves with dashed lines. The transition is reversible within 0.5 °C. Proline significantly reduces the transition temperature.

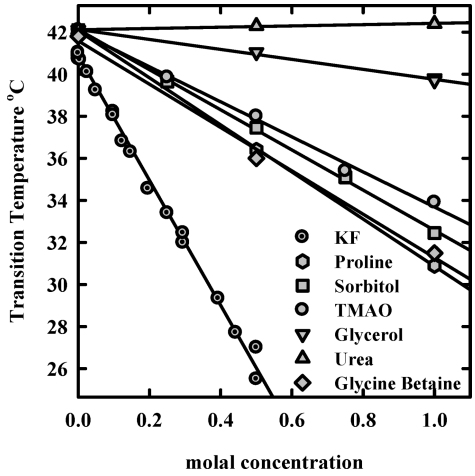

FIGURE 5: Dependence of the precipitation transition temperature on osmolyte concentration. The decrease in precipitation temperature with an increase in the concentration of the protein-stabilizing osmolytes is consistent with exclusion. The slope depends both on the number of excess water molecules released and on the entropy change across the transition as indicated in eq 6.

must necessarily affect the transition temperature. Figure 4 shows the intensity of 90°-scattered light as a function of temperature for dilute HPC (~100 μg/mL) in water and in 1 *m* proline. The midpoint transition without added osmolyte occurs at ~42 °C, which has been observed by many others (*13*). Proline causes a significant decrease in the cloud-point temperature.

Figure 5 shows the dependence of the midpoint transition temperature, T_t, on solute concentration for TMAO, betaine glycine, sorbitol, proline, glycerol, and urea. Data for the strongly kosmotropic salt KF that were reported previously are also included for comparison. The osmolal concentration of KF is approximately twice the molal concentration (within 10%). To a first-order approximation, the dependence of T_t on solute concentration is linear. The correction of molal to osmotic pressure osmolal concentrations for the net neutral solutes is small in the range examined (<15%). Consistent with the X-ray osmotic stress measurements, urea has little effect on the transition. Indeed, the transition temperature actually increases slightly with an increase in urea concentration. Of the other solutes, glycerol is the least effective, also in agreement with the exclusion curves shown in Figure 2.

Table 2: Observed and Calculated Dependencies of HPC Precipitation Temperatures on Osmolyte Concentration[a]

osmolyte	$(dTt/dosmolal)_{obs}$	$(dT_t/dosmolal)_{calc}$
urea	+0.3	0
glycerol	−2.4	−6.4
sorbitol	−9.55	−13.6
TMAO	−8.35	−9.0
proline	−11.2	−13.7
glycine betaine	−9.7	−13.4
KF	−13.9	−15.3

[a] Observed slopes are determined from the data shown in Figure 5 and have an error of 5%. The calculated slopes are determined from eq 6 with a ΔS of 12 cal K^{-1} (mol of glucose monomer)$^{-1}$ as determined previously at 13.9 Å and with ΔN_w determined at 20 °C integrating exclusion curves from 13.9 Å to ∞. Calculated slopes have an error of ~30%.

TMAO, betaine glycine, sorbitol, and proline are all comparable again, in qualitative agreement with Figure 2.

As given in eq 6, the slopes, dT_t/d[osmolal], depend on the transition entropy and the change in the number of excess water molecules between the polymer in dilute solution and in the precipitated aggregate. We have previously determined the distance-dependent entropy from the temperature dependence of the forces between HPC polymers in condensed arrays (*13*). The distance between polymers in the precipitate is assumed to be the same as the 13.9 Å spacing measured by X-ray scattering between polymers in ordered arrays at the 42 °C transition point between repulsion and attraction. Since both ΔN_w and ΔS show similar 3 Å decay length exponential dependences on the distance between HPC chains, the exact spacing in the precipitated assembly is not critical. The observed slopes and slopes calculated using ΔS as previously determined and ΔN_w values from integrating the solute exclusion curves at 20 °C shown in Figure 2 are given in Table 2. A comparison of observed dT_t/d[osmolal] values and calculated slopes is a sensitive test of the osmolyte concentration distribution curves shown in Figures 2 and 3. The calculated slopes are always somewhat larger than the observed slopes. This is likely because exclusion is temperature-dependent. ΔN_w values for betaine glycine, TMAO, proline, sorbitol, and glycerol decrease as the temperature increases. The largest difference between calculated and observed slopes is for glycerol which also has the largest change in ΔN_w between 5 and 20 °C. The smallest discrepancy (10%) is for TMAO that also has the smallest difference in ΔN_w between 5 and 20 °C. The calculated and observed slopes for KF are also within 10%. We previously reported observing no significant difference in ΔN_w between 5 and 20 °C for KF exclusion. The data in Figure 5 are not sufficiently precise, however, to extract an accurate temperature dependence of exclusion. The exclusion of TMAO, glycine betaine, sorbitol, and proline is comparable to that of the strongly kosmotropic salt KF.

DISCUSSION

There is now a vast literature about the interaction of small solutes and salts with macromolecules. Excluded osmolytes can stabilize native protein, RNA, and DNA structures, promote the assembly of macromolecular complexes, and strongly increase ligand affinity (see, for example, refs *4*, *30*, and *31*). The exclusion of small solutes and salts from macromolecular surfaces and cavities necessarily means that

water is included, i.e., a preferential hydration. Different structures, conformations, or assemblies have different numbers of included or excess water molecules. Excluded osmolytes modulate the equilibrium between two states through their osmotic pressure acting on the difference in the numbers of included or excess water molecules (*4, 32*).

The outstanding problems are (1) parsing the preferential hydration contributions from various chemical groups on macromolecules, e.g., the contributions from the peptide backbone, hydrophobic, hydrophilic, and charged side chains to the overall exclusion from proteins, and (2) understanding the physics underlying solute exclusion. Several groups have begun to examine preferential hydration contributions from the dependence of exclusion on the chemical character of the exposed surface (*33–35*). Bolen and co-workers (*11, 36*), for example, have measured the dependence of the solubility of amino acids and amino acid derivatives on solute concentration to estimate the contributions from the peptide backbone and side chains. The Schellman model (*37*) is commonly taken as the physical basis for exclusion, combining steric exclusion or crowding and a relative solute−water binding constant for solvation interactions with the macromolecule. The Kirkwood−Buff formalism links radial distribution functions to the observed inclusion of water or exclusion of solute (*2, 38, 39*).

We have devised a method of measuring changes in solute concentration in ordered condensed arrays of macromolecules. The distance between macromolecules depends on the net osmotic pressure exerted by the bulk solution. Large polymers such as PEG are completely excluded and apply their total osmotic pressure on the ordered array of macromolecules. Solutes that are partially excluded apply only a fraction of their total osmotic pressure. The apparent excess solute osmotic pressure due to exclusion can be determined by relating the change in spacing due to added osmolyte to the equivalent additional PEG pressure necessary to achieve the same spacing in the absence of solute. The change in the number of excess water molecules can then be calculated as a function of the distance between polymers using this excess pressure and eqs 3 and 5. Other measurements of preferential hydration give a single number of excluded water molecules. The excess pressure measurements using the osmotic stress−X-ray scattering approach, in contrast, give a distance dependence of exclusion so that the underlying physics can be probed.

We have previously examined the exclusion of nonpolar alcohols from highly charged DNA arrays (*22, 23*) and of salts from nonpolar HPC arrays (*24*). The salts follow the Hofmeister series (*40*) with KF more excluded than KCl which is more excluded than KBr. Alcohols with a greater excess of alkyl carbons over hydroxyl oxygens were more excluded from DNA. The two systems exhibited common features. The total number of excess water molecules was ~9 per interacting KF-HPC hydroxypropyl group and ~10 water molecules per DNA sodium phosphate−2-propanol interaction. Both KF and sodium phosphate are strong kosmotropes (*40*). The dependence of the change in solute concentration on the distance between DNA helices or HPC polymers was also quite similar. Exclusion from both the DNA and HPC systems could be described by an exponential function with a decay length of approximately 3 Å.

The results presented here extend our observations of a common mechanism for exclusion of small solutes from macromolecular surfaces. In agreement with the measurements of Bolen and co-workers (*11*) and with calculations (*25*), we see no interaction of urea with the hydrophobic groups on HPC at 5 or 20 °C. Urea increases slightly the precipitation transition temperature of HPC, suggesting that urea may be slightly included at elevated temperatures. This non-effect of urea could be due to a cancellation of steric exclusion balanced by specific binding but only if the distance dependence of these two is the same which seems unlikely.

The protein stabilizers, TMAO, glycine betaine, proline, and sorbitol, are all strongly excluded from HPC. The ~20 excess water molecules per saccharide unit for proline, glycine betaine, and sorbitol at 20 °C translate into a transfer energy from water to a 1 osmolal solution of solute of ~200 cal/mol of glucose monomer. If the interaction between these polar or zwitterionic solutes and HPC is dominated by exclusion from the three hydroxypropyl groups that cover the glucose monomer, then the transfer energy is ~65 cal/mol of hydroxypropyl group. Exclusion is somewhat smaller for TMAO at 20 °C corresponding to 50 cal/mol at 1 osmolal. The transfer energy for a hydroxypropyl group into 1 osmolal glycerol is only 30 cal/mol at 20 °C. These energies are similar to those inferred from changes in the solubility of glycylglycine by Auton and Bolen (*41*) for the same solutes and ascribed to exclusion from the peptide bond. If the transfer energies are recalculated for 1 osmolal rather than for 1 M solutions of solute, the peptide unit transfer energies reported by Auton and Bolen (*41*) are ~70 cal mol^{-1} osm^{-1} for TMAO, ~55 cal mol^{-1} osm^{-1} for glycine betaine, ~40 cal mol^{-1} osm^{-1} for proline, ~30 cal mol^{-1} osm^{-1} for sorbitol, and ~12 cal mol^{-1} osm^{-1} for glycerol. Only the exclusion energy for TMAO from the peptide unit is larger than for the hydroxypropyl group of HPC even though the peptide unit and a hydroxypropyl group are approximately the same size. Venkatesu et al. (*42*) estimate also from solubility measurements ~40 cal/mol for the transfer of a single methyl group (three apolar hydrogens) from water to 1 M TMAO or glycine betaine at 25 °C that corresponds to ~1.2 osmolal for both solutes.

Bolen and co-workers (*8, 26, 41*) have assigned the bulk of the transfer energy to the exclusion of these protein stabilizers from the peptide backbone rather than amino acid side chains. We find that exclusion of these polar osmolytes from HPC hydroxypropyl groups results in energies even larger than those estimated for the peptide bond. The exclusion of these polar osmolytes from hydrophobic amino acid side chains should contribute substantially to protein stability. The solubility method used by Bolen and co-workers to determine exclusion assumes that only monomers are present at the solubility limit or, more precisely, that the proportion of monomers compared with associated higher-order species does not change with osmolyte concentration at the solubility limit. It is possible that different solutes, however, can promote to different extents the formation of dimers, for example. This will make interpretation of solubility changes problematic.

The dependence of exclusion on the distance between HPC polymers for these protein stabilizers is consistent with our other measurements of exclusion of salts from HPC and of

alcohols from DNA. To a first-order approximation, exclusion is characterized by an exponential function with a decay length of approximately 3 Å. This functional form is widely observed for the forces at close distances, the last 10−15 Å separation, between many macromolecules from highly charged DNA to completely uncharged HPC and schizophyllan (*13, 14, 21, 43*). We have postulated that this common repulsive force is due to unfavorable restructuring of water between surfaces as they approach. The approximate 10−15 Å range corresponds to approximately two hydration layers on each surface. In contrast to the emphasis on the effect of isolated solutes on water structuring (*44–46*), the hydration force results from the inability of water to hydrate optimally two surfaces at close distances. The structuring of water around one surface affects the structuring around the other. In essence, the hydration force is a distance-dependent measure of the preference of the osmolyte and surface for water. The 3 Å decay length is the water−water correlation length within the hydration force framework. There could, however, be an additional contribution to this decay length from the loss of configurational entropy of the osmolyte in the constricted space between HPC polymers. The amplitude of exclusion is a measure of the extent of disruption of water structuring between the two surfaces. This hydration framework gives a plausible rationale for the exclusion amplitude of salts in the Hofmeister series observed previously (*24*).

As seen in Figure 2, the force amplitude for exclusion of sorbitol is 2-fold larger than for the homologous polyol glycerol that is half the size. This is consistent with our previous observation on the interaction of nonpolar alcohols with DNA (*22*). Exclusion scaled linearly with the number of alkyl carbons in excess of hydroxyl groups for chemically homologous solutes, not size directly. This dependence is not due to a steric exclusion; instead, the magnitude of solute exclusion seems to be a simple sum of the interaction energies of individual constituent chemical groups comprising the solute with the structurally repetitive macromolecular HPC and DNA surfaces. Sorbitol has twice the exclusion magnitude of glycerol because it has twice as many hydroxyl groups interacting with HPC.

If the structuring of the interacting water around either the solute or the macromolecular surface is temperature-dependent, then exclusion will also be temperature-dependent. The effect of excluded salts on the adamantane−cyclodextrin binding reaction has been shown to be primarily enthalpic with little contribution from entropy (*28*). This is in contrast to expectations from hard sphere steric exclusion or crowding that predict exclusion is entropic. The protein stabilizers examined here have a significant temperature dependence of exclusion from HPC. At a solute concentration of 1 osmolal, the free energy change due to exclusion from N_w water molecules per mole saccharide is

$$\Delta G = RT\frac{N_w}{55.56}$$

If we neglect heat capacity terms, then the enthalpy contribution can be crudely approximated as

$$\Delta H = -R\frac{T^2\Delta N_w(T)}{55.56\Delta T}$$

Table 3: Free Energies, Enthalpies, and Entropies of Osmolyte Exclusion at 20 °C[a]

osmolyte	ΔG (cal mol^{-1} osm^{-1})	ΔH (cal mol^{-1} osm^{-1})	$-T\Delta S$ (cal mol^{-1} osm^{-1})
glycerol	100	2300	−2200
sorbitol	200	2650	−2450
TMAO	150	600	−350
proline	200	1700	−1500
glycine betaine	220	1400	−1180

[a] The thermodynamic parameters are calculated for 20 °C per mole of glucose monomer. The error in ΔG is ∼15%. The errors in ΔH and $-T\Delta S$ values are ∼30%.

where $\Delta N_w(T)$ is the difference in the number of excess water molecules between two temperatures, T and $T + \Delta T$. Table 3 summarizes ΔG, ΔH, and $-T\Delta S$ values for the protein-stabilizing osmolytes with HPC at 20 °C based on the difference between 5 and 20 °C. In general, the enthalpic and entropic contributions to ΔG are opposite in sign and much larger than the free energy. The net exclusion free energy is due to the enthalpy magnitude being larger than the entropy magnitude. These thermodynamic properties are quite similar to those reported for the effect of sucrose on the adamantine−cyclodextrin binding reaction (*28*) and of poly(ethylene glycol) on the guanylate kinase−GMP/ATP binding reaction (*29*). This general behavior has been termed entropy−enthalpy compensation (*47, 48*) and has often been attributed to changes in solvation which would be consistent with a hydration force rationale behind exclusion.

CONCLUSIONS

Urea interacts only weakly with the hydrophobic surface of HPC, indicating that the interaction of urea with hydrophobic protein side chains will contribute little to denaturation. The exclusion of the protein-stabilizing osmolytes examined from the hydrophobic side chains of HPC is as large as their suggested exclusion from the peptide backbone, indicating a significant contribution to protein stabilization from the exclusion of these solutes from hydrophobic amino acid side chains. The dependence of exclusion on the spacing between HPC polymers in a condensed array is consistent with an interaction between solute and surface mediated by water structuring energetics, a hydration force. The exclusion of the polar protein-stabilizing osmolytes from HPC is temperature-dependent, suggesting that the energetics of water structuring between solute and surface is temperature-sensitive.

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