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Mechanisms of antifreeze proteins investigated via the site-directed spin labeling technique

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Abstract

The site-directed spin labeling (SDSL) technique was used to examine the antifreeze mechanisms of type-I antifreeze proteins (AFPs). The effects on the growth of seed ice crystals by the spin-label groups attached to different side chains of the AFPs were observed, and the states of water molecules surrounding the spin-label groups were probed via analyses of variable-temperature (VT) dependent electron paramagnetic resonance (EPR) spectra. The first set of experiments revealed the antifreeze activities of the spin-labeled AFPs at the microscopic level, while the second set of experiments displayed those at the molecular level. The experimental results confirmed the putative ice-binding surface (IBS) of type-I AFPs. The VT EPR spectra indicate that type-I AFPs can inhibit the nucleation of seed ice crystals down to $\sim -20^{\circ}\text{C}$ in their aqueous solutions. Thus, the present authors believe that AFPs protect organisms from freezing damage in two ways: (1) inhibiting the nucleation of seed ice crystals, and (2) hindering the growth of seed ice crystals once they have formed. The first mechanism should play a more significant role in protecting against freezing damage among organisms living in cold environments. The VT EPR spectra also revealed that liquid-like water molecules existed around the spin-labeled non-ice-binding side chains of the AFPs frozen within the ice matrices, and ice surrounding the spin-label groups melted at subzero temperatures during the heating process. This manuscript concludes with the proposed model of antifreeze mechanisms of AFPs based on the experimental results.

Keywords

Type-I antifreeze protein; Site-directed spin labeling; VT EPR; Ice crystals; Ice growth inhibition; Ice nucleation inhibition

Introduction

Antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs) protect organisms living in subzero temperatures from freezing damage by inhibiting the growth of seed ice crystals (DeVries and Wohlschlag 1969; DeVries and Komatsu 1970; Komatsu and DeVries 1970; Duman and DeVries 1972; Feeney 1974; Feeney and Yeh 1978; DeVries 1983; Cheng and

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DeVries 1989; Davies and Hew 1990; Duman and Wu 1993). This inhibiting mechanism can lower the freezing points of aqueous solutions more effectively than the colligative properties of water. In addition, such a mechanism imposes an insignificant effect on the osmotic pressure within a cell (Davies and Ewart 1993; Chen and DeVries 1997; Davies and Fletcher 1999). AFPs and AFGPs were also found to inhibit the recrystallization of ice, which can otherwise create large, tissue-damaging ice crystals (Knight and DeVries 1984).

Type-I AFPs have molecular weights in the range of 3.3–4.5 kDa (DeVries and Wohlschlag 1969; Duman and DeVries 1974, 1976; Hew and Joshi 1985). The HPLC6 isoform represents the typical structure of type-I AFPs and thus was the most extensively studied one among all known type-I AFPs (Sönnichsen and Davies 1998; Harding and Ward 1999; Mao and Ba 2006a, b; Mao and Lin 2009; Mao and Jeong 2011). This isoform was isolated from the blood serum of winter flounder inhabiting the nearshore waters off Nova Scotia's coast (Duman and DeVries 1974, 1976). It has an amino-acid sequence of DTASDAAAAA LTAANAKAAAELTAANAAAAAATAR, which contains three 11-residue repeated units, beginning with Thr residues. Its secondary structure is α -helical (Yang and Sax 1988; Sicheri and Yang 1995; Gronwald and Chao 1996; Sicheri and Yang 1996; Sönnichsen and Davies 1998), with the exception of the last peptide unit.

It has been reported that HPLC6 peptides bind to the 12 equivalent bipyramidal planes of ice Ih (hexagonal ice) along the $\langle \bar{1}102 \rangle$ direction (Knight and Cheng 1991). The strong orientational dependence of the binding indicates a structural match between the HPLC6 peptide and the ice surfaces. The 12 AFP-binding planes confine the growth of the ice crystal to a bipyramidal shape (Wen and Laursen 1992; Chao and DeLuca 1995; Haymet and Ward 1999). It was proposed that the putative ice-binding surface (IBS) comprises the Thr side chains and the conserved i + 4 and i + 8 Ala side chains, where "i" denotes the positions of the Thr residues (Chao 1994; DeLuca and Chao 1996; DeLuca and Davies 1998; Graether and DeLuca 1999). Our previous solid-state NMR results are congruent with this proposed putative IBS (Mao and Ba 2006a, b; Mao and Lin 2009; Mao and Jeong 2011).

The Kelvin effect is a popular model for explaining the growth-inhibiting activity of AFPs in seed ice crystals (Wilson 1993). The Kelvin effect states that after AFP molecules are anchored irreversibly at different points on a specific ice surface, the ice could only grow from areas free of the AFP molecules, thereby resulting in interfacial curvatures. The interfacial curvatures made it energetically unfavorable for water molecules to join the ice surface (Raymond and DeVries 1972, 1977). Based on the binding dynamics observed in our NMR experiments, we proposed the mechanism of ice-surface adsorption enhanced colligative effect to explain the inhibition of AFP to the growth of ice (Ba and Wongsakulung 2003; Mao and Ba 2006a, b). Ice-surface adsorption of AFP molecules creates a water–AFP–ice (WAI) interfacial layer in which the concentration of AFPs was much higher than that in the bulk solution, thereby inhibiting the growth of seed ice crystals through the enhanced local colligative effect.

In the present study, the site-directed spin labeling (SDSL) technique (Hideg and Hankovszky 1989; Hubbell and Altenbach 1994; Hubbell and Gross 1998; Hubbell and Altenbach 2000; Steinhoff and Suess 2003; Galiano and Blackburn 2009; Klare and

Steinhoff 2009; Štrancar and Kavalenka 2010; Longhi and Belle 2011) was used to examine: (1) the effects of spin-label groups attached to different side chains of the type-I AFP on their antifreeze activities; and (2) the state of water molecules surrounding the spin-labeled side chains at different temperatures by analyzing the corresponding EPR spectral line shapes. The observed microscopic structures of the ice crystals confined by the spin-labeled AFPs, as well as the molecular information obtained from the EPR spectra, provide complementary information regarding how AFP molecules interact with water and ice to inhibit ice nucleation and the growth of ice crystals in AFP solutions.

Materials and methods

Design of spin-labeled type-I AFPs

The SDSL technique is achieved through cysteine substitution mutagenesis, which is followed by the covalent bond formation of a selective nitroxide reagent with unique sulfhydryl groups (Hubbell and Altenbach 1994; Hubbell and Gross 1998; Hubbell and Altenbach 2000; Steinhoff and Suess 2003; Galiano and Blackburn 2009; Klare and Steinhoff 2009; Štrancar and Kavalenka 2010; Longhi and Belle 2011). MSL (4-maleimido-2,2,6,6-tetramethyl-1-piperidinyloxy) was used in the present study. The structure of the MSL spin label and the resulting chemical modification on the cysteine side chain are shown in Scheme 1.

The residues designed to be single spin labeled are numbered in the following HPLC6 sequence, in which the putative ice-binding residues are underlined: DTASDAAAAAA(11)L(12)TAANA(17)KAA(20)AEL(23)TAANAAAAAAAATAR (Chao 1994; DeLuca and Chao 1996; DeLuca and Davies 1998; Graether and DeLuca 1999). The following MSL spin-labeled HPLC6 mutants were synthesized: (1) L12C, (2) L23C, (3) A20C, (4) A11C, and (5) A17C. The notation of L12C, for example, indicates that the cysteine substitution takes place at the Leu-12 residue. Models of the MSL spin-labeled HPLC6 mutants are shown in Fig. 1. Their structures were optimized using the semi-empirical method of the Gaussian 09 program, and the models were plotted using the Sparta'10 V1.1.0 program. The L12 and L23 residues are at equivalent positions within the first and second repeated units, respectively. The L12 and L23 side chains point up from the putative IBS of the peptide. The side chain of the 20th residue points toward the front side (relative to the position of Fig. 1a HPLC6 peptide), parallel to the putative IBS, while the side chain of the 11th residue points toward the back side, where the salt bridge is formed by the Lys and Glu side chains. The side chain of the 17th residue is located on the putative IBS and would thus contact the ice surface. This spin-labeled mutant was used to confirm the putative IBS because the spin-label groups structurally hinder the peptides from binding to the ice surfaces.

Syntheses of the spin-labeled HPLC6 mutants

The wild-type type-I AFP was purchased from A/F Protein Inc. (Waltham, MA, USA) (99.0%). The cysteine-substituted HPLC6 peptides were synthesized by Biomatik Corporation (Wilmington, DE, USA). Their purities are > 95%. The MSL (4-maleimido-2,2,6,6-tetramethyl-1-piperidinyloxy) spin label was purchased from Sigma-Aldrich (CAS Number 15178–63-9).

A buffer solution (pH = 7.4) was prepared with Phosphate buffered saline tablets (Cas # BP2944–100) purchased from Fisher scientific. One (1.0) mg of cysteine-substituted HPLC6 peptides were dissolved in 0.5 ml of the buffer solution in an Eppendorf vial (2-ml volume), and 1.565 mg of MSL were dissolved in 0.25 ml of ethanol. The two solutions were then mixed together in a closed vial wrapped with aluminum foil. The reaction took place under shaking condition via a vortex mixer for 2–4 h in a cold room kept at 4 °C. After reaction, the sample was lyophilized. Then, the solid powder was dissolved in 0.5 ml of deionized (DI) water and transferred into a dialysis tube with a membrane of 1 kDa mesh size. The dialysis tube was purchased from Sigma-Aldrich (CAS Number PURD10005–1KT). The dialysis was done in DI water at 4 °C. Several changes of water were made, at 1, 2, 4 h, ..., and overnight. The dialyses were conducted until no EPR signal could be detected in the DI water. The purified products were lyophilized and stored in a – 20 °C freezer.

A MALDI-TOF mass spectrometer (Voyager-DE™ STR Biospectrometry™ Workstation) was used to verify the masses of the spin-labeled HPLC6 mutants. The matrix was comprised of alpha-cyano-4-hydroxycinnamic acid, 3% TFA, acetonitrile, and DI water. The mass spectra of the original cysteine-substituted HPLC6 mutants and their spin-labeled versions are provided in the supplemental file.

EPR experiments and line-shape simulations

The EPR experiments were carried out using a Bruker X-Band CW EMX EPR Spectrometer. The following EPR parameters were used: center field: 3360.880 G; sweep width: 300.00 G; resolution: 1024 points; time constant: 0.640 ms; sweep time: 5.243 s; modulation frequency: 100.0 kHz; and modulation amplitude: 1.00 G. X-band frequencies from 9.445 to 9.460 GHz were used. The sample temperatures were controlled using an ER 4141VT-UM nitrogen variable-temperature system (77–500 K). The EPR temperature reading was corrected using a thermo-couple with iced water. The spin-labeled peptide solutions were made at concentration of 2.0 mg/ml using in-house DI water. The sample solutions were placed in capillaries and then sealed. The capillaries were immersed in hexane held in the EPR tubes. The samples were first run at room temperature, then at lower temperatures using heater-regulated cold nitrogen gas generated from a liquid-nitrogen tank. When the assigned temperature was reached (usually in 5–10 min, depending on the cooling conditions), the temperature was maintained for 5 more minutes. We repeatedly ran the EPR spectra at the same temperatures for longer time to ensure the EPR spectra did not change any further after the 5 min.

The rotational correlation time of the spin labels, along with the component line shapes, was calculated for each EPR spectrum. Theoretical simulations of the EPR spectra of the spin-labeled peptides were performed using the Multi-Component EPR Fitting program (version 742) and Labview software. This program was developed by Dr. Christian Altenbach (University of California, Los Angeles, CA) (Altenbach 2018; Couto and Nonato 2011; Takacs and Mot 2013).

Observations of ice crystals

Optical observations of ice crystals in the AFP solutions were carried out using a custom-built Otago nanoliter osmometer, a thermoelectric device with a temperature-controlled cooling stage, an Olympus BX 51 microscope (maximum magnification of 800 $\times$ with a resolution of 1 μ m), and a RETIGA 2000R Color Video Camera. The accuracy of the temperature reading is within ± 0.01 $^{\circ}$ C.

Results

The growth of ice crystals in the spin-labeled peptide solutions and their antifreeze activities

Water and wild-type type-I AFP solutions—In water at atmospheric pressure, ice crystals grow much more rapidly on prism facets than on basal facets, resulting in flat, hexagonal ice crystals (Petrenko and Whitworth 1999). Figure 2a shows our observation of such a phenomenon at 0 $^{\circ}$ C. Water is in thermal equilibrium with ice at 1 atm and 0 $^{\circ}$ C; thus, the freezing point of water and the melting point of ice are identical.

A seed ice crystal in an AFP solution can be initiated by freezing the solution at a very low temperature (~ -20 $^{\circ}$ C), followed by raising the temperature to near 0 $^{\circ}$ C to melt the bulk of the ice, then varying the temperature while keeping it below 0 $^{\circ}$ C. The seed ice crystal will grow to a confined shape at the lowered temperature, which is determined by the facets of the seed ice crystal to which the AFP bind. The seed ice crystals in the type-I AFP solutions grew into bipyramidal shapes at subzero temperatures because the AFP molecules were found to bind to the bipyramidal surfaces of seed ice crystals. The rest of the solutions stayed in super-cooled states due to the insignificant molar concentrations of the AFPs failing to cause any significant colligative effect. Further lowering of the temperature caused the ice crystal to burst, typically starting from its tips and spreading into the whole solution. An example of such a phenomenon is shown in Fig. 2b, which depicts a type-I AFP solution with a concentration of 6.0 mg/ml. The ability of the AFP to hold the bipyramidal shape at subzero temperature reflects its antifreeze activity. Antifreeze activity is defined as the temperature difference between the bursting point of the ice crystal during decrease in temperature and the thermodynamic melting point of the solution (~ 0 $^{\circ}$ C). The activity depends on the properties of the AFP, as well as its concentration in water. The antifreeze activity of the wild-type type-I AFP at different concentrations is represented by black squares in Fig. 3. Our results on the antifreeze activities are consistent with earlier published data by others (DeVries and Wohlschlag 1969; Duman and DeVries 1974, 1976). The corresponding theoretical curve was derived by mapping the data onto an exponential decay curve, based on our opinion that the binding of AFPs to ice surfaces follows the Langmuir theory of surface adsorption (Ba and Wongsakhaluang 2003; Mao and Ba 2006a, b; Ba and Mao 2013).

Spin-labeled HPLC6 mutant solutions—Figure 4 contains photo images of ice crystals in the spin-labeled HPLC6 mutant solutions during the growing process when the temperatures were lowered. The solutions contained: (a) spin-labeled L23C, (b) spin-labeled L12C, (c) spin-labeled A20C, (d) spin-labeled A11C, and (e) spin-labeled A17C. The

concentrations of all the spin-labeled peptides are 8.0 mg/ml. The results show that all the spin-labeled peptides, except the spin-labeled A17C, confined the ice crystals to a bipyramidal shape, and the ice crystals eventually burst into the solutions at lowered temperatures. The antifreeze activity of these peptides versus their concentrations is shown in Fig. 3. The orders of antifreeze activity are as follows: wild-type type-III AFP > spin-labeled L23C > spin-labeled L12C > spin-labeled A20C > spin-labeled A11C. These experimental results indicate that replacement of the L23 and the L12 side chains by MSL still allowed the peptides to retain tremendous antifreeze activity. The spin-labeled L23C has a slightly higher antifreeze activity than the spin-labeled L12C. The A20 residue, as shown in Fig. 1d, is at the front of the peptide, relative to the peptide's IBS. Although MSL in this position does not alter the peptide's IBS, it could contact the ice surface after the peptide bound to the ice surface, which might perturb the ice-binding strength to some extent. The A11 residue, as shown in Fig. 1e, is at the back of the peptide, parallel to the peptide's IBS. Although both the A20 and A11 residues are to the sides of the IBS, the A11 residue is on the same side as the salt bridge. Perturbations to the salt bridge could have a further effect on antifreeze activity.

The growth of ice crystals in the spin-labeled A17C solutions have similar patterns to those in water (i.e., the ice crystals grew faster on the prism facets than on the basal facets). Only at a higher concentration (8.0 mg/ml), the ice crystals' shapes tend to be more irregular than those in pure water, but a hexagonal shape could still be obtained by varying the heat transferred into and out of the solution. As shown in Fig. 1f, MSL was attached to the peptide's putative IBS, which, in turn, hindered the spin-labeled peptide's ability to bind to the ice surface. Thus, it is not surprising that the spin-labeled A17C lost ice binding ability.

VT EPR spectra of the spin-labeled HPLC6 mutants and their antifreeze activities

Solid powders—Figure 5 shows the experimental and simulated EPR spectra of the spin-labeled HPLC6-mutant solid powders (see the supplemental file for more VT EPR spectra). The spectra at lower temperatures (down to 98 K) do not show any significant changes, indicating that even at room temperature, the MSL groups had restricted rotational motions in the solid powders. The restrictions were caused by the tight packing of the spin-labeled peptides in the solid powders. The different patterns were caused by variations in the local structural conformations of the spin-label groups. Generally, a broader line shape has more overlapping lines, due to the broader distribution of local conformations as well as the orientations of the spin-labeled peptides in a solid powder. The correlation times (τ_c) of the spin-labeled L12C and L23C are between 3 and 4 ns, those of the spin-labeled A20C and A11C are between 5 and 6 ns, and that of the spin-labeled A17C is longer (13.3 ns), which indicates that the rotational motion of MSL in the spin-labeled A17C peptide was more restricted. This MSL is the only one that was attached to the ice-binding residue of the type-I AFP.

Spin-labeled L12C and L23C solutions—Figure 6a shows the experimental and simulated VT EPR spectra of the spin-labeled L12C dissolved in water under-going the cooling process (see the supplemental file for more experimental VT EPR spectra). At 287.0 K, the EPR spectral line shape and the corresponding correlation time $t_c = 0.244$ ns)

represent the spin-label groups undergoing fast rotational motion around the chemical bonds (see Scheme 1) and the fast tumbling of the spin-labeled peptides in water. The spectral line shape does not change significantly from room temperature to 251.2 K, at which point the correlation time increased to 0.564 ns, showing slightly slower chemical-bond rotations and molecular tumbling. The spectral pattern at 251.2 K still represents the motions in a solution, although the temperature is much lower than the freezing point of water (273.15 K). However, the EPR line shape changed dramatically at 250.9 K to that of a solid powder, showing restricted (slow) rotational motion, and the corresponding correlation time jumped to 36.5 ns. This dramatic change indicates that the solution was suddenly frozen at this temperature. The formed ice restricted the tumbling of the peptides, and the ice growing to the spin-label groups also hindered the chemical-bond rotations. The exact freezing point should be between 251.2 and 250.9 K (or -21.95 to -20.25 °C). The much lower freezing point than that of water demonstrates the inhibiting effect of the spin-labeled peptides on ice nucleation. Nucleation is the initial process in which a cluster of ions, atoms, or molecules become arranged in a pattern characteristic of a crystalline solid, upon which additional particles are deposited as the crystal grows. Once nucleation occurred in the super-cooled solution, the bulk solution was frozen instantly, although the spin-labeled peptides could inhibit the growth of the seed ice crystals until -0.3 °C (Fig. 3). We refer to this freezing point as the “nucleation-inhibiting freezing point (NIFP),” since freezing point is typically used in a system at thermodynamic equilibrium, whereas the spin-labeled peptide solution was in a super-cooled condition before being suddenly frozen. For comparison, we also tested the same water used to make the peptide solutions in the EPR capillaries, via visual observation. A similar procedure as used in the EPR experiments was used to decrease the temperature, and it was observed that this sample of water froze at 267.2 K (or -6.0 °C). Therefore, the lowered NIFP of the spin-labeled L12C peptide solution compared to the NIFP of water is $T_{\text{NIFPW}} = -16.0$ to -16.2 °C. This comparison demonstrates that the spin-labeled HPLC6 L12C peptides possess the function to inhibit ice nucleation. We also tested the NIFP using the wild-type type-I AFP at a concentration of 2.0 mg/ml, finding that it froze at 254.2 K (or -19.0 °C) and $T_{\text{NIFPW}} = -13.0$ °C, which is 3.0 °C higher than the spin-labeled L12C. The NIFPs and T_{NIFPW} for all the spin-labeled peptide solutions are listed in Table 1. When the temperature was decreased below 242.4 K, the line shape became a little sharper at the top of the line (although insignificant), which indicates that the water molecules became more rigid around the spin-label groups. At 98.2 K, the correlation time became 40.3 ns.

Figure 6b shows the VT EPR spectra of the spin-labeled L12C solution undergoing the heating process (see the supplemental file for more experimental VT EPR spectra). The line shape did not change significantly, until the temperature reached above 258.5 K. The line shapes developed additional features with further increase in temperature. The line shape simulation at 266.9 K shows that the spectrum contains two components: component 1 (72.42%) has a $\tau_c = 12.2$ ns and component 2 (27.58%) has a $\tau_c = 27.58$ ns. These components may represent different local minimum energy conformations among the spin-label groups after being released from the sudden frozen state. The decreased correlation times ($\tau_c = 2.13$ and 2.39 ns) at 269.1 K indicate that the water molecules surrounding the spin-label groups became more mobile, which allowed higher mobility in the chemical-bond

rotations. Although 269.1 and 270.1 K are below the melting point of ice, the EPR line shapes of the spin-labeled peptides did not show a solid powder pattern at these temperatures. If the binding of the spin-labeled peptides to the ice surfaces completely eliminated their tumbling motions, solid powder patterns would have been observable although the spin-label groups' local rotational motions around the chemical bonds of $C_{\alpha}-C_{\beta}$, $C_{\beta}-S_{\gamma}$, $S_{\gamma}-C$, and $N-C$ (see Scheme 1) should also have narrowed the spectral pattern to some extent. This increased mobility may indicate that the spin-label groups were surrounded by mobile water, and the peptides might have tumbled as well, even though the bulk solution was still in the frozen state at these temperatures. The depth of the water layer that the spin label could probe is about 9 Å, according to the model shown in Fig. 1b. At 273.3 K and higher, the EPR line shapes became those of the spin-labeled peptides undergoing fast tumbling in water and fast chemical-bond rotations.

Figure 7a shows the experimental and simulated VT EPR spectra of the spin-labeled L23C dissolved in water undergoing the cooling process (see the supplemental file for more experimental VT EPR spectra). The corresponding correlation times are provided in the legends. At temperatures ranging from 278.0 to 252.2 K, the EPR spectral line shapes represent the mode of fast local chemical-bond rotations and fast molecular tumbling in water. The line shape changed dramatically, to restricted motion in a solid powder, at 251.6 K ($\tau_c = 41.9$ ns). This indicates that the bulk solution was suddenly frozen at this temperature; thus, the NIFP is between 252.0 and 251.6 K (or -21.2 to -21.6 °C) and $T_{NIFPW} = -15.2$ to -15.6 °C (see Table 1). The line shape changed to a more rigid pattern ($\tau_c = 64.33$ ns) when the temperature was decreased to 98.2 K.

Figure 9b shows the experimental and simulated VT EPR spectra of the spin-labeled L23C solution undergoing the heating process (see the supplemental file for more experimental VT EPR spectra). The line shape did not change significantly until the temperature reached above 239.4 K. In spectra of the solution below 272.2 K, the line shape contains two components with different correlation times. Between 269.1 and 270.1 K, the EPR line shape began to show evidence of slow molecular tumbling. Above 273.3 K, the line shape revealed fast molecular tumbling and fast chemical-bond rotations in water.

Similar experimental phenomena were observed for the spin-labeled L12C and the spin-labeled L23C solutions. We know that the L12 and L23 residues are at the identical positions in the first and second repeated units of the HPLC6 peptides, respectively. Therefore, it is reasonable to find that they share similar phenomena.

Spin-labeled A20C and A11C solutions—Figure 8a shows the experimental and simulated VT EPR spectra of the spin-labeled A20C dissolved in water undergoing the freezing process (see the supplemental file for more experimental VT EPR spectra). At temperatures ranging from 278.0 to 252.7 K, the EPR spectral line shapes indicate fast chemical-bond rotations and fast molecular tumbling. The line shape changed dramatically at 252.3 K, to moderately restricted rotational motion ($t_c = 5.6$ ns). This indicates that the bulk solution was suddenly frozen at this temperature; thus, the NIFP should be between 252.7 and 252.3 K (or -20.4 and -20.8 °C) and $T_{NIFPW} = -14.4$ to -14.8 °C (see Table 1). The line shape continuously changed to the modes of more restricted rotational motions

as the temperature decreased. The simulated spectra show that two components evolved with the decrease in temperature. At 175.6 K, for example, one component (79.38%) has a $\tau_c = 30.7$ ns, while the other (20.62%) has a $\tau_c = 2.51$ ns. At 98.2 K, $\tau_c = 142.0$ ns.

Differing from the L12C and L23C spin-label groups, which were frozen quite severely when the solutions reached their NIFPs, the A20C spin-label groups showed much higher mobility after the bulk solution was suddenly frozen from a super-cooled state. As shown in Fig. 1d, the A20 side chain is in the front of the peptide while the L12 and L23 side chains are on top of the peptide. It was obvious that solid water molecules grew directly to the top of the HPLC6 peptides upon reaching the NIFP, which severely hindered the motions of the L12C and L23C spin-label groups. However, water near the A20 side chain still had high mobility, even below the NIFP. The spin-labeled A20C peptides also differed from the spin-labeled L12C and L23C peptides in that the EPR line shapes of the A20C show finer features at low temperatures, which gradually broadened as the temperature decreased. We believe that this was a result of the slower freezing process of the surrounding water, which allowed time for the spin-label groups to adjust their conformations so they could achieve lower local Gibbs energy states. Thus, the conformational distributions of the A20C spin-label groups were narrower than those of the spin-labeled L12C and L23C. At 98.2 K, the correlation time of the A20C spin-label groups is longer (142.0 ns) than the correlation times of the L12C (40.3 ns) and L23C (64.33 ns) spin-label groups, which indicates that the A20C spin-label group was more restricted in movement at this temperature, probably because the spin-label group attached to the A20C side chain was frozen to the rigid ice surface, while water molecules around the L12C and the L23C were not as rigid.

Figure 8b shows the experimental and simulated VT EPR spectra of the spin-labeled A20C solution undergoing the heating process (see the supplemental file for more experimental VT EPR spectra). The line shape did not change significantly until the temperature reached above 157.9 K, becoming narrower as the temperature increased and showing greater freedom of rotational motion. Above 269.1 K, most of the spin-labeled peptides were undergoing fast tumbling in water. The heating process also revealed that the spin-label groups attached to the A20C peptides were more mobile than those of the spin-labeled HPLC6 L23C and L12C.

Figure 9a shows the VT EPR spectra of the spin-labeled A11C dissolved in water undergoing the freezing process (see the supplemental file for more experimental VT EPR spectra). At temperatures ranging from 278.0 to 253.1 K, the EPR spectral line shapes indicate fast chemical-bond rotations and fast tumbling of the spin-labeled peptides in water. At 253.0 K, the line shape changed dramatically to moderately restricted motion. This indicates that the bulk solution was frozen at this temperature; thus, the NIFP should be between 253.1 and 253.0 K (or -20.0 to -20.2 °C) and $T_{\text{NIFPW}} = -14.0$ to -14.2 °C (see Table 1). As the temperature decreased, the line shape continued to change to the mode of more restricted motions. Like in the spin-labeled A20C peptide solution, the water molecules around the spin-label groups attached to the A11C side chains were also more mobile than the water molecules around the L12C and L23C spin-label groups. At 98.2 K, $\tau_c = 37.5$ ns, which is shorter than that of the spin-labeled A20C sample. Although both the A20 and A11 side chains are at the sides of the α -helical structure, parallel to the IBS of the

HPLC6 peptide, the A11 side chain is on the same side as the salt bridge, which might make the water molecules there more mobile in the frozen state.

Figure 9b shows the VT EPR spectra of the spin-labeled A11C peptides undergoing the heating process. The line shape gradually changed to the mode of less restricted rotational motions as the temperature increased. At 266.9 K, the line shape contains two components with different correlation times ($\tau_c = 21.30$ and 1.43 ns). At 268.0 K, the correlation times of the two components became shorter ($\tau_c = 8.31$ and 1.07 ns). The lineshape continuously changed to the mode of less restricted rotational motions with the increase in temperature, and eventually became the lineshape of fast tumbling in water above 269.1 K.

Spin-labeled HLPC6 A17C solution—Figure 10a shows the experimental and corresponding simulated VT EPR spectra of the spin-labeled A17C water solution undergoing the freezing process (see the supplemental file for more experimental VT EPR spectra). At temperatures ranging from 287.0 to 264.8 K, the EPR spectral line shape indicates weakly to moderately hindered chemical-bond rotations and fast molecular tumbling (not a solid-powder pattern). One spectral component was obtained at 287.0 K, with $\tau_c = 4.23$ ns. At lower temperatures, two components could be distinguished (component 1: 33.13%, $\tau_c = 4.92$ ns; component 2: 66.87%, $\tau_c = 5.20$ ns at 265.6 K). Compared with the other spin-labeled peptides, the correlation times of the spin-labeled A17C at temperatures above the NIFP are longer, which indicates that the rotational motion of the spin-label groups are more restricted. The underlying reason is still under investigation. As the temperature decreased, the line shape gradually changed to the mode of more restricted motions. At 264.7 K, the EPR line shape changed dramatically, becoming moderately to severely restricted rotational motion within solid powder; thus, the NIFP should be between 264.8 and 264.7 K (or -8.4 to -8.5 °C) and $T_{\text{NIFPW}} = -2.4$ to -2.5 °C (see Table 1). The NIFP of this peptide is much higher than those of the other spin-labeled HPLC6 peptides. This result is significant in recognizing that the spin-label group attached to the IBS deactivated the antifreeze function, in terms of inhibiting ice nucleation. As observed above, the spin-labeled peptide also lost its ice-binding property. Since the spin-labeled A17C has no ice-binding property, we believe that, at the NIFP, the sudden growth of ice crystals in the bulk solution crushed the spin-labeled peptides together; thus, the restricted motions were primarily due to the hindrance by other peptides, instead of the ice.

Figure 10b shows the experimental and simulated VT EPR spectra of the spin-labeled A17C solution undergoing the heating process (see the supplemental file for more experimental VT EPR spectra). As the temperature increased, the line shape indicated the mode of decreased restrictions in rotational motion. Unlike the other spin-labeled peptides, moderately restricted rotational motion still existed at temperatures higher than 273.3 K, showing that the aggregated spin-labeled peptides had not yet fully dissolved in water, even though the ice had already melted. This melting phenomenon further proves our hypothesis that the spin-labeled A17C peptides were crushed together upon the freezing of the water solution (264.7 K).

Discussion

In this study, we conducted two sets of experiments to observe the effects of the spin-label groups attached to different HPLC6 mutants on the growth of ice crystals, and to probe the states of water surrounding the spin-label groups by analyzing the VT EPR spectral line shapes during the cooling and heating procedures. The first set of experiments revealed antifreeze activities at the microscopic level, while the second set of experiments explored the water environments adjacent to the spin-labeled peptide side chains at the molecular level.

IBSs of type-I AFPs

We have observed that spin labeling on the A17 side chain resulted in the loss of antifreeze activity; however, the other spin-labeled peptides including L12C, L23C, A20C, and A11C retained their antifreeze abilities to a great extent. Thus, we conclude that the putative IBSs of type-I AFPs indeed comprise the Thr side chains and the conserved i + 4 and i + 8 Ala side chains (Chao 1994; DeLuca and Chao 1996; Deluca and Davies 1998; Graether and DeLuca 1999).

Inhibiting the nucleation of seed ice crystals

The VT EPR results indicate that water solutions of the spin-labeled HPLC6-mutants including L12C, L23C, A20C, and A11C contained in the EPR capillaries did not freeze when the temperatures were lowered much below the NIFP of DI water (~ -20 to -22 °C). Obviously, the solutions stayed in super-cooled states above their NIFPs. However, the inactive spin-labeled A17C peptides could only keep their super-cooled solution at temperatures above -8.5 °C. Therefore, we conclude that AFPs can inhibit the nucleation of seed ice crystals in aqueous solutions, which allow the AFP solutions to stay in super-cooled states at much lower temperatures than the bursting points of the bipyramidal ice crystals. We believe that the ice-nucleation inhibiting effect of AFPs plays a more significant role in preventing freezing damage in organisms living in subzero temperatures. In summary, AFPs have two functional roles: (1) inhibiting the nucleation of seed ice crystals and (2) hindering the growth of ice crystals. Conservation of the AFP's IBS is necessary for both of the functions.

Nucleation processes are classified as heterogeneous or homogeneous. The heterogeneous nucleation process occurs on the surface of a different substance, such as a dust particle or a scratch on the wall of a glass container, which acts as the center upon which the first few atoms, ions, or molecules of the crystal are properly arranged. In the homogeneous nucleation process, a few particles happen to be juxtaposed to the crystal in the correct arrangement, upon which larger crystals can grow. The former process allows ice crystals to grow at subzero temperatures close to 0 °C. In the latter process, however, water may stay in a super-cooled state even at very low subzero temperatures. In the present study, the phenomena we observed in the freezing of the spin-labeled HPLC6-mutant solutions were caused by the homogeneous nucleation processes. AFPs may be able to interrupt the nucleation process or interact with the nucleated cluster of water molecules that resemble the early structure of seed ice crystals. The IBSs of the AFPs played a crucial role in inhibiting

ice nucleation. Only at very low temperatures, at which the chemical potential of water is much higher than that of ice, would the APFs fail to prevent the nucleation of seed ice crystals. The exact mechanism of APFs in preventing the nucleation of seed ice crystals should be explored in further investigations.

States of water molecules surrounding the spin-labeled side chains at various temperatures

The VT EPR spectra of the spin-labeled L12C and L23C show that the rotational motions of the spin-label groups were severely restricted at the NIFP. We believe that this was caused by the immediate growth of ice to the spin-label groups. For the spin-labeled A20C and A11C, however, the VT EPR spectra show that the rotational motions of the spin-label groups were less restricted at the solutions' NIFPs. This indicates that the water molecules existing near the A20C and A11C spin-label groups were less rigid. Therefore, we propose that when temperatures are lowered below the NIFPs of the spin-labeled HPLC6 peptides, a layer of the spin-labeled peptides will bind to the ice surface and an ice sheet will grow to the top of the peptide layer. However, water molecules at the sides of the peptides between the two ice sheets remain more mobile, becoming solid only at much lower temperatures. The depth of water that can be detected by the spin-label is about 9 Å, although the actual depth could be much larger.

When the solutions were raised to temperatures above 98.2 K, especially to those higher than the NIFPs, the EPR spectral line shapes indicate that all the spin-label groups became more mobile, which increased as the temperature rose. These phenomena demonstrate that water molecules surrounding the non-ice-binding side chains will become more mobile at higher temperatures. The results of our previous ¹³C relaxation NMR experiments conducted on frozen solutions made of site-directed ¹³C-labeled Ala and Thr side chains in the HPLC6 peptides revealed that the water molecules around the non-ice-binding side chains were mobile, but the ice-binding side chains interacted with rigid water (Mao and Ba 2006a, b; Mao and Lin 2009). The detection distances in our NMR experiments were restricted to the lengths of the corresponding side chains in the wild-type type-I AFPs, while the EPR results in the present study allowed us to probe longer distances. Because the ¹³C-labeled Ala and Thr side chains did not alter the side-chain structures, we were able to detect the solid ice surfaces bound by the peptides in the NMR experiments.

During the freezing process, the changes in mobility among water molecules surrounding the spin labels show that the non-ice-binding side chains interrupted the water molecules joining the ice lattice. During the thawing process, the changes in mobility indicate that the HPLC6 peptides could liquefy the nearby ice, even at temperatures much lower than 0 °C. Mobile components were observed in the EPR line shapes for all the active spin-labeled HPLC6 mutants at temperatures above 269 K and below 273.15 K (or – 4.15 to 0 °C) during the heating processes. This phenomenon shows that the ice matrices of the spin-labeled peptide solutions began to melt locally around the non-ice-binding side chains, and the spin-labeled peptides began to tumble when the temperatures were raised to near 0 °C.

The results of our previous micro-imaging NMR experiments show that frozen type-I AFP solutions at concentrations of 1.0–5.0 mg/ml contained liquid water at temperatures below

– 1.0 °C (Ba and Mao 2013). The EPR observation in the present study is consistent with the previous NMR micro-imaging results. A study involving molecular dynamics simulation revealed that the adsorption of type-I AFP can induce melting in the local ice surface (Calvaresi and Höfinger 2012). In an earlier NMR experiment of ours, we observed adsorption and desorption phenomenon from the type-I AFP molecules to ice surfaces at subzero temperatures, which indicates that the AFP molecules are dynamically bound to the ice surfaces (Ba and Wongsakhaluang 2003).

Figure 11 schematically describes the phenomena observed in the present investigation, as well as our understanding of the behaviors of the HPLC6 peptides and their surrounding water molecules during the cooling and heating processes.

Antifreeze mechanisms

The EPR results of this study indicate that AFP molecules formed an AFP–water layer between two ice sheets at very low temperatures, and the AFP concentration in the layer could be very high due to the fact that liquid water existed provided that colligative effect governs the phenomenon. Such a layer also exists between ice and water (when the top ice sheet does not exist at a much higher temperature). We refer to this AFP–water layer as water–AFP–ice (WAI) interphase. We propose the following model to explain the antifreeze mechanisms: (1) A WAI interphase forms after AFP molecules bind to an ice surface. The driving forces for the binding are primarily due to van der Waals interactions between the involved hydrophobic side chains of the AFP molecules and the water molecules in the ice surface, as well as hydrogen bonding and dipole–dipole interactions between the hydrophilic side chains in the non-ice-binding areas of the AFP molecules and liquid water molecules. Such intermolecular interactions are intrinsically reversible and dynamic. (2) The WAI interphase reduces the ice–water interfacial tension. (3) The thickness of the WAI interphase for the type-I AFP could be larger than 20 Å, which is within the range that can be probed by the spin labels at temperatures ranging between ~ 266 and 269 K. The actual thickness could be larger, especially at temperatures ranging between 269 and 273 K after considering the dynamic effect of the AFP molecules in the WAI interfacial layer. (4) The concentration of AFP in the WAI interphase is much higher than that in the bulk solution, which enhances the local colligative effect on the freezing-point depression. (5) The irregular spatial arrangement and dynamics of the non-ice-binding side chains interrupt the nearby water molecules joining the ice lattice. (6) When heat is transferred to the WAI interfacial layer, ice below the bound AFP molecules will be transformed to a liquid-like layer. The AFP molecules will diffuse to the newly generated ice surface, which will cause further melting of the ice surface. Through this process, AFP molecules can induce melting at subzero temperatures provided that heat is continuously transferred to the system, while super-cooled liquid water is generated behind the newly generated WAI interfacial layer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Scheme 1.

Structure of the MSL spin label and the resulting spin label on the cysteine side chain of a protein

Fig. 1. Molecular models of **a** wild-type type-I HPLC6 (side view with ice surface at the bottom), **b** MSL spin-labeled L12C (side view with ice surface at the bottom), **c** MSL spin-labeled L23C (side view with ice surface at the bottom), **d** MSL spin-labeled A20C (top view with ice surface at the bottom), **e** MSL spin-labeled A11C (top view with ice surface at the bottom), and **f** MSL spin-labeled A17C (side view with ice surface at the bottom)

Fig. 2.

a Photo images of an ice crystal growing in water at 0 °C: (1) seed ice, (2) the ice crystal growing on the prism facets, (3) the ice crystal has formed a hexagonal shape, (4) the ice crystal further growing from the edges of the hexagon, (5) the ice crystal has formed a star-like shape, and (6) the ice crystal burst into the water from a tip of its star-like shape; **b** photo images of an ice crystal confined by the wild-type type-I AFP at a concentration of 6.0 mg/ml, undergoing decreases in temperature from -0.09 , to -0.64 and to -0.65 °C, respectively, from the left to right (the darker circles in the photo images represent air bubbles in the water and the solution)

Fig. 3. Antifreeze activity of the wild-type type-I AFP (black squares), spin-labeled L23C (red discs), spin-labeled L12C (blue, upward-pointing triangles), spin-labeled A20C (purple, downward-pointing triangles), spin-labeled A11C (green diamonds), and spin-labeled A17C (navy blue, leftward-pointing triangles)

Fig. 4.

Photo images of ice crystals confined by **a** MSL-labeled L23C at a concentration of 8.0 mg/ml at -0.15 , -0.63 , and -0.72 °C, respectively, from left to right; **b** MSL-labeled L12C at a concentration of 8.0 mg/ml at -0.08 , -0.34 , and -0.52 °C, respectively, from left to right; **c** MSL-labeled A20C at a concentration of 8.0 mg/ml at -0.10 , -0.65 , and -0.74 °C, respectively, from left to right; **d** MSL-labeled A11C at a concentration of 8.0 mg/ml at -0.05 , -0.23 , and -38 °C, respectively, from left to right; and **e** MSL-labeled A17C at a concentration of 8.0 mg/ml at -0.01 , -0.02 , -0.02 °C, respectively, from left to right (top), and -0.02 , -0.01 , and -0.02 °C, respectively, from left to right (down) (the differences in hue were caused by the filters used when the images were taken)

Fig. 5.
Experimental (black) and simulated (red) EPR spectra of the MSL spin-labeled HPLC6-mutant solid powders at room temperature

Fig. 6. Experimental (black) and simulated (red) VT EPR spectra of the spin-labeled L12C solution (2.0 mg/ml) undergoing **a** the cooling process and **b** the heating process. The temperatures and the correlation times are given in the corresponding legends

Fig. 7. Experimental (black) and simulated (red) VT EPR spectra of the spin-labeled L23C water solution (2.0 mg/ml) undergoing, **a** the cooling process and **b** the heating process. The temperatures and correlation times are provided in the corresponding legends

Fig. 8. Experimental and simulated VT EPR spectra of the spin-labeled A20C water solution undergoing, **a** the cooling process and **b** the heating process. The temperatures and correlation times are given in the corresponding legends

Fig. 9. Experimental and simulated VT EPR spectra of the spin-labeled HPLC6 A11C water solution (2.0 mg/ml) undergoing, **a** the cooling process and **b** the heating process. The temperatures and correlation times are given in the corresponding legends

Fig. 10.

Experimental and simulated VT EPR spectra of the spin-labeled A17C solution (2.0 mg/ml) undergoing, **a** the cooling process and **b** the heating process. The temperatures and correlation times are provided in the corresponding legends

Fig. 11.

Cartoon presentations of the states of water and AFP molecules in the type-I AFP solution at different temperature ranges during the cooling (down) and heating (up) process. The gray color represents hexagonal ice, the blue color represents liquid water, and the orange color represents AFP molecules

Table 1

Nucleation-inhibiting freezing points of the spin-labeled HPLC6 mutants in water (2.0 mg/ml)

	Nucleation-inhibiting freezing point (NIFP) (°C)	Change in NIFP compared to the NIFP of water, – 6.0 °C (°C)
Spin-labeled HPLC6 L12C	– 22.0 to – 22.2	$T_{\text{NIFPW}} = -16.0 \text{ to } -16.2$
Spin-labeled HPLC6 L23C	– 21.2 to – 21.6	$T_{\text{NIFPW}} = -15.2 \text{ to } -15.6$
Spin-labeled HPLC6 A20C	– 20.4 to – 20.8	$T_{\text{NIFPW}} = -14.4 \text{ to } -14.8$
Spin-labeled HPLC6 A11C	– 20.0 to – 20.2	$T_{\text{NIFPW}} = -14.0 \text{ to } -14.2$
Spin-labeled HPLC6 A17C	– 8.4 to – 8.5	$T_{\text{NIFPW}} = -2.4 \text{ to } -2.5$
DI water	– 6.0	$T_{\text{NIFPW}} = -0.0$
Wild-type HPLC6 peptides	– 19.0	$T_{\text{NIFPW}} = -13.0$