Recent experimental results show the irreversible binding of antifreeze proteins to ice surfaces

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Antifreeze proteins (AFPs) are a subset of ice-binding proteins that evolved to arrest ice crystal growth and also inhibit the recrystallization of ice. An accepted theory for their activity, named the adsorption-inhibition hypothesis, posits that AFP binding to ice must be irreversible because the ice crystals do not grow within a freezing hysteresis (FH) gap. This theory has been criticized due to several experimental observations such as the dependence of FH values on the AFP concentration. Here we present recent fluorescence and microfluidics experimental results of our group supporting irreversible binding of AFPs to ice surfaces. i) Photo-bleaching of GFP-tagged AFP residing on the surface of an ice crystal held in the FH gap showed that there is neither exchange nor overgrowth of the bleached AFP. ii) Ice crystals bound by AFPs showed a measurable resistance to melting (melting hysteresis) demonstrating that the AFPs remain surface-bound at temperatures above the equilibrium melting point. iii) Using a temperature controlled microfluidic apparatus we demonstrated that small ice crystals formed in AFP solution stay intact even after the surrounding solution is depleted of AFPs. Additional measurements of surface fluorescence made during and after the exchange procedure showed no decrease in the fluorescence intensity, suggesting that bound AFPs did not leave the ice surface during the process. In light of these results, this review concludes that surface-adsorbed AFPs are the core source of the freezing hysteresis activity of the tested AFPs and that their adsorption to ice is irreversible.

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INTRODUCTION

Some organisms that live in cold climates produce antifreeze proteins (AFPs) to protect themselves from freezing¹,². AFPs depress the
freezing point of the bodily fluid of these organisms and also inhibit recrystallization of ice. Molecular studies have shown that AFPs from different organisms have diverse structures and sequences. This diversity suggests that many AFPs evolved through independent evolution routes. The interaction of these proteins with ice crystals is unique and gives rise to their potential use in the cryopreservation of food and tissues and in cryosurgery.

AFPs adsorb to the surface of ice and make the addition of water to the ice front unfavorable. Their surface adsorption causes a freezing hysteresis (FH) between the lowered freezing temperature and the slightly elevated melting temperature. The difference in FH values of different types of AFPs has led to the classification of AFP as moderate or hyperactive. The hyperactive AFPs (hypAFPs) are 10-100 times more active in depressing the non-equilibrium freezing point compared to moderate AFPs at equivalent micro-molar concentrations. In general, the moderate AFPs have FH of 0.5-1.0 °C at mM concentrations whereas hypAFPs can have FH activities of 2-6 °C at concentrations of ~100 μM. It has been suggested that hyperactivity is related to the affinity of hypAFP to the basal planes of ice.

The adsorption-inhibition model that explains the ability of AFPs to halt ice growth was introduced by Raymond et al. and further developed by Knight et al. This model suggests that AFPs irreversibly bind to ice surface and increase the surface curvature of the ice between bound AFPs. This increase in surface curvature leads to a depression of the freezing point due to the Gibbs-Thomson effect. There have been criticisms of the assumption of irreversible binding of AFPs to ice by many researchers in the field. One of the main arguments against irreversible binding stems from the concentration dependence of the activity of AFPs. Several theoretical works suggest reversible binding kinetics in order to explain concentration dependence of the activity of AFPs. In addition, the existence of a quasi-liquid layer on ice surfaces and surface melting at temperatures below the equilibrium melting point raised doubts about AFP binding to a well-defined ice surface. It is therefore still widely assumed that there must be an exchange between protein molecules bound to ice surfaces and those free in the surrounding solution.

The investigation of AFPs by fluorescence microscopy and microfluidic techniques brought new insights into the field. For instance, Zepeda et al. tagged antifreeze glycoproteins (AFGPs) with fluorescein isothiocyanate (FITC) and examined ice crystal growth in a cell where single ice crystals were grown in a glass capillary from solutions containing low concentrations of AFGPs. Based on the observation of loss of fluorescence intensity on the ice rim, the authors reported that the proteins on the initial ice surface were released upon growth of a new ice layer. Thus, the authors concluded that the attachment of these proteins to ice is weak and further addition of the water molecules to the ice surface ejects the AFGPs from the ice. Based on these findings, they concluded that the AFGPs adsorption is reversible.

We have studied ice crystal growth and melting characteristics as affected by AFPs using a variety of experimental techniques including the nanoliter osmometer, fluorescence microcopy, and newly developed microfluidic devices. In this review we present our results, which show strong evidence for irreversible binding of AFPs to ice.

**MATERIALS AND METHODS**

**Proteins**

The list of antifreeze proteins investigated throughout our studies is summarized in Table 1 along with the abbreviations used in the text. In
addition to wild-type AFPs shown in Table 1, we used green fluorescence protein (GFP) tagged AFPs for fluorescence microscopy studies\textsuperscript{10, 22, 23}. The activity of the AFPs was not affected negatively by this bulky protein because it was attached in such a way as to avoid occluding the ice-binding site of the AFP. Control experiments showed that GFP itself had no interaction with ice.

### Table 1. AFP used in the experiments

<table>
<thead>
<tr>
<th>Protein</th>
<th>Abbrev.</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spruce budworm AFP (501 isoform) \textsuperscript{24}</td>
<td>sbwAFP</td>
<td>Hyperactive</td>
</tr>
<tr>
<td>\textit{Tenebrio molitor} AFP\textsuperscript{25}</td>
<td>\textit{Tm}AFP</td>
<td>Hyperactive</td>
</tr>
<tr>
<td>\textit{Marinomonas primoryensis} AFP\textsuperscript{26}</td>
<td>\textit{M}pAFP</td>
<td>Hyperactive</td>
</tr>
<tr>
<td>Snow flea AFP (long isoform) \textsuperscript{11}</td>
<td>sfAFP</td>
<td>Hyperactive</td>
</tr>
<tr>
<td>\textit{Lolium perenne} AFP\textsuperscript{27}</td>
<td>\textit{L}pAFP</td>
<td>Moderate</td>
</tr>
<tr>
<td>Fish type I AFP\textsuperscript{28}</td>
<td>AFP-I</td>
<td>Moderate</td>
</tr>
<tr>
<td>Fish type III AFP\textsuperscript{29}</td>
<td>AFP-III</td>
<td>Moderate</td>
</tr>
</tbody>
</table>

**Freezing and melting hysteresis experiments**

The FH and MH measurements were done using a custom-designed nanoliter osmometer as previously described in detail\textsuperscript{10-32}. This temperature controlled system provides the opportunity to work with temperatures ranging from -40 °C to room temperature with a precision of 0.002 °C. The experimental cell was placed under the microscope equipped with air objectives and controlled through Labview software\textsuperscript{31}. A detailed description of the FH measurements is also described in ref (34).

**Fluorescence microscopy experiments**

We used an inverted epi-fluorescence microscope and an upright confocal microscope to visualize fluorescently tagged AFPs. The experimental details were done as described in ref.(10, 22, and 23).

**Microfluidics experiments**

The microfluidic devices were fabricated by soft lithography and replica molding techniques\textsuperscript{33, 34} (Fig. 1A). All experiments were done in a temperature-controlled cell (Fig. 1B). Briefly, the cell includes a thermistor in combination with two thermoelectric cooling elements. The thermoelectric coolers are driven by a temperature controller (Model 3140, Newport, Irvine, CA). This custom-designed temperature-controlled cell works in the range from room temperature to -40.00 °C. The microfluidic device is positioned in this temperature-controlled cell on a copper plate.

![Fig. 1. Microfluidic apparatus. (A) Schematic of the microfluidic channel. (B) The experimental cell controlled by thermoelectric coolers. Further details can be found in ref. (34).](image)

**RESULTS AND DISCUSSION**

**Quasi-permanent attachment of type III AFP to ice**

Pertaya et al\textsuperscript{25} used fluorescently tagged fish type III AFP and showed that its attachment to ice was irreversible by using a technique called Fluorescence Recovery After Photobleaching (FRAP). Ice crystals grown in solutions containing tagged AFPs were locally bleached and the fluorescence signal on the ice surface was followed for up to 20 hours. A series of experiments with many such crystals showed that the fluorescent signal at the bleached areas was not recovered, indicating that there was no exchange between adsorbed AFPs and the free AFPs in the surrounding solution (Fig. 2). Careful analysis of the ice surface revealed that the average distance between AFPs
Fig. 2. Confocal microscopy images of ice crystals in type III AFP solution. (A) Images of two ice crystals before bleaching. (B) A part of the top crystal was bleached. (C) A part of the bottom crystal was bleached. (D) After 20 hours of recovery time, there was no observable recovery of the fluorescence signal of the bleached areas for both crystals. Adapted from ref (23).

was on the order of 20 nm, assuming a single layer of AFPs. Zepeda et al.18 also found a similar distance between AFGP molecules bound on an ice rim. Single layer adsorption of type III AFPs was also demonstrated by Hai et al.35. Hai’s neutron reflection studies demonstrated monolayer adsorption of type III AFPs on hydrophilic SiO2 surface at concentrations ranging from 0.01 to 2 mg/ml35.

Superheating of ice crystals in AFP solutions30

Although melting inhibition of ice exposed to AFP solutions was previously reported by Knight and DeVries more than two decades ago30, it was not measured quantitatively until recently30. Our measurements of melting hysteresis with a series of moderate and hyperactive AFPs (see Table 1) strongly suggest irreversible attachment of AFPs to ice surfaces. We performed experiments using a custom-built nanoliter osmometer (Fig. 3) and confocal microscopy and found that depending on the type of AFP, ice can be superheated up to 0.5 °C30.

Exchanging solutions around micron-sized ice crystal34

We developed temperature-controlled microfluidic devices to investigate the role of free AFPs in solution on the freezing point depression and to observe whether the attachment of AFPs to the ice surface is reversible34. This unique experimental setup enables precise control of the temperature and exchange of solution medium in the microfluidic devices without perturbing small ice crystals of 30-50 μm length (Fig. 1). In these experiments, we used GFP-tagged TmAFP and examined whether the fluorescence signal of the surface near the edge of the ice crystal changes when the solution around the crystal is exchanged with solution that does not contain AFPs. Our analysis showed no reduction in the fluorescence signal during the exchange process and after it. The ice crystals incubated in high concentrations of GFP-TmAFP stayed intact even after the solution was exchanged with the AFP-free buffer at temperatures within the FH gap of the samples. We further measured the FH values before and after solution exchange. The measured FH values before and after were comparable (Fig. 4). These findings suggest that surface-bound AFPs are the core source of AFP activity and FH is not a direct function of the proteins in solution.
Fig. 4. Freezing hysteresis measurement in a microfluidic device. (A) The ice crystal was formed in a 20 μM GFP-TmAFP solution and incubated at 0.1 °C below its melting temperature for 10 min. (B) To measure FH activity, the temperature was lowered until growth was observed. The crystal growth (burst) is shown at the non-equilibrium freezing point before the exchange of the solution medium. (C) The initial ice crystal was melted, until a small ice crystal was left. This new crystal was also incubated for 10 min in the GFP-TmAFP solution. (D) The GFP-TmAFP solution was exchanged with AFP-free buffer, and the crystal was found to be stable with no detectable growth at 0.1 °C below the melting point. When the setup was further cooled, the crystal burst at a temperature corresponding to an FH activity of 0.77 °C. The newly grown ice layers are denoted by red arrows. See details in ref (34).

CONCLUSIONS

The present review summarizes our recent findings and newly developed techniques of microfluidic devices and fluorescence microscopy to study the interaction of AFPs with ice crystals. We have demonstrated that there is no exchange of AFPs between the surface and free AFPs in the solution using FRAP. With novel temperature-controlled microfluidic devices and fluorescently-tagged AFPs, we showed that small ice crystals protected by hypAFPs were stabilized in supercooled non-AFP solutions for hours with no observed ice growth. Repeated FH experiments of ice crystals incubated in AFP solutions before and after the exchange of liquids surrounding the crystals in microfluidic devices gave similar FH activities. HypAFPs were found to protect ice against melting as well as freezing, resulting in superheated ice. Ice crystals were remained intact above their equilibrium melting temperatures and remained stable in a superheated state for extended time periods (up to hours). The experimental results of this series of studies provide strong evidence that AFPs adsorb to ice surfaces via irreversible binding. This review shows that the use of microfluidics in combination with fluorescence microscopy has become a valuable technique for studying the binding mechanisms of AFPs to ice and the concentration dependence of AFP activity.

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REFERENCES

4) Chao, H., Davies, P., and Carpenter, J. : Effects


