Effect of the Protein Denaturants Urea and Guanidinium on Water Structure: A Structural and Thermodynamic Study

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Received May 4, 1998

Abstract: The mechanism of the denaturing effects of urea and the guanidinium ion on proteins is still an unsolved and important problem in protein chemistry. Changes in the hydrogen bond network of water in the first hydration shell of urea and guanidinium were analyzed in terms of the random network model using Monte Carlo simulations. Bulk water consists of two populations of hydrogen bonds: a predominantly linear population and a small but significant population of slightly longer and more bent hydrogen bonds. In the first shell of urea, hydrogen bonds between waters solvating the amino groups were shorter and more linear on average than those in bulk water. These changes are caused by a depletion of the more distorted hydrogen bonds. These changes in hydration water structure have previously been seen only around nonpolar solutes of solute groups. Thus urea, being entirely polar, is anomalous in this regard. Hydrogen bonds around guanidinium were longer and more bent than those in bulk water. These distortions are characteristic of a polar solute but are smaller than expected for an ion. The hydrogen bond structural parameters were combined with a random network model equation of state for heat capacity to calculate the hydration heat capacities (ΔC_p) of urea and guanidinium. The value of ΔC_p obtained for urea is positive, characteristic of a nonpolar solute, and in good agreement with the experimental value. Urea and, to a lesser extent, guanidinium are unique among polar molecules in that they are highly soluble yet appear to structure water more like nonpolar solutes. The relevance of this observation to proposed mechanisms of denaturation is discussed.

Introduction

The mechanism by which urea and the guanidinium ion denature proteins in aqueous solutions is still a mystery, although there exists a large literature on the experimental and theoretical studies of denaturation of proteins by these molecules[1−5] (see reviews by Tanford[6] and Pace[7]). It is not certain whether these molecules act directly by binding to peptide groups, thereby weakening internal hydrogen bonds, or indirectly by causing a change in the structure of water's hydrogen bond network around hydrophobic groups in proteins, thereby increasing their solubility and weakening the hydrophobic effect. It is also possible that both mechanisms are operating.

Experimental data from an early study on urea's denaturing ability[1] are suggestive of the mechanism that denaturation could be occurring through the changes in the structure of the hydrogen bond network of water. Wetlaufer et al. studied solubilities of hydrocarbons with chains longer than two carbon atoms and observed that urea increased the solubility of these hydrocarbons. They also found that the solubility of these hydrocarbons depended approximately linearly on the urea concentration, not on the activity of urea, and hence they ruled out solvation of the hydrocarbons solely by urea. They proposed two possible mechanisms: (i) urea changes the hydrogen bond network of water and thus helps the hydration of hydrocarbon molecules and (ii) both urea and water molecules solvate the hydrocarbon molecules. This study concluded that the denaturing effect of urea was partly due to the weakening of hydrophobic effects.

There have been a number of theoretical and experimental studies supporting the more direct mechanism of denaturation, i.e., through better solvation of the protein molecule in the denatured state by urea and guanidinium.[3,5] Schellman proposed a direct binding model for denaturant activity.[3] Since the affinity constant for urea binding to protein required by this model is very low, it has proven difficult to measure directly. Alonso and Dill predicted on theoretical grounds that the denaturants cause unfolding of proteins because the denaturant solutions solvate the hydrophobic groups in the unfolded states of proteins.[4] However, it is not made clear whether denaturing action occurs through the changes in the hydrogen bond network of water or through binding to hydrophobic groups by the denaturant. Myers, Pace, and Scholtz have studied the relation of *m* values (the rate of change of the unfolding equilibrium with increasing denaturant concentration) and heat capacity changes to the changes in the accessible surface area (ΔASA) of protein unfolding by urea and guanidinium ion.[8] They observed that both the *m* values and the heat capacity changes (ΔC_p) correlate with the changes in ASA. They also observed that the *m* values and ΔC_p correlate with each other. They concluded that, for the proteins which undergo denaturation by a two-state mechanism, the accessible surface area exposed to solvent during denaturation is a main factor in the determination of the *m* values for unfolding of proteins by urea and guanidinium. The authors do not give a mechanism by which the

(1) Wetlaufer, D. B.; Malik, S. K.; Stoller, L.; Coffin, R. I. *J. Am. Chem. Soc.* **1964**, *86*, 508.
(2) Makhatadze, G.; Privalov, P. *J. Mol. Biol.* **1992**, *226*, 491−505.
(3) Schellman, J. A. *Biopolymers* **1987**, *26*, 549−559.
(4) Alonso, D. O. V.; Dill, K. A. *Biochemistry* **1991**, *30*, 5974−5985.
(5) Timasheff, S. *Biochemistry* **1992**, *31*, 9857−9864.
(6) Tanford, C. *Adv. Protein Chem.* **1970**, *24*, 1−95.
(7) Pace, N. *Methods Enzymol.* **1986**, *131*, 266−280.
(8) Myers, J. K.; Pace, C. N.; Scholtz, J. M. *Protein Sci.* **1995**, *4*, 2138−2148.

10.1021/ja981529n CCC: $15.00
Published on Web 10/03/1998

denaturant molecules cause unfolding of proteins. Even though the above-mentioned thermodynamic study suggests that direct interaction with urea and guanidinium ions is a plausible mechanism, this study does not rule out the possibility of the indirect mechanism, in which urea changes the structure of water in the hydration shell of proteins. Such an indirect mechanism could still exist because, in this study, the dependence of *m* values is shown to be quite a coarse parameter for quantitating changes in the accessible surface area during the unfolding phenomenon. A calorimetric study by Zou, Habermann, and Murphy on the energetics of dissolution of cyclic dipeptides in different concentrations of aqueous urea solutions concludes that the urea denaturant effect is twofold: it decreases the hydrophobic effect and it binds to the peptide groups via hydrogen bonds.[9] That group further found that the interactions of nonpolar groups with urea are enthalpically unfavorable but entropically favorable, while the reverse is true for urea interactions with polar groups.

None of the experimental studies mentioned above can clearly distinguish whether direct interaction with peptides or indirect changes in the hydrogen bond network of hydrogen bonds, or both, are operating. Because of this, several theoretical and simulation studies have focused on the changes in the water structure around the urea molecule. Franks and Franks, in their study on aqueous solutions of urea, proposed that the water around urea is less hydrogen bonded than bulk water.[10] The first simulation study on aqueous solutions of urea, by Kuharski and Rossky, compared the distribution of pair interaction energies of water molecules in the hydration shell and in the bulk.[11,12] The authors reached the conclusion that the properties of water in the first shell are very similar to those in bulk. Our unpublished results on the distribution of water−water interaction energies in bulk water and in the hydration shells of various nonpolar and polar molecules confirm this observation. However, in a previous study of the random network model (RNM) water structure parameters (the mean and standard deviation of the hydrogen bond length (*d*, *s*) and the root-mean-square hydrogen bond angle (θ)), we observed significant changes in the hydration shell of polar and nonpolar solutes, particularly in θ, with significant but lesser changes in *d*.[13−15] These results show that the RNM parameters are more sensitive to changes in the hydrogen bond network than the water−water interaction energy, and hence it is worthwhile to analyze the difference in the RNM parameters for hydrogen bonds among water molecules in the hydration shell of denaturant molecules, urea and guanidinium.

A recent simulation study on the effect of urea in aqueous solutions of two methane molecules and two ions of the same radius as methane showed that the first peak in the urea-O/water-O radial distribution function is at the same position, i.e., ∼2.8 Å, as the first peak in the water-O/water-O radial distribution peak in pure water.[16] Wallqvist et al. also computed the various O−H radial distribution functions for urea and water oxygens with the urea and water hydrogens. They found that these O−H radial distribution functions match the corresponding pure water O−H radial distribution functions. The authors concluded from this observation that urea does not break the water structure in its aqueous solutions. Previous studies by Tsai et.[17] and Astrand et al.[18] also found little change in the water O−O radial distribution function in urea solutions. These results can be rationalized with our previous studies on water around solutes using the random network model, which showed that the mean hydrogen bond length, *d* (*d* is essentially equivalent to the position of the first peak in the water O−O radial distribution function), is a much less sensitive indicator of structural changes than the hydrogen bond angle between water, θ.[13−15]

Wallqvist et al. also observed that the first peak in the charged-methane/urea-C radial distribution function was bigger than the first peak in the uncharged-methane/urea-C radial distribution function, thus concluding that urea gets absorbed selectively on the hydrophilic groups. The work by Wallqvist et al. is interesting and generates some new questions about the behavior of water in the hydration shell of urea. First, if water does not lose its structure because the oxygen atom in urea behaves like the oxygen atom of water, what happens to the water molecules surrounding the amine groups? Second, do the structures of water around oxygen in urea and of water around amine groups in urea look the same? In other words, how do urea and guanidinium change the structure of hydrogen bond network of water in its hydration shell? These are the questions addressed in the present work. We further ask if we can check the consistency of these observations by relating changes in the hydrogen bond network to measured changes in heat capacity, since we have shown that this quantity is a sensitive indicator of changes in water structure around solutes.

In this work, we have set out to accomplish two goals. The first goal is to analyze the changes in the RNM parameters of the hydrogen bond network in the hydration shells of various groups in urea and guanidinium ions. We also discuss the possible role of these changes in the hydrogen bond network in the denaturation ability of these molecules. The second goal is to compute the heat capacity changes associated with the hydration of these molecules using the methodology developed in our earlier papers. We compare the values for the heat capacity of hydration of these compounds with the experimental estimates. A good agreement with the experimental values would give us confidence in our analysis of the water structure in the hydration shell of these two denaturant molecules.

Methods

Dilute solutions of urea and guanidinium were simulated by inserting one molecule of the solute into a cubic box of 216 molecules of TIP4P water.[19] The box dimension was 18.6 Å per side. Minimum image periodic boundary conditions were used, with a cutoff of 12.0 Å. The OPLS potential function was used for urea.[20] Parameters for guanidinium were taken from Saigal and Pranata.[21] The partial charge distributions for urea and guanidinium are shown in Figure 1. The configurations of the aqueous solutions of urea and guanidinium ion were sampled using a Metropolis Monte Carlo algorithm implemented in the program BOSS.[22] The simulations were performed at 25 °C

(9) Zou, Q.; Habermann, S.; Murphy, K. P. *Proteins* **1997**, *31*, 107−115.
(10) Franks, H. S.; Franks, F. *J. Chem. Phys.* **1968**, *48*, 4746.
(11) Kuharski, R. A.; Rossky, P. J. *J. Am. Chem. Soc.* **1984**, *106*, 5786−5793.
(12) Kuharski, R. A.; Rossky, P. J. *J. Am. Chem. Soc.* **1984**, *106*, 5794−5800.
(13) Madan, B.; Sharp, K. A. *J. Phys. Chem.* **1996**, *100*, 7713−7721.
(14) Madan, B.; Sharp, K. A. *J. Phys. Chem.* **1997**, *101*, 11237−11242.
(15) Sharp, K. A.; Madan, B. *J. Phys. Chem.* **1997**, *101*, 4343−4348.
(16) Wallqvist, A.; Covell, D. G.; Thirumalai, D. *J. Am. Chem. Soc.* **1998**, *120*, 427−428.
(17) Tsai, J.; Gerstein, M.; Levitt, M. *J. Chem. Phys.* **1996**, *104*, 9417−9430.
(18) Astrand, P.; Wallqvist, A.; Karlstrom, G. *J. Phys. Chem.* **1994**, *98*, 8, 8224−8233.
(19) Jorgensen, W. L.; Chandrasekhar, J.; Madura, J. D.; Impey, R. W.; Klein, M. L. *J. Chem. Phys.* **1983**, *79*, 926.
(20) Duffy, E.; Severance, D.; Jorgensen, W. *Isr. J. Chem.* **1993**, *33*, 323−330.
(21) Saigal, S.; Pranata, J. *Bioorg. Chem.* **1997**, *25*, 11−21.
(22) Jorgensen, W. L. *BOSS, Version 3.3*; Yale University: New Haven, CT, 1992.

Table 1. RNM Parameters for Hydrogen Bonds Formed in the Hydration Shell of Urea Molecule and Their Contribution to ΔC_p^{hyd}

H-bond class[a]	d (Å)[b]	s (Å)	θ	no. of H-bonds	ΔC_p/water (cal mol^{-1} K^{-1})	net ΔC_p (cal mol^{-1} K^{-1})
O−O	3.04 ± 0.02	0.220 ± 0.006	38.8 ± 2.4	1.60 ± 0.15	−1.95 ± 0.43	−1.56 ± 0.3
O−N	2.95 ± 0.01	0.224 ± 0.003	31.2 ± 1.5	3.69 ± 0.10	−0.23 ± 0.33	−0.42 ± 0.3
O−C	2.92 ± 0.01	0.219 ± 0.003	29.1 ± 0.9	3.95 ± 0.25	0.41 ± 0.23	0.81 ± 0.5
N−C	2.93 ± 0.01	0.221 ± 0.002	29.0 ± 0.8	6.28 ± 0.17	0.22 ± 0.20	0.69 ± 0.3
N−N	2.92 ± 0.01	0.218 ± 0.002	27.9 ± 0.6	13.9 ± 0.22	0.69 ± 0.17	4.80 ± 0.3
C−C	2.92 ± 0.01	0.220 ± 0.005	28.3 ± 1.2	2.75 ± 0.16	0.53 ± 0.37	0.73 ± 0.5
					ΔC_p^{hyd}	5.0 ± 1

[a] Hydrogen bonds are classified according to the hydration shell to which each water molecule belongs. [b] For bulk water, $d = 2.94$ Å, $s = 0.22$ Å, $\theta = 29.4°$.

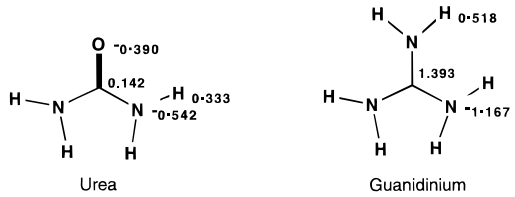

Figure 1. Charge distributions used for simulating urea and the guanidinium ion.

and 1 atm pressure. Flexibility of the solute molecules was not included. The systems were first equilibrated for 5×10^7 Monte Carlo steps, following which data were collected for 10 consecutive runs of 1×10^7 steps each. Error estimates for various average quantities were determined by computing the standard deviations for each average quantity from the 10 runs.

During the data collection runs, an instantaneous configuration of the dilute solution was analyzed every 1000 steps. The values of the various interaction energies, radial distribution functions, $g(r)$, hydrogen bond distance, and angle distributions of the hydrogen bond interactions between the water molecules in the solute hydration shells were computed from the instantaneous configurations. The hydration shell for each group of the solute was determined from the first minimum of the solute-atom/water-oxygen radial distribution function. Two water molecules were defined to be hydrogen bonded if the instantaneous distance between their oxygen atoms was less than or equal to 3.4 Å, which corresponds to the first minimum in the oxygen−oxygen radial distribution function of water. The hydrogen bond angle between two such water molecules is defined as the smallest O−O−H angle formed from the four hydrogens involved. The oxygen−oxygen distances and hydrogen bond angles for hydrogen-bonded water molecules were binned together to obtain histograms for the computation of the probability distribution functions of oxygen−oxygen distances and of the hydrogen bond angles. These probability distribution functions were then used to determine the three random network parameters: average oxygen−oxygen distance between two hydrogen-bonded water molecules, d, the standard deviation in this distance, s, and the root-mean-square hydrogen bond angle between two water molecules, θ.

Hydrogen bonds among water molecules in the first hydration shell were assigned to various classes based on the hydration shell of which solute atom each of the water molecule belonged to. Thus, both intragroup and intergroup hydrogen bonds can occur. The hydrogen bond interactions were further divided into various classes based on the groups solvated by the two waters. The RNM parameters for these different classes are distinguished by the subscripts O, N, and C for the oxygen, amino, and carbon groups, respectively. For example, a hydrogen bond between a water molecule in the hydration shell of the oxygen atom of urea molecule and another water molecule in the hydration shell of the NH2 group of the same urea molecule has parameters X_{O-N}, where $X = d$, s, or θ. If a water molecule was at such a position that it could belong to the hydration shell of two or more different groups, it was assigned to be in the hydration shell of that group which was closest to it. Hydrogen bonds belonging to each of the classes were counted for each snapshot and were averaged over the whole run.

The net heat capacity change due to the hydration effects (ΔC_p^{hyd}) for the two solutes was computed from the changes, with respect to bulk water, in the RNM parameters for the hydrogen bonds in the first hydration shell and from the number of hydrogen bonds in the hydration shell. This procedure has been described in detail in previous papers.[13−15] This method provides ΔC_p^{hyd} contributions from each class of hydrogen bonds. These contributions are then summed to obtain the total heat capacity of hydration of urea and guanidinium ion from the following equation:

$$\Delta C_p^{hyd} = \sum_i N_i [C_p^{rm}(d_i, s_i, \theta_i) - C_p^{rm}(d_o, S_o, \theta_o)] = \sum_i N_i \Delta C_p^{rm}(d_i, s_i, \theta_i) \quad (1)$$

where C_p^{rm} is the contribution to heat capacity arising from a group of N_i perturbed H-bonds with average parameters d_i, s_i, and θ_i, where d_o, s_o, and θ_o are the corresponding values for the bulk water. The detailed form of the equation of state for heat capacity $C_p^{rm}(d_i, s_i\theta_i)$ has been derived in our previous work[13] from the modified version of the random network model of water developed by Henn and Kauzmann.[23]

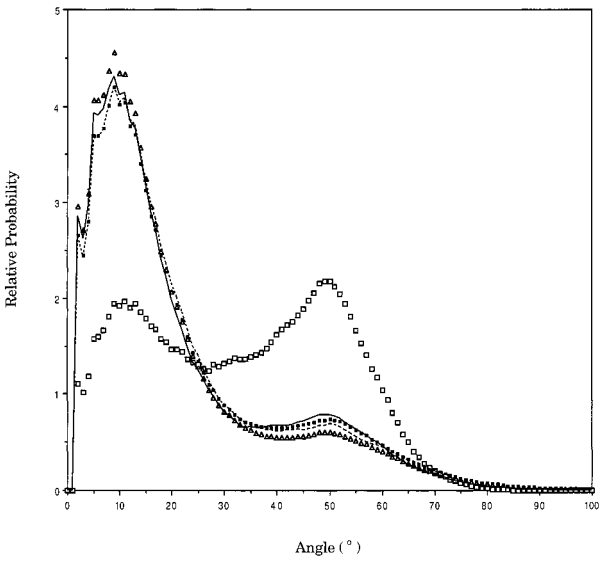

Figure 2. Hydrogen bond angle probability distributions for urea. Data are plotted for selected classes of H-bonds: O−O (□), intrashell N−N (- -), intershell N−N (△), and C−C (—). The distribution for bulk water (■) is shown for comparison.

Results

Urea. The first hydration shell of urea contained, on average, 23 waters, making an average of 32 first shell−first shell water H-bonds. The changes in the characteristics of the hydrogen bond network between water molecules in the hydration shell of urea are shown in the form of the RNM parameters in Table 1 and in the hydrogen bond angle probability distribution in Figure 2. Table 1 shows the RNM parameters for the hydrogen bonds between water molecules in the first shell of each of the

(23) Henn, A. R.; Kauzmann, W. *J. Phys. Chem.* **1989**, *93*, 3770−3783.

three kinds of groups present in the solute molecule. It is observed that the hydrogen bonds between water molecules present in the hydration shell of urea's oxygen atom are similar to those observed previously around polar groups in ethanol and NMA.[15] However, the hydrogen bonds between water molecules around urea's amine groups are more like the hydrogen bonds formed between water molecules around *nonpolar* groups in mixed group molecules such as ethanol and NMA seen in our previous work. This behavior is most evident in θ but is also shown to a lesser extent in d. The average value of θ_{O-O} for urea is 39°, while the value θ_{N-N} for urea is 28°. For comparison, the value of θ_{O-O} for pure water is 29.4°. Similarly, the value of average oxygen−oxygen distances, d_{O-O}, and d_{N-N} for urea are 3.04 and 2.92 Å, respectively, compared to 2.95 for d_{O-O} of pure water. Similarly, the hydrogen bonds between waters solvating the carbonyl carbon of urea (class C−C) and between waters solvating both carbon and amino groups (class N−C) are distorted in the direction characteristic of nonpolar groups, i.e., shorter lengths and smaller angles than those for bulk water. The values of θ and d for hydrogen bonds between water molecules belonging to other classes lie between the two extremes represented by the O−O and N−N/C−C classes. The H-bond geometry is only slightly distorted with respect to that in bulk water, with a barely significant decrease in d and a slight decrease in θ. The changes in length and angle for all classes of groups are highly correlated ($R^2 = 0.8$).

The changes in RNM parameters with respect to those in bulk water for all classes of hydrogen bonds in the hydration shell are used to compute ΔC_p^{hyd} contributions from corresponding groups, as shown in Table 1. The values of hydration heat capacity obtained from the different classes of hydrogen bonds reflect the changes in H-bond length and angle. ΔC_p^{hyd} for O−O H-bonds is −1.6 cal mol^{-1} K^{-1}, while those for the N−N and C−C classes are 4.8 and 0.7 cal mol^{-1} K^{-1}, respectively; i.e., the changes around these polar groups are more characteristic of nonpolar groups. The sum of these individual contributions gives the total heat capacity of hydration for urea, which is 5 ± 1 cal mol^{-1} K^{-1}. Because of the larger number of H-bonds in the N−N, C−C, and N−C classes, they provide the dominant contribution to the heat capacity of hydration, resulting in a net positive ΔC_p^{hyd}. Considering the difficulty of calculating heat capacity changes, our calculated value is in good agreement with the experimentally measured value of 7.4 cal mol^{-1} K^{-1}.[24] This agreement provides confidence that the observed structural changes for the hydration shell water around various groups of urea are realistic.

Mean values of d and θ provide one way to characterize structural changes. However, a more detailed picture of the structural changes is revealed by the H-bond angle probability distribution for different H-bond classes in the hydration shell of urea (Figure 2). We have shown in our previous work[15] that this kind of plot for bulk water clearly allows one to distinguish two hydrogen bond populations: a larger population with quasi-tetrahedral icelike structure, with $\theta_h \approx 12°$, and a smaller population in which a fifth molecule, a mismatch water, comes into the coordination shell of the central water molecule, forming a highly distorted H-bond, with $\theta_h \approx 52°$. We also found in our previous work that nonpolar solutes tend to decrease this second population by competing for the position of the mismatch water molecule. Polar solutes, however, have the opposite effect. The electric field of polar groups tends to align the dipole of water with the intersolute−water axis, producing a large hydrogen bond angle between two water molecules in the hydration shell. Figure 2 shows the dramatic effect of the oxygen atom of urea molecule, acting as a polar group, on the hydrogen bonds between waters in its hydration shell. The probability distribution plot for the hydrogen bond angles between waters around the oxygen atom shows two peaks, a broader peak at 52° and a much smaller peak at 12°. The probability plot for the N−N class shows a small decrease in the 52° peak and an increase in the 12° peak with respect to the bulk water distribution. A similar increase in the 12° peak is seen for the C−C class. This indicates that carbonyl carbon and amine groups, despite having significant partial charge, behave as nonpolar groups. There is no detectable difference in the N−N class of H-bonds between those for waters hydrating neighboring amino groups and the same amino group.

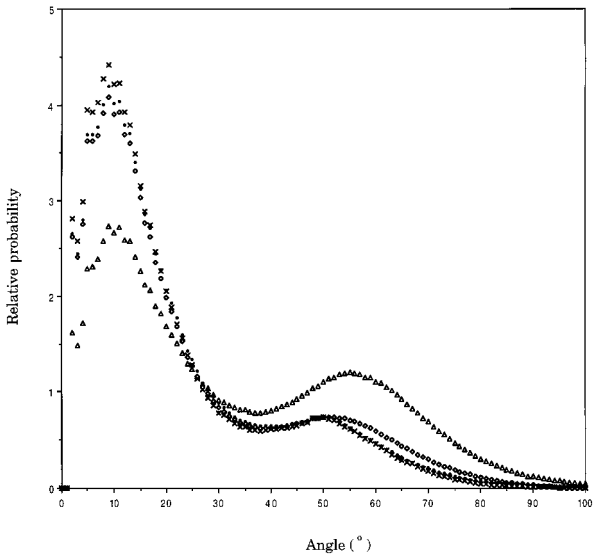

Figure 3. Hydrogen bond angle probability distributions for guanidinium. Data are plotted for selected classes of H-bonds: intrashell N−N (◇), intershell N−N (△), and C−C (×). The distribution for bulk water (●) is shown for comparison.

Guanidinium. The first hydration shell of guanidinium contained, on average, 23 waters, making an average of 30 first shell−first shell water H-bonds. There are only three kinds of hydrogen bonds in the hydration shell of guanidinium: N−N, C−C, and N−C. The N−N class includes H-bonds between waters hydrating neighboring amino groups as well as the same amino group. The changes in the hydrogen bonds in the C−C and N−C classes with respect to those in bulk water are similar to those seen for these classes around the urea molecule: d_{C-C} and d_{N-C} decrease to 2.92 and 2.91 Å, respectively, while the corresponding angles decrease to 28.7° and 28.2°, respectively. The corresponding H-bond probability distributions show increases in the 12° peak similar to those seen around urea (Figure 3). These values suggest that the carbon atom of guanidinium acts on water in a manner similar to that for nonpolar groups. For water−water hydrogen bonds around amine groups (the N−N class), $d = 2.95$ Å and $\theta = 36.4°$, both increasing compared to bulk water. The values for these parameters indicate that the amine groups act as hydrophilic groups, but less strongly than the carbonyl oxygen of urea. This point is emphasized in the H-bond probability distribution, which shows a significant increase in the 52° peak compared to that bulk water, but considerably less than that seen for urea's oxygen (Figures 2 and 3). This behavior is expected because of the uniform delocalization of one positive charge onto three amine groups. Unlike urea, there is a large difference between the

(24) Cabani, S.; Gianni, P.; Mollica, V.; Lepori, L. *J. Soln. Chem.* **1981**, *10*, 563−595.

Table 2. RNM Parameters for Hydrogen Bonds Formed in the Hydration Shell of Guanidinium and Their Contribution to ΔC_p^{hyd}

H-bond class[a]	d (Å)	s (Å)	θ	no. of H-bonds	ΔC_p/water (cal mol^{-1} K^{-1})	net ΔC_p (cal mol^{-1} K^{-1})
N−N	2.95 ± 0.01	0.225 ± 0.002	36.4 ± 0.8	22.4 ± 1.2	−0.51 ± 0.61	−5.7 ± 1
N−C	2.92 ± 0.01	0.218 ± 0.003	28.7 ± 0.6	5.8 ± 0.4	0.73 ± 0.52	2.1 ± 0.5
C−C	2.91 ± 0.01	0.220 ± 0.005	28.2 ± 2.0	1.8 ± 0.2	0.62 ± 0.52	0.56 ± 0.5
					ΔC_p^{hyd}	−3.0 ± 1

[a] Hydrogen bonds are classified according to the hydration shell to which each water molecule belongs.

N−N class of H-bonds for waters hydrating the same amino group (intrashell) and those hydrating neighboring amino groups (intershell). The figure indicates that the larger distortion effect on H-bond geometry is, in fact, due to intershell water molecules around the amine groups. Hydrogen bonds formed between such water molecules increase the population at 52° at the expense of the population at 12°, a behavior which is typical of hydrogen bonds formed between water molecules around polar groups.

Table 2 shows the contributions to ΔC_p^{hyd} from hydrogen bonds formed between different water molecule populations around various groups of guanidinium. The main contribution is from the N−N class because the largest number of H-bonds are formed among water molecules belonging to the hydration shell of the amine groups. Our calculated value for the heat capacity of hydration of guanidinium is -3.0 ± 1 cal mol^{-1} K^{-1}. The negative value of heat capacity of hydration indicates that guanidinium behaves as a polar compound. We could not find any experimental data on the heat capacity of hydration of the guanidinium ion. However, our computed value can be compared to a negative heat capacity of hydration (−17 cal mol^{-1} K^{-1}) for the K^+ ion and a positive heat capacity of hydration (36 cal mol^{-1} K^{-1}) for the TMA^+ ion. Since guanidinium is not as small as K^+ and neither is it as large as TMA^+, nor does it have hydrophobic groups(methyl groups) in it like TMA^+ does, we believe a small negative value for the heat capacity of hydration for guanidinium is a reasonable value. The guanidinium ion also differs from the TMA^+ ion in that the polar atom(s) (nitrogens) are on the outside, not on the inside of the ion. It would be expected that a large ion, especially with hydrophobic groups, would have a positive heat capacity of hydration because, first, the charge is distributed over a large volume and, second, the hydrophobic groups would also contribute to a positive heat capacity change for the hydration.

Discussion

Our results show that the predominantly linear H-bond network of water is maintained, and even enhanced, surrounding the amine groups in urea, while it is distorted surrounding the carbonyl oxygen atom. The hydrogen bonds around the oxygen atom of urea molecule, however, are of both kinds found in bulk water: the more linear hydrogen bonds and more strained hydrogen bonds, but the population of the latter is increased at the expense of the former. Since, on average, there are only 1.6 hydrogen bonds formed around the oxygen atom, the two peaks suggest that water molecules surrounding the urea oxygen atom sometimes form an almost perfect H-bond ($\theta_h < 12°$) and at other times a bent bond ($\theta_h \approx 52°$). The persistence of the first peak at 12° is in agreement with the results of Wallqvist et al.[16] that "urea does not function as structure breaker...", but the peak at 52° suggests that there is an occasional broken hydrogen bond between water molecules surrounding the oxygen atom of urea. Their conclusion was based on the fact that various permutations of O−H radial distribution functions for urea oxygen and hydrogen atoms with water hydrogen and oxygen atoms match the corresponding pure water O−H radial distribution functions. Wallqvist et al.'s results as well as our results (data not shown here) show that the urea-O/water-O radial distribution function has its first peak at 2.8 Å, the same position where the water oxygen−oxygen first peak appears. However, we have observed in our previous works that the hydrogen bond angle is a very sensitive measurement of hydrogen bond characteristics. We believe that examination of radial distribution functions alone is not sufficient to conclude that the oxygen atom of a urea molecule always fits into the network of water molecules, since the probability distribution function of hydrogen bond angles for water molecules around the urea oxygen atom differs markedly from that in bulk water. Hydrogen bond angles also appear to be a more sensitive indicator of structural perturbations in the hydration shell of urea than the distribution of water pair interaction energies studied by Kuharski and Rossky,[11,12] since there are significant differences in the former, but not in the latter. Hydrogen bonds around amine groups of urea are much like those of the bulk water. Similar kinds of changes take place for the hydrogen bonds between water molecules in the hydration shell of guanidinium.

One mechanism postulated for the denaturing activity of urea and guanidinium involves binding to protein groups exposed upon unfolding. Another possible mechanism is through their effect on water structure, and thus on the strength of the hydrophobic effect. Demonstrating that this mechanism, rather than direct binding to protein groups, is sufficient to denature proteins has proved elusive. A necessary condition for the indirect mechanism is that these denaturants affect the structural and thermodynamic properties of water in a unique way (unique in the sense that distinguishes them from the effect on water of solutes that are *nondenaturing*). While our work does not directly address the mechanism of denaturation, the results presented here and in previous work show that analyzing the random network model parameters, particularly the hydrogen bond angle between waters in the first hydration shell, is a powerful way to analyze solute-induced perturbations of water for two reasons: (1) It is sensitive to structural perturbations. Indeed, it may reveal perturbations that do not show up in changes in the radial distribution functions traditionally used to analyze liquid structure (Madan and Sharp, communicated to *Biophysical Chemistry*). This is merely a reflection of the crucial importance of orientational structuring in water, most notably in the tetrahedral nature of the coordination shell. (2) The structural changes may be directly and quantitatively related to a key thermodynamic property, the heat capacity. Ample experimental data have shown that hydration heat capacity change is the most revealing of the common thermodynamic functions (the others being free energy, enthalpy, and entropy) in terms of the differences between hydration of polar and nonpolar (hydrophobic) solutes.

Our previous analysis of 12 solutes of widely differing characteristics, comprising more than 17 different functional

groups, has established an overall pattern of hydration: bulk water contains two populations of H bonds, about 80% that are approximately linear (with an angle of $\theta_h \approx 12°$), the rest being more bent ($\theta_h \approx 52°$) and slightly longer. Both polar and nonpolar solutes perturb water, producing concerted changes in mean H-bond angle and length. Nonpolar groups decrease the mean angle and length by displacing the more bent population of H-bonds. This results in an increase in water's heat capacity. Polar and ionic groups, through the orienting effect of their electrostatic fields, increase the mean angle and length by increasing the population of more bent H-bonds. This results in a decrease in water's heat capacity. In this regard, urea is unique. Although it is an entirely polar molecule (judged by the significant partial charge on all its atoms, Figure 1), the amino groups perturb the water in a way characteristic of nonpolar groups, and the net ΔC_p^{hyd} is positive. No other solute we have examined shows this behavior. Guanidinium is isosteric to urea but differs by the replacement of the oxygen with an amino group, which enables it to support more positive charge, indeed, a formal charge of 1 (Figure 1). Guanidinium shares some of the nonpolar-like effect on water structure with urea, notable in the structuring effect on water hydrating the carbon and carbon/amino groups, although, because of its formal charge, it has a net negative but very small ΔC_p^{hyd}.

We may thus characterize urea, and more tentatively, guanidinium, as sharing both polar and nonpolar characteristics that could be important in their denaturing action. First, they are both highly soluble. This is a requirement since rather large concentrations are required for denaturation, whatever the mechanism. Second, they could, because of their dual nature, interact with both polar and nonpolar groups. The picture of urea that emerges here is consistent with recent work by Zou et al.,[9] which implies that urea binds to both polar and nonpolar groups of peptides, driven by enthalpy and entropy, respectively.

Acknowledgment. Financial support is acknowledged from NIH (GM54105).

JA981529N