Hydrogen bond analysis of Type 1 antifreeze protein in water and the ice/water interface

Paper

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Antifreeze proteins (AFPs) are a group of structurally very diverse proteins with the unique capability of inhibiting ice crystal growth. Although significant progress has been made in the identification of different families of these proteins, the molecular mechanism of their action is unclear. The previously postulated mechanism of hydrogen bonding between the threonine residues of AFP and the water molecules in the ice surface has been disproved by mutation studies with non-polar residues. Currently, the mechanism of antifreeze activity cannot be fully understood from experimental or computational studies. Computational modeling studies have examined protein–ice interactions, mostly *in vacuo*. These studies have neglected the effects of the water phase. It has been shown that the vacuum is a very poor approximation for the water properties. Thus, to gain an insight into the molecular mechanism of these proteins we have computationally modeled a more realistic system comprising of AFP Type I from winter flounder (HPLC6), water and ice without any constraints. The results from this study show that the protein forms hydrogen bonds with the water molecules in the ice/water interfacial region. However, a comparison of the results with the protein in water simulations shows that there is no significant gain of hydrogen bonds for protein in the interfacial region compared to in the solvent. These results support the hypothesis that hydrogen bonding is not the primary reason for interaction of HPLC6 with the ice/water interfacial region.

Introduction

Rapid progress continues in the identification of natural kinetic ice growth inhibitor families, also called thermal hysteresis proteins (THP) or 'antifreeze' proteins (AFP).[1–4] In contrast, the molecular mechanism of their action remains under challenge. The AFP Type I from winter flounder (HPLC6) has 37 residues and forms an alpha helix. The long-claimed[5,6] role of threonine hydroxy groups in Type I AFPs was disproved by mutation studies.[7–11] Previously, water in the ice/water interface has been treated as a vacuum for simplicity. However, supercomputer simulations of this interface show that the vacuum is a poor approximation to the solvation properties of water.[11] The "desolvation" term of at least one side of the antifreeze protein is important and is not treated robustly by the vacuum approximation. The central question is what are the dominant effects (interactions and/or entropy) in the AFP interaction with the ice/water interface?

Haymet and Kay[12] and Brady *et al.*[13,14] reported the first molecular dynamics studies of HPLC6 in a periodically replicated box of water. Several other molecular dynamics simulations have been performed for protein–water interactions.[15–17] These studies found that the structure of water around the threonine residues had reduced mobility and was more "ice like". Comparatively the water remained quite mobile on the opposite, hydrophobic face of the helix. From these observations, it was proposed that the adsorption of the molecule to the ice crystal surface is on account of the hydrogen bonds between the threonine residues and the oxygen atoms and the repelling of water by the hydrophobic alanine residues.

The first modeling study of HPLC6[18] consisted of *in vacuo* energy optimization of the protein. Using the energy-minimized structure and the results from Knight *et al.*'s etching experiments[6] a hydrogen bonding based "binding" mechanism was proposed. Independently, Lal *et al.*[19] proposed that the high affinity of the winter flounder protein for the [2 0 $\bar{2}$ 1] plane derives mainly from the steric compatibility between this plane and the protein molecule, giving rise to a many fold increase in the van der Waals component of the surface–molecule binding energy. Wen and Laursen[5] proposed the "binding" face of the protein to be composed of asparagine, threonine, and leucine. Madura *et al.*[20] modeled the right-handed and left-handed helices on the [2 0 $\bar{2}$ 1] plane. On the basis of those calculations, it was shown that there are stereospecific sites that the protein can recognize on the ice surface. Most recently, Dalal and Sönnichsen[21] have reported using a Monte Carlo rigid body docking of HPLC6 on various ice planes. The study has shown that, although van der Waals interactions are a major source of ice–protein interactions, they cannot be used to completely explain the observed interface specificity.

Several ice/water simulations have been performed.[22–25] Results from these studies indicate that the ice/water interface is a diffuse interface approximately 10–20 Å thick. This interfacial region is observed to be dependent on the ice plane and the order parameters employed to measure it (*i.e.*

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average density, translation order, self-diffusion, and rotational orientation).

Two molecular dynamics studies of ice/protein/water systems have been reported to date for HPLC6. In the first, McDonald *et al.*[26] reported 100 ps of a molecular dynamics (MD) simulation. From this study they concluded that there was significant increased contact between the protein and surrounding water molecules in comparison to the behavior of the AFP in bulk water due to the formation of strong ice–protein hydrogen bonds. They also noticed a significant bend in the protein's helical structure. Finally, in their simulation, the four threonine side chain hydroxy groups were facing away from the interfacial region to test whether an alternate mode of interaction (*i.e.* polar threonine groups not interacting with ice) was possible. In another study, Cheng and Merz[16] performed MD simulations for their ice/protein/water system. In this study they reported 200 ps of MD simulation of the water/protein/ice system at 300 K. They proposed a relation among binding energy, the number of hydrogen bonds, and the activity. They observed that the higher number of total hydrogen bonds between the protein and the ice surface correlates with greater antifreeze activity. Finally, they did not notice any bending of the protein's helical structure.

Unfortunately, in these two studies the ice lattice positions were held fixed and electrostatic interactions were measured using cut-offs resulting in a poor representation of the interfacial region and underestimation of the effects of long-range electrostatic interactions, respectively. Here we report the first molecular dynamics simulation of a complete ice/Type I AFP/water system in the absence of any constraints and using Ewald summation for calculating electrostatic interactions. Furthermore, this simulation is more than twice as long as the previous simulation.[16] In this study we have set out to address the importance of hydrogen bonds for the interaction of the protein with the water molecules in the ice/water interfacial region. We find that the protein interacts with the water molecules in interfacial region through hydrogen bonds. However analysis and comparison of the hydrogen bonds from the protein in water and protein in ice/water interface simulations[14–16,26] lead us to conclude that the primary driving force for the protein–ice–water interaction is not through hydrogen bonding. Furthermore, we discuss the prevalent lattice-matching hypothesis in light of the ice/water interface properties.

Methods

Construction of the ice/water interface was performed utilizing the visualization software, MOE (Molecular Operating Environment, Chemical Computing Group, Montreal, Canada). Periodic images of a box of water containing 216 molecules (18.7 Å^3) were used for the construction of water slabs. Also, the [2 0 $\bar{2}$ 1] ice was constructed using Cerius[2] (Molecular Simulations Inc, San Diego, USA). These ice and water boxes were placed side by side to form the ice/water interface. The detailed analysis of such an interface has been done by Hayward and Haymet.[25] Briefly, the interfacial region wherein the water molecule properties are in between solid ice and liquid water is $\sim$15 Å.

Winter flounder antifreeze protein type I coordinates were obtained from the protein data bank (1WFA) and placed at the ice/water interface. All water molecules within 3 Å of the proteins were removed. The resulting system consisted of an ice slab of 2841 water molecules and 5484 water molecules in the liquid phase. Its dimensions were $40.35 \times 77.55 \times 84.16$ Å^3. Initially, the protein was placed along the $<0\ 1\ \bar{1}\ 2>$ vector on the ice. The protein was placed at the ice/water interface in the water layer next to the ice surface with the Thr–Ala–Asn face of the protein facing the ice. The four threonines (2, 13, 24 and 35) were within hydrogen-bonding distance to the ice as observed from ice/vacuum calculations.[5,20] The other repetitive sequence, which consists of charged Asp5 and polar Asn16 and Asn27, also faced the ice. This configuration places the leucine (12 and 23) side chains in the water layer parallel to the ice surface.

The TIP3P[27] model was used for both phases of water, and the CHARMM parameter set param19 (ref. 28) was used for the proteins. Electrostatic interactions were evaluated using the Ewald summation technique,[29–31] while van der Waals interactions were cut off at a value of 11.0 Å. All X–H bonds were constrained using the SHAKE algorithm.[32] Periodic boundary conditions were used, and all atoms were free to move during the simulation. The molecular dynamics code used to run the simulation was DL_POLY 2.11.[33] 30 ps of simulation time took approximately 10 h on 64 processors of the Cray T3E at the Pittsburgh Supercomputer Center.

After placing the protein in the constructed ice/water system, a 500 step energy minimization was performed with the positions of the protein atoms and the ice oxygen atoms held fixed. The final configuration of this minimization was then subjected to a molecular dynamics simulation of 1 ps at 230 K with the ice oxygen atoms fixed. The configuration at the end of 1 ps was taken as a starting point for another 1 ps simulation at 500 K with the ice oxygen atoms fixed. A simulation of 300 ps at 180 K was performed with the resulting coordinate set. During this and subsequent simulation conditions no constraints were imposed on the system. Subsequently, the temperature was raised to 190 K and a NVT simulation was run for another 900 ps. The trajectory data from the 510 ps of simulation at 190 K were analyzed.

Results

For the first time, a 510 ps molecular dynamics simulation is reported for a 25000 atom ice/protein/water system (Fig. 1). Previous ice/protein/water simulations[16,26] were smaller, half the duration, and utilized constraints on the ice lattice *i.e.* the water molecules in the ice lattice were held fixed. Furthermore, we have employed Ewald summation technique to account for the long range electrostatic interactions as opposed to cut-offs employed in the previous simulations.

To analyze the protein–ice protein–water interactions, we have calculated the average number of hydrogen bonds for the proteins to the water molecules in the ice/water interfacial region, averaged over 510 ps duration of the calculation (Table 1). A hydrogen bond donor–acceptor distance cut-off of 3.5 Å and a donor–hydrogen-acceptor angle cut-off of 120° were applied in examining the protein–water and protein–ice hydrogen bonding. These values are identical to that used by McDonald *et al.*[26] and thus allowing us to directly compare the results. The average number of hydrogen bonds for the

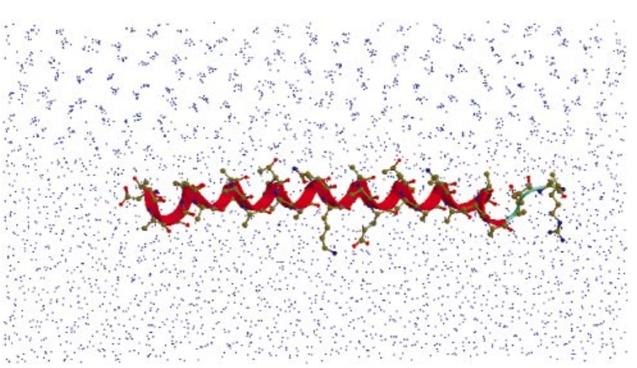

Fig. 1 Snapshot of the system. The oxygen atoms of the ice and water molecules are shown in blue. The ice/water interface is presented with ice on the top. The protein backbone is shown in a red ribbon, with the side chains in ball and stick model.

Table 1 Hydrogen bond analysis and comparison. The total number of hydrogen bonds to the water molecule per residue and comparison with the previous studies for protein in water[14] and protein at ice/water interface[26]

Residue number	Residue	Hydrogen bonds to the interfacial region	Hydrogen bonds from protein in water simulation[14]	Hydrogen bonds from protein at the ice/water interface simulation[26]
1	Asp	8.3	8.6	6.8
2	Thr	4.3	3.0	5.2
3	Ala	2	1.4	1.9
4	Ser	4.2	2.7	4.7
5	Asp	5.8	7.8	5.2
6	Ala	0.6	1.0	1.3
7	Ala	0.6	0.9	0.9
8	Ala	0.6	0.7	1.0
9	Ala	0.8	0.8	1.1
10	Ala	0.3	0.8	0.9
11	Ala	0.6	1.0	1.0
12	Leu	0.3	0.8	0.6
13	Thr	3.2	2.3	3.2
14	Ala	0.1	0.9	0.8
15	Ala	0.8	1.0	0.4
16	Asn	4.8	3.3	4.1
17	Ala	0.9	0.7	0.0
18	Lys	3.6	2.5	2.7
19	Ala	0.3	0.7	1.5
20	Ala	0.5	1.1	2.1
21	Ala	0.8	0.7	0.0
22	Glu	3.1	5.9	6.9
23	Leu	0.3	0.9	0.0
24	Thr	3.1	1.9	0.4
25	Ala	0.6	0.8	1.1
26	Ala	0.6	0.8	1.0
27	Asn	4.4	3.7	3.1
28	Ala	0.4	0.9	1.0
29	Ala	0.6	1.0	1.7
30	Ala	0.7	1.0	2.1
31	Ala	0.9	0.6	0.0
32	Ala	1.1	0.9	2.6
33	Ala	1.3	1.2	1.9
34	Ala	1	1.6	1.1
35	Thr	3.2	3.4	1.8
36	Ala	2.7	1.8	3.2
37	Arg	11.7	12.6	1.9
Total		79.1	81.7	75.2

proteins to the water molecules in the interfacial region is 79.1 (Table 1). In the table one can see that the total number of hydrogen bonds in the interfacial region compares well with the results obtained by McDonald *et al.* for their protein in water[14] (81.7) and protein in ice/water interface[26] (75.2) simulations. We have also estimated similar numbers for other studies[15,16] from their plots. We obtained values of 78.6 (Jorgensen *et al.*[15]) and 72.7 (Cheng and Merz[16]) for the protein in water simulations and 78.8 for the protein at the ice/water interface (Cheng and Merz). These values compare very well with the value of 79.1 obtained by us. Thus, there is no significant increase in the total number of hydrogen bonds between the protein in water and the protein at ice/water interface.

Comparing the values for the Thr (2,13,24,35) and Asx (5,16,27) residues (which are postulated to hydrogen bond to the ice lattice) we observe that there is a total increase of only 3.5 hydrogen bonds ($<5\%$) over these seven residues when the protein is moved from the water (McDonald *et al.*) to an ice/water interface (current simulation). A similar small gain of 2.33 and 3.63 is observed when comparing our results with the protein in water simulations of Jorgensen *et al.* and Cheng and Merz respectively. A stricter distance criterion for hydrogen bonding for these simulations (a donor–acceptor cut-off of 2.4 Å was employed by Jorgensen *et al.* whereas 2.5 Å was used by Cheng and Merz) could account for a percentage of this difference.

The distances of the i, $i+11$ threonine residues average 16.7 Å. The rms fluctuations averaged 0.2 Å. Previous simulations, using a variety of force fields, have yielded different results for the helicity of the protein. McDonald *et al.*[26] noticed bending of the helix in their simulation. On the other hand, Cheng and Merz[16] did not notice any significant helix bending. This could be due to the different forcefields employed in these simulations. McDonald *et al.* employed CHARMM parameters whereas Cheng and Merz used AMBER parameters. To determine in our calculation if there was any substantial bending of the helix we measured the average dihedral values. The average ϕ and ψ values for residues 5–33 are within 10° of −66 and −41° respectively. These values are consistent with the standard values for a helical protein suggesting that there is no helix bending. Thus the differences in the helical bending are not on account of parameterization, since we employed the CHARMM forcefield which was also used by McDonald *et al.* (whereas Cheng and Merz used the AMBER forcefield). The protein is frayed near the termini, more so for the C terminus than the N terminus.

Discussion and conclusion

In this study, we have demonstrated the feasibility of an ice/protein/water system without any constraints. Furthermore, we have also extended the electrostatic interactions by employing Ewald summations instead of cut-offs. These two unique features along with greater than twice the simulation time set this study apart from the previous studies[16,26] which employed fixed ice lattice positions and cut-offs for electrostatic interactions.

The results show that the protein hydrogen bonds with the water molecules in the interfacial region. However, we show that there is no significant gain in the number of hydrogen

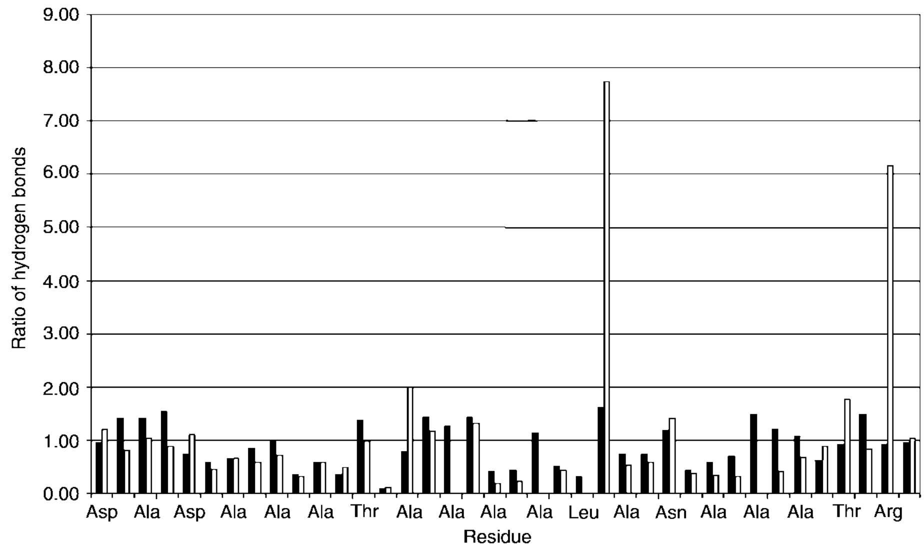

Fig. 2 Ratio of hydrogen bonds between the simulations. The ratio of hydrogen bonds between (a) the current simulation and the protein in water simulation[14] (dark bars) and (b) the current simulation and the protein at ice/water interface simulation[26] (white bars).

bonds when the protein moves from the solvent to the ice/water interfacial region. A comparison of the results obtained by McDonald *et al.* from their two studies also shows that overall there is a smaller number of hydrogen bonds for their simulation in ice/water interface compared to protein in water simulation. The main difference is in the hydrogen bonding capability of Arg37, which could be due to the flexibility of the C terminus in water compared to the ice/water interfacial region. A comparison of the Thr and Asx groups shows that there is a loss of 2.4 hydrogen bonds when the protein is moved from water to the water/ice interface. However, in their protein/ice/water simulation they had the Thr and Asx groups facing away from the ice and all the hydrogen bonds for these groups were derived from the liquid phase water molecules.

A similar comparison of results from Cheng and Merz's protein–water and protein/ice/water simulations reveal that there is a total gain of approximately 6 hydrogen bonds ($<10\%$) when the protein interacts with water/ice interface as compared to solvent. For the polar Thr and Asx residues the gain in total hydrogen bonds is only 1.6.

Fig. 2 illustrates the ratio of hydrogen bonds between our simulation and McDonald *et al.*'s simulations. For a majority of the residues the ratios are between 0.5 and 2, suggesting a correlation between the results from the simulations. For some of the Ala residues the ratio is not in this range. That is because the number of hydrogen bonds is very small, although the number of hydrogen bonds is similar. For example, Ala14 has 0.1 hydrogen bonds in our simulation and 0.9 in McDonald *et al.*'s bulk solvent simulation and 0.8 in the protein at the ice/water interface simulation. These differences in hydrogen bonds, even though not significantly different, result in ratios of 0.11 and 0.12 respectively. In our simulations there are fewer hydrogen bonds for Asp5 and Glu22 compared to the McDonald *et al.*'s protein in water simulation, and again in our simulation Glu22 had less hydrogen bonds compared to the McDonald *et al.*'s protein at the ice/water interface simulation. Interestingly, there are more hydrogen bonds in our simulation for Thr24 and Arg37 compared to the protein at ice/water interface simulation. McDonald *et al.* find that Thr24 and Arg37 form hydrogen bonds with the protein atoms resulting in fewer hydrogen bonds with water molecules. Furthermore in their simulation the ice lattice was held rigid and hence lacked a proper interfacial region. This rigid ice lattice lead to steric hindrance for the protein causing fewer hydrogen bonds with water for the termini residues.

The analysis presented here lead us to conclude that the primary driving force for the protein–ice–water interaction is not hydrogen bonds. There have been previous protein–ice simulations by us[21] and others[19] that have suggested that the affinity of the protein–ice interaction is derived from the van der Waals interactions. According to our knowledge, this is the first ''full system'' modeling study that demonstrates that the interaction of the protein with the ice/water interface is not due to hydrogen bonding. This conclusion corroborates the recent experimental results that show little or no importance for hydrogen bonding interactions in the antifreeze mechanism.[7–11,34]

As far as the helicity of the protein is concerned we found that the protein remains fairly helical through the simulation, consistent with Cheng and Merz[16] but in contrast to McDonald *et al.*[26] However, we did notice some fraying at the termini, more for the C than the N terminus. Doig *et al.*[35] measured N and C capping preferences for all 20 amino acids in alpha-helical peptides. They found that the peptide with Asp at the N terminus has 0.527 fraction helicity compared to 0.490 for Arg at the C terminus. Thus, with the AFP sequence one would expect a relatively larger fraying at the C compared to the N terminus.

The Thr side chain angle (χ_1) is populated at three rotamer values (-60, 60 and 180°). We do not observe any jumping of the Thr rotamer from one value to another. This is consistent with previous simulations[16,26] where the rms fluctuations were 10–15° for Thr χ_1 values. A longer simulation time could result in jumps of χ_1 values (on the order of 60°).

Thus, in conclusion we have not only demonstrated the simulation of a protein/ice/water system without any constraints, but also employed Ewald summation instead of cut-offs, thus calculating the long-range electrostatic interactions. We have also shown that the protein interacts with the solid and liquid phase water molecules in this interfacial region and is in a position to ''bind'' to an ice plane or direct the expression of an ice plane. However, there is no gain of hydrogen bonds when the protein desolvates and interacts with the ice/water interfacial region and thus we conclude that the mechanism of protein–ice–water interaction is not dominated by hydrogen bonding.

The prevalent lattice-matching hypothesis has directed the belief that the antifreeze protein (especially Type I) binds to the ice 'lattice' through a distance matching criterion. Inherent in this hypothesis is the belief that there exists an ice/vacuum interface, *i.e.* ice can exist as a perfect solid crystal. Nothing could be further from truth, experimental studies have shown that the ice/water interface is highly dynamic and more than 20 Å wide. Since, in this region the ice crystal is poorly defined there is no defined ice structure periodicity to match with the protein's 11-residue repeat. Thus it is vital that the alternative hypothesis account for the fact that the protein interacts with the water molecules that are in between liquid and crystal form (depicted as an interfacial region in this study) and modeling studies such as this will help in understanding of the molecular mechanism.

It is worth collecting here some final thoughts on the terminology used in this field. For many years authors have talked of thermal hysteresis proteins "binding" to ice. While this nomenclature is a natural one for ice/vacuum interfaces, we see from the above discussion that the molecular-level explanation for the action of these kinetic ice growth inhibitors lies in a full consideration of the protein, ice and water. More correct terms for the accumulation of the kinetic inhibitors at the interface between phases are found in more closely related fields, such as "poisoning" of metallurgical interfaces, and even, from a purely thermodynamic example, the accumulation of a detergent at the water/oil interface.

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