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Chemistry of Hofmeister Anions and Osmolytes

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Key Words

hydrophobic collapse, lower critical solution temperature (LCST), liquid-liquid phase separation, vibrational sum frequency spectroscopy (VSFS), urea, protein denaturation

Abstract

The study of the interactions of salts and osmolytes with macromolecules in aqueous solution originated with experiments concerning protein precipitation more than 100 years ago. Today, these solutes are known to display recurring behavior for myriad biological and chemical processes. Such behavior depends both on the nature and concentration of the species in solution. Despite the generality of these effects, our understanding of the molecular-level details of ion and osmolyte specificity is still quite limited. Here, we review recent studies of the interactions between anions and urea with model macromolecular systems. A mechanism for specific ion effects is elucidated for aqueous systems containing charged and uncharged polymers, polypeptides, and proteins. The results clearly show that the effects of the anions are local and involve direct interactions with macromolecules and their first hydration shell. Also, a hydrogen-bonding mechanism is tested for the urea denaturation of proteins with some of these same systems. In that case, direct hydrogen bonding can be largely discounted as the key mechanism for urea stabilization of uncollapsed and/or unfolded structures.

1. INTRODUCTION

Hofmeister series: a ranking of the physical properties of ions in aqueous solutions discovered by Franz Hofmeister (1850–1922)

Kosmotropic ions: strongly hydrated ions, sometimes referred to as water structure makers

Chaotropic ions: weakly hydrated ions, sometimes referred to as water structure breakers

Over the past 12 decades, a wide variety of phenomena, from protein folding and enzymatic activity to colloidal assembly and protein crystallization, has been shown to follow the Hofmeister series (1–11). The series ranks the relative influence of ions on the physical behavior of a wide variety of aqueous processes. This behavior is more pronounced for anions than cations and is quite general. The typical order for the anion series is as follows:

$$CO_3^{2-} > SO_4^{2-} > S_2O_3^{2-} > H_2PO_4^- > F^- > Cl^- > Br^- \sim NO_3^- > I^- > ClO_4^- > SCN^-.$$

Ions on the left are called kosmotropes, which tend to precipitate proteins from solution and prevent protein unfolding, whereas ions on the right are chaotropes, which increase solubility and promote the denaturation of proteins. Chloride is usually considered the dividing line between these two types of behavior. In recent years, there has been an explosion in research papers tied to the Hofmeister series (12–54).

Despite its ubiquity, a molecular-level understanding of the Hofmeister series is still lacking. It was originally believed that ions affected the physical behavior of aqueous macromolecular systems by making or breaking water structure (3). Kosmotropic ions are referred to as water structure makers as they are supposed to strengthen the hydrogen-bonding network of bulk water, whereas chaotropic ions supposedly help break the hydrogen-bonding network. However, recent studies cast serious doubts on this notion. Specifically, Bakker and coworkers (18–21) studied the hydration shell of different anions in water by means of femtosecond two-color pump-probe spectroscopy. They found that the hydrogen-bonding network of the solution is not significantly changed by the ion's presence. Using pressure perturbation calorimetry, Pielak and coworkers (22) monitored the effect of various salts, stabilizers, and denaturants on bulk water structure. These studies showed that the effects of different species on bulk water structure appeared to be uncorrelated with the Hofmeister series. Ninham and colleagues (23–26) have introduced a dispersion potential into DLVO (Derjaguin-Landau-Verwey-Overbeek) theory and employed their modified model to help interpret many ion-specific phenomena. Most recently, Saykally and coworkers (42) combined Raman spectroscopy and theory to study water's OH vibrations in the presence of salts. They found that the ions do not have long-range effects on bulk water structure.

Particular attention has been paid over the past few years to ion-partitioning behavior at the air/water interface. Jungwirth and Tobias (10, 32–35) have performed simulations of air/electrolyte systems in the presence of salts in which they showed clearly that larger, less hydrated anions have a preference for the interfacial region. Pegram & Record (44–46) employed a salt ion-partitioning model to interpret and predict Hofmeister ion effects at the air/water and protein/water interfaces. These studies also showed that more chaotropic anions generally partition to the interface to a greater extent than other species. This notion has also been experimentally demonstrated by high-pressure X-ray photoelectron spectroscopy (41).

Additionally, work has been carried out to understand the influence of salt at the protein/water and lipid/water interfaces. For example, Jungwirth and coworkers (8, 37, 38) have run molecular dynamic simulations at the protein/water interface. Leontidis and coworkers (47, 50, 51) investigated lipid monolayer at the air/water interface and suggested that specific anion effects on this lipid model system were mostly related to ion size. Finally, our laboratory studied water structure and Langmuir monolayers by nonlinear optical spectroscopy (55). These studies demonstrated that the physical properties of the monolayer may directly follow a Hofmeister series even when the adjacent water structure does not. All the evidence above suggests that bulk water structure making and breaking are not responsible for phenomena related to the Hofmeister series. This

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requires alternative hypotheses to be formulated. One of the best ways to do this is through the appropriate choice of model systems, which we highlight in the discussion below.

Our laboratory has employed a variety of approaches to elucidate the molecular-level mechanism of osmolytes and Hofmeister anions on the behavior of macromolecules in solution. Many of these experiments have involved monitoring the hydrophobic collapse of thermoresponsive polymers (13, 14, 56) and peptides (15), as well as the aggregation of proteins (16) by temperature gradient microfluidics. Employing this strategy allows the phase-transition temperature of collapse and aggregation to be obtained rapidly and repeatedly with excellent precision. Based on the data abstracted from these model systems, we proposed a mechanism for the influence of Hofmeister ions on hydrophobic collapse and protein aggregation. Complementary data are abstracted with lipid, polymer, and protein monolayers at the air-water interface by vibrational sum frequency spectroscopy (VSFS), a surface specific vibrational spectroscopy (17, 55, 57–65). VSFS can provide crucial corroborating evidence for understanding polymer-ion interactions because it is capable of examining interfacial water structure as well as the ordering of molecules at the interface (66).

In this review, we focus on the chemical specificity of Hofmeister anions and the molecular-level mechanism for urea denaturation. In Section 2 we discuss the mechanisms for the Hofmeister series on the aggregation behavior of thermoresponsive polymers and peptides. In Section 3 we turn our attention to the urea denaturation of proteins, again using model systems. Finally, we briefly discuss the future prospects for this type of research.

Osmolytes: small net neutral organic solutes that can affect the physical properties of macromolecules in aqueous solutions

VSFS: vibrational sum frequency spectroscopy

PNIPAM: poly(*N*-isopropylacrylamide)

Hydration entropy of an ion: the degree of order or disorder created by adding an ion to an aqueous solution

Surface tension increment: the change in surface tension per unit concentration of added solute

2. HOFMEISTER ANIONS

2.1. Uncharged Model Systems: The Hydrophobic Collapse of Thermoresponsive Macromolecules

Proteins can undergo both cold and thermal denaturation. When the temperature of the solution is raised, a protein will denature as thermal energy starts to break hydrogen bonds, activate low-lying vibrational modes, and unravel the macromolecule (67, 68). On the other hand, cold denaturation occurs because of the enthalpically favorable interactions between the more hydrophobic interior of the protein and solvent water molecules (69, 70). To mimic the cold denaturation of proteins, we have employed poly(*N*-isopropylacrylamide) (PNIPAM) (71). This thermoresponsive polymer, which is an isomer of poly(isoleucine), consists of a hydrocarbon backbone with a pendant amide group. This macromolecule is highly soluble in aqueous solution at temperatures below ~31°C, but rapidly collapses, aggregates, and precipitates at higher temperatures (72, 73), as shown in **Figure 1*a***. **Figure 1*b*** illustrates the structure of this molecule, along with its interactions with anions, which are discussed below.

The influence of Hofmeister anions on the hydrophobic collapse of PNIPAM can be explained on the basis of direct interactions of anions with the macromolecule and its first hydration shell (13, 14). **Figure 1*b*** shows a model consisting of three interactions among anions, PNIPAM, and hydration water. An anion, X^-, can polarize a water molecule that is directly involved in hydrogen bonding with the amide. The ability of an anion to polarize the first-hydration-shell water of the polymer is manifest quantitatively in the hydration entropy, ΔS_{hydr}, of each anion. Second, anions can interfere with the hydrophobic hydration of PNIPAM by increasing the surface tension at the hydrophobic/aqueous interface. As the salt concentration increases, the surface tension is increased at the aqueous/polymer interface (44–46). Moreover, the energy for cavity formation at the interface will be raised (74, 75). The surface-tension increase is measured quantitatively by the surface tension increment, σ. Both the water polarization and the surface tension effect

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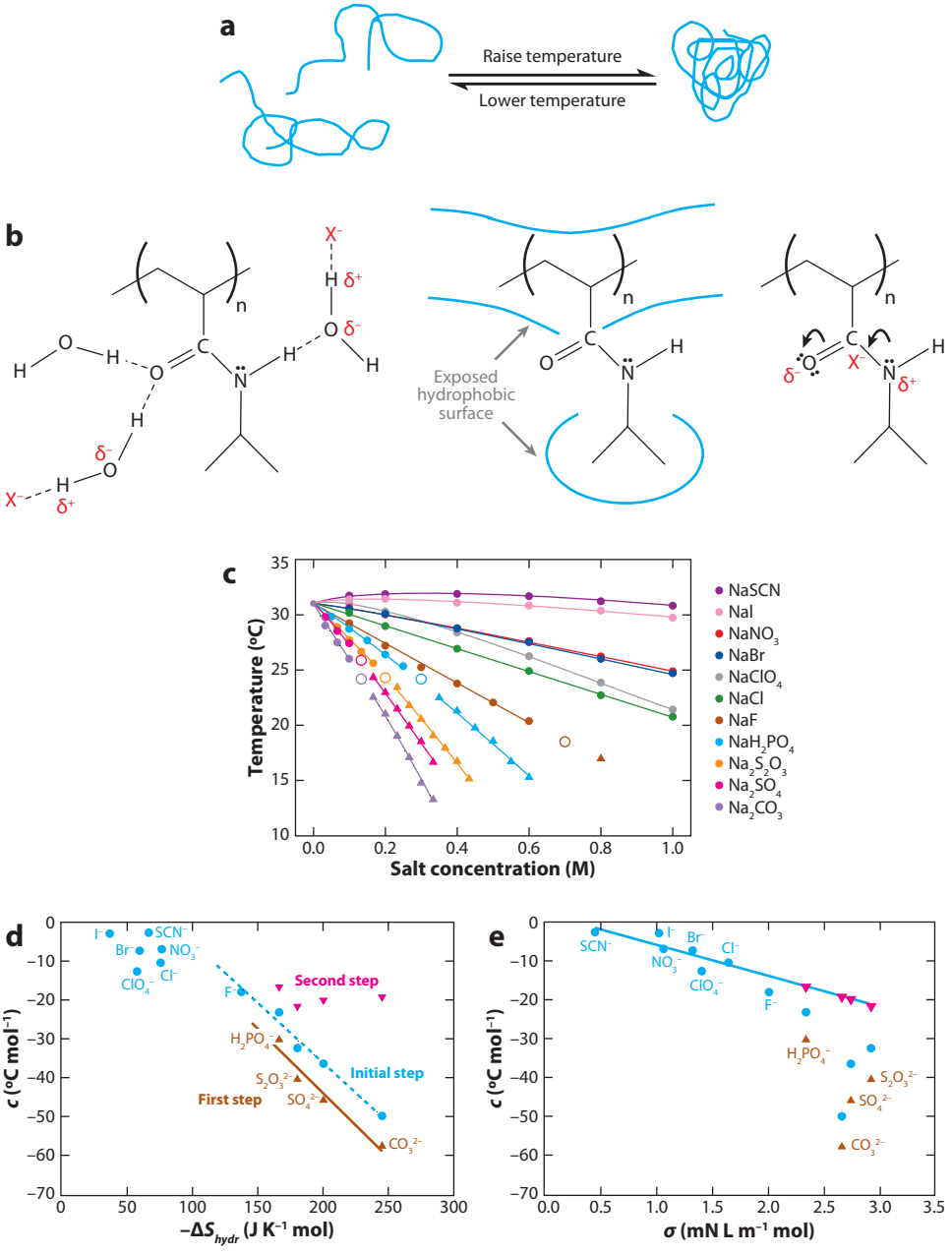
LCST: lower critical solution temperature

cause a depression in the lower critical solution temperature (LCST) of PNIPAM as the salt concentration is increased. These effects should vary roughly linearly with salt concentration at least up to moderate concentrations (76). By contrast, anions can also bind to the amide moieties in PNIPAM. This causes an increase in the LCST because it charges the polymer surface. Moreover, it is a saturation phenomenon.

As shown in **Figure 1*c***, the effect of 11 anions in the Hofmeister series on the hydrophobic collapse of PNIPAM was investigated (13). The perturbation of PNIAPM's LCST by chaotropic



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anions was modeled with a simple equation that includes a constant, a linear term, and a Langmuir isotherm:

$$T = T_0 + c[M] + \frac{B_{\max} K_A [M]}{1 + K_A [M]}, \quad (1)$$

where T_0 is the LCST of PNIPAM in pure water and $[M]$ is the molar concentration of salt. The constant, c, has units of temperature/molarity. K_A is the apparent binding constant of the anion to the polymer, and $B_{\max}$ represents the maximum LCST increase at saturation ion binding. ClO_4^- exhibited the strongest salting-in effect, followed by SCN^-, I^-, Br^-, and NO_3^-. On the other hand, kosmotropes showed a linear dependency on salt concentration:

$$T = T_0 + c[M]. \quad (2)$$

The parameters in Equation 2 have the same physical meaning as in Equation 1. Beyond a certain concentration, however, a two-step transition is observed for the kosmotropic anions (CO_3^{2-}, SO_4^{2-}, $S_2O_3^{2-}$, $H_2PO_4^-$, and F^-), and both a low and high temperature change in the light-scattering data is observed. The first step in the two-step phase transition has a steeper slope than that initially observed, whereas the second step has a shallower dependency on salt concentration. By plotting c values with the properties of anions (**Figure 1*d*,*e***), the initial slope and the slope from the first step of the two-step phase transition correlate well with hydration entropy, ΔS_{hydr}, for kosmotropes. However, the rest of the data do not correlate well with ΔS_{hydr}. Yet when the same c values are plotted against the surface tension increments of anions, σ, the data for chaotropes and the second step of the two-step phase transition are correlated, whereas the rest of the data are not (**Figure 1*e***).

The results shown above indicate that strongly and weakly hydrated anions affect the LCST of PNIPAM by different mechanisms. The chaotropes decrease the LCST via a surface-tension effect, which causes hydrophobic collapse. For kosmotropic ions, the polarization of hydration-shell water molecules and surface-tension effects are both at work. Specifically, the first step of the two-step phase transition in the presence of kosmotropes results from the perturbation of solvation waters hydrogen-bonded to the amide, whereas the second step involves the dehydration of the hydrophobic portion of PNIPAM. On the other hand, direct ion binding is a saturation phenomenon, which leads to the salting-in of PNIPAM. Cl^- and all kosmotropes do not show any binding to the polymer. Moreover, the molecular-weight effect on the salt dependency of PNIPAM's LCST was investigated with four PNIPAM samples of different molecular weights (14). Although the molecular weight of the polymer affects the individual interactions among PNIPAM molecules, water, and anions, the overall mechanism is the same for different molecular weights.

We note that the thermodynamic dissociation constants for chaotropic anions binding to PNIPAM abstracted from the LCST measurements are only apparent binding constants

Figure 1

(*a*) The hydrophobic collapse of PNIPAM as a function of temperature. (*b*) Schematic diagrams of the interactions among anions, PNIPAM, and hydration waters. (*c*) Plot of the lower critical solution temperature values for PNIPAM as a function of salt concentration between 0.0 and 1.0 M for a series of 11 Hofmeister salts. The triangular symbols for the kosmotropes are for the lower-temperature phase transition of a two-step phase transition. The higher-temperature phase transition for kosmotropes is not shown. The order of the curves (*from top to bottom*) corresponds to the color legend at the right of the figure. The solid lines are fits to the experimental data points. (*d*) Plot of hydration entropy of the 11 anions versus the fitted c values from Equations 1 and 2. (*e*) Plot of surface tension increment of the 11 anions versus c values obtained from Equations 1 and 2. Hydration entropy and surface tension increment values were obtained from References 2, 79, 133, and 134. Figure reproduced with permission from Reference 13. Copyright 2005, American Chemical Society.

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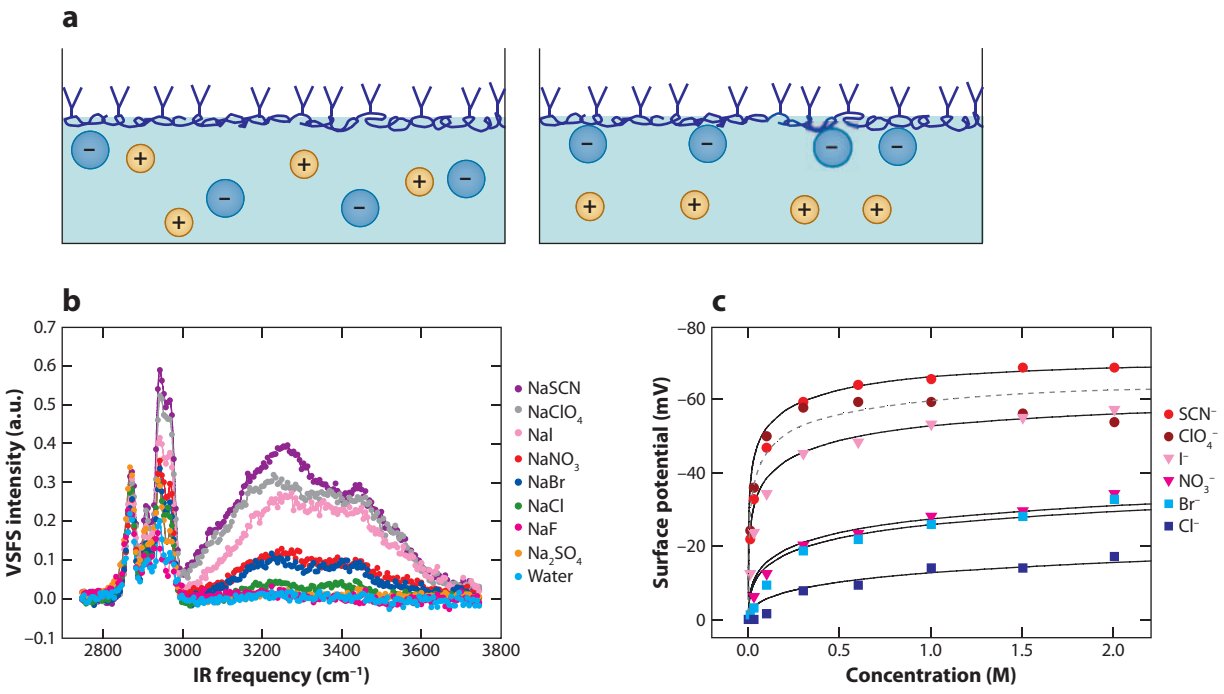

Figure 2

(*a*) Schematic diagram of anion adsorption onto a PNIPAM monolayer adsorbed at the air/water interface. (*b*) Vibrational sum frequency spectroscopy spectra showing specific anion effects on PNIPAM adsorbed at the air/water interface. Each subphase contained 1 M of a given salt as indicated in the legend except for NaF and Na_2SO_4, which are measured with saturated solutions (~0.8 M for both salts). (*c*) Change in the surface potential as a function of salt concentration in the subphase. The curves represent fits using the Gouy-Chapman-Stern model. Figure reproduced with permission from Reference 17. Copyright 2007, American Chemical Society.

because they were not obtained isothermally. They were based on the temperature change as a function of chaotropic anion concentration. To measure the binding constants of chaotropic anions to the macromolecule in an isothermal fashion, Cremer and coworkers (17) studied specific ion effects on interfacial water structure next to PNIPAM by VSFS (**Figure 2*a***). From the data (**Figure 2*b***), one can see that only very weak OH stretch peaks were observed in the absence of salts. When chaotropic anions were introduced, however, the water peaks became much more prominent. The most chaotropic anion, SCN^-, induced the greatest water-peak intensity, whereas the most kosmotropic species, SO_4^{2-}, had virtually no influence on the water peaks. The interfacial water structure is therefore ion specific, and the intensities of both OH peaks (3200 cm^{-1} and 3400 cm^{-1}) followed the Hofmeister series:

$$SCN^- > ClO_4^- > I^- > NO_3^- \approx Br^- > Cl^- > \text{pure water} \approx F^- \approx SO_4^{2-}.$$

The oscillator strength of the 3200 cm^{-1} peak was employed as a measure of the surface potential. This allowed binding-constant information for chaotropic anions to be obtained by varying their subphase concentrations (**Figure 2*c***). The shape of the binding curves was quite reminiscent of a Langmuir isotherm, which suggested saturation-binding behavior. Because of electrostatic repulsions with increased binding, however, a Langmuir absorption isotherm is physically inadequate. Instead, a modified isotherm based on Gouy-Chapman-Stern theory is superior (17, 77, 78). The binding constants of anions to PNIPAM obtained at the air/water interface fell in the same

rank order as that from the LCST measurements, except for perchlorate, which was anomalous because of its larger size compared with other anions.

Anion adsorption behavior at the PNIPAM interface can be viewed as a partitioning of anions between the polymer-covered surface and the bulk solution (44–46). The equilibrium constant dictates the distribution between these two states. For an anion to be adsorbed at the interface, it needs to undergo partial desolvation. The chaotropic anions have relatively low solvation free energies (79). Therefore, a smaller desolvation penalty is paid compared with the kosmotropic anions. However, varying the identity of the cations in this system made almost no difference.

ELP: elastin-like polypeptide

The mechanism proposed for the hydrophobic collapse of PNIAM can also be applied to explain Hofmeister effects on the LCST of uncharged elastin-like polypeptides (ELPs) (15). **Figure 3** illustrates the mechanism for the influence of anions on the hydrophobic collapse of ELPs. ELPs are based on a repetitive pentapeptide motif, Val-Pro-Gly-Xaa-Gly, in which the guest residue, Xaa, is any amino acid except proline. One of the most commonly employed guest residues is valine, which is the one shown in **Figure 3**.

Upon heating an ELP solution through its LCST, the macromolecules collapse to form molecular aggregates without specific tertiary structure, but with β-spiral secondary structure (80, 81). ELPs have an advantage over PNIPAM as a model system in that their primary sequence is identical to that of protein systems. Additionally, their sequence and chain length can be precisely controlled by genetic expression in bacteria using recombinant DNA technology (82, 83).

Two ELPs, ELP[$V_5A_2G_3$-120] and ELP[V-120], were employed for Hofmeister anion studies (15). The value, 120, in the formula represents the number of pentameric repeats in the 600 residue macromolecules. In ELP[V-120], all the guest residues are valine, whereas in ELP[$V_5A_2G_3$-120], 50% of the guest residues are valine with 20% alanine and 30% glycine. The overall mechanism for Hofmeister effects on the LCST of ELPs was nearly the same as that for PNIPAM. However, the LCST did not break up into two distinct steps in the presence of kosmotropic anions, even at high salt concentration. Moreover, several key differences were observed between these two ELPs. First, the initial LCST of ELP[$V_5A_2G_3$-120] in the absence of salts is approximately 14°C higher than that of ELP[V-120]. This results from the presence of less hydrophobic residues such as alanine and glycine. Second, the salting-in effects from chaotropic anions were more pronounced for ELP[$V_5A_2G_3$-120] than for ELP[V-120]. This is because the accessibility of anions to the backbone of the polymer increases as bulky valine residues are replaced with alanine and glycine. The ELP work lends credence to the idea that the molecular-level mechanism for Hofmeister effects originally proposed for PNIPAM can be applied more widely. It appears from these results that the mechanism can indeed be applied to at least other noncharged polymer systems that undergo hydrophobic collapse and aggregation. The next question that must be addressed is whether this mechanism continues to be valid for charged systems, which is discussed in the next section.

2.2. Positively Charged Systems: Liquid/Liquid Phase Separation of Proteins

Most proteins possess a net charge in aqueous solutions. It has been suggested that the relative efficacy for anions to influence the physical properties of proteins follows distinct Hofmeister series depending on whether the macromolecules bear a net negative or net positive charge (27, 84–87). Specifically, when the pH of the solution is above the pI of the protein, the direct series is followed. Conversely, an inverse Hofmeister series is typically observed when the solution pH is below the pI of the protein. As such, negatively charged proteins are thought to obey a direct Hofmeister series, and positively charged proteins are thought to follow an inverse Hofmeister series. Recently, we studied the liquid-liquid phase separation of lysozyme in the presence of

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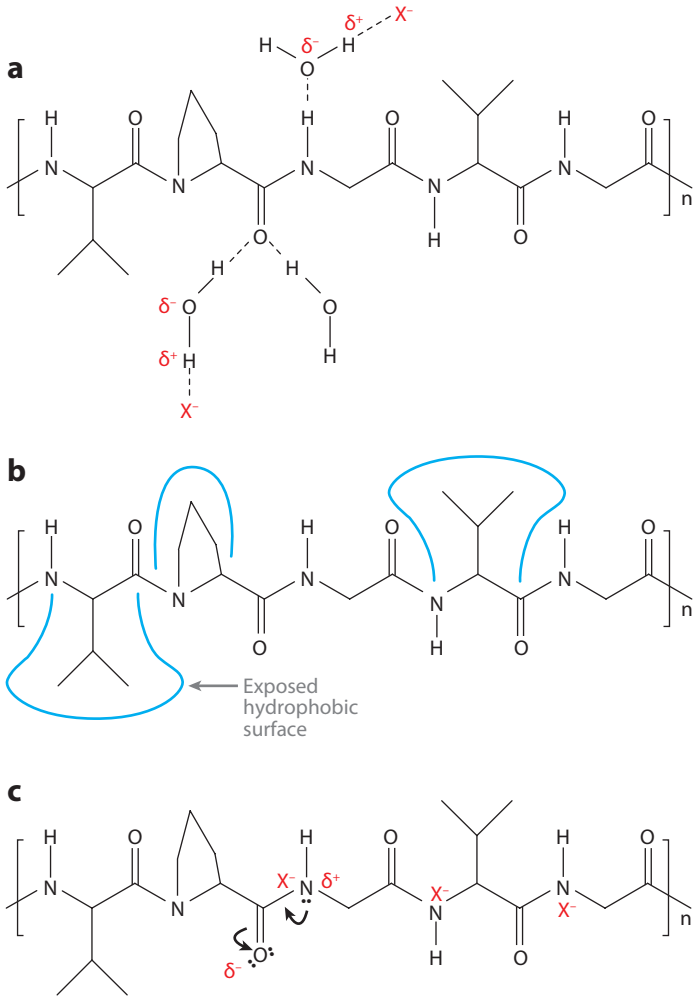

Figure 3

Proposed mechanisms for specific anion effects on the hydrophobic collapse of elastin-like polypeptides. (*a*) Direct interactions of anions with water molecules involved in the hydrogen bonding of the amide. (*b*) The blue lines represent the hydrophobically hydrated regions of the biomacromolecule. The cost of such hydration increases as salt is added to solution. (*c*) Direct ion binding of chaotropic anions to the amide moieties along the backbone of the polypeptide should cause a salting-in effect. Figure reprinted with permission from Reference 15. Copyright 2008, American Chemical Society.

chaotropic salts (16). This system was chosen because it is probably the most widely known and explored model of protein-protein interactions for positively charged biomacromolecules. An inverse Hofmeister series was followed at low salt concentration, as has previously been observed (27, 84–86). However, at higher salt concentrations (above 200 to 300 mM monovalent salt), the system reverted back to a direct Hofmeister series.

Liquid-liquid phase-transition behavior can be found for numerous concentrated protein systems in water, especially if polyethyleneglycol is also added to the system. When these systems are cooled below the phase-transition temperature, they form micrometer-sized droplets of aggregated proteins. The process, which is usually reversible, is called a liquid-liquid phase separation

because the solution separates into two-coexisting liquid phases: one rich in protein and one poor in protein. When this occurs, the solution becomes cloudy. Hence, the transition temperature is referred to as a cloud point (88–90). Studying the cloud-point temperature of proteins in concentrated solutions is an effective way to determine the strength of protein-protein interactions (91).

Polarizability: relative tendency of a charge distribution to be distorted by an external electric field

Figure 4*a* shows the cloud-point behavior for a 90.4 mg ml^{-1} lysozyme solution at pH 9.4 as a function of anion type for a series of sodium salts. It can be readily observed that the system follows an inverse Hofmeister series at low salt concentrations and a direct Hofmeister series at high salt concentrations. The anion effects on the cloud-point temperature can be modeled

$$T = T_0 + \frac{B_{\max}[M]e^{-b[M]}}{K_d + [M]e^{-b[M]}} + c[M], \quad (3)$$

where T_0 is the cloud-point temperature of lysozyme in the absence of salt, and $[M]$ is the molar concentration of salt. The constant b is an electrostatic interaction factor that is related to the surface potential of lysozyme. The constant $B_{\max}$ represents the maximum increase in the cloud-point temperature under saturation conditions. Both $B_{\max}$ and b measure the effectiveness of a specific anion to screen the electrostatic repulsion between the charged macromolecules. The constant c characterizes specific anion effects on the interfacial tension at the protein/water interface. Equation 3 can also be applied to neutral systems, such as those described in the previous sections (13–15). In those cases, however, the surface potential would be zero and, therefore, $b = 0$. As can be clearly seen from the data, the inverse Hofmeister series is dominant at low salt concentrations, and the order reverts to a direct Hofmeister series at high salt concentrations.

The inverse and direct Hofmeister series shown for the lysozyme system can be more easily visualized by breaking up the data in **Figure 4*a*** into a linear and a saturation contribution by fitting the curves with Equation 3. Indeed, when the linear term is subtracted from the data, the series of binding curves can be directly observed in the residuals (**Figure 4*b***). These residuals follow an inverse Hofmeister series from the most effective to least effective salting-out anion: $ClO_4^- > SCN^- > I^- > NO_3^- > Br^- > Cl^-$. This series results from the differing abilities of the anions to associate with the positively charged lysozyme surface, and it is correlated with the size of the hydrated anions, as shown in **Figure 4*d*,*e***.

It is known from the literature that the size of hydrated anions is directly related to their hydration free energy (79) and that bigger anions have a lower hydration free energy. Therefore, it appears that these larger anions are more readily able to shed their hydration shells and interact with binding sites on the positively charged proteins. Such a phenomenon would, of course, be expected to show saturation behavior as there are a fixed number of charged sites on the protein molecules. By contrast, a direct Hofmeister series can be revealed by subtracting off the binding curve portion of the data in **Figure 4*a*** and plotting the linear residuals (**Figure 4*c***): $Cl^- > NO_3^- > Br^- > ClO_4^- > SCN^- > I^-$. Such a direct Hofmeister series is to be expected as the anions modulate the surface tension at the protein/aqueous interface.

Curiously, the slopes of these lines in **Figure 4*c*** are not correlated with changes in surface tension at the air/water interface. Instead, an excellent correlation is found to the polarizability of the anions (**Figure 4*f***). As can be seen, more polarizable anions have a stronger ability to partition to the protein/aqueous interface and decrease the interfacial tension. This actually inhibits the formation of the aggregated phase. On the other hand, the least polarizable anion, Cl^-, increases the interfacial tension and facilitates the cloud-point formation. The behavior of these anions at the protein/water interface stands in stark contrast to their behavior at the air/water interface, where they all are known to increase the surface tension (44, 45, 76). The difference in behavior at the protein/water interface stems from the macromolecules having a much higher dielectric constant

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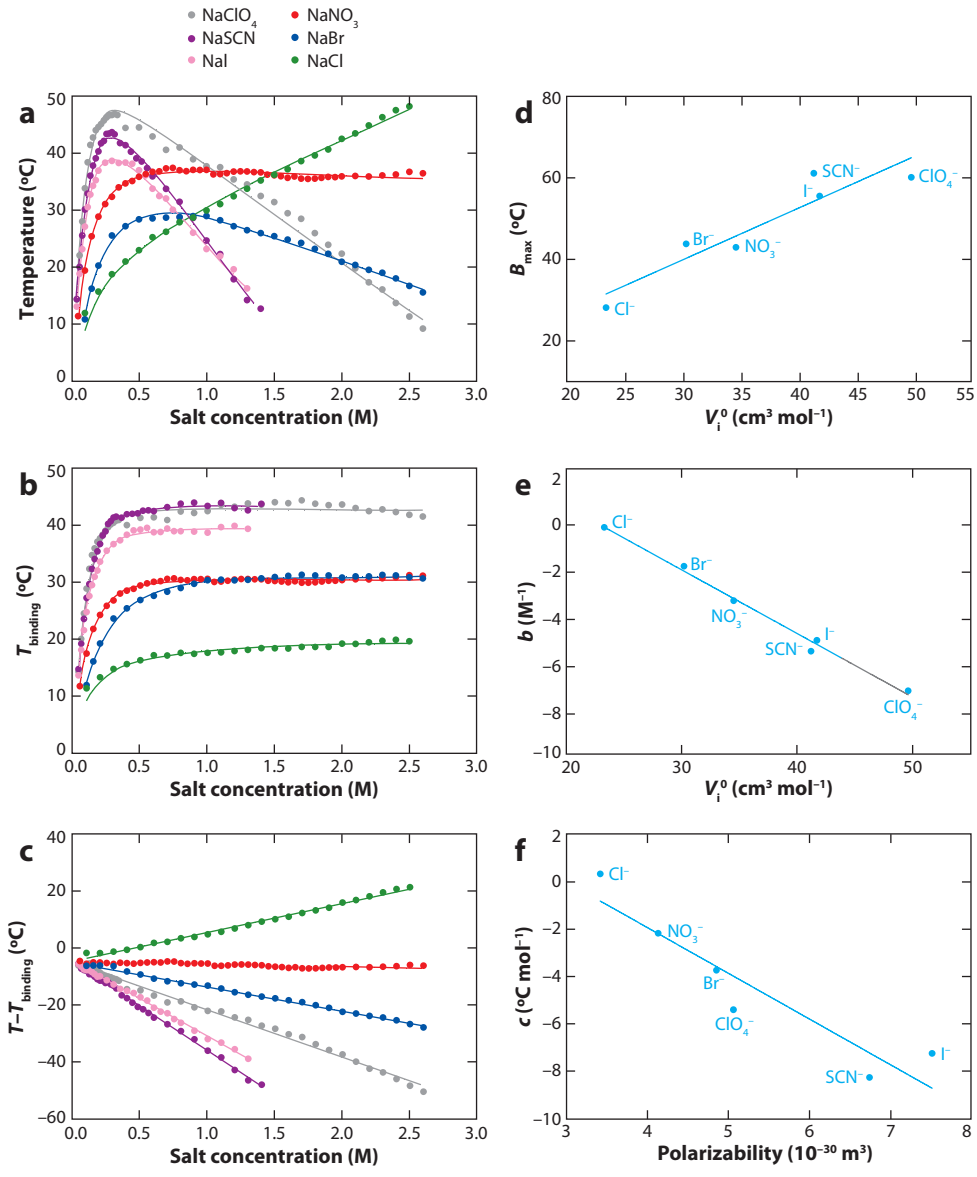

Figure 4

(*a*) Cloud-point temperature data for the liquid-liquid phase transition of lysozyme as a function of anion type and concentration. All experiments were performed with 90.4 mg ml^{-1} lysozyme in 20 mM tris buffer at pH 9.4. The solid lines are curve fits to Equation 3. (*b*) The residual data after subtracting the linear portion contribution from Equation 3. (*c*) The residual data after subtracting the binding isotherm contribution from Equation 3. (*d*) Correlation of standard partial molar volumes of the anions versus the constant B_{max}. (*e*) Correlation of standard partial molar volumes for the anions versus the constant *b*. (*f*) Plot of the polarizability of the anions versus the constant *c*. Standard partial molar volumes and polarizability of the anions were obtained from Reference 79. Figure reproduced from Reference 16.

than air (92, 93). Indeed, it is well-known that the most chaotropic anions decrease the surface tension at oil/water interfaces (94, 95). Their ability to do this correlates to their octanol-water partition coefficient, which is also dependent on the polarizability of the anion (96, 97).

In summary, experiments on the liquid-liquid phase separation of lysozyme show that ion-macromolecule binding and interfacial tension changes dominate this positively charged system. This may be a general phenomenon for positively charged systems, whereby an inverse Hofmeister series will be obeyed at low salt concentrations and a direct Hofmeister series will take over as the salt concentration is increased. In fact, preliminary data suggest that this is also the case for positively charged ELPs (Y. Cho, Y. Zhang & P.S. Cremer, unpublished data).

3. OSMOLYTES

Like salts, osmolytes have significant effects on protein folding and stability (98, 99). For example, urea is widely used as a denaturant, whereas trimethylamine *N*-oxide (TMAO) is a powerful stabilizer (100, 101). Work studying the effects of osmolytes on protein stabilization/denaturation goes back more than half a century (102). Despite this, there is still no consensus as to the mechanism by which molecules like urea and TMAO affect protein structure at the molecular level (103–125). Putative mechanisms described in the literature can be summarized into two categories: direct effects and indirect effects. Here we define direct mechanisms to involve hydrogen bonding between osmolyte molecules and peptide backbones and/or polar and charged side chains. On the other hand, we define indirect effects as influences of osmolytes on the solvation of hydrophobic portions of the protein or changes to bulk water structure.

Based in part on the work shown above, the effects of Hofmeister ions are believed to be direct. However, the influence of osmolytes on macromolecules may be indirect, at least in the case of urea. One piece of evidence supporting an indirect hypothesis is the substantial concentration of osmolytes typically required to influence protein structure. For example, proteins are often denatured in 8 M urea solutions (126, 127), whereas it is known that even 0.1 M salt is sufficient to influence protein stability (128). This difference is noteworthy because 76% of water molecules should be in contact with at least one urea molecule in a 4 M urea solution (112). Therefore, little bulk water is left. This means that changes in the solution properties via high concentrations of osmolytes could substantially influence the ways in which polypeptides are solvated. According to this hypothesis, denaturation agents like urea should favor the unfolded state because an increase in solvent accessible area is thermodynamically favorable in comparison to pure aqueous solutions (108). Recent work by Bolen and coworkers (106, 109) points to the importance of solvent interactions with the polypeptide backbone as opposed to the side chains. Molecular dynamics simulations by Bennion & Daggett (112) indicate direct backbone interactions as well as indirect effects. Furthermore, Berne and coworkers (123) have demonstrated that the interactions between denaturant molecules and hydrophobic side chains may be a key consideration. Finally, Kuharski & Rossky (129) have suggested that urea denaturation may involve the displacement of several water molecules by each urea from the apolar solvation shell based upon entropic considerations.

3.1. Testing Hydrogen-Bonding Models of Protein Denaturation by Urea

To understand why urea denatures proteins, it is first important to determine if the osmolyte works through direct hydrogen-bonding interactions or whether indirect effects are dominant. Although protein denaturation by urea has been widely investigated in the literature, spectroscopic information for urea interacting with proteins is still largely missing. This is the result of the physiochemical complexity of proteins. For example, amide I band Fourier transform infrared

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(FTIR) data is commonly used to study the structural changes of proteins and urea interactions with biomacromolecules. However, proteins and peptides typically show many overlapping peaks, which makes it difficult to distinguish the spectroscopic signature of urea binding from related changes in protein secondary and tertiary structure. Recently, we employed the protein-mimetic species PNIPAM as a model system to directly investigate hydrogen-bonding interactions between urea and amide moieties by using amide I band FTIR spectroscopy (56). We chose this system because PNIPAM's amide I band structure is substantially simpler than most proteins and, therefore, easier to interpret. Along with FTIR data, thermodynamic measurements of PNIPAM's hydrophobic collapse and Stokes radius were performed. Additional experiments were also conducted with methylated urea.

Figure 5*a* shows the LCST values for PNIPAM with increasing concentrations of urea measured by temperature gradient microfluidics. The decrease in the LCST of PNIPAM with increasing urea concentration is quite curious. Indeed, this indicates that urea actually stabilizes the collapsed and aggregated state of the polymer. This result is exactly opposite to the behavior found for the vast majority of protein systems, in which urea stabilizes the unfolded state relative to the hydrophobically collapsed state. Indeed, urea helps stabilize the collapsed state of PNIPAM because the NH_2 groups on urea interact with the pendant amide groups on PNIPAM in a bivalent manner and thereby work like a cross-linking agent. This brings adjacent chains into closer proximity and thereby stabilizes the collapsed state (**Figure 5*b***).

The direct binding of urea to the amide groups in PNIPAM was proven by amide I band FTIR measurements (**Figure 5*c***). The amide I band of PNIPAM in 10 mM phosphate buffer at 10°C consisted of a single peak at ~1625 cm^{-1}, which indicated that all the carbonyl groups were hydrogen bonded to water (130). Once 6 M ^{13}C-labeled urea was introduced into the PNIPAM solution at 10°C, a second peak appeared at ~1652 cm^{-1}. This peak was indicative of C=O–H-N hydrogen bonding (131) between the H-N moieties in urea and the C=O moieties in PNIPAM. The assignment of this peak was confirmed by a series of isotopic labeling experiments.

The fraction of urea bound to PNIPAM as a function of urea concentration was determined from FTIR spectra as shown in **Figure 5*d***, and it was strongly inversely correlated to the decrease in the LCST value. Therefore, the observed changes in the phase-transition temperature are directly coupled with the hydrogen bonding of urea to PNIPAM. Moreover, the binding of urea to the macromolecule is highly cooperative, which is consistent with a cross-linking mechanism. This mechanism was further elucidated by Stroke radius measurements. These studies showed that the ratio between the Stroke radius of PNIPAM in increasing concentrations of urea decreased until approximately 5 M and then rose sharply (**Figure 5*e***). Cross-linking by urea binding at low urea concentration induced the hydrophobic collapse of PNIPAM and decreased the Stokes radius

Figure 5

(*a*) Lower critical solution temperature (LCST) trends for PNIPAM with increasing urea concentration. (*b*) Proposed cross-linking mechanism for PNIPAM by urea. (*c*) Fourier transform infrared (FTIR) spectrum of the amide I band for PNIPAM at 10°C in phosphate buffer (*top panel*) and in the presence of 6 M ^{13}C-labeled urea (*bottom panel*). The orange circles represent the data, the red curves are the overall fit to the data, and blue curves are the individual Gaussian contributions to the fits. (*d*) The fraction of urea bound to PNIPAM as abstracted from fitting the FTIR data. The fraction represents the area under the curve for the 1652 cm^{-1} peak divided by the area for both the 1625 cm^{-1} and 1652 cm^{-1} peaks. (*e*) Stokes radii measurements for PNIPAM as a function of urea concentration by gel filtration column chromatography. (*f*) LCST trends for PNIPAM as a function of osmolyte concentration for methylated ureas. The data for each osmolyte are color coded. Figure reproduced with permission from Reference 56. Copyright 2009, American Chemical Society.

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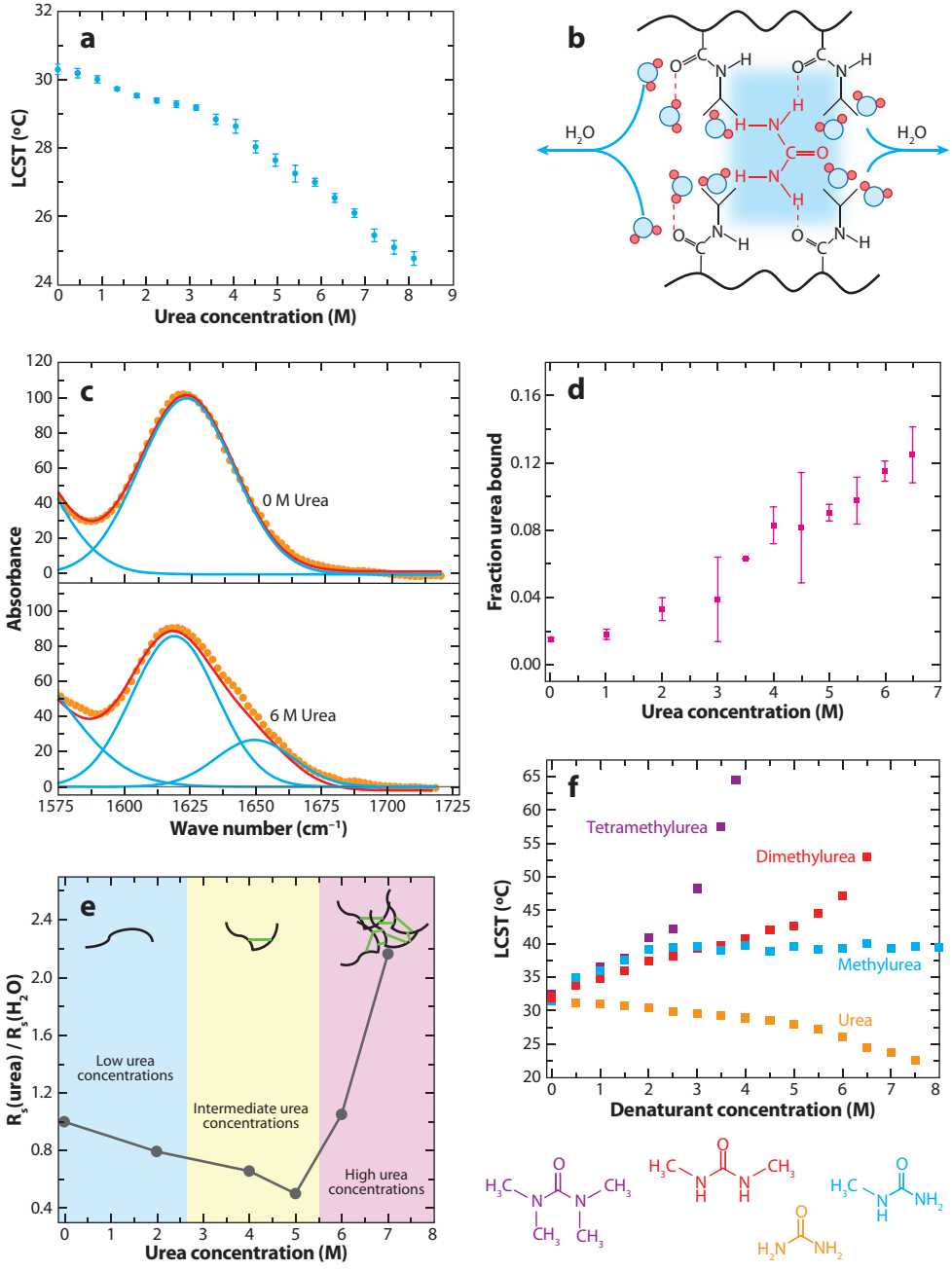
of the polymer. At higher urea concentrations, intermolecular cross-linking became dominant and increased the size of the polymer aggregates.

A final piece of evidence to demonstrate the cross-linking mechanism involves methylating the urea used in the above experiments. These studies showed that increasing the methylation of urea increased the LCST of PNIPAM at constant osmolyte concentration (**Figure 5*f***). In fact,



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BSA: bovine serum albumin

methylated urea compounds eliminate hydrogen-bonding possibilities and strongly inhibit urea's cross-linking ability. Therefore, the uncollapsed state of PNIPAM was favored, and the LCST values rose. In summary, urea interacts with the pendant amide groups in PNIPAM through hydrogen bonding in a bivalent manner and thereby stabilizes the folded and aggregated state of the polymer. Methylated urea molecules do not hydrogen bond to PNIPAM and therefore help stabilize the uncollapsed, soluble state of the polymer. The findings of these studies are consistent with an indirect, hydrophobic mechanism for urea-based protein denaturation. This is because direct hydrogen binding seems to favor the collapse state of macromolecules. It should of course be more difficult for urea to bind with the backbone of polypeptides compared with the pendant amide groups of PNIPAM. Therefore, far less hydrogen bonding is expected for proteins. The question still remains, however, exactly how an indirect mechanism for urea-based denaturation would operate. Such a mechanism may involve the displacement of several water molecules by the larger osmolyte molecules (129), changes in interfacial water structure to increase the solubility of proteins (132), and interactions between denaturant molecules and hydrophobic side chains (123).

3.2. Urea Orientation at the Protein/Water Interface

To further understand the mechanism for protein denaturation by urea, we investigated the interfacial orientation of these osmolytes at protein surfaces. VSFS, an intrinsically surface-specific technique, was used to address this issue (57). In VSFS spectra, the net orientation of the transition dipole moment of a vibrational mode can be determined to be either up or down relative to other oscillator moieties in the system (58–60). Therefore, the absolute orientation of interfacial urea can be directly measured under the appropriate circumstances. To do this, we measured VSFS spectra next to a saturated monolayer of bovine serum albumin (BSA) at the air/water interface at various pH values in the presence and absence of urea. The results demonstrated that (similar to water molecules) (66) interfacial urea flips its orientation as the system goes through the isoelectric point of BSA. It appears that the orientations of both urea and water are determined by the net surface charge of the protein interface (**Figure 6**). When the solution pH is higher than the isoelectric point, BSA molecules are negatively charged, which induces both urea and water molecules to orient with their hydrogen atoms pointing toward the protein and their oxygen atoms pointing away from it. At the isoelectric point, pH 5, urea still orients with its hydrogen atoms facing toward the protein surface, but the net orientation is substantially weaker. Finally, when the solution pH is below the isoelectric point, the urea molecules flip to become weakly orientated with their oxygen atoms facing toward the interface.

Like the PNIPAM studies, the flipping of interfacial urea molecules also suggests an indirect mechanism for protein denaturation. Indeed, a vast number of different proteins are denatured in

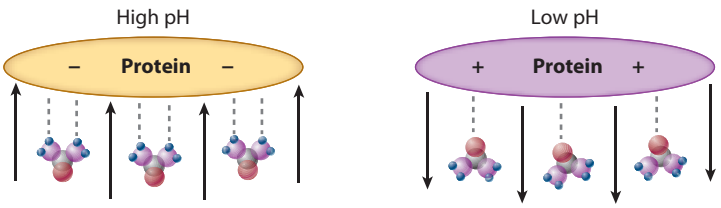

Figure 6

Schematic diagram of urea orientation at the protein/water interface for two different surface charge states (pH values). The arrows represent the local electric fields. Figure reprinted with permission from Reference 57. Copyright 2007, American Chemical Society.

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urea solution regardless of the net charge on the biomacromolecules. Therefore, urea probably adopts different orientations under various conditions. This would be inconsistent with a simple mechanism whereby a preferential orientation of urea helps induce protein unfolding.

4. FUTURE PROSPECTS FOR HOFMEISTER AND OSMOLYTE STUDIES

With some data in hand about the effects of ions and osmolytes, the question becomes what to investigate next. Perhaps the most important unsolved problem is the role that cations play in Hofmeister chemistry. Indeed, cations are key biological players with high intercellular concentrations of K^+ and high extracellular concentrations of Na^+. We suggest that an effective means of studying the cationic Hofmeister series would be to employ simple model systems, as was done with anions. The next key question to be addressed is whether mechanisms for the Hofmeister series described here are applicable to a wide range of phenomena. Indeed, these mechanisms were derived from studies of macromolecular aggregation. It is simply unknown whether they would be useful for explaining Hofmeister effects in enzymology, atmospheric science, or a myriad of other phenomena.

Future studies of osmolytes will also be crucial. As noted above, some osmolytes stabilize protein structure, whereas others destabilize it. The use of model systems to understand the molecular-level details of these phenomena should certainly be pursued. Specifically, it is now important to understand exactly how an indirect mechanism for urea-based protein denaturation should operate. More investigations can be done for other osmolytes as well, including TMAO, trehalose, other sugars, and amino acids. Spectroscopic studies, thermodynamic measurements, and simulations will all be necessary.

SUMMARY POINTS

1. In uncharged systems, the anionic Hofmeister series can be explained on the basis of the direct interactions of anions with macromolecules and their immediately adjacent hydration shells. The series is related to the anions' hydration entropy, surface tension increment, and ability to bind to the macromolecules. Kosmotropic and chaotropic anions typically work through separate mechanisms.
2. Positively charged systems follow both inverse and direct Hofmeister series depending on salt concentration. At low salt concentrations, electrostatic interactions follow an inverse Hofmeister series, which is correlated with the size and hydration of the anions. After the positive charges are neutralized, the systems obey a direct Hofmeister series, which is affected by interfacial tension at the protein/aqueous interface in the presence of chaotropic anions.
3. Direct hydrogen bonding between urea and protein molecules is not the central mechanism for protein denaturation by urea. Instead, denaturation occurs through indirect mechanisms that may involve changes to water structure or hydrophobic interactions.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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9. Summarizes some studies that undermine the idea that Hofmeister effects work by changing bulk water structure.

10. Reviews comprehensively the Hofmeister series at the air/water interface.

13. Proposes a molecular-level model to explain the Hofmeister series that only involves the ions' direct interactions with macromolecules and their first hydration shell.

16. Presents a general mechanism for the anionic Hofmeister series that can be applied to both neutral and positively charged systems.

17. Demonstrates that anion binding to a polymer adsorbed at the air/water interface causes adjacent water molecules to align according to the Hofmeister series.

18. Shows that water structure outside the hydration shell of an ion is not influenced by the ion.

22. Demonstrates in a thermodynamic analysis that no correlation can be found between a solute's impact on water structure and its effect on protein stability.

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