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The Dominant Interaction Between Peptide and Urea is Electrostatic in Nature: A Molecular Dynamics Simulation Study

Abstract: The conformational equilibrium of a blocked valine peptide in water and aqueous urea solution is studied using molecular dynamics simulations. Pair correlation functions indicate enhanced concentration of urea near the peptide. Stronger hydrogen bonding of urea-peptide compared to water-peptide is observed with preference for helical conformation. The potential of mean force, computed using umbrella sampling, shows only small differences between urea and water solvation that are difficult to quantify. The changes in solvent structure around the peptide are explained by favorable electrostatic interactions (hydrogen bonds) of urea with the peptide backbone. There is no evidence for significant changes in hydrophobic interactions in the two conformations of the peptide in urea solution. Our simulations suggest that urea denatures proteins by preferentially forming hydrogen bonds to the peptide backbone, reducing the barrier for exposing protein residues to the solvent, and reaching the unfolded state. © 2003 Wiley Periodicals, Inc. *Biopolymers* 68: 359–369, 2003

Keywords: denaturation; unfolding; computer simulations; potential of mean force; hydrophobicity; electrostatic interactions

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INTRODUCTION

Most *in vitro* folding and unfolding experiments are initiated by varying the concentration of denaturants such as urea. Varying urea concentration creates different stable thermodynamic "intermediates,"¹ whose structural characterization is required to map the folding pathways. Despite the extensive use of urea as an unfolding agent, the mechanism by which it induces unfolding is still unclear. In the folded states of most proteins hydrophobic residues are clustered in the core while polar and charged amino acids are exposed to the solvent. This suggests a kinetic argument that urea may disrupt the interactions between surface residues and water, before it could access the hydrophobic core. From this perspective, denaturation may involve an "outside-in" action in which urea binds to the backbone of the solvent exposed residues. This picture is implied in the earlier experimental studies of Robinson and Jencks² and the more recent work of Makhadate and Privalov.³ A mechanism of urea denaturation that is based on enhanced stability of apolar molecules in aqueous solution⁴ does not explain how the initial (kinetic) barrier is overcome.

Computer simulations are expected to shed light on the detailed molecular mechanisms of denaturant-induced unfolding of proteins. A few computational studies investigated the interactions of urea with proteins, model proteins, and water.

Wallqvist, Covell, and Thirumalai⁵ studied the effect of 6*M* urea solution on the interactions between simple solutes. Small model systems can be studied in detail to obtain statistically converged results. Based on these simulations a number of interesting observations were made: (a) *Urea is well solvated in water.* It is therefore unlikely that urea favors phase separation and reduces the hydrophobic effect. This explains the large concentration (typically >4*M*) of urea needed to fully denature proteins. (b) The lack of "hydrophobic effect" in urea denaturation is supported by a potential of mean force (PMF) calculation between two hydrophobic spheres. The PMF suggests that *urea enhances the hydrophobic interactions and does not diminish it*, which is in accord with the enhanced solubility of side chains of amino acids in aqueous urea solution.^{2,3,6} (c) Based on computations of charge particle interactions in aqueous urea solution, *a denaturation model was proposed that is based on the disruption of surface charge-solvent contacts.*

The results on simple model systems are consistent with recent experimental data on solvation of cyclic peptides.⁴ However, the lack of structure of the spherical solutes makes it difficult to predict the corresponding effects in proteins. It is not known how the

geometry, chain connectivity, and the detailed interactions of a protein chain may affect the conclusions.

Besides the above studies on simple solutes, two simulations of a protein interacting with water-urea solution have been reported. Tirado-Rives et al.⁷ and later Caffisch and Karplus⁸ simulated the unfolding of barnase in urea. Partial unfolding of the protein was observed at elevated temperatures (360 K) and essentially no unfolding at room temperature. Analysis of the simulation results⁷ suggested that a key factor in denaturation is the interaction of the urea molecules with polar groups. On the other hand, Caffisch and Karplus⁸ argued that both hydrophobicity and polar interactions are important in the denaturation by urea. This conclusion was based on the decomposition of the stability energies due to interactions of urea and water molecules in the first solvation shell around barnase. The analyzed data was from a molecular dynamics simulation of 870 picoseconds. The maximum stability of barnase in 8*M* aqueous solution of urea arises predominantly from charged residues, backbone moiety, and polar groups (Table 7 of Caffisch and Karplus⁸). Moreover, the contribution to stability of barnase from nonpolar groups is less than kT . The observations on the importance of electrostatic interactions would seem to support earlier computational² and experimental results.^{2,4}

The attractive feature of these studies is that they were done for protein molecules that have secondary and tertiary structures. However, the potential sampling problems and the use of high temperatures to accelerate the simulations make it difficult to assess their validity in describing the denaturation mechanism.

It is surprising that similar simulation protocols on the same protein do not appear to give qualitatively similar results for the denaturation mechanism.^{7,8} In particular, the emphasis on the role of hydrophobic interactions in denaturation of proteins in urea solution is in qualitative disagreement with experiments.^{2,4} It is therefore of interest to perform investigation of a simple system with significant resemblance to proteins for which thermodynamic averages can be computed accurately.

The computations described below are an attempt to bridge the gap between studies of model systems⁵ and proteins.^{7,8} The system investigated (a blocked valine peptide) has the characteristic chain-like properties of a protein. It is clearly too small to form internal hydrogen bonds and secondary structure, and as such one may expect that urea effects will be small. However, as was shown experimentally for small peptides, and is also demonstrated in the present computation, a significant solvation effect is observed. The present simulations also provide insights into the

recent experiments on cyclic peptide solvation.⁴ Studies on peptides have the potential of improving our understanding of *protein* folding, and at the same time making it possible to perform quantitative comparison between theory and experiment.

Dipeptide conformational flexibility (so-called Ramachandran or φ , ψ map⁹) plays a crucial role in determining plausible secondary structures in proteins. The map suggests an upper bound for the range of conformations we may obtain, as primarily determined by van der Waals clashes. The nearby geometry of solvent molecules is influenced by aqueous urea solvation. A probe of the conformational transition between the two minima, influenced by urea, allows us to propose a mechanism of denaturation in proteins.

The goal of the present manuscript is to examine atomically detailed interactions of urea and the peptide, using statistically significant calculations at room temperature, and to compare them to the interaction with water molecules. We consider both solvent structure near the peptide and the free energy of peptide conformational transitions. While some global cooperative effects that are enhanced with size can be missed when interpolating from peptides to proteins, factors that are significant for small peptides are likely to be significant also for the much larger proteins.

We focus on solvation structure along a "folding coordinate" (the ψ dihedral angle). We find very small changes in the potential of mean force for the ψ torsion angle of the peptide calculated at aqueous and 8*M* urea solutions. These small changes are not inconsistent with the known small free energy differences for protein unfolding in urea.¹ More striking is the significant hydrogen bonding of the peptide amides to urea donor or acceptor groups, which we analyze using pair correlation functions. The highly structured hydrogen bonding of the amide groups to urea (sharp peaks in the pair correlation functions) that replaces hydrogen bonding to water supports the electrostatic model mentioned earlier.

On the other hand, we found no indication for a significant hydrophobic effect on the conformation or the solvent environment of the small peptide. Hydrophobic effects are proportional to the exposed apolar surface area of the proteins. They are expected to be larger for longer peptides by (at most) a factor of *N*, the number of amino acids. The hydrogen bonding of urea and peptide backbone scales similarly with the protein size, suggesting that our study of a small peptide is relevant also to proteins.

In the next section we discuss the simulation methodology and point to potential ergodicity problems even in this small system. Straightforward sampling using direct molecular dynamics simulations at room

temperature are therefore difficult if not impossible to do in urea solution. Higher temperature simulations are useful for qualitative interpretation but are likely to distort equilibrium distributions. Our analyses and conclusions are therefore extracted from simulations with a biasing (umbrella) potential¹⁰ and are presented in the Results section. Our model for urea-induced-unfolding is presented in the Discussion.

METHODS

Simulation Protocol

All the computations described below were done using the package of programs MOIL,¹¹ which is available for free download at www.tc.cornell.edu/CBIO/moil. The force field in MOIL is the combination of AMBER (Assisted Model Building with Energy Refinement)¹² and OPLS (Optimized Potential for Liquid Simulation)¹³ extended atom force fields.

A blocked valine peptide (methyl-CO-NH-CH[CH(CH₃)₂]-CO-NH-methyl) was immersed in a cubic box with volume 8000 Å³. Periodic boundary conditions were used. The electrostatic interactions were calculated with the Particle Mesh Ewald,¹⁴ with a grid in *k*-space of 32 × 32 × 32 points. The Lennard-Jones interactions were truncated at 10 Å, and the nonbonded list was updated every 20 steps. The equations of motion were integrated using the Velocity Verlet algorithm with a time step of 3 fs. The lengths of bonds to hydrogen atoms were kept fixed with the Rattle algorithm.¹⁵ The dielectric constant was one, and the 1-4 scaling factors were 2 and 8 for electrostatic and Lennard-Jones interactions respectively. We used the TIP3P model for water¹⁶ and the interaction parameters for urea were taken from Ref. 17. The temperature was maintained at the desired value using velocity scaling. The scaling was employed only when the kinetic temperature deviates more than 10° from the desired temperature. A typical time interval between velocity scaling events was a picosecond. Structures were saved every 150 fs.

Molecular Dynamics Simulations

We briefly discuss two straightforward molecular dynamic simulations at 300 and 320 K that are *not* used in the detailed analysis. To obtain different starting configurations that are uncorrelated with each other at 300 K, we used the following procedure: trajectories at 360 K, for both aqueous and 8*M* urea solution at constant volume, were run for 50 ps. Ten structures equally spaced in time from the high temperature runs were used to initiate 3 ns trajectories at a temperature of 300 K. None of the trajectories in aqueous urea showed φ , ψ transitions, suggesting that the simulations are not ergodic, and adequate sampling of conformational space in a single or a few trajectories was not achieved. The straightforward molecular dynamic simula-

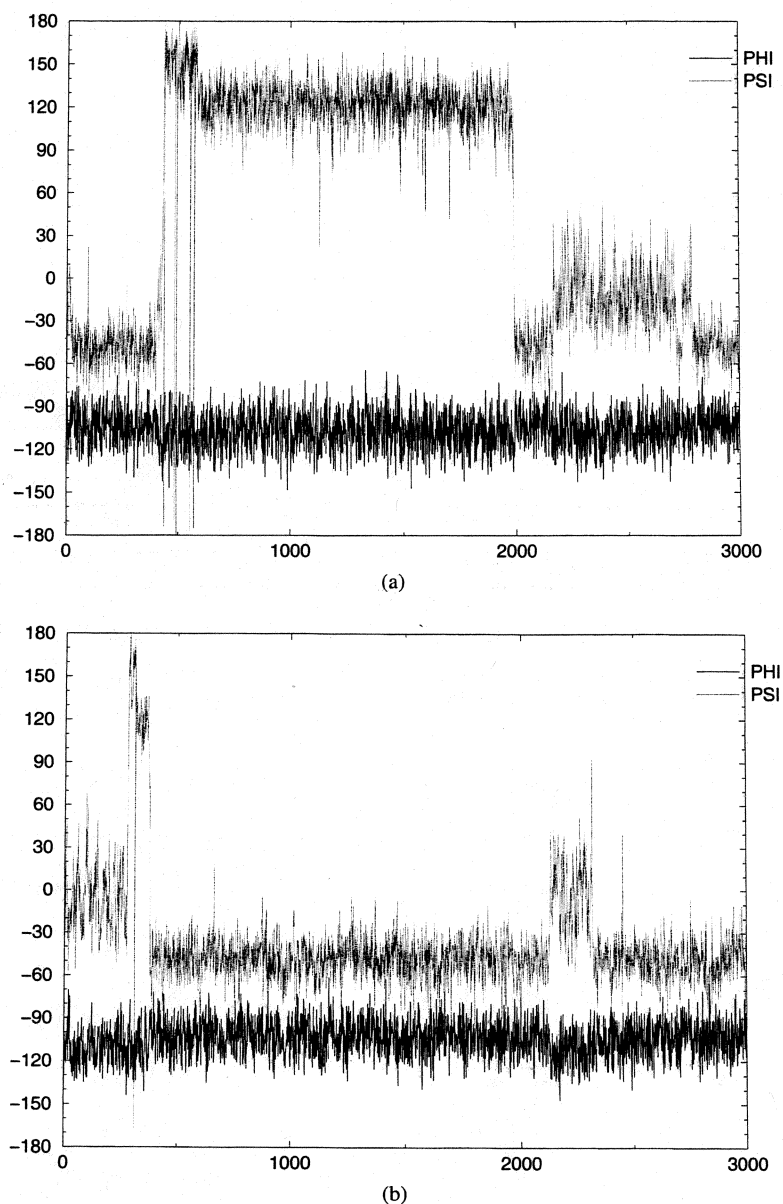


FIGURE 1 (a) A time history (3 ns) for the ϕ and ψ dihedral angles of the blocked valine peptide in water. Solid line is the ϕ dihedral, dashed line ψ . The temperature is 300 K. (b) The same as (a), except that 8M urea is used for a solvent. Note the very limited fluctuations of the ψ dihedral angle. Solid line is the ϕ dihedral, dashed line ψ . Other trajectories show similar "activity."

tions in solution of 8M urea at 300 K do not sample adequately the configuration space on the time scale of nanoseconds. This is illustrated in Figure 1b, which shows (at best) a single ψ transition. This is insufficient to probe the equilibrium between the two conformations. In contrast a transitions is observed in pure water (Figure 1a). The enhancement in sampling cannot be achieved even if multitrajectory sampling protocol is used.¹⁸

At 320 K a significant number of dihedral angle transitions are observed. At this temperature we generated two 4 ns trajectories. In Figure 2 we plot the time dependence of the ψ dihedral angle of the peptide solvated in 8M urea

solution. In contrast to the simulation at 300 K, the present simulation at 320 K shows significant activity of this important torsion, suggesting that sound statistical estimates of the weight of different conformations can be made. However, since 320 K is not the temperature of interest we focus on a calculation with umbrella sampling.

Computation of the Potential of Mean Force $W(\psi)$

The $W(\psi)$ calculations were performed using the umbrella sampling protocol¹⁰ with the ψ dihedral angle as a reaction

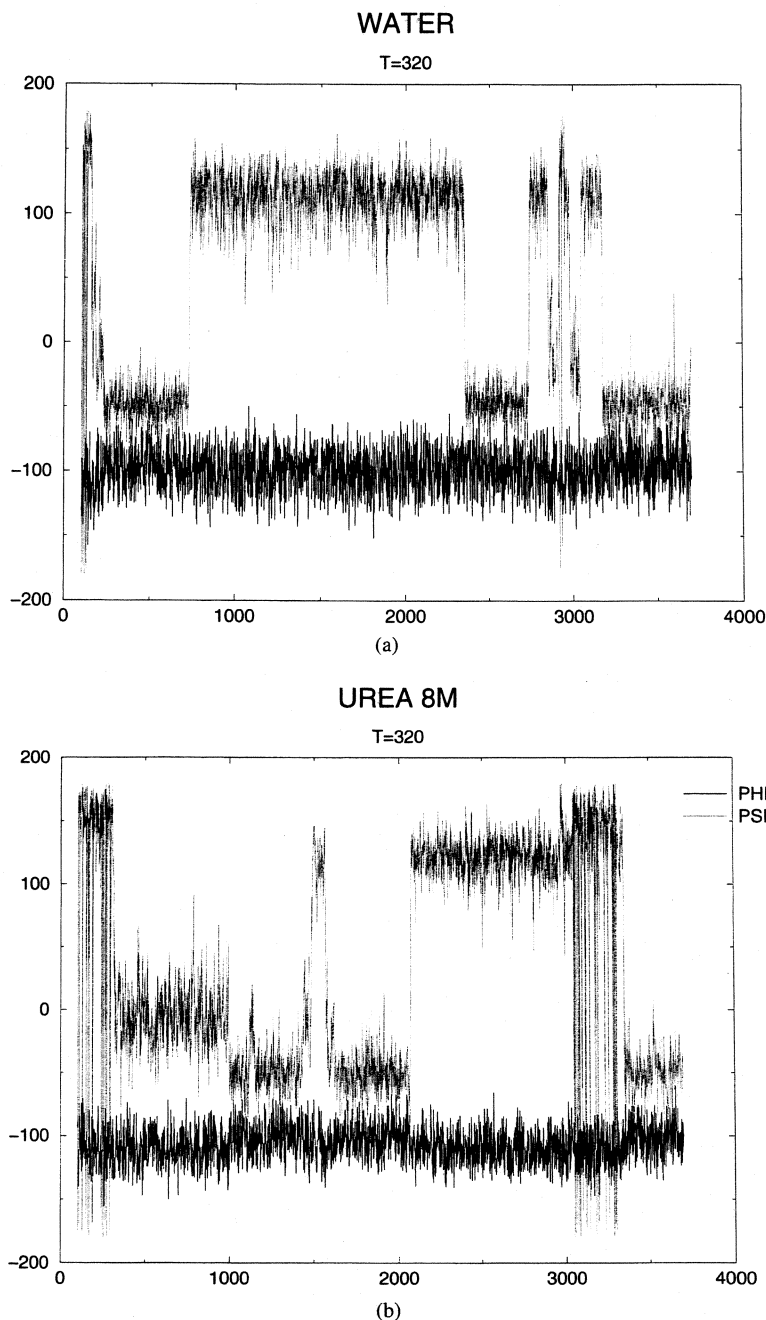


FIGURE 2 The time history for ψ dihedral angle for the valine peptide solvated in aqueous solution of 8M urea at 320 K. Note the significant “activity” of the dihedral angle in contrast to the simulation at 300 K. Here the trajectory length is of 4 ns.

coordinate. As is shown in the direct molecular dynamics simulations, the φ torsion does not change significantly (Figure 1), and therefore focusing on ψ transitions to observe the urea-induced unfolding is appropriate. The φ dihedral angle was left unconstrained in the potential of mean force (PMF) calculations. Even without additional constraint the φ dihedral angle remained at the left side of the Ramachandran plot throughout the simulation. The χ_1 dihedral angle of the valine side chain remains near 60° .

To compute $W(\psi)$ we divided the interval $-180 \leq \psi \leq 180$ into equally spaced bins of 5° . To enhance the population at the i th bin, a harmonic biasing potential $V(\psi) = k(\psi - \psi_i^0)^2$ was used. The force constant k was $10 \text{ kcal} \cdot \text{radian}^{-2} \text{ mol}^{-1}$. Each window was equilibrated for 1 ps. This short period was found to be sufficient since the ψ spacing was small and the fluctuations at each window were larger (exceeded 20°). The large fluctuations and the small steps along the reaction coordinate mean that the next step

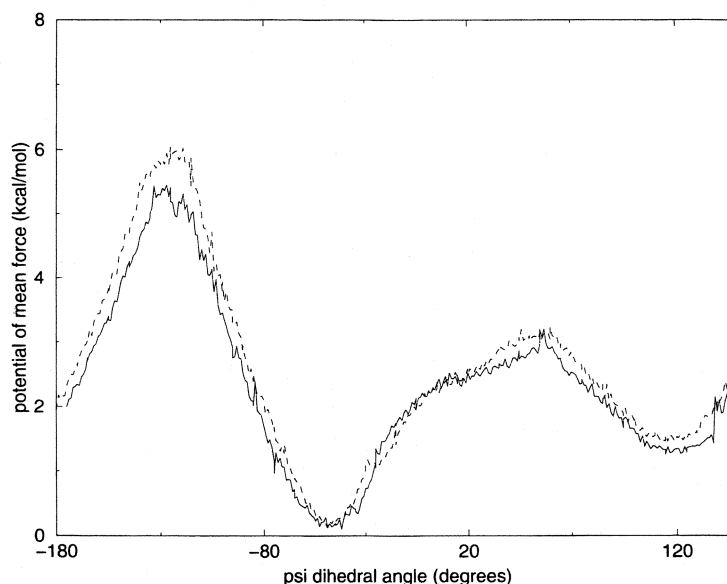


FIGURE 3 The potential of mean force for the ψ dihedral angle of a blocked valine peptide in two solvents: (a) water (dashed line) and (b) 8M urea (solid line). The energy is in kcal/mol. The temperature of the simulation was 300 K.

is initiated with a configuration close to equilibrium. The data was collected for 600 ps (at each window), making the total simulation length $0.600 \times 72 = 43.2$ ns. Structures were saved every 0.150 ps, providing 4000 configurations at each window for further analysis. The peptide was maintained at the center of the box by an additional harmonic term that constrained the geometric center of the peptide to the middle of the box. The force constant for the last restraint was $50 \text{ kcal/mol } \text{\AA}^{-2}$.

We spent considerable efforts to test the overlaps of the biased distributions. The matches were done first manually, and then using automated protocols. We have tried different scoring schemes in which (a) all the bins with significant statistics were considered, or (b) only bins "in between" were used, i.e., if bins i and $i + 1$ are matched only the counting between ψ_i and ψ_{i+1} are considered. Another test of the matching was to overlap the distributions using 10° intervals (instead of 5°). The extensive sampling we generated makes it possible for us to reduce the data size by a factor of two and still obtain adequate matching. The differences were found using alternative matching protocols were small and of the same order of magnitude as the matching errors (Figure 3).

RESULTS

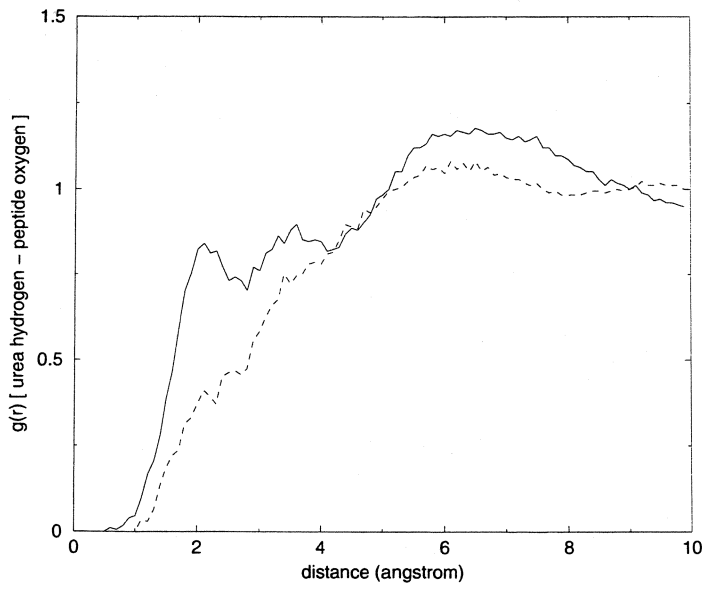
The model peptide system, a valine dipeptide (Methods) can be characterized in terms of (φ, ψ) that specifies the secondary structure. Because the φ angle does not change significantly in the conformational transition $\alpha \rightarrow \beta$, the observed transition can be

described by one dimensional reaction coordinate ψ . In terms of ψ the urea-induced transition occurs from the upper left corner in the Ramachandran plot ($\psi > 0$) to the lower left ($\psi < 0$) corner. For convenience we refer to the set of conformations $\psi > 0$ ($\psi < 0$) as β (α) respectively.

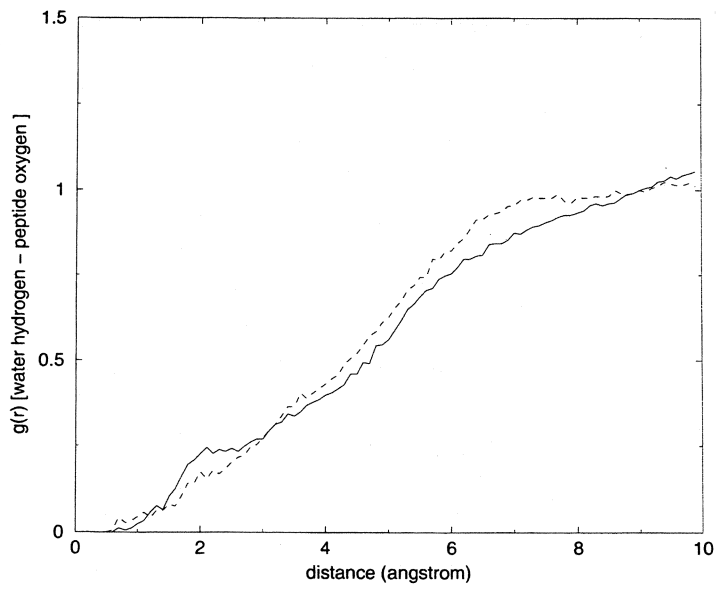
Potential of Mean Force $W(\psi)$ at 300 K

We computed $W(\psi)$ for a conformational transition of the blocked valine peptide from $\beta \leftrightarrow \alpha$ state at 300K. For reasons given in Methods $W(\psi)$ at 0 and 8M urea concentration are computed using ψ dihedral as the reaction coordinate. The effect of urea on the potential of mean force is small and is within the error bars of the matching procedure. In particular the differences observed between the potential of mean force computed in water and the potential of mean force computed in 8M urea solution is comparable to the differences we observed when employing different

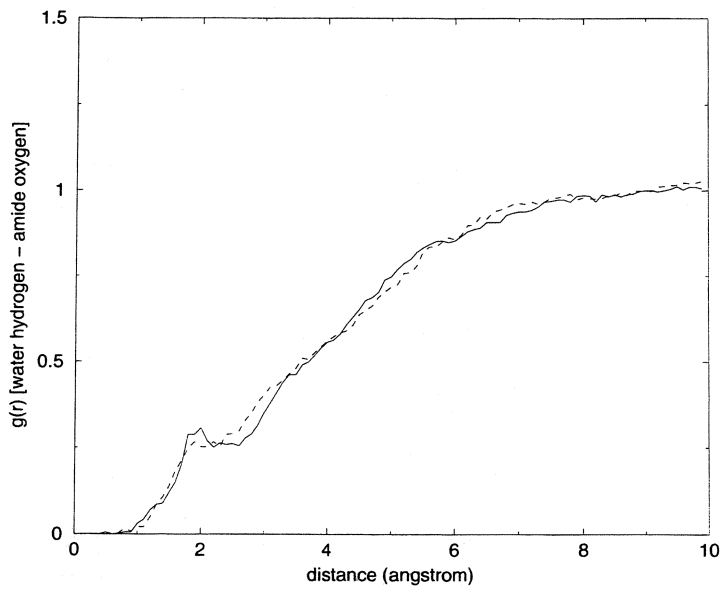
FIGURE 4 Pair correlation functions of solvent hydrogen and peptide carbonyl oxygens. (a) Urea hydrogens and peptide oxygens in 300 K, computed for two peptide conformations (solid line $\psi = -60$, dashed line $\psi = 120$). (b) The same as in (a), this time for water hydrogens in 8M urea. (c) The same as in (a), this time for water hydrogen for simulation of peptide in pure water. See text for more details.



(a)



(b)



(c)

matching procedures as explained in the Methods section. This is (perhaps) not surprising since the overall energetic effect on *protein* folding is small and is on the order of a few kcal/mol.¹ The effect of urea on a peptide is therefore not larger than our error bars and a different method of analysis is required to recover the unfolding mechanism induced by urea.

Solvent Structure Near the Peptide in Solution of 8M Urea

To probe the local density of urea and water molecules near the peptide we calculated the solute-solvent pair correlation function, $g(r)$. To make the comparisons more transparent we normalize all the pair correlation functions to one at large distance (9 Å). This does not take into account differences in concentration (e.g., for 8 moles of urea we have about 55 moles of water). However, it makes it easier to identify groups with higher tendencies to form hydrogen bonds with the peptide.

We considered the oxygen/polar hydrogen atoms of the peptide and the oxygen/hydrogen atoms of urea and water. We examined these distributions separately for the α and the β conformations, attempting to identify urea-induced preference for a specific secondary structure. To analyze the α state we examined the 4000 conformations sampled with an umbrella potential centered at $\psi = -60^\circ$. To analyze the β state we considered the conformations biased toward 120° . Since the umbrella potential is quite soft, significant sampling was obtained in the neighborhood of the biased angles with a range of roughly $\pm 15^\circ$.

In Figure 4(a) we show the pair correlation function for the distance between the urea polar hydrogens and peptide carbonyl oxygens. Significantly sharper first and second solvation peaks are observed for the helical conformation (solid line) as compared to the extended state (dashed line). The water hydrogens and the peptide oxygens (for pure water solvation and the water molecules in urea aqueous solution) are significantly less hydrogen-bonded (Figure 4b and 4c). There is also a depletion of density of water hydrogen near the peptide oxygen "contact" in 8M urea solution as compared to pure water. Note also the small differences between the extended and helical conformations (solid and dashed lines) on one hand and the small difference between pure water and water in urea solution on the other hand (figures 4b and 4c).

Qualitatively similar characteristics of hydrogen bonding are seen in Figure 5. In Figure 5 we examine

the pair correlation function of the oxygen of the solvent and the hydrogen of the solute. The strong first peaks of urea oxygen in Figure 5a are striking, especially compared to the much weaker "signal" for water molecules in figure 5 b. Note also the additional reduction in peak height when we compare water molecules in pure water and in urea solution (Figures 5b and 5c).

DISCUSSION

The most striking observation of the present simulations is a strong effect of urea on the solvent structure and hydrogen bonding near a small peptide. The increased concentration of urea molecule near the peptide and its protein like hydrogen bonding (amide to amide hydrogen bonding) is likely to disrupt the protein secondary structure and to lead eventually to unfolding. The limited flexibility of the peptide suggests that the urea-induced equilibrium shift should be small. There is no significant loss of internal hydrogen bonding in a peptide with two sequential amide planes and the hydrophobic interactions do not seem to play a role since the exposed surface area (see below) is similar in the two conformations.

It is surprising at first sight that urea binds more strongly to the helix conformation. However, we must keep in mind that the system simulated is only a dipeptide and the "helix" conformation has no internal hydrogen bond. Therefore, the hydrogen-bonding groups (amides) are exposed to the solvent. In contrast, some internal hydrogen bonding (in the form of a γ turn) is available at the extended chain conformation.

Hydrophobic Effect Does not Account for Urea-Induced Equilibrium Shift

Because the major driving force for forming globular structures in proteins is the hydrophobic effect it was argued by Tanford⁶ that urea-induced unfolding should also be dominated by alterations in the hydrophobic interactions between the folded and unfolded states. A simple estimate of the change in hydrophobicity is based on the exposed surface area in the two conformations. The hydrophobic effect is assumed to be proportional to the solvent exposed surface area, weighted by the properties of the different amino acids. Roughly speaking, the larger the exposed surface area, the more significant is the hydrophobic effect. The hydrophobic contribution is estimated as $F_{\text{hyd}} = \gamma \cdot A + C$, where γ mimics the surface tension ($\gamma = 0.00486 \text{ kcal/mol } \text{Å}^{-2}$), A is the exposed surface

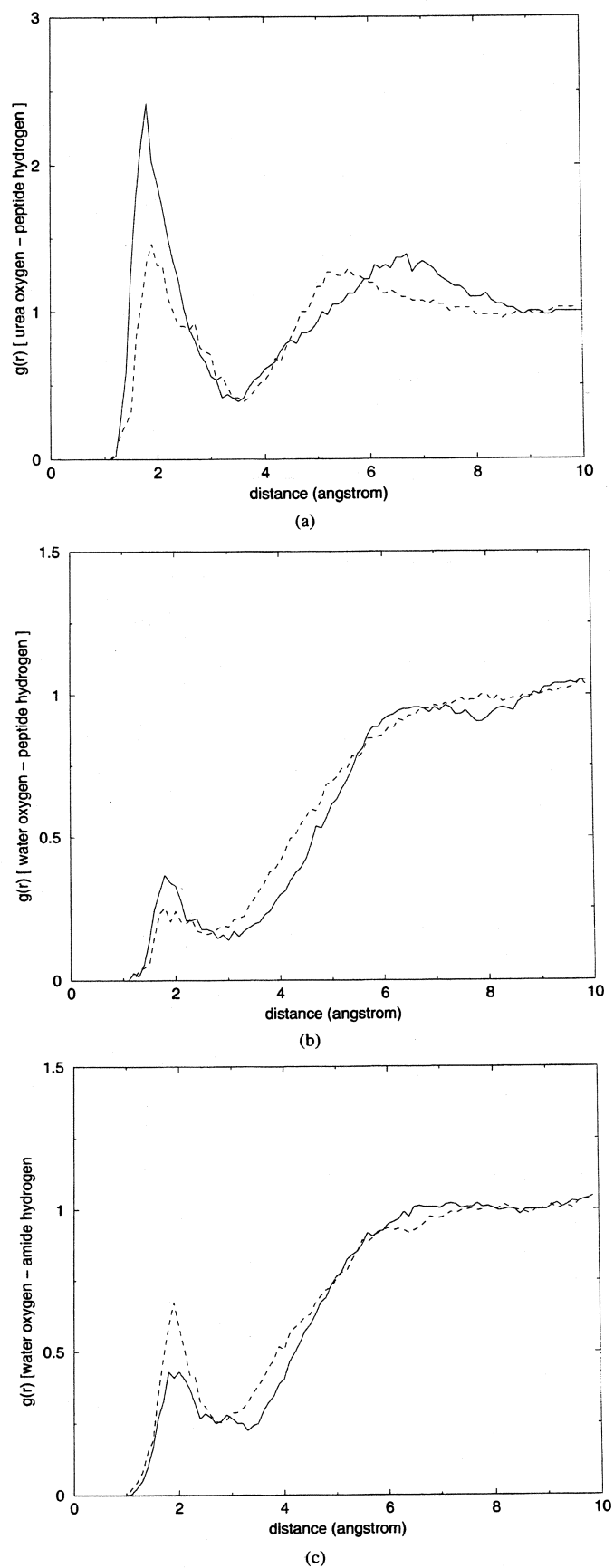


FIGURE 5 Pair correlation functions for solvent oxygens and peptide amide hydrogen. (a) Urea oxygen and peptide amide hydrogen: solid line $\psi = -60$, dashed line $\psi = 120$. (b) The same as in (a), this time for water oxygen (in 8M urea solution) and peptide amide hydrogen. (c) The same as in (a), this time for pure water oxygen interaction with peptide hydrogen.

area (in \AA^2), and $C = 1.092$ kcal/mol is a constant that accounts for finite volume effects.¹⁹

To estimate F_{hyd} , we computed A in two typical conformations, α and β , adopted by the peptide. The surface area of the two conformations was computed with a probe radius of 1.77\AA (Ref. 20). The difference of the *total* exposed surface area of the two conformations was 11\AA^2 and the difference in the hydrophobic surface area was 7\AA^2 . The total difference in surface area is an upper bound since it includes also polar atoms. Even for the upper bound the free energy difference is remarkably small (0.05346 kcal/mol). This difference is too small to explain the significant changes in the solvent structure and hydrogen bonding found in $8M$ urea (Figures 4 and 5). Parameters from another program (the surface term of Macromodel is $\gamma = 0.0072$) provide a comparable small free energy difference (0.0792 kcal/mol).

Conformational Transition and Hydrogen Bonding Between Urea and the Peptide

A different proposal for the denaturation of proteins by urea is based on the observation that urea is similar to the peptide group, so that it can engage in better hydrogen bonding with the protein backbone than water. In this model the denaturation mechanism is electrostatic in origin. The two conformations of the peptide do not form strong internal hydrogen bonds. However, while in the α conformation the two amide planes do not interact directly, they do have weak interactions in the β conformation, potentially forming a γ turn.

We examine the hydrogen-bonding pattern using the correlation functions for the solvent and solute (Figure 5). If we examined the hydrogen atoms of the solvent (either urea or water molecules), then a significant preference for urea is observed. This preference is even stronger for the solvent oxygen-peptide hydrogen case. Because the charge on the urea oxygen (-0.39) is smaller than the charge on the water molecule (-0.834). The enhancement of urea solvation must be due to higher electric moments and steric effects. Since the oxygen is a relatively large for atom solvation, preferences of urea compared to water will be more significant in open conformations. In open conformations the oxygen atom can approach more easily the polar hydrogens at the peptide backbone.

Hence, the above analysis suggests that the primary origin of the shifts in the equilibrium of peptide conformations in urea is due to the different hydrogen bonding. The hydrophobic effect was estimated to be too small to account for the observed large changes in

the pair correlation functions for the two conformations of the peptide.

CONCLUSIONS

Using two different studies of peptide solvation in water and $8M$ urea, we have demonstrated that the major factor that influences the conformational equilibrium of the peptide are the variation in electrostatic forces and hydrogen bonding. In the peptide we found no evidence for a significant contribution of the hydrophobic interactions to the urea-induced conformational transition. Our results are in qualitative agreements with the experimental studies of peptides solvation in urea solution.²

The present simulations and previous computations⁵ suggest that urea unfolds proteins by direct interactions with the peptide backbone and other solvent-exposed charged residues. An immediate prediction of the proposed denaturation mechanism proposed here is the following: If a methyl group, for example, is attached to the carbonyl oxygen of urea, [i.e., $(\text{NH}_2)_2 \text{COCH}_3$], then the hydrogen bonding capacity of this molecule would be greatly diminished. On the other hand, we expect that methylation of polar hydrogen of urea will have a smaller effect. Methylation of the oxygen would weaken the ability to denaturate proteins if our model is valid. This prediction can be tested experimentally and by computer simulations.

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