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Salting Effects on Protein Components in Aqueous NaCl and Urea Solutions: Toward Understanding of Urea-Induced Protein Denaturation

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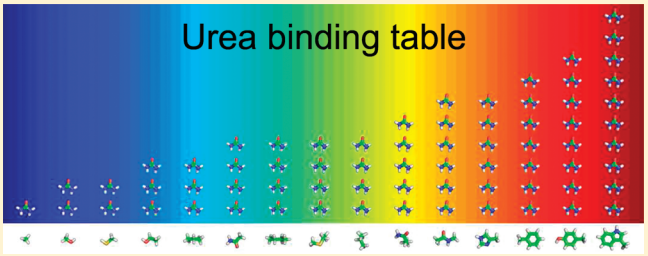
Supporting Information

ABSTRACT: The mechanism of urea-induced protein denaturation is explored through studying the salting effect of urea on 14 amino acid side chain analogues, and *N*-methylacetamide (NMA) which mimics the protein backbone. The solvation free energies of the 15 molecules were calculated in pure water, aqueous urea, and NaCl solutions. Our results show that NaCl displays strong capability to salt out all 15 molecules, while urea facilitates the solvation (salting-in) of all the 15 molecules on the other hand. The salting effect is found to be largely enthalpy-driven for both NaCl and urea. Our observations can explain the higher stability of protein's secondary and tertiary structures in typical salt solutions than that in pure water. Meanwhile, urea's capability to better solvate protein backbone and side-chain components can be extrapolated to explain protein's denaturation in aqueous urea solution. Urea salts in molecules through direct binding to solute surface, and the strength is linearly dependent on the number of heavy atoms of solute molecules. The van der Waals interactions are found to be the dominant force, which challenges a hydrogen-bonding-driven mechanism proposed previously.



INTRODUCTION

For more than a century, urea has been commonly used as an agent for protein denaturation. The use of urea as a probe plays a fundamental role in the science of biology which helps us know about protein folding and unfolding, binding, and aggregation. However, the mechanism of how denaturation happens has been a historical puzzle and has been reviewed from time to time. Two mechanisms have been proposed. In the "direct interaction mechanism",[1−4] direct interactions between protein and denaturants are supposed to be important. For urea denaturation, a two-stage unfolding of the proteins has been proposed.[4] In the first stage, urea molecules accumulate in the vicinity of the protein surface. These urea molecules replace water to "solvate" the protein surface and initiate protein unfolding. In the second stage, urea penetrates into the interior of the protein, forming hydrogen bonds with backbone atoms and breaking the hydrophobic interaction. Thermodynamically, the urea-binding biases the folded/unfolded equilibrium toward unfolding and finally causes denaturation. On the contrary, the "indirect interaction mechanism"[5−11] emphasizes the effect of changing of water structure through addition of denaturents. The direct interaction mechanism is becoming widely accepted while the latter one is lacking experimental support.[12−16]

The attracting force between urea and protein is quite controversial. Because urea has a carbonyl group and two amide groups, it potentially can form hydrogen bonds and other electrostatic interactions when encountering side-chain and backbone atoms.[1,2,17−19] It is therefore not surprising that the action of urea denaturation was attributed to the direct hydrogen bondings.[1] There are numerous experimental works showing that urea does form hydrogen bond with protein.[17,20] However, the direct hydrogen bonding was found not necessary to induce denaturation.[21] Apart from the electrostatic interaction, van der Waals (vdW) or dispersion force is another driving force that mediates urea−protein interaction.[22,23] In one recent study, neutral residues, rather than the charged ones, attract more urea in their surroundings.[24] Also, it is reported that mutation of charged residues to neutral ones can accelerate urea denaturation through computer simulations.[25] Thus, it is still an open question whether the denaturation is mainly induced by the electrostatic interaction/hydrogen bonding or by the vdW interaction.

From another point of view, urea can be treated as a cosolvent and compared with simple ions. Ions' position in the *Hofmeister* series[26,27] reflects their salting effects on protein stability and solubility. The order of anions is usually given as $SO_4^{2-} > HPO_4^{2-} > Cl^- > Br^- > I^- > ClO_4^- > SCN^-$. Anions on the left decrease the solubility of hydrophobic molecules and

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Table 1. Model Molecules Used in This Study and Solvation Free Energies (in kcal/mol) in Pure Water in This Study and from Previous Literature

3-letter code	1-letter code	model compound	$\Delta G_{\text{AMBER charges}}$	$\Delta G_{\text{RED charges}}$	$\Delta G_{\text{Shirts,}}$[35]	$\Delta G_{\text{EXP1,}}$[44]	ΔG_{EXP2}[45]
NMA	B	*N*-methylacetamide	−8.20	−7.80	–	–	–
ALA	A	methane	2.45	2.45	2.57	1.94	2.00
CYS	C	methanethiol	0.06	−0.37	0.11	−1.24	−1.24
PHE	F	toluene	0.54	−0.35	0.10	−0.76	−0.89
HIE	H	4-methylimidazole	−5.31	−7.46	−8.98	−10.27	–
ILE	I	butane	2.47	2.52	2.84	2.15	2.08
LEU	L	isobutane	2.41	2.41	2.72	2.28	2.32
MET	M	methylethyl sulfide	0.22	0.28	0.91	−1.48	–
ASN	N	acetamide	−6.97	−8.49	−7.80	−9.68	−9.71
GLN	Q	propionamide	−10.75	−7.72	−7.69	−9.38	–
SER	S	methanol	−4.61	−4.45	−4.37	−5.06	−5.11
THR	T	ethanol	−3.40	−3.84	−3.83	−4.88	−5.01
VAL	V	propane	2.33	2.46	2.69	1.99	1.96
TRP	W	methylindole	−3.60	−4.76	−4.88	−5.88	–
TYR	Y	*p*-cresol	−1.92	−4.90	−4.23	−6.11	−6.14

precipitate proteins from solution ("salting-out"). By contrast, anions on the right increase the solubility of hydrophobic molecules ("salting-in") and promote the denaturation of proteins.[22] One approach to explore the mechanisms of cosolvent effects on protein structure and stability is to determine the effects of cosolvent on model molecules of protein components.[28,29] One key physical quantity relating to the solubility is the transfer/solvation free energy (ΔG). The solvation free energy of model molecules can not only be calculated with high accuracy but also be decomposed into physical components, which can provide unambiguous evidence on the underlying mechanism.

Motivated by the above considerations, we explored the protein denaturation through comparative study of the salting effects from aqueous urea and NaCl solutions. Fifteen model molecules were used to mimic protein backbone and side chains. The solvation free energies in these two electrolytes and those in pure water were used to evaluate the salting effect from cosolvents. Our study has successfully reproduced the salting-out effect of NaCl and salting-in effect of urea on these protein compounds. By careful analysis of the thermodynamics properties of the salting effects, the mechanism of urea-induced denaturation is revealed in an unambiguous and unique way.

MATERIALS AND METHODS

All the molecular dynamics (MD) simulations were performed with GROMACS[30] version 4.0, using the *NPT* ensemble with pressure 1 bar and temperature 300 K. SHAKE constraints[31] were applied to all bonds involving hydrogen atoms. The MD integration time step was 2 fs. The van der Waals interactions were treated with a typical distance cutoff of 12 Å, with a long-range correction employed (for distances greater than the cutoff). The electrostatic interactions, on the other hand, were handled with the particle mesh Ewald algorithm.[32,33] The nonbonded interaction pair list was updated every 10 fs. Periodic water box contained 1 solute molecule, 900 TIP3P[34] water in the pure water simulation. For the salting simulation, additional 35 NaCl or 70 urea molecules were added corresponding to molar concentrations of 2.05 M NaCl and 3.56 M urea, respectively. Thermodynamic integration (TI)[35−37] was used to compute the solvation free energy through computing the ensemble average of $dH/d\lambda$ at a number of values of λ (λ-windows). The electrostatic interactions were first decoupled linearly with 21 λ_C windows from 0.0 to 1.0 with a step length of 0.05. When turning off the Lennard-Jones (LJ) interactions, the soft-core potentials[38] were used to avoid the poor convergence. In addition to the same 21 λ windows in the first step, 32 λ_{LJ} windows have been added (see Supporting Infomation). At each λ value, a short (0.4 ns) simulation was first conducted for system to pre-equilibrate, which is followed by a 2 ns production run. Other than the free energy calculations, normal molecular simulations were also conducted for 200 ns for dynamics analysis. The 14 neutral amino acid side chain analogues considered are shown in Table 1. Other than these, *N*-methylacetamide (NMA), which is a secondary amide that mimics the protein backbone, was also considered. Topologies of the solute molecules were adopted from AMBER(ff03).[39] Both AMBER-derived atomic partial charges[35] and RED-generated (using RED tools[40]) partial charges (through Gaussian03[41] with HF/6-31G* basis set; a list can be found in the Supporting Information) were used. AMBER-supplied urea model was used. For Na^+ and Cl^- ions, it is well-known that AMBER force fields usually induce salt crystallization well below their solubility limit. New van der Waals parameters for Na^+ and Cl^- (new-NaCl) ions developed by In Suk Joung[42] have also been used in this study. Our results show that the inaccuracies caused by the force fields do not impact the salting effects qualitatively.

Kirkwood−Buff (KB) theory is a statistical theory of solutions.[43] The principal quantities of interest are the KB integrals

$$K_{ij} = 4\pi \int_0^\infty [g_{ij}^{\mu VT}(r) - 1] r^2 \, dr$$

where r is the distance between two species, i and j. $g_{ij}(r)$ is the radial distribution function (RDF) for species j around the center of species i. These integrals can be used to relate the thermodynamic properties of a solution to the molecular distributions within it. One gets $\Delta\mu_s^{ex} \propto -(\Delta K_{\text{solute,cosolvent}} - \Delta K_{\text{solute,water}})$ which gives a direct link of solvation free energy change ($\Delta\Delta G$) to the KB integral change from pure water to the solution in study.

RESULTS AND DISCUSSION

The solvation free energies in pure water (ΔG_{water}) are shown in Table 1. For most molecules, the values are within a range of

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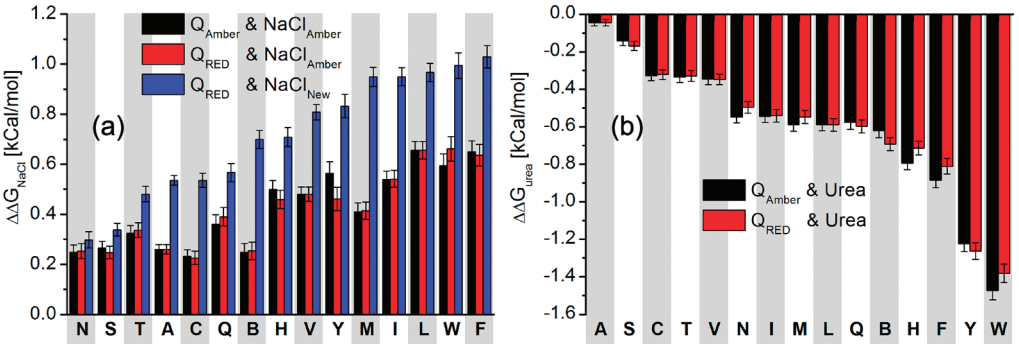

Figure 1. Solvation free energy changes ($\Delta\Delta G_{NaCl}$) of 15 molecules from pure water to NaCl solution (a). Three different combinations of force fields were tested: AMBER derived molecule charges with AMBER NaCl (black), RED-generated molecule charges with AMBER NaCl (red), and RED-generated molecule charges with new-NaCl model (blue). Solvation free energy changes ($\Delta\Delta G_{urea}$) from pure water to aqueous urea solution (b). AMBER-derived molecule charges (black) and RED-generated molecule charges (red) were tested.

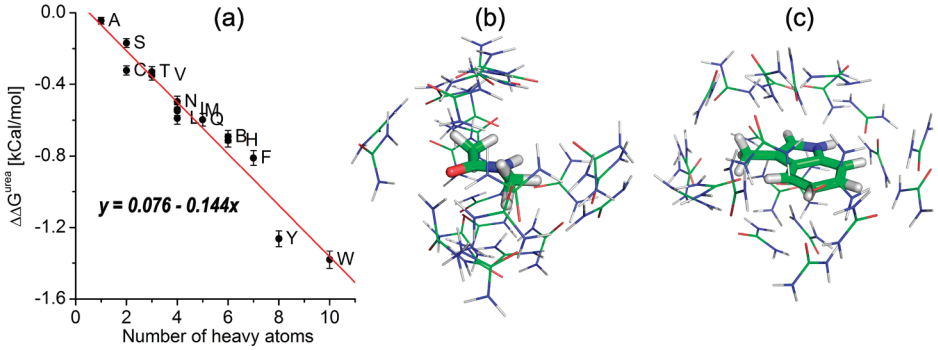

Figure 2. Linear relationship between the solvation free energy change in aqueous urea solution and the number of heavy atoms of the solute molecule (a). The representative binding patterns of urea around (b) NMA and (c) TRP. The structure snapshots were obtained from the top 25 highest populated clusters, where the clustering method is introduced in the Supporting Information.

−10−3 kcal/mol. The RED charges and AMBER charges give similar ΔG_{water} for most of the molecules examined, where RED charges show better consistency with the experiment values of PHE, HIE, ASN, TRP, and TYR. Overall, the values are well consistent with the experimental and previous theoretical results.[35,44,45] When cosolvent was added, the solvation free energy change was defined as follows (in the case of NaCl):

$$\Delta\Delta G_{NaCl} = \Delta G_{NaCl} - \Delta G_{water}$$

According to this definition, a positive value of $\Delta\Delta G$ means worse solvated (salting-out effect), a negative value means better solvated (salting-in effect).

NaCl Salts out All 15 Molecules. In order to check the sensitivity of the force fields used, we have performed three sets of simulations with force field combination of (1) AMBER partial charges of solute molecules with AMBER NaCl model, (2) RED partial charges of solute molecules with AMBER NaCl model, and (3) RED partial charges of solute molecules with the new-NaCl model. The results were shown in Figure 1a. All the 15 molecules are uniformly salted-out by NaCl, which is independent of the force fields used. It is found that two sets of partial charges principally do not affect the salting effect (this phenomenon will be discussed later) when the same AMBER NaCl model is used. It is obvious that new-NaCl model results in stronger salting-out effect for all the molecules. Our data show that small and polar molecules like SER and ASN are relatively weakly salted-out, while large and nonpolar molecules like LEU, PHE, and TRP are most significantly salted-out. This is in line with our previous study on 121 model molecules.[46] The salting-out effect could be described as preferential binding of ions and water to solutes.

Urea Salts in All 15 Molecules. In the case of urea, we have performed two sets of simulations with the force field combination: (1) AMBER partial charges of solute molecules with urea, and (2) RED partial charges of solute molecules with urea. As shown in Figure 1b, all the molecules are uniformly salted-in. In contrast to the case of NaCl, the strength of the salting effect depends on the size of solutes rather than the chemical polarity. For example, ALA, SER, and CYS are the smallest molecules which are weakly salted-in, while TYR and TRP are the largest molecules which are strongly salted-in. The dependence of $\Delta\Delta G_{urea}$ with respect to the number of heavy atoms of the solute molecules is shown in Figure 2a. A linear correlation was clearly observed with fitting curve as $y = 0.076 - 0.144x$. The linear correlation provides a hint that the salting effects could not mainly be of electrostatic or hydrogen-bonding nature which will be elaborated more below. It needs emphasizing that the fitting curve does not go across zero point when $x = 0$. This implies that a positive $\Delta\Delta G$ is expected for even tiny molecules. In fact, methane molecule (an ALA analogue) is an exception and was found being salted-out by urea based on an early study.[5] However, our calculation predicts a weak salting-in effect (−0.04 ± 0.02 kcal/mol) for methane. We realized that ΔG of methane in pure water is 2.45 ± 0.01 kcal/mol in our calculation, which is larger than the experimental value of ∼2.0 kcal/mol.[44,45] The methane model used here overestimates the positive solvation free energy. Further improvement of the methane model is needed.

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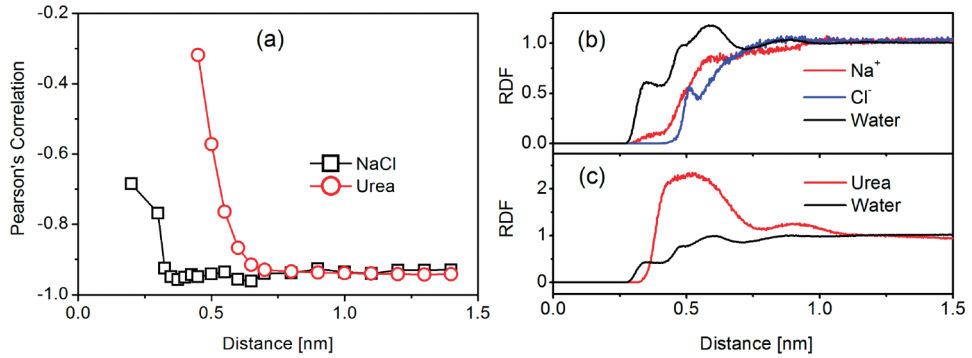

Figure 3. Correlation of differential KB integral with the values of $\Delta\Delta G$ (a). (b,c) Radial distribution functions of Na^+, Cl^-, urea, and water around a TRP molecule.

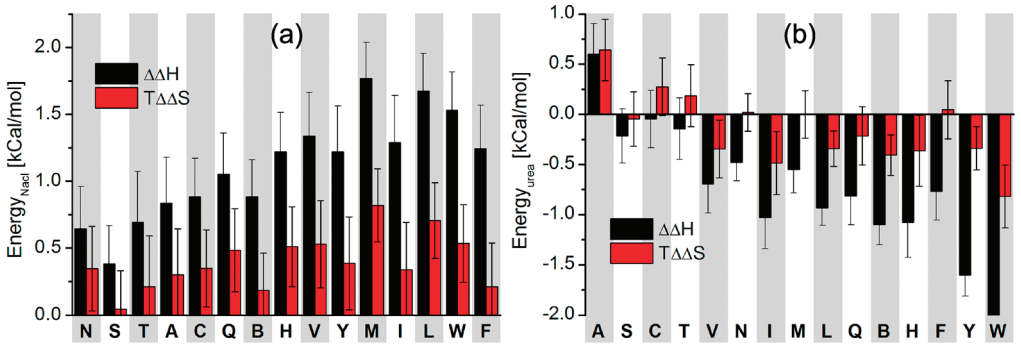

Figure 4. Enthalpic (black) and entropic (red) contributions to solvation free energy changes from pure water to (a) NaCl solution and (b) urea solution. The order of the protein components is $|\Delta\Delta G|$ increasing (weak salting effect to strong salting effect).

The top four salted-in molecules are TRP, TYR, PHE, and HIE analogues, which are all aromatic residues and have planar structures. Urea's position around NMA and TRP are shown in Figure 2b and Figure 2c, where the highest populated residue-urea interacting patterns were illustrated. One finds that the orientation of urea relative to the solute molecule is quite random. There is no clear indication of favorable hydrogen-bond interaction.

Interpret $\Delta\Delta G$ with Kirkwood–Buff Integral. The results of $\Delta\Delta G$ can be interpreted through KB integral which describes the salting effect purely through a statistical point of view.[43,47] In our previous study, a direct relationship, $\Delta\Delta G \propto \Delta K_{SC} - \Delta K_{SW}$, among 121 model solutes in NaCl solution, was established,[46] where ΔK_{SC} and ΔK_{SW} are changes of KB integral of cosolvent (C) and water (W) around solute (S) from pure water to the NaCl solution, respectively.

KB integrals were calculated based on MD simulation trajectories (200 ns) using RED partial charges and the new-NaCl model. Pearson's correlation[48] between $\Delta\Delta G$ and $\Delta K_{SC} - \Delta K_{SW}$ is shown in Figure 3a, where a high correlation is observed at distance larger than 0.35 nm (NaCl solvent) and 0.70 nm (urea solvent), respectively. The radial distribution functions (RDF) of Na^+, Cl^-, urea and water around TRP are also shown in Figure 3, b and c. It is clear that, around a TRP, there is a discernible water shell while Na^+ and Cl^- ions are repelled away. On the contrary, for a TRP in urea solution, urea accumulates nearby and water is repelled away. Correlation value does not change at larger distances, which implies that the modulation of water structures does not contribute to salting-effect. This phenomenon supports the direct interaction mechanism in which the preferential binding of urea results in denaturation.

Salting Effect Is Enthalpy Driven. In the frame of direct interaction mechanism there are still controversies regarding the main driving force for the denaturation action of urea.[4,19] Therefore, it is valuable to decompose the free energy changes to enthalpic and entropic contributions. For a solvation process, the enthalpy change (ΔH) is defined as the change of potential energy from the decoupled state (isolated solute with solvent, $\lambda_{LJ} = 0$, $\lambda_C = 0$) to the coupled state (solvated complex, $\lambda_{LJ} = 1$, $\lambda_C = 1$). The potential energies were computed by averaging over the 200 ns trajectories of the respective states. The entropy change of the solvation process, ΔS, is derived from the relation $\Delta G = \Delta H - T\Delta S$.

The relative enthalpy change from pure water to NaCl solvent is defined as $\Delta\Delta H_{NaCl} = \Delta H_{NaCl} - \Delta H_{water}$ (similar formulation for entropy). The values of $\Delta\Delta H$ and $T\Delta\Delta S$ are shown in Figure 4. For the case of NaCl, all the $\Delta\Delta H$ are positive which indicates that the solvation is enthalpy unfavorable. On the other hand, all $T\Delta\Delta S$ values are positive playing a counteracting role on the free energy. The absolute values of enthalpy increases overwhelm those of entropy increases. Hence for NaCl, the salting-out effect is clearly enthalpy driven.[49] In the case of urea, the $\Delta\Delta H$ of all molecules, except ALA, are negative which indicates that the urea solution stabilizes the solvation state energetically. Most of the entropy decreases (negative $T\Delta\Delta S$), however, with the absolute value less than that of enthalpy. Overall, the salting effect of urea is also enthalpy driven,[23] similar to that of NaCl. It is interesting to find that most $T\Delta\Delta S$ have the same sign as $\Delta\Delta H$ which demonstrates the well-known entropy–enthalpy compensation.[50−52] This compensation is commonly observed in thermodynamic analysis of proteins, nucleic acids–ligands interaction.

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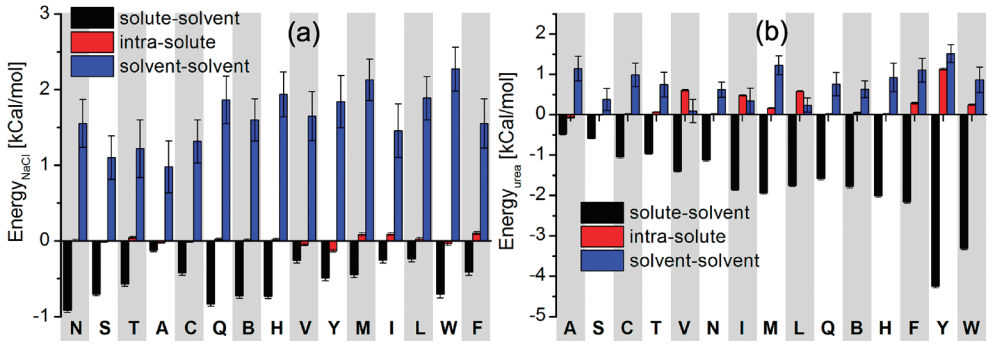

Figure 5. Decomposition of the enthalpy change into solute−solvent, intrasolute, and solvent−solvent interaction energy terms for (a) NaCl solvent and (b) urea solvent.

To gain more insights into the underlying mechanism, the enthalpy changes are decomposed further into changes of solute−solvent, intrasolute, and solvent−solvent interaction energy terms as shown in Figure 5. Here, the solvent includes water molecules and cosolvent (NaCl or urea). Because all the 15 molecules are nearly rigid, it is not surprising that the changes of intrasolute term are very small and can be safely ignored. The physical meaning of the solvent−solvent term is related to the energy required to create a cavity inside the solvent to accommodate the solute molecule. The solute−solvent term is the coupling between solute and its environment.

It has been reported that both NaCl and urea can increase the surface tension of water.[53] This effect is reflected in our present calculations by the positive values of all the solvent−solvent energy change terms. These energy-unfavorable terms reflect harder cavity formation in both NaCl and urea solutions compared to that in pure water. On the other hand, the coupling term of solute−solvent is energy favorable in both the cases. This phenomenon is interesting especially in the case of NaCl solutions which tend to salt-out solute molecules. Addition of NaCl has strengthened the interactions between solute molecules with its surroundings. However, this solute−solvent energy decrease cannot counterbalance the increasing of solvent−solvent energy, which finally results in salting-out effect. On the contrary, in urea solution, the solute−solvent energy decrease successfully overwhelms the unfavorable solvent−solvent interaction, which results in salting-in effect.

Furthermore, the favorable solute−solvent interaction in urea solution is found to come mainly from the van der Waals interactions, and the electrostatic interactions only play a very minor role as shown in Figure 6, which is consistent with

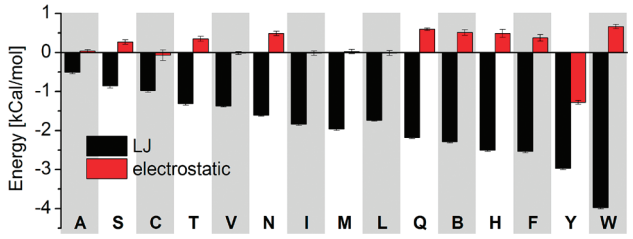

Figure 6. Decomposition of the solute−solvent interaction in urea solvent into the van der Waals (LJ) and the electrostatic terms.

previous studies on protein lysozyme in 8 M urea[4] and carbon nanotubes in 8 M urea.[54] This phenomenon challenges a hydrogen-bonding-driven mechanism in urea-induced protein denaturation. We also realize that in this study the salting effects of urea on the stability of intramolecular hydrogen bonds are not directly addressed. Such work is underway in our lab.

CONCLUSION

To conclude, we have successfully reproduced the salting-out effect of NaCl and salting-in effect of urea on protein compounds. The capability of urea to better solvate protein backbone and side chain analogues can be extrapolated to explain protein's denaturation in aqueous urea solution. By careful analysis of the thermodynamics properties of the salting effects, the mechanism of urea-induced denaturation is revealed in an unambiguous and unique way. For NaCl, polar molecules are less affected while large and hydrophobic molecules are significantly salted-out. For urea, the strength of salting-in effect is linearly dependent on the number of heavy atoms, and salting-out effect is expected for smaller molecules like methane. For both NaCl and urea solutions, the salting effects are found to be enthalpy driven. The enthalpy change of solvation can be decomposed into two terms: an energy-favorable solute−solvent interaction term and an energy-unfavorable solvent−solvent interaction term. The advantage of urea is that the decrease of solute−solvent interaction energy term counterbalances the increase of solvent−solvent interaction energy term, which finally facilitates the solvation. The van der Waals interactions between urea molecules and protein compounds are found to be the attraction force, which challenges a hydrogen-bonding-driven mechanism for the urea denaturation proposed previously.

ASSOCIATED CONTENT

Supporting Information

λ values used in our calculation, verification of the free energy calculations, RED-generated atomic charges of all the solute molecules, and generation of urea's representative binding patterns. This material is available free of charge via the Internet at http://pubs.acs.org.

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