Partitioning of Fish and Insect Antifreeze Proteins into Ice Suggests They Bind with Comparable Affinity†

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ABSTRACT: Antifreeze proteins (AFPs) inhibit the growth of ice by binding to the surface of ice crystals, preventing the addition of water molecules to cause a local depression of the freezing point. AFPs from insects are much more effective at depressing the freezing point than fish AFPs. Here, we have investigated the possibility that insect AFPs bind more avidly to ice than fish AFPs. Because it is not possible to directly measure the affinity of an AFP for ice, we have assessed binding indirectly by examining the partitioning of proteins into a slowly growing ice hemisphere. AFP molecules adsorbed to the surface and became incorporated into the ice as they were overgrown. Solutes, including non-AFPs, were very efficiently excluded from ice, whereas AFPs became incorporated into ice at a concentration roughly equal to that of the original solution, and this was independent of the AFP concentration in the range (submillimolar) tested. Despite their >10-fold difference in antifreeze activity, fish and insect AFPs partitioned into ice to a similar degree, suggesting that insect AFPs do not bind to ice with appreciably higher affinity. Additionally, we have demonstrated that steric mutations on the ice binding surface that decrease the antifreeze activity of an AFP also reduce its inclusion into ice, supporting the validity of using partitioning measurements to assess a protein's affinity for ice.

Many organisms, including insects, plants, bacteria, and polar fish that survive in cold climates, produce antifreeze proteins (AFPs)[1] in response to the challenge of preventing or better tolerating freezing (*1*). AFPs from different species are highly diverse in sequence and structure but act through a common mechanism. AFPs adsorb to the surface of ice crystals, inhibiting the addition of water molecules to the ice lattice by the Kelvin effect (*2*), thereby lowering the freezing temperature below the melting temperature (*3*). The amount that the nonequilibrium freezing temperature is depressed (C°) below the melting temperature is referred to as thermal hysteresis (TH) activity.

AFP concentrations in the micromolar to millimolar range are effective at inhibiting the growth of ice because the AFP adsorbs to the ice at a sufficiently high density to realize the Kelvin effect. At the lower end of this concentration range, AFPs will bind the ice but do so at a lower density that allows the formation of a large radius of curvature between bound AFP molecules. This coverage is not sufficient to prevent the addition of more water molecules to the ice surface as the solution becomes undercooled. Under these conditions, AFPs are overgrown and become included into the ice. In contrast, other solutes (including macromolecules such as bovine serum albumin) are excluded from ice and become concentrated in the liquid phase (*3*).

AFPs bind to specific crystallographic planes of ice, sometimes in preferred orientations (*4*) that appear to be determined by the surface−surface complementarity of the protein's ice binding face and the structural features of the particular plane of the ice lattice (*5*). Knight et al. (*4*) exploited the inclusion of AFPs into ice to determine these binding planes. A single, oriented ice crystal was grown into a hemisphere in the presence of a low concentration of AFP, and the hemisphere was then allowed to sublime. The sites where protein binding occurred were revealed as opaque patches on the ice surface, termed an ice etch. Different AFP types adsorb to different planes of ice and hence produce distinct etch patterns (*6*).

We have recently applied a modified ice hemisphere method to the purification of fish type III AFP (ice affinity purification, IAP) based on its affinity for ice (*7*). Because binding planes were not being determined, it was not necessary to grow the hemisphere as a single crystal, a technique that is time-consuming and challenging. Instead, to promote the inclusion of AFP molecules over the entire ice surface, a coldfinger was nucleated with multi-crystalline ice, which was then grown into a hemisphere in a dilute solution of protein. This slowly growing ice efficiently excluded solutes and non-AFPs, while the AFP adsorbed and became included. This differential partitioning allowed the AFP to be purified away from other contaminants. Using

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[1] Abbreviations: AFP, antifreeze protein; Cf, *Choristoneura fumiferana*; FPLC, fast-performance liquid chromatography; HPLC, high-performance liquid chromatography; IAP, ice affinity purification; PI, partitioning index; TH, thermal hysteresis; Tm, *Tenebrio molitor*; wt, wild-type.

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two successive rounds of IAP, recombinantly expressed fish type III AFP was purified to homogeneity from *Escherichia coli* cell lysates, and type III AFP was also successfully used as an affinity tag for the purification of a larger protein, *E. coli* maltose binding protein (MBP) (*7*).

Although IAP was effective for the isolation of this fish AFP, the yield of AFP captured in the ice was lower than would be expected for an affinity-based technique. Insect AFPs have 10−100-fold higher TH activity than those from fish (*8*−*10*) when compared on a molar basis. It is conceivable that they could have a higher affinity for ice and thus might partition into ice more efficiently than the type III AFP. The ice binding faces of the characterized insect AFPs are larger, more highly complementary to their binding planes (*10*, *11*), and more ordered (*12*) than those of fish, which may offer a mechanistic rationale for enhanced ice binding. On the other hand, it has been argued that to achieve TH activity, all AFPs must bind irreversibly to ice because any significant off-rate would allow water to join the ice lattice (*13*). This implies that insect AFPs cannot have higher affinity for ice than those of fish. In the absence of a viable method to measure the affinity of an AFP for ice, we have assessed this indirectly by examining how proteins partition between the liquid and the solid (ice) fractions during the growth of a multicrystalline ice hemisphere.

In the current series of experiments, we have compared the partitioning of previously purified AFPs from two fish and two insect species to investigate the basis of the hyperactivity of insect AFPs. To further explore how partitioning relates to antifreeze activity, and to validate using partitioning to assess an affinity for ice, we have examined the partitioning into ice of AFPs with steric mutations that reduce TH activity.

MATERIALS AND METHODS

Preparation of AFPs and Controls. Four different AFP types (two from insects and two from fish) were used. The insect AFP from spruce budworm, *Choristoneura fumiferana* (CfAFP; isoform 337) (*14*), was expressed in *E. coli*, refolded from inclusion bodies, and purified on an *S*-Sepharose FPLC column, followed by reversed-phase HPLC on a C18 column (*15*). Recombinant AFP from the meal worm beetle, *Tenebrio molitor* (TmAFP; isoform 4−9) (*16*), was expressed in *E. coli* and allowed to refold and oxidize in the cellular lysate until the TH activity stabilized, at which point oxidized glutathione was added. The protein was then purified by size-exclusion chromatography on Sephadex G-75, followed by reversed-phase HPLC (C-18) (*16*, *17*). HPLC-pure recombinant TmAFP contains some misfolded protein, which is difficult to remove by chromatography. To select for well-folded protein, IAP was used for the final purification of this material. The TmAFP mutant T41L was purified identically to the wild-type. The mutants, T41Y and T41K, were purified by two successive rounds of IAP, followed by HPLC. The characterization of these mutants has been described previously (*16*).

Fish type II AFP was enriched from sea raven serum by two successive rounds of size-exclusion chromatography on Sephadex G-75 (*18*) followed by final purification by IAP. The fish type III AFP used was M1.1, a variant derived from the ocean pout QAE isoform HPLC 12 (*19*). The protein was expressed in *E. coli*, recovered from the inclusion bodies, refolded, and purified by FPLC using an *S*-Sepharose column (*19*). Control samples that lacked antifreeze activity were myoglobin (type I from equine skeletal muscle), bovine serum albumin (BSA, fraction V, 96−99% pure), the glycoprotein fetuin (prepared by ammonium sulfate fractionation), and blue dextran (MW, 2000 kDa), purchased from Sigma Chemical Company (St. Louis, MO). All proteins and the blue dextran were lyophilized or obtained in powder form and dissolved in 20 mM ammonium bicarbonate for partitioning experiments.

Ice Hemisphere Growth and Analysis of Partitioning. IAP was performed by growing polycrystalline ice from a coldfinger, through which a cold ethylene glycol solution was circulated by a temperature programmable water bath (Neslab) (*7*). The coldfinger was seeded with a thin layer of ice (∼1 mm) by nucleating it with cold distilled water containing ice, and it was then immersed into 50 mL of prechilled (0−1 °C) AFP or control solution in an insulated 100 mL beaker. While gently mixing the solution with a magnetic stir bar, the temperature of the coldfinger was gradually lowered at a linear rate (usually −0.5 to −2.5 °C over 20 h) to promote the freezing of ∼1/2 to 2/3 of the volume into a hemisphere (Figure 1).

At the end of this period, the coldfinger and attached hemisphere were removed from the liquid, and the ice surface was allowed to melt for ∼10−15 min to remove any protein (in ∼1−2 mL) that was nonspecifically associated with the surface film of liquid. The ice hemisphere was then detached from the coldfinger, and the ice was melted. The volumes of the ice and liquid fractions were measured, and each was analyzed for optical absorbance, conductivity, and TH activity. Absorbance was measured over a spectrum of wavelengths (200−420 nm for proteins and 500−800 nm for blue dextran) using a Shimadzu UV-1601 spectrophotometer.

Conductivity was measured using a Radiometer CDM 2d conductivity meter (Copenhagen). Thermal hysteresis was measured using a Clifton nanoliter osmometer, as described previously (*20*). It should be noted that the exclusion of solutes from ice is so efficient that the ice fraction frequently loses buffering capacity. If the AFP is prone to aggregation or denaturation at low ionic strength or neutral pH, it may be desirable to melt the ice into a small volume of concentrated buffer. This was not necessary with the proteins used here.

Based on the absorbance at 280 nm of the ice and liquid fractions, a partitioning index (PI) was calculated to assess the degree of inclusion of proteins into ice for each sample. (Absorbance at 620 nm was used for blue dextran.) This partitioning index was calculated as (fraction of protein in ice)/(fraction of water in ice) to allow for the variable extent of ice growth. Quantification of protein was based on approximate extinction coefficients for each protein, which cancel out in the calculation of the fraction of protein in ice and thus do not affect the index. PIs were determined twice for each solution, and the means with ranges are presented.

RESULTS

Non-AFPs and Solutes Are Very Effectively Excluded from Ice. To provide a baseline against which the partitioning of

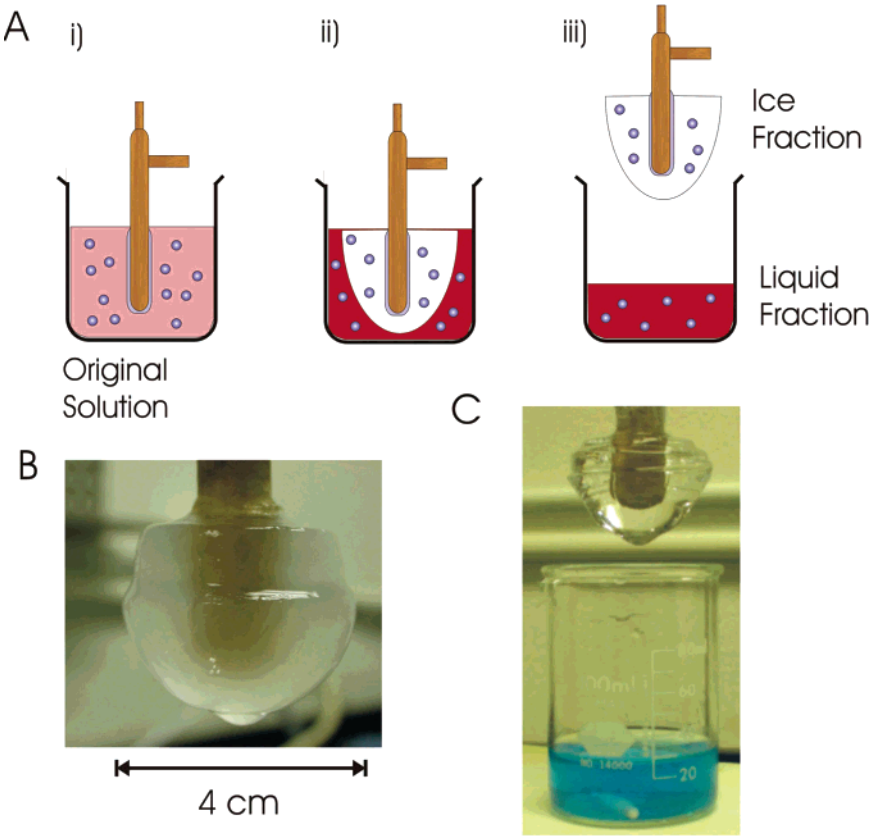

FIGURE 1: Partitioning apparatus and method. (A) Ice affinity purification: (i) the brass coldfinger is seeded with ice and immersed into the protein solution; here myoglobin is represented in red, and AFP molecules are shown as dots. (ii) The temperature of the coldfinger is lowered to promote the growth of an ice hemisphere, from which myoglobin is excluded, but into which AFPs are incorporated. (iii) The coldfinger and ice hemisphere are removed from the liquid and can be melted to recover the AFP. Myoglobin is concentrated in the liquid fraction. (B) Photograph of the ice hemisphere produced from a 0.1 mg/mL solution of CfAFP demonstrating the typical cloudiness of ice grown in the presence of AFPs. (C) Photograph of the ice and liquid fractions following hemisphere growth in the presence of 0.4 mg/mL blue dextran illustrating the exclusion of non-AFP molecules from the ice hemisphere and the clarity of the ice when grown without AFPs present.

Table 1: Partitioning of AFPs and Non-AFPs into Ice[a]

protein	$A_{280\ original}$	$A_{280\ ice}$	$G_{original}$ (μmho)	G_{ice} (μmho)	PI
myoglobin	0.214 ± .021	0.006 ± .004	1400 ± 100	25 ± 10	0.028 ± .019
BSA	0.162 ± .007	0.002 ± .001	1300 ± 500	15 ± 2	0.010 ± .004
fetuin	0.127 ± .013	0.006 ± .006	2700 ± 200	60 ± 20	0.05 ± .05
type III AFP	0.171 ± .033	0.161 ± .010	1300 ± 100	255 ± 15	0.94 ± .02
Cf AFP	0.112 ± .011	0.157 ± .003	1500 ± 0	175 ± 5	1.248 ± .055
carbohydrate	$A_{620\ original}$	$A_{620\ ice}$	$G_{original}$ (μmho)	G_{ice} (μmho)	PI
blue dextran	0.187 ± .002	0.001 ± 0	2700 ± 200	32 ± 14	0.0055 ± .0005

[a] Absorbance (280 or 620 nm) and conductivity (μmho) of the original and ice fractions for the partitioning experiments of ~0.2 mg/mL myoglobin, bovine serum albumin (BSA), fetuin, type III AFP, spruce budworm (Cf) AFP, and ~0.4 mg/mL blue dextran. For each sample, a partitioning index (PI) was calculated as described in the Materials and Methods. Values are averages of two experiments with ranges.

AFPs could be compared, we first determined how efficiently a slowly growing ice mass excludes nonantifreeze macromolecules including two proteins, a glycoprotein, and blue dextran. Ice hemispheres were grown from solutions of 0.2 mg/mL myoglobin, BSA, and fetuin and 0.4 mg/mL blue dextran. After the ice had grown to ~1/2 to 2/3 of the total volume, it was melted, and the absorbance and conductivity of the ice and liquid fractions were measured. On the basis of the absorbance at 280 nm, a partitioning index (PI) was calculated as (fraction of protein in ice)/(fraction of water in ice). A protein that partitions evenly between liquid and ice, producing an equal concentration in both fractions, would give an index of 1.0, while the index of a protein that is excluded from ice would be near zero.

The absorbance and conductivity of the ice fraction demonstrate very efficient exclusion of the nonantifreeze macromolecules, as well as the buffer, from the ice fraction (Table 1). For the control proteins myoglobin and BSA, the ice fraction contained <0.5% of the protein accumulated in the liquid fraction, with resulting PIs of 0.03 and 0.01, respectively. Similarly, the conductivity of the ice fraction was 170−200-fold lower than that of the liquid and 50−100-times lower as compared to the original sample. The glycoprotein fetuin and the polysaccharide blue dextran

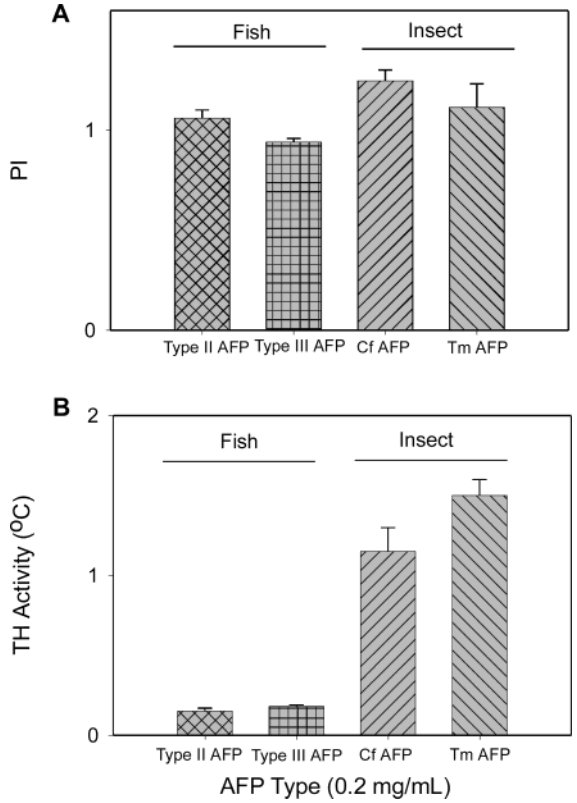

FIGURE 2: Partitioning indices (PI) and thermal hysteresis (TH) of insect vs fish AFPs. (A) PI values {calculated as (fraction of protein in ice)/(fraction of water in ice) as described in the Materials and Methods} for fish and insect AFPs: type II (sea raven) 0.16 mg/mL, type III (ocean pout) 0.2 mg/mL, Cf (*Choristoneura fumiferana*) 0.2 mg/mL, and Tm (*Tenebrio molitor*) 0.07 mg/mL. (B) TH activities of each of these proteins at 0.2 mg/mL.

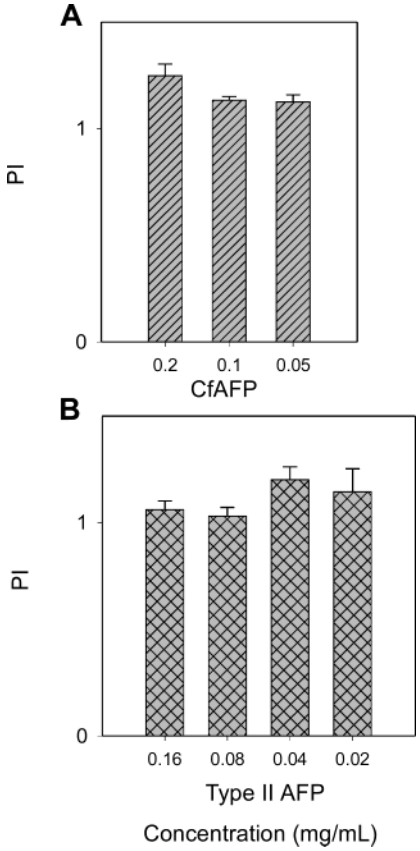

FIGURE 3: Effect of AFP concentration on the partitioning indices (PI) for (A) CfAFP (0.2−0.05 mg/mL) and (B) type II (sea raven) AFP (0.16−0.02 mg/mL).

behaved similarly to the protein controls, exhibiting PI values of 0.05 and 0.006, respectively. The ice grown from these control solutions was crystal clear and smooth (Figure 1C). Myoglobin and blue dextran were particularly useful controls because their colors gave a visual demonstration of their exclusion from ice and concentration in the liquid fraction (Figure 1C).

Fish and Insect AFPs Partition into Ice to a Similar Degree. In contrast to the non-AFP control proteins, a substantial portion of purified type III AFP at 0.2 mg/mL was included in the ice fraction, which contained about the same protein concentration as the original solution, producing a PI value of 0.94 ± 0.02 (Table 1). Although the conductivity of the ice fraction produced with type III was very low, it was almost 10-fold higher than the values for the non-AFPs. This could be due to the trapping of counterions with the AFPs and/or the nonspecific inclusion of ions due to the structuring of the ice by the AFP. The latter is supported by the observation that the ice grown in the presence of AFP was typically cloudy with a surface that appeared roughened (Figure 1B). Hemispheres were also grown with a second fish AFP (type II from sea raven), resulting in a similar PI value of 1.06 ± 0.04 (Figure 2A).

The insect AFPs that have been well characterized to date are 10−100 times more active in TH than fish AFPs (*8*−*10*). To investigate whether the drastic difference in the antifreeze activity of fish versus insect AFPs is reflected in the partitioning of these proteins, hemispheres were also grown with two insect AFPs (Cf and Tm). The PIs for CfAFP and TmAFP were 1.25 ± 0.05 and 1.12 ± 0.12, respectively, indicating that there was a modest preferential accumulation in the ice fraction. The PIs for the hyperactive insect AFPs were on average ∼18% higher than the average PI for the fish AFPs (Figure 2A). This difference is small as compared to the many-fold difference in TH activity measured at the same [AFP] used in the partitioning experiments (Figure 2B).

Partitioning of AFPs between Ice and Liquid Fractions Is Independent of AFP Concentration. Under the conditions of the previous experiment, the PI values for all four AFPs were close to one, indicating that each AFP partitions approximately evenly between the ice and the liquid fractions. We wanted to see if reducing the AFP concentration led to an increase in PI values. At lower AFP concentrations, the same number of AFP binding sites would be available to fewer AFP molecules, which might allow the adsorption of a larger proportion of the AFP present. Hemispheres were grown from CfAFP at 1/2 and 1/4 of the previous concentration, but all three concentrations resulted in similar PIs of 1.25 ± 0.05, 1.13 ± 0.02, and 1.13 ± 0.03 (Figure 3A), indicating that, over the examined range (0.2−0.05 mg/mL), ice partitioning is largely independent of protein concentration. We repeated this analysis with a fish AFP (type II), which was examined over an 8-fold concentration range, and like insect AFP, it partitioned independently of concentration (Figure 3B) with PIs of 1.06 ± 0.04, 1.03 ± 0.04, 1.20 ± 0.06, and 1.15 ± 0.11 for the dilution series. In these dilution series, the difference in PI of the fish and the insect AFPs was even smaller than that observed in Figure 2A.

Mutations that Reduce TH Activity Also Decrease Partitioning into Ice. The introduction of large side chains into

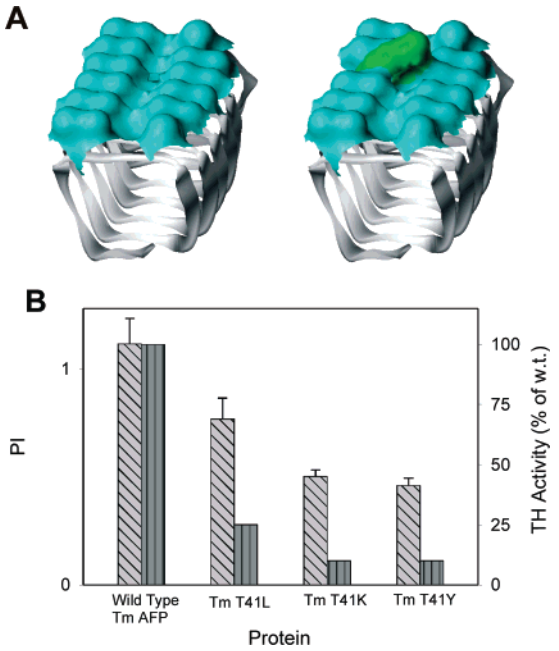

FIGURE 4: Effect of steric mutations on the partitioning index (PI) and thermal hysteresis (TH) of TmAFP. (A) Models of wild-type TmAFP (left) and the mutation T41Y (right) with the protein backbone shown as a gray ribbon and the surface of the Thr-rich ice binding face in cyan. The substituted Tyr residue is green. (B) PI (diagonal lines, left) and TH expressed as a percentage of wild-type TmAFP activity (vertical lines, right) for TmAFP and three steric mutations. Partitioning experiments were carried out at 0.07 mg/mL for wild-type, 0.10 mg/mL for T41L, and 0.22 and 0.23 mg/mL for T41K and T41Y, respectively. TH was compared at 1 mg/mL.

an AFP's ice binding site by site-directed mutagenesis significantly disrupts TH activity, likely because the steric mutation spoils binding to the ice surface. In TmAFP, for example, Thr 41 has a central location in the Thr-rich ice binding site (Figure 4A) (*16*). Three steric mutations of this residue that reduce the TH activity of TmAFP were tested for their effect on partitioning. The mutation T41L, which resulted in a 75% loss of TH activity (*16*), decreased the PI by 30% (Figure 4B). As well, the mutations T41K and T41Y, both of which reduced TH activity to about 10% of the wild-type levels, decreased the PI to 40 and 45% of the wild-type values.

DISCUSSION

To investigate whether the hyperactivity of insect AFPs is due to increased binding to ice, we have used a modified ice hemisphere technique to measure the partitioning into ice of AFPs from insects and fish. To establish a control against which the partitioning behavior of AFPs could be compared, we initially determined how several non-AFP controls partition under our ice growth conditions. The proteins myoglobin and BSA were very efficiently excluded from the slowly growing ice hemisphere, with partitioning indices of ≤ 0.03. The exclusion of every *E. coli* protein from the hemisphere during ice affinity purification of recombinant AFPs from cell lysates (*7*) demonstrates that exclusion from ice is a general property of non-AFPs, even though only two were examined as purified proteins here. We also investigated the partitioning of two other control macromolecules: the glycoprotein fetuin and the polysaccharide blue dextran, which were also efficiently excluded from ice. Like macromolecules, small molecule solutes were also well excluded from ice, as demonstrated by the low conductivity of the ice fractions. In contrast, all four AFPs were included in the ice at a concentration equal to or slightly higher than the original concentration.

Effect of Concentration. After the initial experiments in which the PIs of fish and insect AFPs were compared and found to be similar, we considered the theoretical possibility that this reflected strong binding at few available sites for one AFP (insect) versus weak binding at many sites for the other AFP (fish). Therefore, partitioning of Cf and type II AFPs were investigated over 4- and 8-fold concentration ranges, respectively. (It should be noted that the range that can be experimentally assayed is limited. If AFPs are too concentrated, the growth of the ice hemisphere is inhibited, but low concentrations cannot be assayed spectrophotometrically due to the paucity of aromatic residues in these AFPs.) There appears to be little or no competition for binding sites on the ice surface at the AFP concentrations tested since the PIs did not change appreciatively with dilution. Indeed, the large growing ice mass would be expected to present an almost infinite number of potential docking sites on a surface that is constantly being renewed. The concentration independence also supports the now generally accepted view that these AFPs bind to ice independently (*21*).

Partitioning and the Affinity of AFPs for Ice. Insect AFPs are 1−2 orders of magnitude more active than AFPs from fish (*8*−*10*). Despite this huge difference in TH activity, the PI values for insect AFPs are only slightly higher than those obtained with fish AFPs. Thus, insect AFPs become incorporated into a growing ice mass only marginally more extensively than fish AFPs, suggesting again that insect AFPs do not bind to ice more readily, nor do they bind more tightly. Indeed, if AFPs must bind ice irreversibly to effectively inhibit ice growth (*13*), logically insect AFPs cannot bind ice more tightly than fish AFPs.

We have recently proposed that the superior thermal hysteresis of insect AFPs does not reflect a difference in their affinity for ice but rather better coverage of the growth-susceptible surfaces on the ice crystal (*22*). In the presence of fish AFPs, ice crystals form hexagonal bipyramids that are susceptible to growth and bursting from the vertexes, along the *c*-axis. In contrast, ice etching showed that CfAFP binds to both the prism and the basal planes of ice (*10*), and modeling of TmAFP docking to ice suggests that this AFP may also bind to these two planes (*11*). By providing a more complete coverage of ice crystals, insect AFPs are more effective than fish AFPs at inhibiting ice growth. Thus, the slightly higher PIs for insect AFPs may simply reflect the greater likelihood that these proteins will encounter a potential binding plane (basal or prism) on the multi-crystalline ice surface.

Although fish and insect AFPs may bind ice with similar avidity, it is possible to spoil this interaction by disrupting the ice binding site with steric mutations. When T41, in the middle of the TmAFP ice binding face, was replaced with the bulkier amino acids Leu, Lys, or Tyr, these steric mutations reduced the PI down to about 1/2 to 2/3 of what it is with the wild-type. This reduction of PI with mutation helps validate the idea that wild-type fish and insect AFPs, which have comparable PIs, do indeed have similar binding

affinities for ice. We suggest that these mutants either reduced the probability of ice binding, or increased the probability of unseating, resulting in partitioning properties intermediate between wild-type AFPs and non-AFPs. When assessed by TH activity, the effect of the mutations were more severe. However, the phenomenon of TH requires that the entire surface of the supercooled ice crystal be protected from growth. The unseating or overgrowth of an AFP molecule at any one of a myriad of binding sites on the crystal could present a surface from which ice growth can occur and bring TH to an end. Thus, a defect in the ice binding face might be expected to have a greater effect on an AFP's TH activity than its partitioning into ice.

Single versus Multiple Crystalline Ice Hemispheres. All of the partitioning experiments reported here were performed with a random multi-crystalline hemisphere. The coldfinger was seeded with chilled water containing ice to generate multiple nucleation sites giving rise to many separate ice crystals in different orientations. Upon transferring this seeded coldfinger to the AFP solution, AFPs that make contact with their preferred binding plane on the ice surface would adsorb. Since AFP binding impedes ice growth, this would likely influence the distribution of ice planes found at the surface as the hemisphere grows. AFP-inhibited supercooled ice crystals viewed in a nanoliter osmometer express the binding planes of the AFP (e.g., ref *23*); thus, it is possible that the presence of an AFP would bias the hemisphere so that more of that AFP's binding plane would be exposed. Alternatively, in a multi-crystalline ice mass, the inhibited plane could be overgrown and covered by other, more rapidly growing ice crystals. As a result, the partitioning of an AFP may be slightly different for multi-crystalline ice versus single-crystal hemispheres.

Multi-crystalline hemispheres were chosen in these experiments for simplicity. Single ice crystals are extremely difficult and time-consuming to produce and mount on the coldfinger, and the considerable volume of the initial seed crystal would dilute the ice fraction upon melting. Because IAP is also performed with multi-crystalline ice, the results of this study can be applied to optimizing this purification method.

Why Do AFPs Partition Evenly between Ice and Liquid? Each of the wild-type AFPs partitioned approximately evenly between the ice and the liquid phases following multi-crystalline hemisphere growth. On the basis of an irreversible binding model, it might be expected that as the hemisphere grows, AFPs would be continually adsorbed to the constantly renewing ice surface until virtually all of the AFP molecules have been incorporated, particularly at low AFP concentrations. One explanation for even partitioning could be that the quasi-liquid layer of partially ordered water molecules at the ice/liquid interface acts as a barrier to the diffusion of AFPs. A quasi-liquid layer has been observed at the ice/air, ice/vacuum, and ice/solid interfaces using a number of independent experimental techniques, and the surface properties of ice have become an important field of study in ice physics (reviewed in ref *24*). While the ice/water interface is less accessible to spectroscopic techniques and has not been studied as extensively, an interfacial layer with unique properties has been observed experimentally between the liquid and the solid phases of water using ellipsometry (*25*). The ice/liquid interface has also been simulated in computer models using a number of different methods (e.g., refs *26*−*28*). Hayward and Haymet's models (*27*, *28*) of the ice/liquid interfacial layer demonstrate that successive layers of water molecules become more ordered and oriented and more closely resemble ice as the layer that forms the surface of the ice lattice is approached. The rate of diffusion of water molecules was found to steadily decrease and approach 0 with proximity to the ice lattice. The rate of diffusion of an AFP molecule would steadily decrease as it approaches the ice lattice and may be drastically reduced before the AFP encounters a layer with properties resembling ice strongly enough to provide a binding site. Thus, AFP molecules circulating in the liquid may have difficulty traversing the quasi-liquid layer to encounter a binding site. However, as the hemisphere grows, water in the quasi-liquid layer joins the ice, trapping any AFPs there. The boundary of the partially ordered water advances in concert with the ice front to engulf any AFPs that are encountered. Thus, the concentration of AFPs in ice equals that in the layer of partially ordered water, which is in turn the same as the AFP concentration in solution, explaining the observation of even partitioning, regardless of AFP concentration and type. It would be interesting to examine whether much slower rates of ice growth promote greater inclusion of AFPs into ice, potentially by allowing more diffusion of AFPs into the quasi-liquid layer of water.

During the review of this paper, an alternative hypothesis was put forward to explain why AFPs partition evenly between ice and liquid. It was proposed that partitioning into ice is determined by the rate of adsorption of AFP together with the growth rate of the ice. The growth rate of the ice, however, is dependent on the concentration of AFP at the interface, which is in turn modulated as AFP is engulfed by ice. Thus, there is potentially a feedback between partitioning and growth rate, which could lead to the establishment of a steady state in which the concentration of AFP engulfed in the ice is equal to that in the bulk liquid. When the seeded coldfinger is introduced into an AFP solution of uniform, low concentration, the ice would initially grow relatively rapidly. Because AFP molecules have little time to adsorb to a rapidly growing ice surface, AFP molecules would tend to be excluded from ice and accumulate on the liquid side of the interface, establishing a high local AFP concentration that would subsequently begin to inhibit ice growth. The increased AFP concentration and decreased growth rate would promote the inclusion of more AFP into ice until a balance is reached, leading to a steady (or oscillating) state in which AFPs tend to partition evenly between the ice and the liquid.

Regardless of whether the even partitioning of AFPs between ice and liquid is caused by this type of steady-state equilibrium or by limited access to the ice surface through the quasi-liquid layer, the similar partitioning of fish and insect AFPs strongly suggests that they bind with similar affinity.

Other Applications. The exclusion of non-AFPs such as *E. coli* proteins (*7*), BSA, and myoglobin from ice suggests that IAP could find practical application for the concentration of non-AFPs in the liquid fraction, by effectively removing water into an ice hemisphere. This could be used to concentrate proteins that may adsorb to concentrator membranes or large proteins that cannot be lyophilized. The

advantages of this approach are that small-molecule cofactors and volatile compounds would not be lost, labile macromolecules would be protected as the procedure is done at ice temperature (~0 °C), and the method should prove to be scalable to industrial size volumes. The success of IAP suggests that it may be feasible to develop analogous methods to select macromolecules with affinity for other crystalline substances. For example, it may be possible to purify biomineralization proteins by growing calcium carbonate or hydroxyapatite crystals in the presence of tissue extracts or peptide libraries.

Conclusions. In conclusion, the two insect AFPs examined here partitioned into ice to a similar, albeit slightly greater degree than the two fish AFPs, suggesting that the hyperactivity of insect AFPs is not the result of markedly superior ice binding activity. The ability of insect AFPs to bind more than one plane of ice could explain their efficient partitioning into multi-crystalline ice and is likely the basis of their exceptional TH activity. All of the wild-type AFPs partitioned approximately evenly between ice and liquid, which is consistent with a model of irreversible binding where access to ice is limited by a quasi-liquid barrier to diffusion. Steric mutations that decrease TH activity also caused a reduction in the PI, which reinforces the idea that the steric residues interfere with ice binding and validates the concept that PI values reflect affinity for ice.

The growth of ice hemispheres has already been shown to be useful for the purification of antifreeze proteins (IAP), and the determination of binding planes (ice etching). Here, we have described a third application of ice hemispheres in which the partitioning of pure proteins is quantified. We propose that this technique can provide insight into the mechanisms of AFP activity and will prove to be useful for characterizing novel ice-active proteins. For example, it will be interesting to determine whether plant or bacterial AFPs that exhibit weak TH activity but potent ice recrystallization inhibition will partition into ice as efficiently as the fish and insect AFPs.

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