Superheating of ice crystals in antifreeze protein solutions

Yeliz Celik*, Laurie A. Graham, Yee-Foong Mok, Maya Bar, Peter L. Davies, and Ido Braslavsky

Department of Physics and Astronomy, Ohio University, Athens, OH, 45701; Department of Biochemistry, Queen's University, Kingston, ON, Canada K7L3N6; and Department of Structural Biology, Weizmann Institute of Science, Rehovot 76100, Israel

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It has been argued that for antifreeze proteins (AFPs) to stop ice crystal growth, they must irreversibly bind to the ice surface. Surface-adsorbed AFPs should also prevent ice from melting, but to date this has been demonstrated only in a qualitative manner. Here we present the first quantitative measurements of superheating of ice in AFP solutions. Superheated ice crystals were stable for hours above their equilibrium melting point, and the maximum superheating obtained was 0.44 °C. When melting commenced in this superheated regime, rapid melting of the crystals from a point on the surface was observed. This increase in melting temperature was more appreciable for hyperactive AFPs compared to the AFPs with moderate antifreeze activity. For each of the AFP solutions that exhibited superheating, the enhancement of the melting temperature was far smaller than the depression of the freezing temperature. The present findings clearly show that AFPs adsorb to ice surfaces as part of their mechanism of action, and this adsorption leads to protection of ice against melting as well as freezing.

Superheating of solids is defined as the absence of melting at temperatures higher than the equilibrium melting temperature. A solid can exist in this metastable phase up to a maximum superheating temperature at which it will lose its stability and melt catastrophically. The maximum superheating was calculated to be around 20% above the equilibrium melting temperature (1, 2). In contrast to the supercooling of liquids, which is commonly encountered, solids embedded in a liquid can rarely withstand even slight superheating because the liquid/solid interface promotes melting (1, 3, 4). Also, for crystals that are surrounded by a gas or solid, surface melting is known to occur even at temperatures below the bulk melting point (i.e., premelting (5). Nevertheless, recent studies demonstrate that solids can be superheated if surface melting is circumvented (4, 6–9). For example, high superheating values were achieved by embedding nanocrystals of one substance into another material with a higher melting point (4, 8). Additionally, superheating inside the bulk of a material may be observed while melting occurs at the surface. For instance, superheating of ice was experimentally observed by shock heating of micron-sized crystals up to temperatures of 60 °C for 1.3 ns (10). Knight and DeVries predicted that ice crystals in a solution containing antifreeze proteins (AFPs) would remain stable when superheated (11). They reasoned that because AFPs stop ice crystals from growing by irreversibly binding to their surfaces, such AFP adsorption should also inhibit melting. An illustration of an ice surface pinned by AFPs, with ice fronts bulging out between the AFP molecules during cooling and receding inward during heating, is shown in Movie S1. AFPs are structurally diverse proteins evolved in marine and terrestrial organisms to help survival in freezing conditions (12). They share the ability to lower the nonequilibrium freezing temperature of an ice crystal in an aqueous medium to below the melting point. This property of AFPs has been called thermal hysteresis but will be referred to here as freezing hysteresis (FH) to distinguish it from melting hysteresis (MH), which is the elevation of the nonequilibrium melting temperature above the melting point. Knight and DeVries observed the melting shape of a hole drilled in a single ice crystal (∼3 cm in diameter) filled with either pure water or a solution containing AFPS (11). In the absence of AFPS, the hole remained circular during melting due to symmetrical heat flow, whereas in the presence of AFPS, the hole became asymmetrical and faceted. Additionally, they observed that melting of ice, initiated near air bubbles within the ice, tunneled toward the melted hole containing the AFP solution (11). These observations showed that the ice around the drilled hole was superheated. To date, however, no direct measurements of the superheating of ice crystals in AFP solutions have been described, and no experimental evidence has been reported showing that ice crystals can be stabilized at temperatures above the equilibrium melting point, T_m*, for long periods.

Here we report measurements of the superheating of ice crystals in solutions containing hyperactive and moderately active AFPS. AFPS are classified as moderate and hyperactive according to their FH activities (13, 14). Moderate AFPS are defined here as having FH activity of 0.5 to 1.0 °C at millimolar concentrations. Hyperactive AFPS (hypAFPs) are an order of magnitude more active in FH than moderate AFPS in micromolar concentrations, and some depress the freezing point by at least 6 °C at millimolar concentrations. In the present study, the melting behavior of small ice crystals (∼3–40 μm) was examined as a function of temperature and solution content. We found that ice crystals in certain AFP solutions remained stable above their equilibrium melting temperatures for up to several hours.

Results

Experiments were conducted using a custom-designed nanoliter osmometer, and samples were observed by confocal microscopy (15). We examined four hypAFPs and four moderate AFPS. These AFPS, and the abbreviations used to denote them, are summarized in Table 1.

Freezing and Melting Hysteresis. The superheating phenomenon was particularly evident during growth and melt cycles of ice crystals in solutions containing Marinomonas primoryensis (MPAFP) (16) or Tenebrio molitor (TmAFP) (17), both of which are hypAFPs. In Fig. 1A, a single ice crystal grown in MpAFP solution is shown. When the temperature was dropped to a value below the FH gap of 1.87 °C, rapid ice growth propagated from the surface of the crystal (designated as “burst” growth, Fig. 1B). Once the sample was completely frozen, the initial crystal was still...
visible and remained intact even when the sample began to melt back (Fig. 1C). As the temperature was raised to the \( T_m \), all of the newly formed ice slowly melted (Fig. 1D and E). The original crystal remained intact for more than 30 min after the rest of the ice had melted. Even when the temperature was increased at a rate of 0.01 °C/30 s, the crystal did not undergo any melting until it eventually melted rapidly at 0.18 °C above \( T_m \) (Fig. 1F). In this instance, the MH value was roughly one-tenth of the FH value. A recording of this experiment is shown in Movie S2. These findings suggest that the ice crystal grown in AFP solution was protected from melting as well as from freezing.

**Observation of Proteins Adsorbed on Ice.** To visualize adsorbed proteins on ice surfaces, we tagged MgAFP with green fluorescent protein (GFP). An ice crystal exposed to a 2 μM solution of the fluorescent fusion protein GFP-MgAFP exhibited uniformly bright surfaces with intensely fluorescent edges, consistent with surface adsorption of the AFP (Fig. 2). When the temperature was raised to 0.04 °C above the \( T_m \) of the crystal, the crystal melted and the bound GFP-MgAFP was released into the liquid, dispersing away with a diffusion coefficient of 20 ± 10 μm^2/s. Unlike the uniform melting we observed for crystals in pure water, melting started at one point on the ice surface and then proceeded through the crystal. This can be seen in Fig. 2F where melting is evident only on the left surface. Thus we consider the initiation of the melting of the surface as melting nucleation.

**Table 1. AFPs used in the experiments; the maximum MH/FH values were measured at different protein concentrations for the different AFPs**

<table>
<thead>
<tr>
<th>AFP</th>
<th>Species</th>
<th>MH/FH (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgAFP (hyperactive)</td>
<td>Marinomonas primoryensis (16)</td>
<td>0.44/2.0</td>
</tr>
<tr>
<td>TmAFP (hyperactive)</td>
<td>Tenebrio molitor (17)</td>
<td>0.18/1.7</td>
</tr>
<tr>
<td>sbwAFP 501 (hyperactive)</td>
<td>Spruce budworm (18)</td>
<td>0.04/4.1</td>
</tr>
<tr>
<td>Dc hemolymph (hyperactive)</td>
<td>Dendroides canadensis (19)</td>
<td>0.20/6.2</td>
</tr>
<tr>
<td>Type II AFP (moderate)</td>
<td>Sea raven (20)</td>
<td>0.02/0.5</td>
</tr>
<tr>
<td>Type III AFP (moderate)</td>
<td>Ocean pout (21)</td>
<td>0.02/0.8</td>
</tr>
<tr>
<td>Type I AFP (moderate)</td>
<td>Winter flounder (22)</td>
<td>0.02/1.3</td>
</tr>
<tr>
<td>Lp AFP (moderate)</td>
<td>Lolium perenne (23)</td>
<td>0.00/0.8</td>
</tr>
</tbody>
</table>

**Melting Behavior of Ice Crystals.** In some AFP solutions we observed multiple distinct ice crystals. In particular, multiple crystals readily formed at high AFP concentrations, where the crystals were stabilized faster than in low concentration solutions. Having a group of ice crystals enabled us to observe the melting nucleation of superheated ice crystals in the same droplet and gather statistical information on the melting behavior in relation to superheating. In Fig. 3a, the ratio of the number of crystals that had melted (\( N_m \)) relative to the initial number of crystals (\( N_0 \)) as a function of superheating (\( T - T_m \)) is presented for a system starting with 56 crystals (5–40 μm) in a drop of 20 μM (0.18 mg/ml) TmAFP solution. The temperature was increased at an average rate of 0.01 °C/7.5 s. A movie of this experiment is shown in Movie S3. The melting behavior can be fitted into a sigmoidal plot where half of the crystals melt at \( \Delta T_{50%} = 0.1 °C \). Our observation of a cumulative distribution of melting resembles experiments on crystal nucleation in which samples comprised of small separate droplets were observed (24–26). In the case of melting nucleation, the crystals can be in the same droplet due to the fact that melting of one crystal does not induce melting of a nearby crystal. A possible shortcoming of this approach is that the temperature drop associated with the melting of one crystal may influence nearby crystals. This can be explained by considering that the diffusion coefficient of heat in water is 140,000 μm^2/s (27), which means that within 0.1 s heat can diffuse to the melting crystal from hundreds of microns away. However, if the latent heat of the melting crystal and the heat capacity of the larger volume influenced by this melting are taken into account, we estimate that the temperature drop associated with the melting of a single crystal is less than 0.01 °C. This is supported by our observation that several crystals melted at around \( \Delta T_{50%} \), an unlikely event had the drop cooled significantly. In addition, the temperature-controlled stage compensates for small temperature changes within 2 s. Similar cluster melting behavior was observed in a drop of 36 μM (1.25 mg/ml) MgAFP solution containing 23 ice crystals (Fig. S1).

**Stability of Superheated Ice Crystals.** To investigate the stability of superheated ice crystals in a drop of AFP solution, we performed experiments on a group of small ice crystals (3–24 μm in size) in 36 μM (1.25 mg/ml) MgAFP solution. After the formation of the ice crystals, we slowly raised the temperature at a rate of 0.01 °C/30 s. After half of the ice crystals melted (25 out of 51), corresponding to \( \Delta T_{50%} = 0.22 °C \) above \( T_m \), we maintained the temperature without change for 1 h. During the initial 10 min of temperature maintenance, half of the remaining crystals (13 out of the 26) melted, but further melting was not observed in the following 50 min at this temperature. Then, we slowly increased the temperature further at an average rate of 0.01 °C/8 min. During the next 3 h the crystals melted sporadically. The last ice crystal (~4 μm in size) melted at a temperature of 0.44 °C above the \( T_m \), that is, after it was superheated for more than 4 h. The crystal size was plotted as a function of superheating and is shown in Fig. S2. This experiment suggests that there is a weak correlation between crystal size and superheating, but it is still not clear why some similarly sized crystals withstand superheating for longer periods and higher temperatures than others.

**Melting Velocities of Superheated Ice Crystals.** The melting behavior was further analyzed by measuring the melting velocities of ice
The circled data point corresponds to the experiment described in Fig. 1. The results of additional experiments with individual crystals in multiple crystal samples were comparable to those obtained using single crystals with different concentrations. Note that the melting velocities recorded in solid squares represent data points from experiments using tals stabilized in AFP solutions. Open squares correspond to the crystals in tals formed in AFP solutions. (Fig. 3.) At a superheating of 0.04 °C, melting of the superheated ice crystal started at a point on the surface and proceeded through the crystal. (G–J) After melting commenced, the GFP-tagged AFPs that had been adsorbed on the ice diffused away.

**Fig. 2.** Fluorescence images of ice superheated in a GFP-MpAFP solution. (A–E) A series of images recorded while the crystal was warmed slowly to temperatures above the equilibrium melting point. (F) At a superheating of 0.04 °C, melting of the superheated ice crystal started at a point on the surface and proceeded through the crystal. (G–J) After melting commenced, the GFP-tagged AFPs that had been adsorbed on the ice diffused away.

**Fig. 3.** Analysis of melting nucleation and the melting velocities of ice crystals as a function of superheating in the presence of TmAFP, MpAFP, or GFP-MpAFP (Fig. 3B). The melting velocities are clearly proportional to the observed MH. For instance, when an ice crystal grown in pure water was heated to 0.01 °C above $T_m$, it melted with a velocity of ∼3 μm/s, whereas at 0.15 °C above $T_m$, the superheated ice melted at velocities of 40–200 μm/s. This clearly demonstrates that the crystals are in a superheated state. In Fig. 3B, the line of best fit for each sample is linear and extends to a point very close to the origin. At very low superheating, however, the melting velocity may not be a linear function of superheating. It is also evident that the melting velocities are faster in the presence of TmAFPs than MpAFPs, indicating that the AFPs influence the melting of the ice even after melting has commenced. These findings suggest that surface kinetics and not just temperature affect the melting velocity. Nevertheless, the mechanism underlying the AFP-induced modulation of the melting velocity remains unclear.

**Raman Spectroscopy of Superheated Ice.** To ascertain whether there were any changes in the ice structure as the temperature was increased, we examined the crystals using Raman spectroscopy (28, 29). We did not observe any significant change in the ice spectrum as the crystal was superheated, indicating that the ice maintains its integrity and crystalline form in this state. See Figs. S3 and S4 for further details.

**Melting and Freezing Hysteresis as a Function of AFP Concentration and Type.** The effect of AFP concentration on both MH and FH was examined for GFP-MpAFP. The FH activity showed a sigmoidal increase with a plateau at around 1.7 °C (Fig. S5), whereas the MH activity increased at a slower rate and reached a plateau at around one-tenth of the maximal FH value (Fig. 4A). This difference is evident in the plot of FH vs. MH in Fig. 4B. Similar behavior was also observed for Dendroides canadensis hemolymph (Fig. S6), which is a mixture of AFPs and other incompletely characterized cofactors/enhancers that exhibits extremely high antifreeze activity (30). In the present experiments we measured FH values of up to 10.5 °C. FH and MH were also measured with purified 501 isoform from the spruce budworm (sbw) AFPs (sbwAFPs) (Fig. S7). Although the 501 isoform was extremely active with a FH greater than that observed with the MpAFP or the TmAFP, the FH activity of the 501 isoform was far lower compared to those of MpAFP and the TmAFP.
Finally, we conducted experiments to measure superheating in the presence of moderately active AFPs. Although all three fish AFPs tested exhibited MH, the values were lower than those observed with the hypAFPs. For example, type I AFP showed a MH of only $0.011 \pm 0.003 \, ^\circ C$, at a far higher concentration (12 mM, Fig. S8) relative to the concentrations used for the hypAFPs (Figs. S6 and S7). At lower concentrations no MH was detected for type II and III AFPs. The ice crystals melted smoothly into round shapes during a temperature increase of as little as 0.002 °C (close to the instrumental resolution, 0.001 °C) above $T_m$. When the temperature was lowered into the FH gap, the crystals grew into hexagonal bipyramidal shapes that could be melted back into the round shape. At higher AFP concentrations, however, type II AFP (7.1 mg/mL, 0.48 mM) and type III AFP (9 mg/mL, 1.28 mM) showed MHs of up to 0.02 °C. This may be an underestimate as it was extremely difficult to accurately measure $T_m$. In systems with either type I AFP or low concentrations of most of the hypAFPs mentioned above, during the initial melt, all of the crystals in the droplet decreased in size uniformly from all sides when the temperature was held at just above $T_m$. If the melting process was halted, even for a few seconds, it could not be resumed at the same temperature and MH was evident from the melting behavior of the crystals: The crystals melted one at a time, rapidly and nonuniformly. However, with higher concentrations of types II and III AFP as well as sbwAFP isomorph 337, initial smooth melting could not be achieved; rather, the crystals melted, one at a time, rapidly and nonuniformly. This suggests that these crystals were already superheated. Once a single crystal remained, variable MH readings of up to 0.02 °C were obtained. In contrast, Lolium perenne (Lp) AFP (LpAFP), which shows comparable FH activity to type I AFP, failed to show MH at concentrations up to 20 mg/mL (1.5 mM).

**Discussion**

Our experiments on a series of hyperactive and moderately active AFPs demonstrate that the degree of superheating of ice could be easily measured in the presence of most hypAFPs, and it is also detectable with some moderate AFPs. Given that the MHs of the hypAFPs are much lower than their FHs, it is not surprising that the MHs of the moderate AFPs are significantly lower than those of the hypAFPs, and in the case of LpAFP, absent. It is also apparent that at the higher concentrations necessary for type II and III AFPs to show measurable MH activity, they appear to bind to the newly formed surfaces of the melting ice so rapidly as to affect the melt and prevent determination of accurate $T_m$ values.

While superheating of ice in solutions containing AFPs was first discussed two decades ago (11), it has yet to become established. For instance, Kristiansen and Zachariassen argued that AFPs may not irreversibly bind to ice surfaces based on the absence of superheating of ice crystals in AFP solutions (31). According to their two-step model, AFPs bind reversibly to ice surfaces at $T_m$ and bind irreversibly only when the temperature is dropped to values in the FH gap (31). Moreover, Zepeda et al. (32) observed that antifreeze glycoproteins (AFGPs) were not incorporated into an ice body as the ice surface slowly grew. Therefore, they concluded that AFGP adsorption must be reversible and the AFGPs do not adsorb to ice surfaces strongly. On the other hand, the model suggested by Knight and DeVries (11) to explain superheating of ice by AFGPs requires that the AFGPs attach strongly to the ice surface in order to modify the ice surface curvature and to protect the ice from melting.

One factor that has been postulated to explain the difference in FH activity between hypAFPs and moderately active fish AFPs is that while both types bind to prism or pyramidal planes, only the former binds to the basal planes (13, 15). Although ice growth or melting tends to be slower on the basal plane, a lack of binding to this surface could lead to easier melting of ice crystals (11). Indeed, Knight and DeVries suggested that AFGPs dynamically stop the basal plane growth by attaching to nearby prism planes (33). Nevertheless, other factors are doubtlessly involved as sbwAFP has been shown to bind to the basal plane (15) yet shows MH only marginally higher than that of the moderate AFPs.

According to the adsorption-inhibition model, the ice surface is pinned by AFPs and the surface curvature is directly proportional to the degree of supercooling based on the Gibbs-Thomson effect (34). Sander and Tkachenko (35) developed a theoretical explanation of the concentration dependence of FH activity based on the adsorption-inhibition model. A major parameter in their analysis is the maximum slope of the ice ($\chi_{\text{engulfment}}$) at the edge of an adsorbed AFP molecule that will resist engulfment of the AFP by the ice. If such engulfment occurs, it will result in further ice growth. A critical slope for engulfment has also been described by other groups (31, 36). Knight and DeVries (11) suggested that the AFP-induced melting inhibition should be achieved in the same manner as the freezing point depression by the Gibbs-Thomson effect but with a negative curvature between bound AFPs rather than positive. Thus, a unique critical value of the slope $\chi_{\text{superheating}} < 0$ should be determined by the resistance of the AFP molecules to leave the surface (Fig. S9). Our results clearly show that observed MH values are smaller than FH values at the same concentrations. We note that such a difference would occur if $|\chi_{\text{superheating}}|$ values are only a fraction of the corresponding $\chi_{\text{engulfment}}$ values. The increased outward curvature of the ice surface during supercooling may allow the AFP to increase its area of contact with the ice, leading to irreversible binding (37). During superheating, however, the surface would curve in the opposite direction, preventing further stabilization and perhaps allowing the AFP to desorb at a much lower curvature than that required for overgrowth. Such a difference in $\chi_{\text{engulfment}}$ and $|\chi_{\text{superheating}}|$ could account for the observation that MH is consistently lower than FH.

**Fig. 4.** Comparison of FH and MH activity for GFP-MpAFP. (A) MH activity as a function of concentration. (B) The measured MH as a function of FH at the corresponding concentrations.
Several researchers consider a transition area between ice and water as continuous (32, 38) and thus do not accept the notion of irreversible attachment. One possible resolution to the different approaches could be found in recent molecular dynamic simulations of a sbwAFP (39). In the simulated system, the AFPs organized the water molecules around the ice binding face, and the mobility of the water molecules was decreased in that region. Therefore, it is reasonable to assume that the binding face of the AFP bridges through the transition layer and results in AFP attachment to the ice that gives rise to both FH and MH as described above.

The inhibition of ice melting has direct biological importance in the inhibition of ice recrystallization, which is a mechanism many organisms use to tolerate freezing. The resistance to melting, although small, will be of value particularly because it is accompanied by resistance to ice growth and will exert an effect during each of the many freeze-thaw cycles these organisms encounter during overwintering.

In conclusion, we showed that ice can be superheated in AFP solutions under isothermal conditions, and we measured the amount of superheating in the presence of moderate and hyperactive AFBs. Although AFBs were discovered more than 40 years ago, it is still unclear how they function, and suggested theories regarding their binding kinetics to ice crystals remain inconclusive. The results presented here provide strong evidence of the irreversible binding of several AFBs to ice surfaces. We hope that our results will stimulate further experimentation and theorizing and contribute to the understanding of how AFBs function at the ice/water interface. Additionally, this work implies that specific surface impurities can be used as a method to achieve superheating in solids.

Materials and Methods
Proteins. The AFBs used in this work were produced as recombinant proteins in Escherichia coli unless otherwise mentioned. They are as follows: the AFB domain from MpaAFP (16); TmAFP isomorph 4–9 (40); sbwAFP isomers 337 and 501 (18); synthetic winter flounder HPLC6 (type I AFB) (22); sea raven type II AFP (16); bay mussel (17); and needlefish (Rana esculenta) (13). The AFP from the perennial ryegrass (LpAFP) (23). Dendroides canadensis larvae (19) were collected at the Queen's Biological Field Station at Lake Opinicon in late winter, their hemolymph extracted, pooled, and diluted 1:1 (vol/vol) in 50 mM Tris-HCl (pH 7.9), 150 mM NaCl, 2 mM phenylthiocarbamide (Sigma-Aldrich). All the AFPs except TmAFP were produced in the laboratory of Peter L. Davies (Queen's University), whereas TmAFP was produced in the laboratory of Deborah Fass (Weizmann Institute of Science) (40). To conduct fluorescence microscopy studies, GFP-tagged MpaAFP (GFP-MpaAFP) was expressed and purified in the laboratory of PLD in the same manner as the other tagged AFBs (41) and was not adversely affected by the fusion. Spruce budworm AFBs were kept and diluted in 20 mM Tris-HCl (pH 8). Type I, type III, and LpAFP were kept and diluted in 100 mM ammonium bicarbonate (pH 8). TmAFP was kept in 20 mM ammonium bicarbonate buffer (pH 8), whereas GFP-MpaAFP, a Ca2+–dependent AFP, was kept in a buffer solution containing 20 mM CaCl2 and 25 mM Tris-HCl (pH 8).

Nanoliter Osmometer Experiments. Freezing hysteresis and melting hysteresis experiments were performed using a Clifton nanoliter osmometer (Clifton Technical Physics) and a custom-designed nanoliter osmometer. Both systems were validated to give comparable results. The nanoliter osmometer experiments were conducted in a cell that has been described previously in detail (14, 42, 43). The temperature of the custom-designed nanoliter osmometer was controlled through a LabView interface developed by Ido Braslavsky. Images were recorded to a personal computer hard drive using a Sony CCD-IRIS video camera connected to a video frame grabber (IMAQ-PCI-1407, National Instruments). The images were collected at a rate of 30 frames/s.

The procedures for the measurements of thermal hysteresis values (FH and MH) were as follows: AFP solutions with submicrometer volumes were placed in immersion oil and cooled to −35 °C to induce freezing. The samples were then warmed to close to the melting point. Accurate determination of the Tm in the presence of AFBs is a challenging process due to the stabiliza-
Supporting Information

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SI Text

Raman Spectroscopy. Experimental procedure. The temperature controlled cell was observed with a WiTec Raman/near-field scanning optical microscope (WiTec Instruments Corp.) with an illumination line of 532 nm. A Nikon Air 50× (NA 0.55, long working distance) objective was used to collect Raman spectra of the ice and solution phases. The laser intensity was adjusted to 5 mW. The 5 μl sample was sandwiched between two cover glasses and placed on a temperature controlled metal plate. The sample was frozen by cooling the stage to −18 °C, then melted back to form individual ice crystals. The sample was heated slowly at a rate of not more than 0.01 °C/s, and the temperature was held constant at each stage of data collection. Raman spectra of ice below the melting point and ice in the superheated state were collected. Raman images were obtained by scanning an area and summing over it the ice OH stretching peak of the spectrum; see details in ref. 1. It took 4 min to collect a Raman intensity map of an area of 30 μm × 30 μm. For a full Raman spectrum from a single point, acquisition time was set to 1 min. In addition, bright light microscopy connected to a video camera was used and images were captured with a frame rate of 25 frames/s, which enabled us to measure melting velocities of superheated ice crystals.

Results. The series of images in Fig. S3A show an ice crystal formed in 72 μM Marinomonas primoryensis antifreeze protein (MpAFP) solution at different superheating temperatures. This crystal was incubated for 45 min at 0.05 °C below its Tm after which the temperature was slowly increased and images and single spectra were taken at different temperatures. The series of images in Fig. S3B show the Raman intensity map of a 30 μm × 30 μm section of the ice crystal before and after it was superheated. The melting for this particular crystal occurred at superheating of 0.37 °C, and the melting velocity of the ice front at the actual melting temperature (Tms) was 106 μm/s. Raman spectra of ice were collected from the center of the crystal at different temperatures to observe whether there were any spectral changes prior to it going through its phase transition (Fig. S4). The spectrum of the crystal, with a local maximum around 640 nm, was virtually unchanged upon superheating, clearly indicating that the crystal is indeed superheated and still has an ice Ih structure. The transition in the spectrum from the ice phase to the melt phase was confirmed with repeated experiments (n > 10). Furthermore, the spectrum obtained just after the crystal melted (Fig. S4, purple line) was indistinguishable from the spectrum of the supercooled solution close to the crystal (Fig. S4, black line). The Raman spectra of the ice observed in these experiments are consistent with those obtained by others for ice Ih; see details in ref. 2).


Fig. S1. Statistical analysis of melting as a function of superheating. A group of ice crystals (N0 = 23) grown in MpAFP solution are observed for their melting behavior at different superheating temperatures. The plot shows the ratio of the number of crystals that were melted (Nm) relative to the initial number of crystals (N0) as a function of superheating (T − Tm). Half of the crystals melted when the sample was already 0.18 °C superheated.
Fig. S2. Size distribution of the superheated ice crystals. A group of ice crystals \( (N_0 = 51) \) grown in MpAFP solution were observed for their melting behavior over time as the temperature was increased slowly (see the main text for detailed information). Superheating values are shown as a function of the size of the ice crystals melted.

\[
\Delta T = +0.37 \, \text{C}^\circ \\
\Delta T = +0.14 \, \text{C}^\circ \\
\Delta T = -0.05 \, \text{C}^\circ \\
\Delta T = +0.06 \, \text{C}^\circ \\
\Delta T = +0.14 \, \text{C}^\circ
\]

Fig. S3. Superheated ice crystal in MpAFP solution. A single ice crystal at different temperatures is shown in images A1–A3. The scale bar shown in A2 is equivalent to 30 \( \mu \text{m} \) and applies to images A1–A3. The dashed rectangle indicates the area corresponding to the Raman intensity map of the 30 \( \mu \text{m} \times 30 \mu \text{m} \) section of the ice crystal shown in images B1–B4. The pseudocolors in B1–B4 represent the intensity of the Raman spectrum around the ice peak (640 nm) at each point. Dark areas correspond to water, while bright areas represent ice. The red circular dot in the center of the crystal in A2 indicates where the laser was pointed to collect each individual Raman spectrum shown in Fig. S4.
Fig. S4. Raman spectra of ice in MpAFP solution. (A) The Raman spectra were obtained for solutions near the ice crystal, at the center of the ice crystal at different temperatures, and after ice melted at 0.37 °C above the melting temperature (purple line).

Fig. S5. Freezing hysteresis (FH) activity for GFP-MpAFP: FH activity as a function of concentration is shown. These data were used in Fig. 4.

Fig. S6. Comparison of FH and melting hysteresis (MH) activity for Dendroides canadensis hemolymph. The measured MH values as a function of FH values at the corresponding concentrations are shown.
**Fig. S7.** Concentration dependence of MH activity for spruce budworm AFP (sbwAFP). MH activity for sbwAFP as a function of concentration.

**Fig. S8.** Concentration dependence of MH activity for type I AFP. MH activity for type I AFP as a function of concentration.
Fig. S9. Schematic model of the surface curvature of ice under supercooling and superheating conditions with adsorbed AFPs. (A) Under supercooling conditions, the ice develops a positive slope. (B) Under superheating conditions, a negative slope is formed. Dots represent adsorbed AFPs on the ice surface. The slopes are defined as $\chi_{\text{engulfment}} = \tan(\theta_1)$ and $\chi_{\text{superheating}} = \tan(\theta_2)$. An illustration of the time evolution of the surface curvature as a function of temperature is presented in Movie S1.

Movie S1. Illustration of surface curvature as a function of temperature. AFPs adsorb to the ice surface (red semicircles), causing ice growth or melting to occur along curved fronts in which the curvature increases as a function of supercooling or superheating. When the sample is supercooled, these ice fronts are concave, whereas they become convex when the sample is superheated.

Movie S1 (WMV)
Movie S2. FH and MH experiment in a drop of MpAFP solution. A movie of the experiment described in Fig. 1.

Movie S2 (WMV)

Movie S3. Melting experiment on a group of ice crystals. Ice crystals a solution containing the hyperactive AFP from Tenebrio molitor were examined in terms of their melting behavior in the same droplet while the temperature was slowly increased. The movie emphasizes the fast melting rates of the ice crystals above the $T_m (= -0.05 \, ^\circ\text{C})$. The last ice crystal melts at 0.18 °C above the $T_m$. The temperature of the sample is noted (Upper Left), and the real time during measurement is presented (Lower Left).

Movie S3 (WMV)