Aggregation of Antifreeze Protein and Impact on Antifreeze Activity

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Antifreeze protein type III aggregates once the concentration exceeds a critical value, the so-called critical aggregation concentration (CAC). It was found for the first time that the aggregation of antifreeze protein exerts a direct impact on the antifreeze efficiency. It follows from our measurements that the AFP III above CAC will enhance the antifreeze activity because of the increase of the kink kinetics barrier of surface integration. This is attributed to the optimal packing of AFP III molecules on the surface of the ice nucleus as well as ice crystals above CAC. This study will extend our understanding of the antifreeze mechanism of antifreeze protein monomers as well as antifreeze aggregates on ice nucleation and shed light on the selection of antifreeze agents.

Introduction

Many fish, plants, insects, and other organisms have involved unique adsorptive mechanisms that allow them to survive in harsh environments at the extremes of temperature.[1−4] Antifreeze proteins (AFPs), known as thermal hysteresis proteins,[5] can prevent or reduce the damage caused by freezing to living organism by lowering the freezing point of their blood and other internal fluids. These properties have attracted significant interest for their potential applications in medicine and industry where low-temperature storage is required and ice crystallization should be avoided.[6] In addition, the applications include improved protection of blood platelets and human organs at low temperatures,[7] increasing the effectiveness of the destruction of malignant tumors in cryosurgery,[8] and improvement of the smooth texture of frozen foods.[9]

One of the most accepted descriptions for the antifreeze mechanism by which AFPs lower the freezing point of water is the adsorption-inhibition mechanism.[10] According to this mechanism, the freezing point depressing activity depends on the ability of AFPs to adsorb onto the ice crystal surface. Early models of AFP−ice interaction suggested the H-bond as the primary driving force behind such interactions.[10−12] Recent experimental evidence, however, suggested that hydrophobic interactions could be the main contributor to AFP−ice binding.[13−15] Even with the acquisition of several AFP three-dimensional structures and the definition of their ice-binding sites by mutagenesis, the effect of the adsorbed AFPs on the kinetics of ice formation remains a mystery.[16] Recently, a new model has been put forward to explain the antifreeze mechanism of AFPs on ice crystallization.[17] Ice crystallization can be regarded as two major stages: nucleation and growth. Nucleation is associated with the creation of new ice crystallites whereas growth refers to the increase in the volume of the existing ice crystals. In the process of freezing, the nucleation of ice plays a very crucial role as it determines the initiation of freezing (or the freezing temperature). Even at the stage of ice growth, primary and secondary ice nucleation still allow the number of ice crystallites to increase. Therefore, the control of ice nucleation is one of the key challenges for antifreeze action.

Our recent studies[18] show that AFPs can inhibit the ice nucleation process by adsorbing onto both the surface of ice and that of foreign particles. In other words, the antifreeze activity is related to the surface activity of antifreeze proteins, which determines the adsorption behavior of antifreeze proteins. It should be noticed that the adsorption of AFP molecules on the surface of ice crystals exerts a similar effect on both nucleation and growth kinetics. In this sense, the impact of AFPs on the growth kinetics of ice can also be understood by examining the kinetics of nucleation.

Many proteins have the tendency of self-aggregation/assembly due to their amphiphilicity. They often change their biofunctionality after aggregation or assembly. It would be very desirable to examine the impact on the antifreeze efficiency of AFP at and above the aggregation or assembly concentration of AFP. In this paper, we will examine the effect of the antifreeze protein aggregation in water on ice nucleation kinetics based on a newly developed model.[17,18] This model allows us to quantitatively analyze the correlation between the antifreeze effect and the adsorption of the antifreeze protein on ice nuclei as well as that of foreign particles. As the surface activity of amphiphilic molecules is related to the aggregation in solution, we thus examine the aggregation of AFPs in terms of surface tension.

Experimental Methods

Surface Tension Measurement. The surface tension measurements were performed for all 10 AFP III solutions with different concentrations, using a Single Fibre Tensiometer (Model K14, Kruss). All AFP III solutions were freshly prepared with AFP III powder sample and were filtered by a 20 nm filter before the surface tension measurement. In these measurements, the Dynamic Wilhelmy method, which is a universal method especially suited to check surface tension over long time intervals, is applied. This method uses a vertical plate of known

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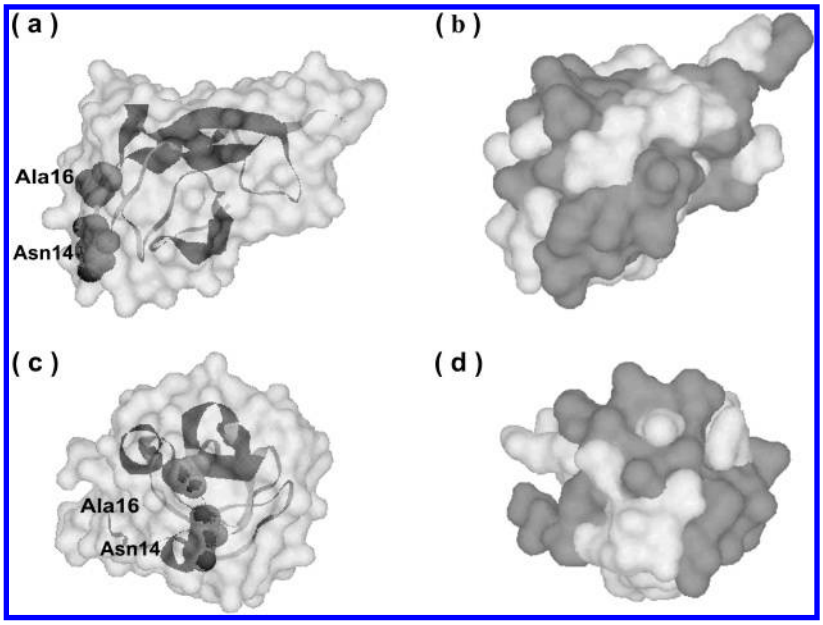

Figure 1. (a) Structure of AFP III. The ice binding face is on the left. Ala 16 is in the center of the ice binding site, Asn 14 is on the edge. (b) Molecular surface in the same orientation as structure a and (c) rotated 90° to show binding face. (d) Molecular surface in the same orientation as part c. In structures b and d, the hydrophobic residue surface is dark gray and the hydrophilic residue surface is white.

perimeter that is attached to a balance to detect the force change due to the wetting when the plate touches the solution surface. Then the surface tension can be calculated by the detected forces. All our measurements last 3 h for AFP III solutions to reach their equilibrium on the surface as well as in the bulk solution.

Ice Nucleation Kinetics Measurement. In our experiments, AFP III (A/F Protein Canada) was used to examine the antifreeze effect. This 6.5 kDa protein has a compact, angular structure in which the overall fold comprises numerous short β-strands and one turn of α-helix.[19] To measure the induction time of ice nucleation, the so-called double oil layer microsized crystallization technique[17] was employed. This technique can minimize the influence of the container and dust particles on ice nucleation, and also allows us to examine the effect of antifreeze proteins on ice nucleation kinetics quantitatively. For a better understanding of the ice nucleation kinetics for AFP III monomers and aggregates, we measured 10 concentrations of a protein solution from 0.5 to 5 mg/mL.

The experiments of microsized ice crystallization were performed in a microsized water droplet, which was suspended between two layers of immiscible oil in a circular quartz cell. First, the lower oil layer (Silicon Oil AR 1000 from Fluka), which has a higher density than water, was injected into the quartz cell up to one-half of its volume. Second, a drop of pure water or AFP III solution was carefully injected onto the surface of the oil with a microsyringe. Third, an oil (200/500cS Fluid from Dow Corning) with a lower density than water was injected to fill the cell, so as to cover the water droplet and the lower oil layer. A glass cover slip was then placed on top of the cell to prevent evaporation. Due to the density difference, the water droplet is suspended between the two layers of immiscible oils. Notice that our measurements show the melting point of the water droplet will not be influenced by their volume on the curvature within our experimental conditions, which turns out to be the same as bulk water (0 °C).

To minimize the effect of dust particles, before the water and oils were injected into the cell, they were filtered twice with 20 nm filters to remove big particles. The water used in the experiments was in a highly pure deionized form (18.2 MΩ). The ice crystallization was controlled by a Linkam THMS 600 Heating and Freezing stage, which is capable of controlling the temperature where the cell was mounted within 0.1 °C with the range −192 to + 600 °C. Nucleation was observed by using a polarized transmission microscope (Olympus, BX60-F) to which a 3 CCD color video camera (Panasonic, KY-F55BE) with an S-VHS video recorder (Panasonic AG-MD830) was attached. Any ice crystal occurring in the drop could immediately be detected by a polarized microscope.

Theoretical Basis

Surface-active agents are amphiphilic molecules, normally consisting of separate hydrophobic and hydrophilic portions in a molecule. Since proteins are composed of both hydrophobic and hydrophilic amino acids, most of them have an amphiphilic nature.[20] By making a comparison of the overall hydrophobicity of AFP III (fish antifreeze proteins type III, see Figure 1) with other proteins of similar size, it is found AFP III has a 62% apolar surface.[21] This proportion is very high, and comparable to one of the most hydrophobic soluble globular proteins known, Crambin, which has a 64% apolar surface. The binding face is even more hydrophobic, but does contain some polar regions (Figure 1d). Since the entropic penalty for hydrating nonpolar molecules is high, the aqueous solution will seek ways to minimize contact with the hydrophobic groups on the protein surface. As a consequence, the protein will behave as an amphiphilic molecule and exhibit surface activity by adsorbing at the hydrophobic/hydrophilic interface, such as an air/water interface (Figure 2a).

According to the Gibbs equation[22]

$$\Gamma_{\text{add}} = -\frac{1}{RT}\frac{\mathrm{d}\gamma}{\mathrm{d}\ln a_{\text{add}}} \tag{1}$$

(R is the gas constant; a_{add} is the activity of AFP in the aqueous solutions, $a_{\text{add}} = C_{\text{protein}}$ for dilute protein solutions; and Γ_{add} is the surface excess of AFP in the chosen dividing surface), the adsorption of the AFP on the surface of the water will give rise to a decrease in the surface tension. Similarly to normal amphiphilic molecules, if the AFPs start to aggregate, a change

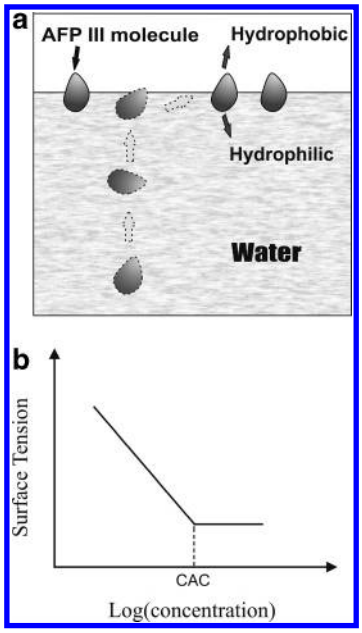

Figure 2. (a) Illustration of AFP III adsorption at the air−water interface. (b) Use of Gibbs equation to determine surface adsorption from the variation of surface tension with concentration. The accumulation of AFP III on the surface leads to the lowering of the surface tension γ. When the surface is fully occupied (saturated) by AFP III molecules, γ will then reach its minimum. The minimal point of the curve is defined as the critical aggregation concentration (CAC) of AFP.

in surface tension $\gamma - \ln c$ should be observed and it allows one to determine the critical aggregation concentration (CAC) (cf. eq 1 and Figure 2b). Similar analysis is used in the study of the correlation between protein aggregation and surface tension in refs 23−25.

As mentioned, freezing is an ice crystallization process. The formation of a new crystalline phase from the ambient phase proceeds via nucleation followed by growth.[26] This implies that in the case of ice crystallization, nucleation is the initial and one of the most important steps toward creating ice. Without this step, ice will never occur in supercooled water. In the following discussion, we will examine ice nucleation based on the model published in refs 17 and 18.

For ice crystallization, a positive thermodynamic driving force is required, which is defined as[27]

$$\frac{\Delta\mu}{kT} = \frac{\mu^{f} - \mu^{s}}{kT} = \frac{(\Delta H_m/T_m)\Delta T}{kT} \tag{2}$$

where μ^f and μ^s are the chemical potential of solute molecules in the fluid phase and in the solid phase, respectively; ΔH_m denotes the enthalpy of melting per molecule and T_m the melting temperature; ΔT is supercooling ($\Delta T = T_m - T$, T is the actual temperature); and k is the Boltzmann constant.

The nucleation process can be regarded as a kinetic process for ice nuclei to overcome a kinetic barrier, the so-called nucleation barrier, under a given thermodynamic driving force $\Delta\mu/kT$.[27] Taking into account the effect of foreign particles on nucleation,[18] the nucleation rate of ice, which is defined as the number of nuclei generated per unit of time−volume, is given according to the model as[17,18,28−31]

$$J = 4\pi a\beta_{knk}[(R^s)^2N^0]\, f''(m)[f(m)]^{1/2}B \exp[-\kappa f(m)/(T(\Delta T)^2)] \tag{3}$$

with

$$\kappa = 16\pi\gamma_{cf}{}^3\Omega^2/3kS_m{}^2 \tag{4}$$

$$B = 14\pi a^2\Omega\left(\frac{\gamma_{cf}}{kT}\right)^{1/2} \tag{5}$$

$$r_c = 2\Omega\gamma_{cf}/\Delta T\Delta S_m \tag{6}$$

where γ_{cf} denotes the specific interfacial free energy between the crystals and the mother phase, Ω is the volume of the growth units, S_m is the entropy of melting per molecule, a is the dimension of a growth unit, r_c is the radius of a critical nucleus, m is a function of the interfacial free energy difference between the different phases

$$m = (\gamma_{sf} - \gamma_{sc})/\gamma_{cf} \tag{7}$$

(γ is the interfacial free energy, subscripts f, c, and s denote the fluid phase, the cluster of the crystalline phase, and the foreign body, respectively), and

$$f(m) = 1/4(2 - 3m + m^3) \tag{8}$$

$$f''(m) = 1/2(1 - m) \tag{9}$$

Notice that $f(m)$ is a factor describing the lowering of the nucleation barrier ΔG^* due to the occurrence of foreign bodies (or substrate).

$$f(m) = \Delta G^*/\Delta G^*_{homo} \tag{10}$$

In the equations, ΔG^* is the actual nucleation barrier and ΔG^*_{homo} is the homogeneous nucleation barrier, and m ($-1 \le m \le 1$) can be approximated by cos θ (θ is the contact angle between the nucleating phase and the substrate[28,29]). Both $f(m)$ and $f''(m)$ change from 0 to 1, depending on the correlation and the structure match between the nucleating phase (ice) and the substrate (foreign bodies).[30,31] When the interaction between the nucleating phase and the substrate is optimal, one has $m \to 1$ and $f(m) \to 0$ (cf. eq 8). On the other hand, if the interaction between the nucleating phase and the substrate is very poor, one has $m \to -1$ and $f(m) \to 1$ (cf. eqs 8 and 10), meaning that the substrate exerts almost no influence on nucleation. Under such conditions, the nucleation of ice will become very difficult.

As demonstrated by our experiments,[18] we cannot completely eliminate the influence of foreign bodies, such as dust particles, which will in most cases promote ice nucleation. Therefore, to inhibit ice nucleation any antifreeze agent should be able to disrupt the interaction between ice nuclei and foreign bodies.

Apart from overcoming the nucleation barrier, the nucleation of ice is also affected by the incorporation of H_2O molecules onto the surface of the ice nuclei. The rate of kink kinetics is described by β_{kink}. β_{kink} is associated with $\Delta G^{\ddagger}_{kink}$, the energy barrier to be overcome to remove other adsorbed molecules, and is given by

$$\beta_{kink} \approx \exp(-\Delta G^{\ddagger}_{kink}/kT) \tag{11}$$

where $\Delta G^{\ddagger\prime}_{kink}$ denotes the kink kinetics barrier attributed to the adsorption of impurities/additives on the surface. Obviously, the adsorption of additives on the surface of ice will enhance $\Delta G^{\ddagger}_{kink}$ by $\Delta(\Delta G^{\ddagger}_{kink}) = \Delta G^{\ddagger\prime}_{kink} - \Delta G^{\ddagger}_{kink}$ (see Figure 3). Consequently, the integration of H_2O units into ice crystals will be significantly slowed or even terminated due to a very low β_{kink} (or high $\Delta G^{\ddagger}_{kink}$) (cf. eq 11).

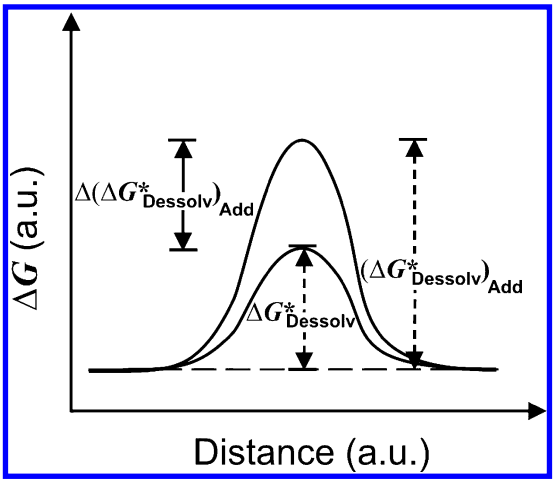

Figure 3. Enhancement of the kink kinetics barrier $\Delta(\Delta G^{\ddagger}_{\text{kink}}) = \Delta G^{\ddagger\prime}_{\text{kink}} - \Delta G^{\ddagger}_{\text{kink}}$ by the adsorption of AFP III at the kink site.

One of the most common ways used to access the nucleation is to measure the induction time of nucleation t_{nucl} at different supercoolings.[28] However, due to the crystallization sequence, what one then measures is the induction time t_i for crystallization, which is defined as the meantime elapsing before the appearance of an observable amount of the new phase. Actually, t_i includes the time t_g necessary for the crystals to grow to an observable size, and t_{nucl}. Since the free energy barrier for 3D nucleation is much higher than that in 2D nucleation, the growth of crystals is then much easier than nucleation in most cases. This is exactly the case for ice crystallization: once ice nuclei occur, the rapid growth rate leads to complete freezing of the whole water droplet in less than 0.5 s. This implies that we have $t_g \ll t_{\text{nucl}}$, and then $t_i \approx t_{\text{nucl}}$. According to the definition of the nucleation rate, one has

$$J = 1/(t_{\text{nucl}}V) = 1/(t_i V) \tag{12}$$

where V is the volume of the water droplet in the experiment.

Combining eqs 3, 11, and 12 yields

$$\ln(t_{\text{nucl}}V) = \kappa f(m)/(T(\Delta T)^2) + \Delta G^{\ddagger}_{\text{kink}}/kT - \ln\{f''(m)[f(m)]^{1/2}B'\} \tag{13}$$

where $B' = 4\pi a(R^s)^2 N^0 CB$, which remains constant under a given condition (C is constant).

It follows from eq 13 that for ice nucleation, the plot $\ln(\tau V) - 1/(T(\Delta T)^2)$ will give rise to a straight line for a given $f(m)$ (and $f''(m)$), and $f(m)$ can be utilized to derive the key parameters associated with the kinetics of ice nucleation (see the discussions in the following sections). This equation will be applied in the following discussion to analyze the antifreeze effect of AFPs.

Results and Dicussion

The surface activity of AFP III is schematically indicated by the drop of the surface tension with the concentration shown in Figure 4. The minimum point in Figure 4 indicates the saturation of the AFP III packing on the water−air interface and the aggregation of AFP III inside the bulk phase. This concentration at which the surface tension reaches its minimum is defined as the Critical Aggregation Concentration (CAC). For AFP III, the CAC is 2.5 mg/mL as shown in Figure 4. For globular proteins, such as lysozyme, they may decrease the surface tension of water from 72.8 (pure water at 20 °C) to 60 mN/m.[25] The even lower surface tension of the AFP III solution (Figure 4) to 40.2

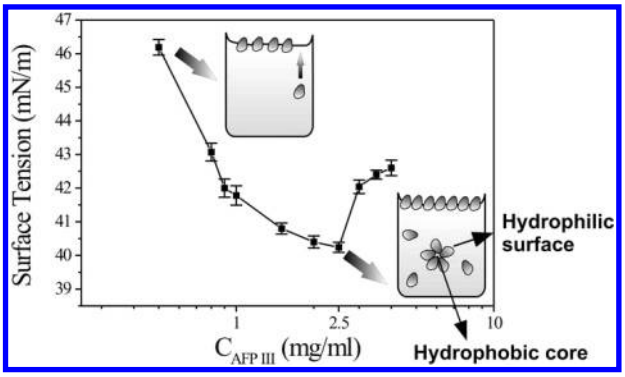

Figure 4. Surface tension as a function of AFP III concentration. The accumulation of AFP III on the surface leads to the lowering of the surface tension γ until the surface is fully occupied (saturated) by AFP III molecules at 2.5 mg/mL (CAC).

mN/m indicates the high surface activity of the AFP III molecule due to its high hydrophobicity. The increase of surface tension γ above CAC in the plot of the surface tension against concentration is often taken as an indication of the presence of impurities in the proteins.[32]

Among the various factors that may affect the surface activity of proteins, hydrophobicity is a dominant parameter.[20] It is known that the hydrophobicity of AFP III is quite high, as judged by the amino acid compositions. Thus it is not surprising that AFP III has a high surface activity presented by the low surface tension of its solution. At the same time, the hydrophobic groups on the AFP III surface gather together to minimize the net hydrophobicity area exposed to water as an entropically favorable process. This process apparently drives protein aggregation in the solution. Recently, it was suggested that hydrophobic residues of AFP III may contribute to the protein−ice binding interactions.[15] Therefore it is necessary to consider the occurrence of antifreeze protein aggregates caused by the hydrophobic interactions between AFP III molecules and study their correlation to the antifreeze mechanism.

As reported in our previous publication,[18] it is almost impossible to eliminate the influence of foreign particles under normal crystallization conditions. In their function as a substrate for ice nucleation, foreign particles always lower the nucleation barrier by a factor f (cf. eq 10). Therefore, these foreign particles can be regarded as ice nucleators (INs). In fact, in biological systems, ice nucleation is promoted because of the presence of ice nucleators.[33] Due to the fact that it is impossible to eliminate all the INs in real systems, antifreeze proteins and/or other antifreeze agents are required to prevent freezing by modifying the interaction between INs and the nucleating phase. In the case of ice nucleation promotion, the adsorption of AFPs on INs will improve the interaction and/or the structure match between the INs and the nucleating ice. This will then result in $m \to 1$ and $f \to 0$. Since for a given nucleation system, κ is constant (see eqs 4 and 13), such a change can then be identified from the lowering of the slope and the increase of the intercept of $\ln(\tau V) - 1/(T(\Delta T)^2)$ plot (cf. eq 13). Conversely, if the adsorption of AFPs leads to a stronger repulsion and an interfacial structure mismatch between the INs and the nucleating phase, one has then $m \to -1$ and $f \to 1$. This corresponds to an increase in the nucleation barrier (cf. eq 10). The effect can be identified from the increase in the slope of $\ln(\tau V) - 1/(T(\Delta T)^2)$ and the decrease of the intercept. Apart from the aforementioned effect, AFPs may also adsorb onto the surface of ice nuclei. According to eq 13, the variation in the intercept of the $\ln(\tau V) - 1/(T(\Delta T)^2)$ plot at a constant $f(m)$ corresponds to the change in $\Delta G^{\ddagger}_{\text{kink}}/kT$.

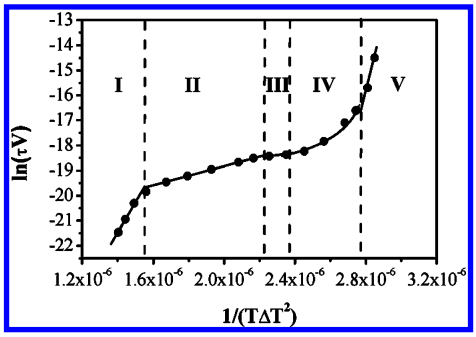

Figure 5. The correlation between $\ln(\tau V)$ and $1/(T\Delta T^2)$ for a 2.5 mg/mL AFP III solution. The plot is divided into 5 regions according to the supercooling ΔT.

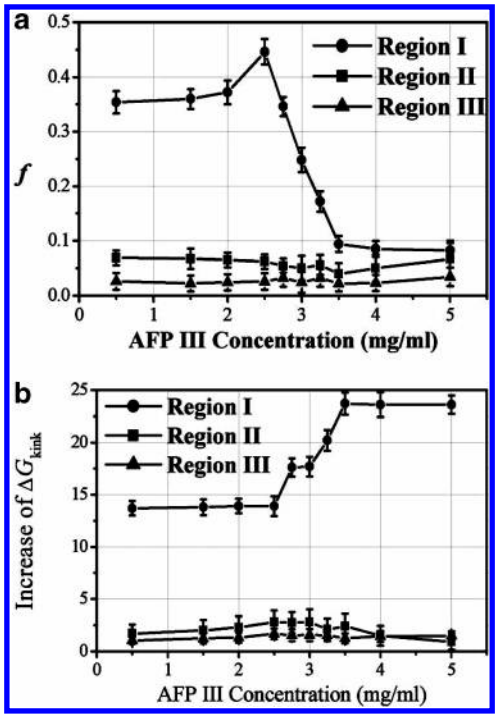

Figure 6. The plot of (a) $f(m)$ and (b) $\Delta(\Delta G^{\ddagger}_{kink}/kT)_{add}$ changed with AFP III concentration.

In our experiments, AFP III was added to deionized water at 0.5, 1.5, 2, 2.5, 2.75, 3, 3.25, 3.5, 4, and 5 mg/mL concentration. When AFP molecules adsorb on these INs, the interaction and the structure match between INs and the nucleating phase will be significantly altered. As mentioned before, this adsorption on the INs and the impact on nucleation can be quantified from the $\ln(\tau V) - 1/(T(\Delta T)^2)$ plot (cf. Figure 5). According to eq 13, the slope of the $\ln(\tau V) - 1/(T(\Delta T)^2)$ plot is $\kappa f(m)$. In Regime V, inverse homogeneous-like nucleation takes place ($f(m) = 1$), one then has $\kappa f(m,R) = \kappa$.[14] For the other regions, $f(m)$ can be calculated by dividing the slope by κ. By eliminating the influence of the change of $f(m)$ ($f''(m)$), $\Delta(\Delta G^{\ddagger}_{kink}/kT)_{add}$ can be calculated from the intercept of the $\ln(\tau V) - 1/(T(\Delta T)^2)$ plot. Thus from the slopes and intercepts resulting from the linear regression for these systems, $f(m)$ and $\Delta(\Delta G^{\ddagger}_{kink}/kT)_{add}$ were calculated as plotted in Figure 6a,b.

The adsorption of AFP III molecules on INs turns out to strongly disturb the wetting between the nucleating ice and the INs. This can be identified from the variation of $f(m)$ in region I in Figure 6a. The other two plots for Regions II and III in Figure 6a show little variation. This can be explained based on the size of the critical nucleus at these low supercoolings. According to eq 6, the radius of the critical nucleus r_c is

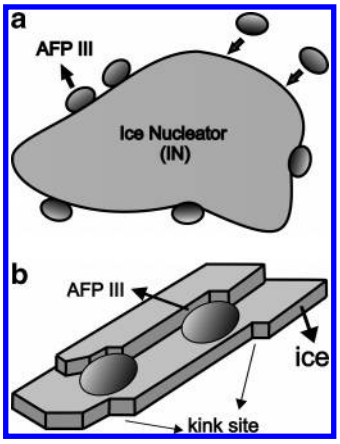

Figure 7. A simplified illustration of adsorption of AFP III molecules (a) on the interface of ice nucleator (IN) and liquid and suppression of water molecules approaching the IN surface (b) on ice surface and suppression of water molecules approaching the ice surface.

proportional to $1/\Delta T$. Thus r_c is large at low supercoolings (Regions II and III), then the effect of the AFP III in changing the wetting between the ice nucleating phase and the INs is slight. On the other hand, the results given in Figure 6b show that the AFP III will also adsorb onto the growing ice nuclei. The adsorption will suppress the incorporation of water molecules into the ice nuclei so as to inhibit ice crystallization. This can be seen from the increase in the desolvation kink kinetics barrier $\Delta G^{\ddagger}_{kink}$ at Region I in Figure 6b. In a similar way, the effect of AFP III in Regions II and III is small.

It is noted that in Region I, f reachs its peak value at 2.5 mg/mL concentration (CAC). Interestingly at the same time, $\Delta G^{\ddagger}_{kink}$ goes through a sharp rise at the same concentration. This suggests that the adsorption-inhibition mechanism has been greatly changed by the AFP III at CAC. At lower concentrations (<2.5 mg/mL), when no aggregate is formed in the solution, the adsorbed AFP III molecules on the INs are few (cf. Figure 7a). With the increase of the AFP III concentration, more such AFP III monomers adsorb onto the INs. The adsorption of AFP III monomers on INs turns out to strongly disturb the structural match between the nucleating ice and the INs (cf. Figure 8a), thus $f(m)$ increases. However, with the further increase of the AFP III concentration (>2.5 mg/mL), as shown from the result of the surface tension, some AFP monomers will aggregate to form oligomers. Due to the fact that the result of the aggregation is to minimize the aqueous solution contact with hydrophobic groups on the protein surface, the surface of those AFP III aggregates is hydrophilic. This results in the occurrence of new ice nucleators—AFP III aggregates. Because this effect is more dominant, the wetting between ice and INs becomes better (f decreases).

In contrast, as shown in Figure 6b, $\Delta G^{\ddagger}_{kink}$ shows a different behavior from that of f at 2.5 mg/mL concentration (CAC). As mentioned in the section on Theory, $\Delta G^{\ddagger}_{kink}$ refers to the kink kinetics barrier attributed to the adsorption of impurities/additives (AFP III molecules) onto the ice surface. The integration of H_2O units in the ice nucleus will be significantly slowed or even terminated due to a high $\Delta G^{\ddagger}_{kink}$ (see Figures 3 and 8b). Consequently, the sharp rise of $\Delta G^{\ddagger}_{kink}$ at CAC in Figure 6b suggests that more AFP III molecules adsorb onto the surface of ice nuclei especially at the kink site, and the optimal packing of AFP III molecules on the ice surface begins to form. As shown in Figure 6b, this optimal packing is accomplished above CAC (around 3.5 mg/mL). As illustrated by Figure 8b, the covering by the AFP III of the ice nuclei surface will stop the further addition of water molecules and thus the antifreeze activity will

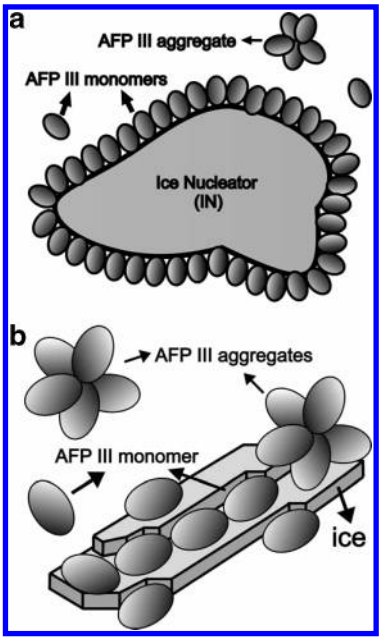

Figure 8. (a) The new ice nucleators (INs)−AFP III aggregates formed at CAC. These new INs will improve the wetting between ice and the INs. (b) The optimal packing of the AFP III molecules on the ice surface is accomplished above the CAC. This optimal packing suppresses the further addition of water molecules on the ice surface effectively.

be enhanced at CAC. In the same way, the growth of ice crystals could also be inhibited by this optimal packing of AFP III molecules on ice crystals at or above CAC.

Conclusions

In summary, AFP III molecule will aggregate in aqueous solution at CAC due to its high surface hydrophobicity. Similar aggregation in aqueous solutions might happen to other antifreeze proteins because the high hydrophobicity is a general feature of all four types of fish antifreeze proteins.[34] It is found that the ice nucleation kinetics can be modified at CAC by two effects. First, the aggregates appearing in solution may work as ice nucleators. Thus if the AFP is to inhibit ice nucleation, the aggregation should be suppressed. The second effect, which is the dominant effect, is that more AFP III will adsorb onto the surface of ice nuclei and reach their optimal packing to inhibit ice nucleation at or above CAC. In this way, AFP III exhibits an enhanced inhibition effect on ice nucleation at CAC.

It should be noticed that as the surface integration remains the same during the stages of nucleation and growth, the impact of AFP III on the surface integration during ice growth will be the same as that during ice nucleation. Therefore, based on the above result, we can conclude that the growth inhibition efficiency of AFP III will be significantly enhanced at CAC. This conclusion is particularly important for the selection of the optimal concentration of AFP in our antifreeze experiment. Another important point to be highlighted here is that as the type and concentration in an aqueous solution of electrolyte as well as the pH often affect the value of CAC, we can also anticipate that the changes of the above conditions will inevitably modify the antifreeze efficiency of the AFPs. Further work in this respect is the subject of a forthcoming publication.

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