Adsorption of α-helical antifreeze peptides on specific ice crystal surface planes

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ABSTRACT The noncolligative peptide and glycopeptide antifreezes found in some cold-water fish act by binding to the ice surface and preventing crystal growth, not by altering the equilibrium freezing point of the water. A simple crystal growth and etching technique allows determination of the crystallographic planes where the binding occurs. In the case of elongated molecules, such as the α-helical peptides in this report, it also allows a deduction of the molecular alignment on the ice surface. The structurally similar antifreeze peptides from winter flounder (Pseudopleuronectes americanus) and Alaskan plaice (Pleuronectes quadritaberulatus) adsorb onto the [2021] pyramidal planes of ice, whereas the sculpin (Myoxocephalus scorpius) peptide adsorbs on [2110], the secondary prism planes. All three are probably aligned along (0112). These antifreeze peptides have 11–amino acid sequence repeats ending with a polar residue, and each repeat constitutes a distance of 16.5 Å along the helix, which nearly matches the 16.7 Å repeat spacing along (0112) in ice. This structural match is undoubtedly important, but the mechanism of binding is not yet clear. The suggested mechanism of growth inhibition operates through the influence of local surface curvature upon melting point and results in complete inhibition of the crystal growth even though individual antifreeze molecules bind at only one interface orientation.

INTRODUCTION

Polar and many northern fishes inhabit sea water at its freezing point, −1.9°C. To avoid freezing, these fish synthesize antifreeze glycopeptides (AFGP) or antifreeze peptides (AFP) in their livers, and secrete them into the blood where the concentration can reach as high as 35 mg/ml. Because the antifreezes (AF) have molecular weights typically between 3,000 and 30,000 D, the maximum concentration is roughly 0.01–0.001 M. They do not appreciably affect the equilibrium freezing point of the fish serum, which is −1°C, the melting temperature of ice crystals in fish serum, but they do prevent ice crystal growth down to −2.2°C or below, which is substantially lower than the temperature of ice-laden seawater (DeVries, 1982, 1988). The separation of the equilibrium freezing point and the temperature of ice crystal growth is referred to as freezing point hysteresis. The details of the mechanism of the antifreeze effect are unknown, though it is known that adsorption of AF molecules to the ice is involved (Raymond and DeVries, 1977).

Impurity effects on crystal growth rate are commonplace, but that by the antifreezes on ice is unprecedented. The antifreezes act on crystal growth from the melt, and they do not just retard ice growth, but stop it completely over a supercooling temperature range that is a function of their concentration in solution. DeVries and Lin (1977) showed that a polycrystalline ice seed could be kept for many days in a 10 mg/ml aqueous solution of purified AFGP, supercooled by 0.6°C, without detectable change in shape or size. At this supercooling, ice-growth rate in pure water or an ordinary solution is ~1 mm min−1 (Hobbs, 1974). Harrison et al. (1987) measured free growth rate of ice in uniformly supercooled AFGP solution (5 mg/ml), and found it to be zero until a threshold supercooling is reached (i.e., the nonequilibrium freezing point), at which temperature the growth rate increased very suddenly. More recently, using large single ice crystals, Raymond et al. (1989) observed that within the hysteresis gap, antifreeze (0.5–20 mg/ml) inhibited growth on the prism faces, but allowed some growth on the basal plane leading to the formation of hexagonal pits as growth proceeded. Ice growth completely stopped when the basal plane became fully pitted or when the crystal assumed the shape of a hexagonal bipyramid.

The inhibition of ice crystal growth by these antifreezes does not involve the same kind of kinetic effects involved in ordinary chemical reactions, which are controlled by the activation energy of breaking or making individual chemical bonds. The existence of a very sharp, rate threshold (the nonequilibrium freezing point) suggests that the process is much more like crystal nucleation, in which the energy barrier is related to creating new surface and involves tens or even hundreds or more
of molecules. Accordingly, it has been suggested that the antifreeze effect is due to the AF molecules blocking step growth on the ice surface (Raymond and DeVries, 1977) or blocking growth normal to the surface (Knight and DeVries, 1989). In both of these mechanisms, the energy barrier for growth is analogous to that for nucleation and would produce a growth rate threshold like that observed. Indeed, water molecules are so small and so mobile that the freezing inhibition must be an effect that operates this way in principle: an effect that constrains the ice surface so that “local” growth requires an increase in free energy, even though bulk growth would provide a decrease.

Adsorption of AF molecules to ice surfaces has been clearly demonstrated (Raymond and DeVries, 1977), but the particular ice faces involved in the adsorption have only been inferred, not identified. A match between the spacing of potential hydrogen-bonding side chains in some antifreezes and the water molecular spacing parallel to the a-axes in ice was used by DeVries and Lin (1977) and DeVries (1984) to suggest that they may bind to the primary prism faces, [1010]. Expression of [101x] faces on the growing ice crystal in the presence of AFGP (Knight et al., 1984; Raymond et al., 1989) and other AF (Raymond et al., 1989) has been observed, suggesting that these may be the adsorption surfaces. Yang et al. (1988) have reinforced this interpretation for the winter flounder AFP they studied, but their proposed crystallography of adsorption differs from the data presented below.

It is clear that understanding the crystallography of the adsorption is vital in the understanding of how AF molecules interact with ice to stop ice crystal growth. We have developed a simple method to determine the crystal planes of AF adsorption. The experimental design is to grow a single ice crystal in a dilute antifreeze solution into a large hemispherical single crystal, in such a way that all interface orientations are present during growth. The crystallography of the adsorption is then determined from measurement of the interface orientations at which antifreeze is incorporated into the growing crystal. Sufficiently low AF concentrations are used to allow virtually unretarded ice growth. The method was based upon the reasoning that the adsorption of antifreeze molecules to ice must be essentially permanent in order to inhibit ice growth completely. That is, there must be virtually no dynamic exchange between adsorbed molecules and those in solution, as desorption would allow supercooled water to join the ice lattice instantly, resulting in ice growth. Thus, when supercooling is sufficient to force growth on an adsorption plane, the adsorbed molecules must be incorporated in situ within the growing ice crystal, rather than being pushed along with the moving interface.

The detection of AF incorporation is by evaporation etching. Accumulation of the nonvolatile AF molecules left behind by evaporation results in visible etched regions on the ice surface. The orientations of these etched regions are then determined using a universal stage. We argue that the direction of alignment of the AF molecules on the adsorption plane can also be deduced in certain cases, as described for the AFP in this report.

The AFP from the winter flounder, Alaskan plaice, and short-horn sculpin are used in this study because they have been well characterized with respect to their primary and secondary structure. Based on circular dichroism they are largely α-helical (Raymond and DeVries, 1977; Hew et al., 1985; DeVries, unpublished data). Helical wheel constructions show them to be amphiphilic helices. The winter flounder AFP in crystalline form has been confirmed to be a single helix by x-ray crystallography (Yang et al., 1988).

**METHODS AND MATERIALS**

**Antifreeze peptides**

The antifreeze peptides from the winter flounder (*Pseudopleuronectes americanus*), Alaskan plaice (*Pleuronectes quadrituberculatus*), and short-horn sculpin (*Myxocephalus scorpius*) were purified from fish serum by Sephadex G75 gel filtration chromatography followed by reverse-phase HPLC, sequenced and characterized by the methods of Cheng and DeVries (1989).

**Preparation of single ice crystal seeds**

Seven liters of deionized and filtered water (resistivity 16–18 MΩ cm) were placed in a well insulated, open glass container (20 cm diameter, 25 cm height) and allowed to freeze slowly from the top in a constant temperature chamber at −1°C. A weighted, plastic container with a hole in its bottom had been placed in the glass container, to prevent the breaking of the glass or cracking of the ice during freezing. Volume expansion during ice formation pushed unfrozen water into the plastic container, compressing the air within. (This artifact also results in ice growth with few included air bubbles. The prevention of high pressure in the liquid and of the sudden pressure drops when the ice cracks minimizes bubble nucleation at the growth interface.) After two days, the ice slab formed on top was 3–4 cm thick, and usually contained a large, single crystal with a top surface area about half that of the ice slab and with its c-axis, [0001], vertical, as determined by crossed polaroids. Approximate orientation of the a-axes, (1120), was deter-

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1 The segregation coefficient (the partitioning between crystal and liquid) for these AF materials has not been measured quantitatively in the experimental conditions of the work to be described. Its orientation dependence will be the major result, but quantitatively it is expected to depend upon both crystal growth rate and concentration in solution.
mined by growing a little frost on the ice surface and observing the location of prism faces, which are parallel to the a-axes. Sections of ice ~2 cm in width were cut from this single ice crystal with a band saw, such that the cut surfaces were approximately parallel to a prism plane. When these sections were stored with the cut surfaces in contact, prism facets developed, and allowed accurate orientation with a reflecting goniometer. The oriented ice section was then cut into ~2 cm cubes that were used as seed crystals for the growth experiment.

Single ice crystal “hemisphere” growth experiment

The crystal growth apparatus (Fig. 1) consisted of a brass cold finger onto which an oriented, single-crystal seed was frozen. The ice seed was first hollowed out on one side using a warmed, rounded brass cylinder set onto the needle of a hypodermic syringe so that as it melted into the ice, the melt water could be drawn away. The hollowed-out seed crystal was then held in a special jig to maintain its orientation, and pressed onto the prewarmed cold finger (the coolant to it temporarily shut off). When enough melting had occurred to expel all the air, the coolant circulation (−6°C) was restarted, and the seed soon became frozen fast to the cold finger, with good thermal contact. Unless specified otherwise, the seed crystal was oriented with a prism face horizontal: that is, normal to the long axis of the cold finger.

The cold-finger-ice-crystal assembly was then dipped into an insulated, cylindrical container of dilute AFP solution (3 × 10⁻⁷ mg/ml for the winter flounder and Alaskan plaice AFP, 8 × 10⁻⁴ mg/ml for sculpin AFP) having an initial temperature ~+15°C. The seed first melted back and then grew in six hours into a single ice crystal “hemisphere” ~6 cm in diameter. (Actually, the ice surface was not hemispherical but quite flattened, because of temperature stratification in the solution.)

Etching and determination of the adsorption plane

The ice hemisphere was removed from the solution and transferred to a cold room at −10 to −15°C. The hemisphere surface was then carefully scraped with a sharp blade to remove the frozen surface film of antifreeze solution, and then left exposed in the cold room to etch by evaporation for several hours. (Without scraping, the etch pattern is often obscure.) During evaporation, the roughened surface of the ice hemisphere becomes mirror smooth, except where the AFP has been incorporated into the crystal. There, the surface becomes etched, appearing similar to finely ground glass, as a “matrix” of AFP molecules accumulates at the surface. That the etched regions do represent incorporation of AFP molecules has been directly confirmed by our previous work using radioactively-labeled antifreeze glycopeptides (Knight and DeVries, 1988), and by the slight growth retardation that occurs even at these low AF concentrations, which correlates perfectly with the etching, as illustrated in the following section. No etching is observed on similar ice hemispheres grown from either pure water or dilute solutions of ordinary solutes or nonantifreeze polypeptides. The orientations of the centers of the etched regions were determined with a universal stage, accurate within ~3° in any direction, and were identified as the adsorption planes.

The etch patterns of the curved ice surface were very obvious to the eye, but difficult to photograph adequately. To eliminate extraneous reflections, the back side of the ice was frozen to a flat-black surface. The rounded, etched surface was photographed using a ring light between it and the camera.

RESULTS

The adsorption planes

A photograph of the etch pattern on the surface of an ice hemisphere grown from winter flounder AFP is shown in Fig. 2a. There are two centrally located, oval etched regions, as well as two partial oval etched regions at both the right and left edges. Etched cross-sections through the ice hemisphere revealed AFP incorporation wherever the growth interface orientation had been the same as that of a surface etched region, except of course in the seed crystal. Fig. 2b shows a side view of another hemisphere grown from the same flounder AFP solution, this one with the c-axis parallel to the long axis of the cold finger and not scraped: the hemisphere was taken from the growth solution and photographed in the cold room at −15°C with no special treatment. The same interface orientations at which the etching occurs show incipient faceting, confirming the correspondence of the etching with growth retardation.

The winter flounder and Alaskan plaice AFP have nearly identical etch patterns, sketched in Fig. 3a from photographs, and Fig. 3b is a sketch of the etch pattern of sculpin AFP. These are the etch patterns on the curved surface of the ice, which has the flattened shape shown in Fig. 2b. Thus, the etched regions in Fig. 3 do not appear as they would on a true hemisphere. The patterns do have sixfold symmetry in orientation, but this is distorted in the views of Fig. 3, a and b.

The important relationship here is between the etching and the interface orientations with respect to the ice structure because it is orientation that determines the arrangement of the molecules at the ice surface. The orientations of the etched regions were determined with a universal stage and found to be low-index planes of ice, as sketched in Fig. 3, c and d. (Because the etched
regions are actually curved, the adsorption planes are identified as tangent at the centers of the etched regions. This is discussed more fully below.) The adsorption planes are the 12 equivalent [2021] bipyramidal planes for the flounder and plaice, and the six equivalent [2T10] secondary prism planes for the sculpin.

Fig. 4 is a standard stereographic projection of the traces of two of the 12 equivalent F/P (flounder/plaice) adsorption planes, namely (2021) and (2201), and one of the six equivalent S (sculpin) adsorption planes, namely (2T10), and their corresponding poles. Rough outlines of the etched regions are shown. The three adsorption planes intersect along [01T2], and other sets of adsorption planes for F/P and S could have been chosen to intersect along five of the other 11 equivalent directions of [01T2]. These are not shown in Fig. 4 for the purpose of clarity of illustration.

The etched regions are consistently elongated (Figs. 2, 3, a and b), and the elongation directions for both the F/P and S etched regions are similar. In fact, the normals to the axes of elongation measured in the adsorption planes (i.e., at the center of the etched regions) are found to be identical and within measurement uncertainty of a low-index direction. For the three etched regions selected for Fig. 4, this common direction is [0112], corresponding to the line of intersection of the three adsorption planes in Fig. 4. This direction is deduced to be the direction of alignment of the AFP molecules on the adsorption plane, for reasons discussed below.

Two topics have been simplified in this discussion for the sake of easier communication. First, the orientation measurements. An enlarged version of the petrologist’s universal stage was used, with the entire hemisphere mounted at the center. Two horizontal mutually perpendicular rotation axes allow tilts to be measured. To measure the winter flounder adsorption plane orientation, the hemisphere oriented as in Fig. 2 a was placed with the c-axis parallel to the N-S rotation axis of the stage, and a horizontal rod (also parallel to the c-axis) was positioned close to the top point of the hemisphere, tangent to the surface there. The centers of the two etched regions along the N-S axis were marked with a scribe, and the hemisphere was rotated about the E-W rotation axis until the horizontal rod was judged to be tangent at one of the marked points. That angle reading was noted, and the specimens were rotated to determine the angle of the tangent at the other adsorption plane. The sum of these two angles (0° is horizontal and deviations are noted as positive either way) is twice the deviation of the adsorption plane from (10T0). This procedure corrects for the c-axis not being exactly horizontal on the stage.

Four successive sets of measurements of the angle
difference between the tangents to two etched regions corresponding to the central pair in Fig. 3a, separated by rotating the hemisphere 180° on the stage, are (12°, 20°), (12, 20), (12, 21) = 32.3°; (17, 15), (18, 15), (19, 15) = 33.0°; (14, 20), (13, 21), (12, 19) = 33.0°; and (17, 15), (18, 14), (18, 13) = 31.7°. Each was read three times, and the average difference is 32.5°, which compares with 29.8° for the difference between the two low-index [2021] planes identified as the adsorption planes (using \(c = 7.3616 \text{ Å}\) and \(a = 4.5190 \text{ Å}\)). The otherwise lowest-index competing plane of this type would be [5053], for which the comparable angle is 35.4°. Thus, the specific conclusion does rest on the assumption that the adsorption plane has low indices. The measurement of the alignment directions (described later) follows similar procedures and is subject to a little more uncertainty, but is adequate as long as that direction also can be assumed to have low indices.
Second, there are more complex features of the etching than have been described. In both sculpin and winter flounder, etched cross-sections of the ice hemisphere reveal some planar etching features that appear to arise from small-scale facetting of the curved, growth interface. In fact, Fig. 2a shows some of these features at the etched, hemisphere surface. These have not been studied adequately, for lack of material. By analogy with studies of an AFGP (unpublished data), such features become much more marked at higher AF concentration, at which the growth interface becomes stepped at a macroscopic scale, rather than smoothly curved. These planar features appear to be parallel to the deduced adsorption planes. Yet another complexity left out of account here is the asymmetry of the individual etched regions; each etched patch is elongated, but the two ends are usually distinctly and consistently different. In fact, strictly, the centers of the etched regions can be slightly but consistently displaced from the low-index adsorption plane. The overall symmetry of the etch patterns that is consistently maintained, is the sixfold axis. These complexities are considered to have secondary significance in the framework of this paper, and they are neither well-understood nor well-studied as yet.

**DISCUSSION**

Two basic variables in this experiment are the AF concentration and the ice crystal growth rate. Lowering the AF concentration produces less and less obvious etch patterns, but the geometrical aspects do not change. The etched regions do shrink in size, but they disappear before becoming very small. The patterns are still detectable at concentrations <10^{-2} times those illustrated here, although the etching time required to reveal them becomes many hours. Higher concentrations of these AFP have not been studied for lack of material. The ice growth rate was standardized here, with a constant cold finger temperature of 6°C, and varied from ~0.1 mm min^{-1} at the start to 0.03 mm min^{-1} at the end (e.g., Fig. 2) of the 5–6 h of growth time. Growth rates were varied outside this range, but again there were no qualitative changes of the etch patterns. A quantitative study of growth rate effects is planned, because it may be fundamental to understanding the antifreeze phenomenon.

**The etching symmetry**

The most immediately striking and surprising feature of both of the etch patterns in Figs. 2 and 3, to a crystallographer, is that their symmetry is different from that of ice. Ice has a mirror plane oriented north-south that bisects the ice hemispheres shown in Figs. 2, 3, a and b along the c-axis and normal to the page. This mirror plane symmetry is absent for the etch patterns; if present, it would give each of the etched regions the shape of a “fat X.” This result indicates that the adsorbed molecules are not aligned along a line of mirror symmetry on the adsorption plane, and that the chirality, i.e., the lack of centrosymmetry, of the AFP molecules (right-handed α helices), is significant to the geometry of the adsorption.

Because these molecules are incorporated into the growing ice crystal, the bulk ice in these sectors undoubtedly loses its center of symmetry as well. Symmetry lowering from foreign molecules incorporated systematically into crystals has only very recently become the object of considerable attention (Vaida et al., 1988; Weissbuch et al., 1989).

This symmetry lowering by the adsorbed AF molecules gives rise to a notation ambiguity that should be pointed out. The notation (00T2) normally means all equivalent directions according to the crystal symmetry, of which there are 12. Here, it means all equivalent directions according to the symmetry of the crystal with the adsorbate, of which there are only six.

**The etched region elongation: the adsorbate alignment**

The elongated shapes of the etched regions on the curved ice surface are interpreted to be indicators of the alignment of the adsorbed molecules on the ice surface, as follows.

The orientations of the centers of the etched regions on the curved surface have been identified as the adsorption planes: that is, the adsorption planes are tangent to the ice surface at the centers of the etched regions. We assume that the individual AFP molecules adsorb only at this orientation; in other words, that a structural match with the surface is necessary for adsorption. The fact that the etched regions extend over appreciable areas of the curved ice surface does not contradict this because the scale of this curvature is so much larger than that of the adsorbed AF molecules.²

²It is worth emphasizing for those who have not thought about crystal surfaces in this context, that the structure of a crystal surface does not change gradually with orientation at a small scale. In fact, at the scale of the adsorbed molecules being discussed here (roughly 10 × 50 Å), in a sense the interface orientation itself cannot change gradually, because the molecules in the crystal are rather strictly fixed at certain lattice positions. In a time-average sense, the interface between a crystal and its melt can have any orientation because lattice sites at an interface may be occupied a fraction of the time, from 0 to 1. However, this time-averaged view of the interface is not appropriate for thinking about adsorption. The interface between the crystal and an adsorbed molecule is not free to fluctuate in this manner.
The AFP of winter flounder is a rodlike molecule roughly 10 Å in diameter and 50 Å in length. In order to adsorb, this molecule needs to find at the ice surface an area of adsorption plane at least \(10 \times 50\) Å oriented to correspond with the alignment along which it fits. Because the radius of curvature of the ice hemisphere itself is enormously larger, on the order of \(10^6\) Å, the AF molecules are readily accommodated on very small-scale (and probably quite transitory) facets on the macroscopically curved ice surface near the point where the adsorption plane is tangent. Moving away from the point of tangency, appropriate sites become more and more scarce. This is illustrated in Fig. 5, which also shows the reasoning for associating the molecular alignment with the elongation of the etched regions. Section (A–A) is parallel and section (B–B) normal to the elongation of one of the flounder etched regions (middle top of Fig. 3a). The etched region (at any instant, see footnote) can be viewed ideally as having concentric steps with treads of the same orientation as the adsorption plane, (20\(2\overline{1}\))

in this case. The peptides adsorb on the (20\(2\overline{1}\)) step treads if there is enough room. When an etched region is elongated, the rodlike molecules must have been able to find treads with sufficient area to fit farther away from the central (20\(2\overline{1}\)) plane along the elongation direction, than perpendicular to it. This is the natural result if the molecules are aligned normal to the elongation axis of the etched region, that is, parallel to the steps when viewed in section (A–A), or normal to the steps when viewed in section (B–B), in Fig. 5. The molecular alignment directions of the three different AFP were determined according to this reasoning, and were found to be (01\(1\overline{2}\)) for all three, as has been seen in Fig. 4.

The ice surface in the absence of AFP adsorption is probably much rougher than Fig. 5 suggests, with the step locations fluctuating back and forth and islands and holes forming and disappearing on the treads that are drawn to be flat. However, the thesis still holds: the probability of the peptide finding a site where it fits varies with macroscopic orientation in the same manner as illustrated in Fig. 5, even if the interface actually fluctuates randomly on a small scale.

**Fitting the AFP molecules onto the ice surface**

The strong orientation dependence of the adsorption shows that a structural match between the AF molecules and the ice surface is involved. The repeat spacing in ice along the alignment direction, (01\(1\overline{2}\)), is 16.7 Å, and this must correspond to some regularity along the \(\alpha\)-helical AFP molecule if the direction is correct.

The AFP of the winter flounder and Alaskan plaice contain three sequence repeats of 11 amino acids, each ending with the polar residue, threonine (Fig. 6; Scott et al., 1987). Each sequence repeat constitutes a distance

Winter Flounder:

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DTASDDAALTAANAAAAKLTADNAAAAATAA
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Alaskan Plaice:

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DTASDDAALTAANAAAAKAAEKTRADAAAAATAAAAAR
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Short-horn Sculpin:

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MDGETPAOAKAARAAAAALAAKTADAAAAAKAAAA
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**FIGURE 5** Schematic illustration of why the alignment of the antifreeze peptide molecules is deduced to be normal to the elongation of the etched regions on the curved ice surface (see text). Section A–A is parallel and section B–B is normal to the elongation of one of the winter flounder etched regions (middle top in Fig. 3a). The AFP molecules adsorb only on the adsorption plane, and when the overall surface orientation is slightly different, it is on the “treads” of steps: it is not at the steps themselves in the sense of needing contact with both the riser and the tread. The slanting lines in each cross-section represent the edges of the “cone” of incorporation of the AFP into the growing crystal.

**FIGURE 6** The amino acid sequences of the AFP from winter flounder, Alaskan plaice, and short-horn sculpin. The 11-residue repeats in the peptide are indicated by brackets.
of 16.5 Å along the helix, which nearly matches the 16.7 Å periodicity in ice along (01\(\overline{1}\)2). Because 16.5 Å is also very close to the distance of three complete helix turns (16.2 Å), the threonine residues are almost collinear on the same side of the helix. It is therefore likely that the flounder/plaice AFP adsorbs via hydrogen bonding between the polar side chains of threonines and water molecules in the ice lattice along (01\(\overline{1}\)2). The rotational freedom of the threonine side chains, and/or the flexibility in the helix itself, might readily compensate for the 0.2 Å difference between the 16.5 Å threonine spacing and the 16.7 Å ice periodicity along (01\(\overline{1}\)2), and for the slight deviation from collinearity of the threonines. Sequence repeats are not as apparent in the amphiphilic helical AFP from the sculpin, but there are two potential hydrogen-bonding amino acids 11 residues apart, namely, arginine at position 12, and lysine at position 23.

Whereas the close correspondence of the 16.7 Å ice repeat spacing with the 16.5 Å 11-amino acid repeat spacing is almost certainly significant in AFP adsorption, at least two major questions concerning the adsorption remain to be answered. First, why is it so permanent? We speculate that this has to do with the size of the adsorbed molecules, and that the lack of exchange between adsorbed molecules and those in solution (itself a deduction, not yet directly demonstrated) results from the rather large contact area between the AF molecules and the ice surface. If the AF molecules are rigid enough to be unable to peel off the ice surface by breaking one bond at a time, they can only separate from the ice by breaking a number of bonds simultaneously. This is likely to be an extremely rare event. Second, why is it asymmetric? The 16.7 Å repeat spacing along (01\(\overline{1}\)2), which is deduced to be the adsorption alignment, is also present along (01\(\overline{1}\)2), but the AFP are not aligned along that direction. Modeling the structures has not provided a clear reason for the preference of one over the other, and until that is explained, any specific model of the binding onto the ice remains in doubt. The “secondary” features of the etching patterns also remain, both to be described thoroughly and to be explained.

**Stopping the ice crystal growth**

The major scientific objective of the biophysical studies of antifreeze action is to explain quantitatively the freezing point hysteresis. Two models have been proposed, that of Raymond and DeVries (1977) and that of Knight and DeVries (1989). They are identical in principle, both considering the energy barrier that arises from the need to increase surface area locally in order for the crystal to grow, but they differ in geometrical detail.

The models are illustrated in Fig. 7. Fig. 7a shows in perspective a growth step on an ice surface, at and somewhat <0°C, pinned in place by antifreeze molecules. This is the Raymond and DeVries concept, and it can be valid if the crystal growth occurs by steps advancing across the adsorption plane. Fig. 7b is a cross-section of an ice surface with AF molecules pinning growth normal to the surface. In perspective this would look like the surface of a mattress, with the buttons representing the AF molecules (known as “felting”). Growth is inhibited in both cases, because locally it requires an increase of the ice-water interfacial area. The Kelvin (or Gibb-Thompson) effect of interfacial curvature lowers the local freezing point, but not the bulk freezing point, and produces the freezing hysteresis. In both cases the AF molecules must be stuck tightly to the ice on one side and they must resist contact (be “icephobic”) on the other side.

The fact that the adsorption planes can be fairly high index (neither the prism nor the basal planes) rather strongly favors the three-dimensional picture of Fig. 7b, because if the crystal surface on which step growth is inhibited (Fig. 7a) is the adsorption plane as identified here, there is no evident reason why growth normal to the adsorption plane would not occur. Ice growing in pure water never shows facets of these orientations.

Complete inhibition of ice growth requires that growth
at all surface orientations be stopped, not just normal to the adsorption plane. Both models of Fig. 7 can provide this. As envisioned here, the adsorption at the scale of the individual AF molecule is at one specific orientation, but the growth inhibition applies straightforwardly to surfaces whose overall orientation differs by at least several degrees from that. Note that the surface in Fig. 7a contains a step, so its overall orientation already differs from the adsorption plane, and a greater step density makes the difference greater. In Fig. 7b, the AF molecules need not reside exactly at the same lattice layer for the mechanism to function, though in the drawing they are arranged in a plane. Knight et al. (1984) first reported that the facets induced by a glycopeptide were curved, and the same can be seen in Fig. 2b, for the winter flounder AFP. Such curved facets can completely enclose a crystal. (Note that the curvature is necessary for sculpin, but not for flounder and plaice because the 12 adsorption planes themselves could define a bounded volume.) Exactly the same inhibition mechanism can function at the edges and corners. Fig. 8 illustrates an idealized edge between inhibited planes at a 120° angle. Just as on the face, at the edge the growth is inhibited because it would require the local radius of curvature of the ice surface to decrease. 

The surface of an inhibited crystal is everywhere in metastable equilibrium with the supercooled melt: the surface is everywhere curved such that its radius of curvature is the critical radius for nucleation at that supercooling. The function of the adsorbed AF molecules is to require that local growth leads to a decrease in the radius of curvature, and hence to an unstable configuration. Thus, inhibition of the growth of complete crystals by adsorption of AF molecules at a single, specific orientation is not a conceptual difficulty. However, there will be considerable difficulty in quantifying this mechanism. The interface shape is very difficult to represent in three dimensions, being a surface of constant \((1/r_1 + 1/r_2)\) where \(r_1\) and \(r_2\) are the principal radii of curvature, and the constraints, the AF molecules, are probably more or less randomly spaced, not in a regular array. This is more than enough technical difficulty without even contemplating the possibility that the surface energy of ice may be significantly anisotropic.

**CONCLUSION**

The experimental results show that the three antifreeze peptides adsorb onto particular ice surfaces and strongly suggest particular alignment directions. A general picture for a structural match between the AFP molecules and ice becomes clear, but the specifics remain to be elucidated. The mechanism for the antifreeze effect is perhaps understood in principle, but certainly not in specific terms.

Two different adsorption planes are found among the three AFP studied in this report. We have also studied adsorption of antifreeze glycopeptides from *Dissostichus mawsoni*, and their behavior (to be reported elsewhere) is crystallographically different from these AFP, and is much more complex. Furthermore, the adsorption of the sea raven and eel pout AFP is different yet from all of the above. This complexity is surprising. It is interesting that none of the antifreezes adsorb on or near the basal plane, which is consistent with the occurrence of limited basal plane growth in the presence of several of these AF, reported by Raymond et al. (1989). Because the basal plane has threefold symmetry, linear molecules adsorbed upon it would not be aligned, and thus might not produce a significant antifreeze effect.

A better understanding of these phenomena may contribute insights to biocrystallization in general, and perhaps even to the interaction of peptides and proteins with membrane surfaces, which have a great deal in common with crystal surfaces.

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