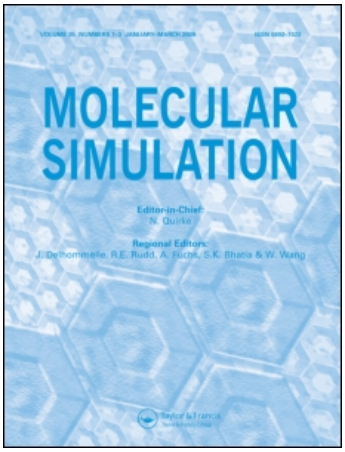

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MOLECULAR DYNAMICS STUDY ON WINTER FLOUNDER ANTIFREEZE PROTEIN AND ITS BINDING MECHANISM

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In order to study the antifreezing mechanism of the HPLC6 antifreeze protein and its structural effects on the antifreezing activity, molecular dynamics simulations for this protein in aqueous solution were carried out at two different temperatures (300 K and 253 K). The results of these simulations showed that the binding mechanism of the antifreeze protein is mainly due to the adsorption of the molecule to the ice crystal surface and thereby inhibiting the ice growth. The hydrogen bonds between the threonine residues and the oxygen atoms in the ice play an important role in this mechanism. Mutation study was carried out to verify the mechanism. In addition, hydrophobic alanine residues are located on the opposite side of threonine residues and they repel water molecules to prevent water molecules from approaching the ice surface.

Keywords: Antifreeze protein; molecular dynamics simulation; mutation study; amphiphilicity; water structure

1. INTRODUCTION

Recently investigations of the structural relationship to the function of proteins are active research themes of molecular biologists, structural biologists, and chemists. In this regard, antifreeze protein (AFP) is one of excellent examples showing direct relationship between the structure and the mechanism of function of molecules. Antifreeze proteins have been reported to be contributors for survival of some polar fishes against cold environment. Winter flounder AFP from *Pseudopleuronectes americanus* is one of

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the best characterized AFP and some investigations about the antifreeze mechanism have been reported [1–2]. It has been separated into at least seven components by high performance liquid chromatography. One of the seven components, known as HPLC6, contains 37 residues and alanine is the main component.

Recently the crystal structure of HPLC6 was resolved and it was reported as an α-helix [3]. And the periodicity of its backbone sequences is thought to be important for the antifreezing function.

Asp-Thr-Ala-Ser-Asp-Ala-Ala-Ala-Ala-Ala-Ala-
Leu-Thr-Ala-Ala-Asn-Ala-Lys-Ala-Ala-Ala-Glu-
Leu-Thr-Ala-Ala-Asn-Ala-Ala-Ala-Ala-Ala-Ala-
Ala-Thr-Ala-Arg

This periodicity brings about an imperfect 11-residue repeat which places hydrogen-bonding groups in specific spatial arrangements that have been found to coincide with oxygen sites along certain vectors in the ice lattice. And recent experiments identified the adsorption plane in ice of the structurally similar antifreeze proteins from winter flounder (*P. americanus*), Alaskan plaice (*P. quadriaberulatus*), and the sculpin protein (*Myxocephalus scorpius*) [4]. These are probably aligned along the [01 $\bar{1}$2] direction in ice, where the repeat distance of the oxygen atoms is 16.6 Å and it is theorized that the spacing of threonine side chains should match this distance thereby explaining the mode of binding.

Some computer simulations of HPLC6 have been done to see the behavior of the protein itself [5], either in an effective dielectric field or in explicit solvent so far [6, 7]. However, to see the direct influence of water solvent, which is thought to be important according to the mechanism stated before, molecular dynamics simulations at two different temperatures (300 K and 253 K) were carried out and the structures of water solvent influenced by AFP were analyzed. And the mutation study of AFP was also performed. In the mutation study, active threonine residues was exchanged into hydrophobic valine residues, which would significantly eliminate the antifreeze activity of AFP molecule if the present binding mechanism were correct.

2. METHOD

All simulations were done using the CHARMm program (Version 24.0) modified for use on the SGI INDIGO2 workstation [8]. All interactions

were calculated between pairs of atoms closer than 15 Å. Trajectories were calculated using Verlet algorithm with a time step of 1 fs. Initial structure is X-ray structure of winter flounder HPLC6 peptide. To mimic real solution structure water molecules were added explicitly. Total 2333 water molecules were generated in the box of the size of 60 Å × 30 Å × 40 Å. TIP3P potential of water model [9] was used.

Before molecular dynamics simulation on the system, it was relaxed by 1000 steps of steepest descent minimization and 2000 steps of adopted basis Newton-Raphson minimization to eliminate the initial strains in the system. A heating of 24 ps (up to 600 K) and an annealing of 12 ps (down to 300 K) were done. Again the ongoing annealing down to 253 K for 2 ps was done for the analysis of subzero system. Equilibrations were done at each temperature (300 K and 253 K). After the production dynamics of 100 ps, we obtained the trajectories of systems, and analyzed those to get the structure of protein and solvent. The structural characteristics of proteins are suggested by root-mean-square fluctuation (rmsf) and Ramachandran map, and the structure of solvent is mainly analyzed by radial distribution function (RDF).

The three threonine residues were mutated to valine residues, because the side chain of valine is not able to make hydrogen bonds and has the hydrophobicity. The detailed procedure of calculation and analysis for the mutant was the same as above.

3. RESULTS AND DISCUSSION

According to the supposed mechanism of antifreeze activity, AFP molecule should have structural regularity so that the orientation and distances of hydroxyl oxygen atoms of threonine residues should coincide with those of oxygen atoms in ice nuclei. Thus, we calculate the structure of AFP to find out whether the above mechanism is reasonable. Molecular dynamics simulations at two different temperatures result in the following structural features. At first, the calculated distances of threonine oxygen atoms in average structure are around 16 ~ 17 Å, all of which are within 1 Å of the distances between oxygen atoms of ice in specific alignment direction [01 $\bar{1}$2]. The repeat spacing in ice along this direction is 16.7 Å [10]. To make hydrogen bonds with ice oxygen atoms the threonine hydroxyl groups should be equally spaced with this distance and also lined up to one direction. The angles made by the serial three threonine oxygen atoms show that they are almost collinear and aligned to specific direction (See Tabs. I and II).

TABLE I Inter-oxygen distances of hydroxyl group of Thr side chains

	distance 1[a] *(Å)*	*distance 2*[b] *(Å)*	*distance 3*[c] *(Å)*
X-ray structure	15.90	16.43	16.35
300 K	16.58	17.69	16.46
253 K	17.56	17.56	17.63

[a] distance 1 : Thr 2 O^{γ} – Thr 13 O^{γ}.
[b] distance 2 : Thr 13 O^{γ} – Thr 24 O^{γ}.
[c] distance 3 : Thr 24 O^{γ} – Thr 35 O^{γ}.

TABLE II Angles (degree) between two lines connecting oxygen atoms of Thr side chains

	line 1[a] *– line 2*[b]	*line 2 – line 3*[c]
X-ray structure	172.0	169.9
300 K	159.0	170.0
253 K	175.2	171.7

[a] line 1 : Thr 2 O^{γ} – Thr 13 O^{γ}.
[b] line 2 : Thr 13 O^{γ} – Thr 24 O^{γ}.
[c] line 1 : Thr 24 O^{γ} – Thr 35 O^{γ}.

Another important figure to point out about the calculated structure is the temperature dependency of AFP molecule. To investigate this temperature dependency, we have conducted annealing molecular dynamics [10, 11]. After the initial X-ray structure is solvated, the system is heated to high temperature and again cooled down slowly to the target temperature. Although working temperature of AFP molecule is slightly under the freezing point of water, $-2°C \sim -3°C$, in order to obtain solvent environment with the more ice-like structure, the temperature is set to 253 K, supercooled environment. For the comparison, another simulation is done at the room temperature. After total 160 ps of simulation, unexpected structural change was found. As can be seen in Ramachandran map in Figures 1 to 3, the initial structure of AFP is single α-helix, but in room temperature the structure is changed to a loose helix with a loop in the middle of the helix. And after further annealing down the structure restores original single α-helix again (See Figs. 1 to 4). This structural dependency on the temperature seems due to Lys 18 – Glu 22 salt bridge pair. At low temperature this salt bridge pair is relatively significant enough to keep the structure as α-helix. As the temperature increases, the thermal motions of solute and solvent molecules are more activated and the salt bridge pair is not stable enough to keep the molecule from the loss of relatively long helix structure composed of 37 amino acids. This structural change is in good accordance with experimental data [13]. The structural change may be related to the environmental selectivity of AFP molecule.

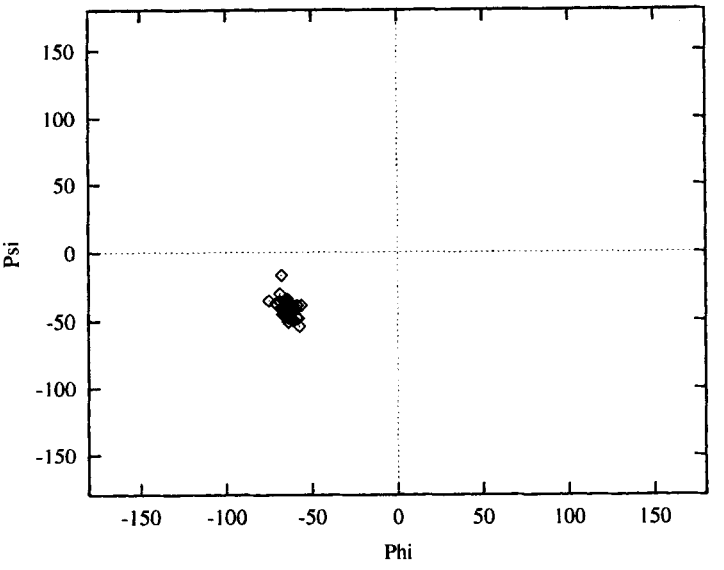


FIGURE 1 Ramachandran map of X-ray structure of AFP.

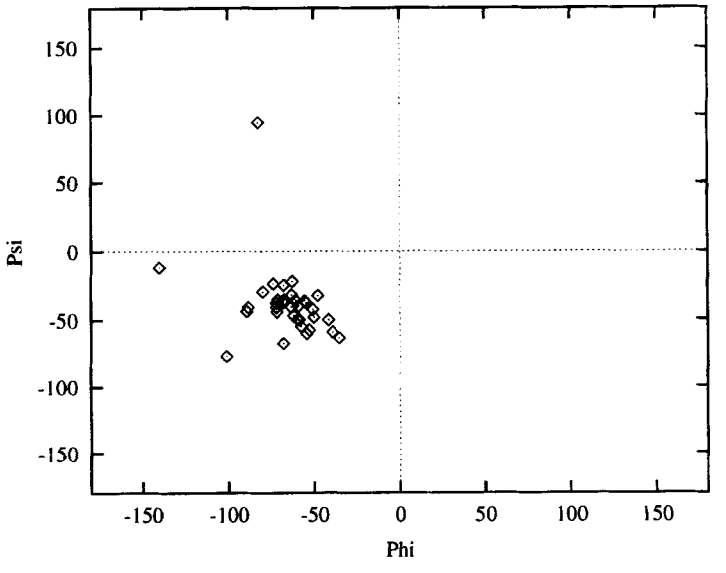


FIGURE 2 Ramachandran map of AFP at 300 K.

Root-mean-square fluctuations (rmsf) of AFP molecule at both temperatures are calculated to see the flexibility of molecule (See Fig. 5). When compared with the rmsf calculated from B factor of crystal structure,

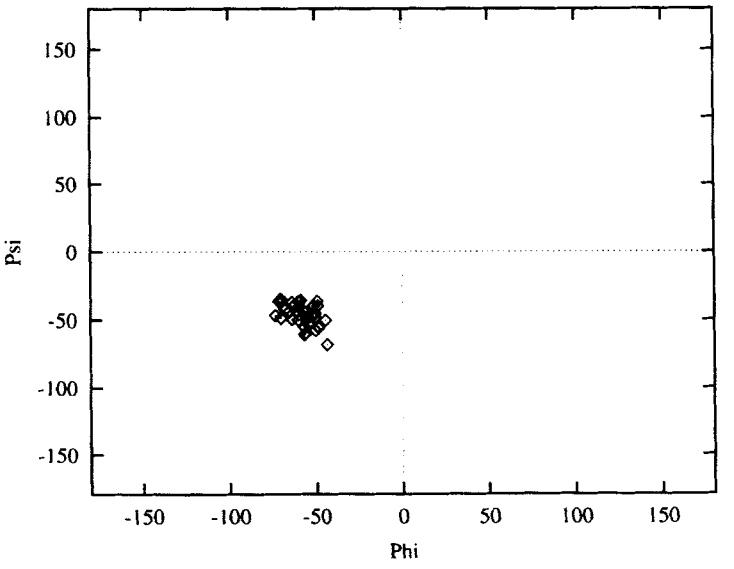
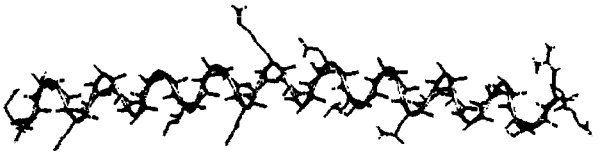
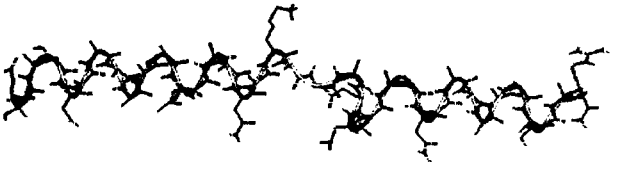


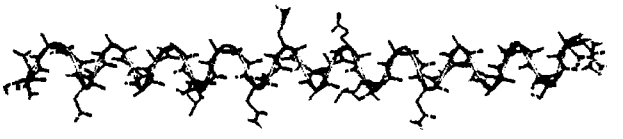
FIGURE 3 Ramachandran map of AFP at 253 K.



(a) X-ray structure



(b) 300 K



(c) 253 K

FIGURE 4 Structure change of AFP helix at different temperatures.

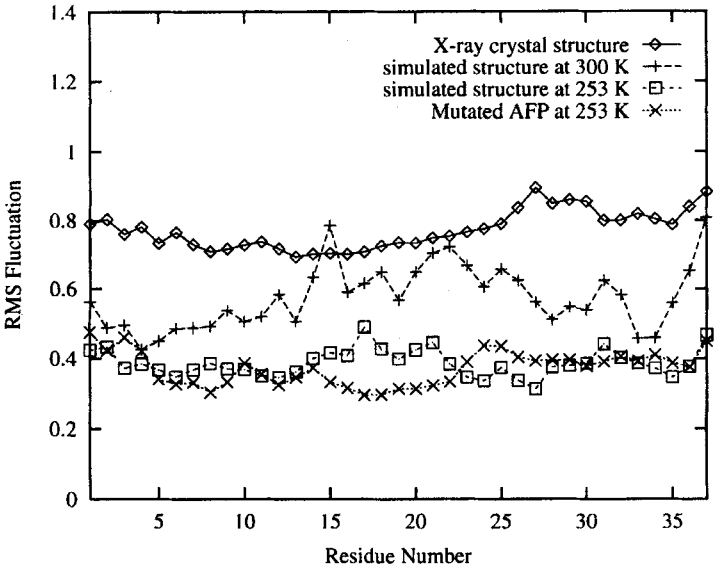
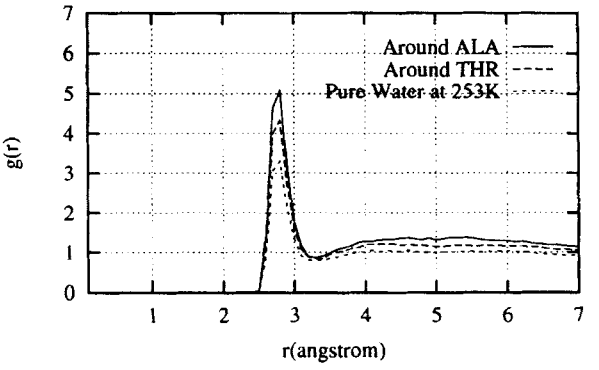


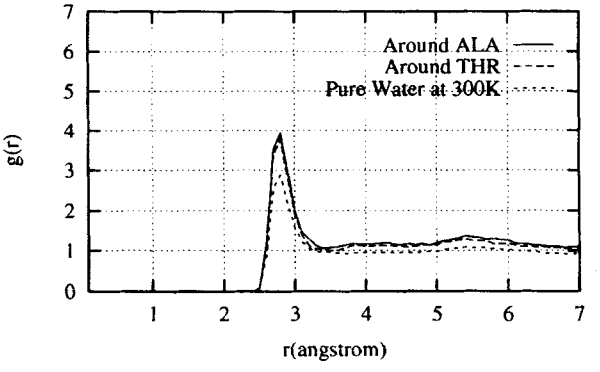
FIGURE 5 Root-mean-square fluctuations of AFP.

rmsf of simulation shows similar tendencies. At 253 K, the molecule is very rigid with rmsf range from 0.3 to 0.5. At 300 K, much different pattern can be seen, but the rmsf values are still low. Due to the thermal motion, the terminal residues have relatively high flexibility and the breakage of salt bridge in the middle of the helix also results in high rmsf values in corresponding residues. Mutation of threonines to valines is done and rmsf is also calculated at 253 K. When the rmsf's are compared with regard to dynamic motion of molecule, mutant shows little change from the wild type AFP.

The function of AFP is closely related to water solvent, because the interaction of AFP with water can be said to be responsible for the activity of AFP. Therefore the structure of water solvent is as important as that of protein itself for the investigation of mechanism. We choose the supercooled water as the environment of AFP molecule. The structures of water solvent molecules are mostly studied by radial distribution function (RDF). To see the detailed structure of water in the proximity of active threonine residues, we select water molecules within 5 Å from each oxygen atom of threonines. And for the comparison, water molecules within 5 Å from β-carbons of four randomly chosen alanines on the opposite side of the helix are also selected. Figure 6(a) shows the solvent–solvent inter-oxygen RDF, $G(r)_{00}$ at 253 K, where water molecules around hydrophobic alanine residues tend to be



(a) solvent-solvent RDF of AFP solution at 253 K



(b) solvent-solvent RDF of AFP solution at 300 K

FIGURE 6 Solvent–solvent radial distribution function of AFP solution.

more structured to make icebergs and solvent molecules around hydrophilic threonine residues on the opposite side are more structured than pure solvent also. At room temperature the difference of the contact value, the height of first peak, between water molecules around Thr and Ala residues is disappeared (See Fig. 6(b)). This result can be explained as follows. As the temperature is increased, the hydrophobic nature of Thr overrules the hydrophilic nature so the contact value is increased. Mutated AFP shows the same result as that of 300 K, although the contact values are higher due to the hydrophobic nature of valine (See Fig. 7).

Direct interaction between threonines and water molecules are shown by solute-solvent RDF (See Fig. 8). As can be seen in the RDF plot of threonine oxygen atoms and solvent molecules, the first peak appears near 2.8 Å, which is almost the same distance between oxygen atoms of two

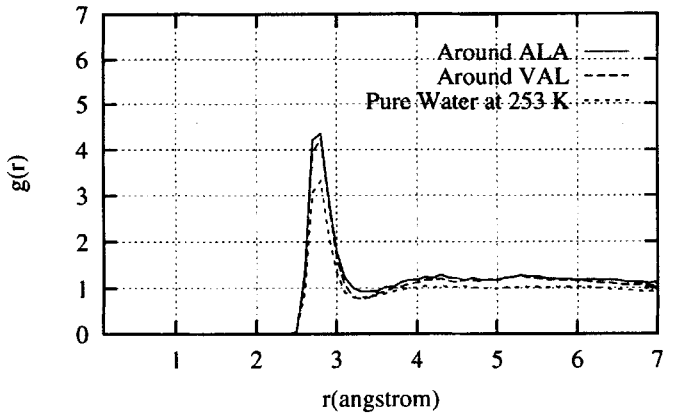
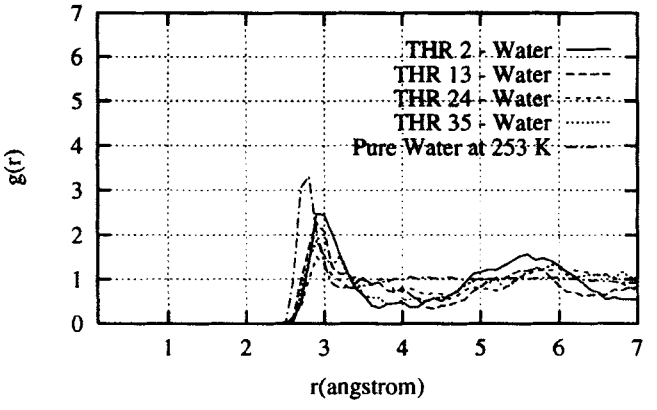


FIGURE 7 Solvent–solvent radial distribution function of Thr's→Val's mutant at 253 K.

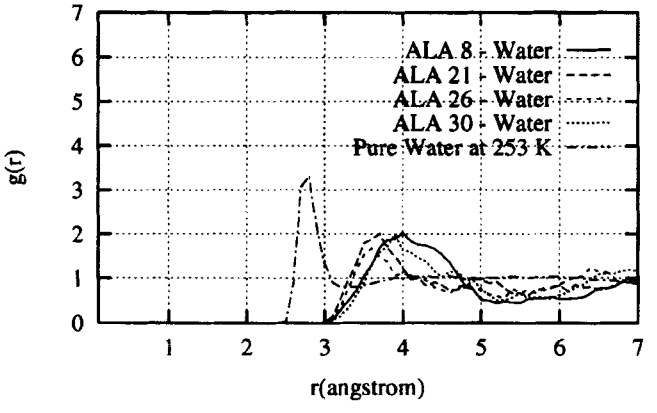
neighboring water molecules. As a result, threonine oxygen atoms bind specific water molecules, or specific oxygen atoms on the ice crystal surface. On the other hand the hydrophobic residues, most of which are located on the opposite side of threonine residues, prevent other water molecules from approaching to grow ice crystal. The solute-solvent RDF plot for alanine and water molecules coincides with this explanation. In this RDF, the location of the first peak is shifted to the right by about 1 Å compared to the RDF plot of Thr's and their proximate water molecules. This indicates that for the hydrophobic nature of alanine residues water molecules are repelled. Thus, the amphiphilicity of this molecule is very important feature for the function of AFP. Mutated AFP, where active hydrophilic threonine residues are mutated to hydrophobic valine, does not have this amphiphilicity. It cannot bind any growing ice crystal, rather enhances water molecules to make clathrates and seeds for ice crystal nucleation [14]. The solvent–solvent and solute-solvent RDF data are in accordance with this explanation (See Figs. 7 and 9). From these results we may deduce the antifreezing mechanism of AFP. First, oxygen atoms of threonine residues anchor at ice crystal surface, and the alanine residues on the opposite side inhibit the approach of water molecules. This function results in suppressing the growth of ice crystal.

4. CONCLUSION

We have carried out molecular dynamics simulations to investigate the relationship between the structure of AFP and water structure. Analysis of



(a) solute-solvent RDF of Thr and water of AFP at 253 K

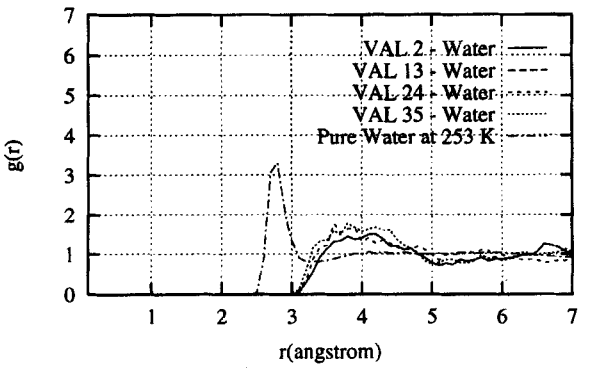


(b)Solute-solvent RDF of ALA and Water of AFP at 253 K

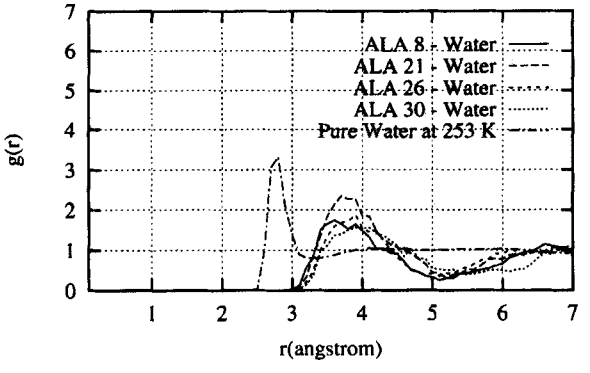
FIGURE 8 Solute–solvent radial distribution function of AFP solution at 253 K.

the structure of water solvent molecules support the binding mechanism stated above.

Threonine side chains can make hydrogen bond with water molecules and are the important active sites for the function. The water structure around these residues is similar to that of the bulk water. This means the oxygen atoms of threonine residues can be attached to the ice crystal. When threonine residues are mutated to hydrophobic valine residues the structure of water solvent is much different from that of wild type AFP, i.e. the adsorption of AFP to ice cannot be performed so that the inhibition of the growth of ice crystal is impossible. On the opposite side of threonine



(a) solute-solvent RDF of VAL and water of mutated AFP at 253 K



(b) solute-solvent RDF of ALA and water of mutated AFP at 253 K

FIGURE 9 Solute–solvent radial distribution function of Thr's→Val's mutant solution.

residues, there are many hydrophobic alanine residues. These residues keep water molecules apart from the AFP. This function of alanines makes the growth of ice crystal harder.

In conclusion, according to our theoretical calculation HPLC6 AFP molecule has an unique favorable structure for the antifreeze activity. Especially the amphiphilicity of AFP molecule is an important origin of antifreezing activity.

Acknowledgement

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