Adsorption to ice of fish antifreeze glycopeptides 7 and 8

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ABSTRACT Experimental results show that fish antifreeze glycopeptides (AFGPs) 8 and 7 (with 4 and 5 repeats respectively of the Ala-Ala-Thr backbone sequence) bond onto ice prism planes aligned along a-axes, and inhibit crystal growth on prism planes and on surfaces close to that orientation. The 9.31-Å repeat spacing of the AFGP in the polyproline II helix configuration, deduced from NMR studies, matches twice the repeat spacing of ice in the deduced alignment direction, 9.038 Å, within 3%.

A specific binding model is proposed for the AFGP and for the α-helical antifreeze peptide of winter flounder. For AFGP 7–8, two hydroxyl groups of each disaccharide (one disaccharide is attached to each threonine) reside within the ice surface, so that they are shared between the ice crystal and the disaccharide. This provides 24 hydrogen bonds between AFGP 8 and the ice and 30 for AFGP 7, explaining why the chemical adsorption is virtually irreversible and the crystal growth can be stopped virtually completely. The same scheme of sharing polar groups with the ice works well with the α-helical antifreeze of winter flounder, for which an amide as well as several hydroxyls are shared. The sharing of polar groups with the ice crystal, rather than hydrogen-bonding to the ice surface, may be a general requirement for adsorption-inhibition of freezing.

INTRODUCTION

The nonequilibrium antifreezes found in many cold-water fish have been studied for over two decades. Recent reviews are by DeVries (1984), Cheng and DeVries (1991), and Davies and Hew (1990). These antifreezes (AFs) inhibit growth of existing ice crystals from solution or from pure water, to temperatures ~1.5°C below the equilibrium freezing point at concentrations of ~2 wt%. They prevent ice crystal growth in the fish down to temperatures as low as −2.2°C, which is 1.2°C below the equilibrium freezing point of the fish blood.

The AFs are either peptides (AFPs) or glycopeptides (AFGPs). The AFGPs are periodic and come in a wide range of lengths. This paper presents and interprets results on the adsorption plane and alignment on ice crystals of the shortest two, numbers 7 and 8. The methods and reasoning are much the same as in an earlier report that concerned α-helical AFPs (Knight et al., 1991), but the specifics of the adsorption are different. Specific bonding schemes are proposed for AFGP 7–8 and for the α-helical, winter flounder AFP, in which OH or other hydrogen-bonding groups of the antifreeze molecules reside within the ice surface, providing three hydrogen bonds between the AF molecule and the ice for each such shared group.

METHODS

In order to stop ice growth completely, the AF molecules must adsorb irreversibly to the ice at the ice-water interface. If ice crystal growth is forced to occur by lowering the temperature, the adsorbed AF molecules become incorporated into the ice crystal. Therefore, determining the growth-interface-orientation-dependence of AF incorporation into ice single crystals is a way of determining the orientation dependence of the adsorption: a basis for determining adsorption planes (if such exist) and also, as it turns out, the alignments of the adsorbed molecules with respect to the ice orientation.

A single, oriented ice crystal is frozen onto the end of a cold finger maintained at −6°C by circulating coolant. The crystal is partly submerged into 100 ml of AFGP solution at +15°C, in an insulated container. The seed crystal melts back to a shell ~0.5 cm thick around the end of the cold finger, and then grows slowly to form a single crystal that is approximately hemispherical. Since ice has a mirror plane of symmetry, all possible crystal interface orientations are exposed for growth in the one experiment. The long axis of the cold finger is normal to the solution surface. The crystals were grown with a prism plane of the ice parallel to the surface of the solution; that is, perpendicular to the long axis of the cold finger. Thus the c-axis, [0001], and one a-axis, $[\bar{1}2\bar{1}0]$, also lie perpendicular to the long axis of the cold finger. Final hemisphere diameter is ~6 cm after 6 h of growth.

Etching by evaporation is used to determine the location of any AFGP incorporated into the crystal. The ice surface to be examined (either a cut through the hemisphere or the rounded hemisphere surface itself) is scraped clean and evaporated at −15 to −20°C for minutes or hours. The AFGP accumulates at the surface as ice evaporates; the time required for clearly visible etching varies with AFGP concentration. Evaporation of a scraped surface of pure ice produces a completely smooth surface in an hour or two, but a ground-glass-like etching appears where AF molecules are incorporated. (It is best to use a low etching temperature with the smallest AF molecules, including those studied herein, because at temperatures not far below 0°C they may form a liquid film of solution on the surface instead of etching.)

The etching pattern indicates the orientations at which adsorption occurs, but is qualitative in the amount adsorbed and incorporated. The first version of this experiment used tritium-labeled AFGP and did give some quantitative results. An example is given in the Appendix.

MATERIALS

The AFGP was isolated from the blood serum of the Antarctic cod *Dissostichus mawsoni*. The AFGP fraction was separated from other blood proteins and fractionated into three major size classes using ion exchange chromatography according to the method of DeVries et al. (1970). The smallest size class contains approximately equal amounts of AFGP 7 and 8. The molecules consist of a repeated sequence of the three amino acids, Ala Ala Thr, with a disaccharide bonded to each Thr, as shown in Fig. 1 (see Shier et al., 1975, for the final determination of the structure). The molecular weight of each such unit is 608 D. AFGPs 7 and 8 have five and four repeat units, and contain some

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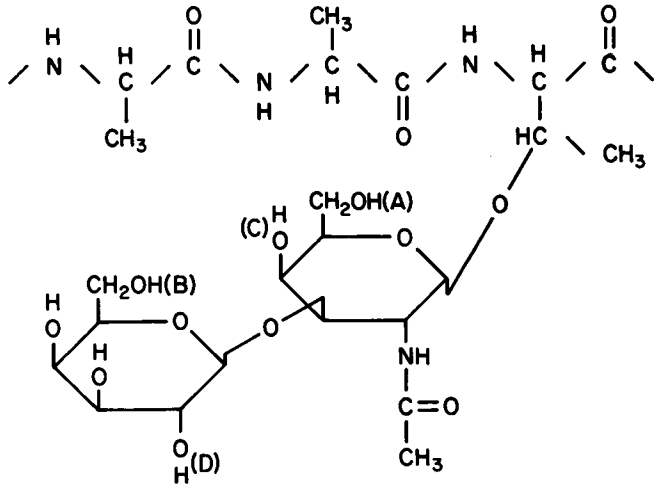

FIGURE 1 Basic repeating structural unit of the antifreeze glycopeptide. The polypeptide backbone is composed of repeated -Ala-Ala-Thr- with a disaccharide, β-D-galactopyranosyl-(1 to 3)-2-acetamido-2-deoxy-α-D-galactopyranose, joined to every threonine through a glycosidic linkage. The letters *A*, *B*, *C*, and *D* represent hydroxyl groups possibly involved in attachment to the ice, as discussed in the text.

substitutions of Pro for Ala beginning at position 7 and sometimes at every third residue thereafter. The molecular weights of numbers 7 and 8 are ~3,500 and 2,600 D.

The solution structures of the AFGPs have been studied with circular dichroism and NMR (Raymond et al., 1977; Franks and Morris, 1978). The NMR studies suggest a three-fold, left-handed helical configuration similar to the polyproline II helix. In this configuration the disaccharides are aligned 9.31 Å apart along one side of the helix (Rao and Bush, 1987). The proline substitutions probably have no special significance in the bonding to ice.

RESULTS

The orientation-dependent incorporation of AFGP 7–8 into the ice hemispheres can be detected easily at concentrations of 10^{-2} mg/ml in the original solution, if the evaporation etching lasts many hours at a low temperature. At this concentration, the rounded surface of the hemisphere reveals faint, oblong etched regions centered at the points of tangency of the prism planes to the hemisphere surface. These etched regions are elongated in the *c*-axis direction. There is no discernable effect upon growth shape, and the etched regions extend ~5° away from the prism orientation toward each adjacent prism plane, and ~22° away toward the basal plane, along the *c*-axis direction.

At higher concentration there is both a clear effect of AFGP 7–8 on growth shape and a very marked etching effect. Fig. 2 illustrates the results at 2 mg/ml, which corresponds to an overall freezing-point depression of ~0.1°C (DeVries, 1984). The somewhat curved facets are centered at each prism plane orientation, and, of course, indicate growth inhibition at these orientations. Note that the curvature is substantially greater in the section cut parallel to the *c*-axis (Fig. 2, *b* and *c*) than in the section normal to the *c*-axis (Fig. 2, *d* and *e*) which is consistent with the incorporation pattern at lower concentrations. The incorporation pattern indicated by the etching in Fig. 2 corresponds with the facets and, therefore, also with the growth inhibition, providing direct, visual confirmation of the expected link between adsorption, incorporation, and growth inhibition.

INTERPRETATION

The adsorption plane for molecules of AFGP 7–8 is the prism plane $\{10\bar{1}0\}$, and their alignment direction on the prism plane is deduced to be parallel to the *a*-axis, normal to the *c*-axis. The adsorption plane deduction is obvious, but that of the alignment is less so. The premise is that AFGP molecules must find sites at which they fit in order to adsorb. They fit on the prism plane aligned in one direction, so each adsorption site is a strip of prism-plane orientation elongated in that direction. Such sites are available both on surfaces oriented exactly parallel to the prism plane and on surfaces at small angles to that orientation. Because the sites need to be several times longer parallel to the alignment orientation than normal to it, the axis of elongation of the etched region on the curved ice surface is expected to be normal to the alignment direction.

As was the case for the α-helical, winter flounder AFP, this deduction gives an alignment with a good match between a periodicity in the adsorbed molecule and a repeat spacing of ice, though both adsorption plane and alignment are different. The periodicity of the polyproline helix is 9.31 Å (Rao and Bush, 1987), and the repeat spacing in the ice in the deduced alignment direction, along an *a*-axis, is 4.519 Å. Twice this is 9.038 Å, which differs from 9.31 Å by only 3%. The existence of this lattice fit was also pointed out recently by Avanov (1990). This fit, along with that already reported for the winter flounder AFP, provides a good deal of confidence in the deduction of alignment from the shapes of the etch patterns. This simple test of alignment deductions by matching periodicities is only possible, of course, when the antifreeze is periodic. Many AFs are not, and for them the deduction of alignment orientation from this kind of experimental evidence is testable only by finding the details of the binding to the ice.

Given the adsorption plane and alignment direction, three issues need to be addressed: (*a*) the nature of the ice-water interface and its relevance to the adsorption, (*b*) the nature of the individual bonds that connect the AFGP with the ice, and (*c*) the specific bonding arrangement.

1. The ice-water interface

The first problem in specifying the details of the attachment of any AF molecule to ice, assuming that hydrogen bonds are involved, is how to deal with the ice-water

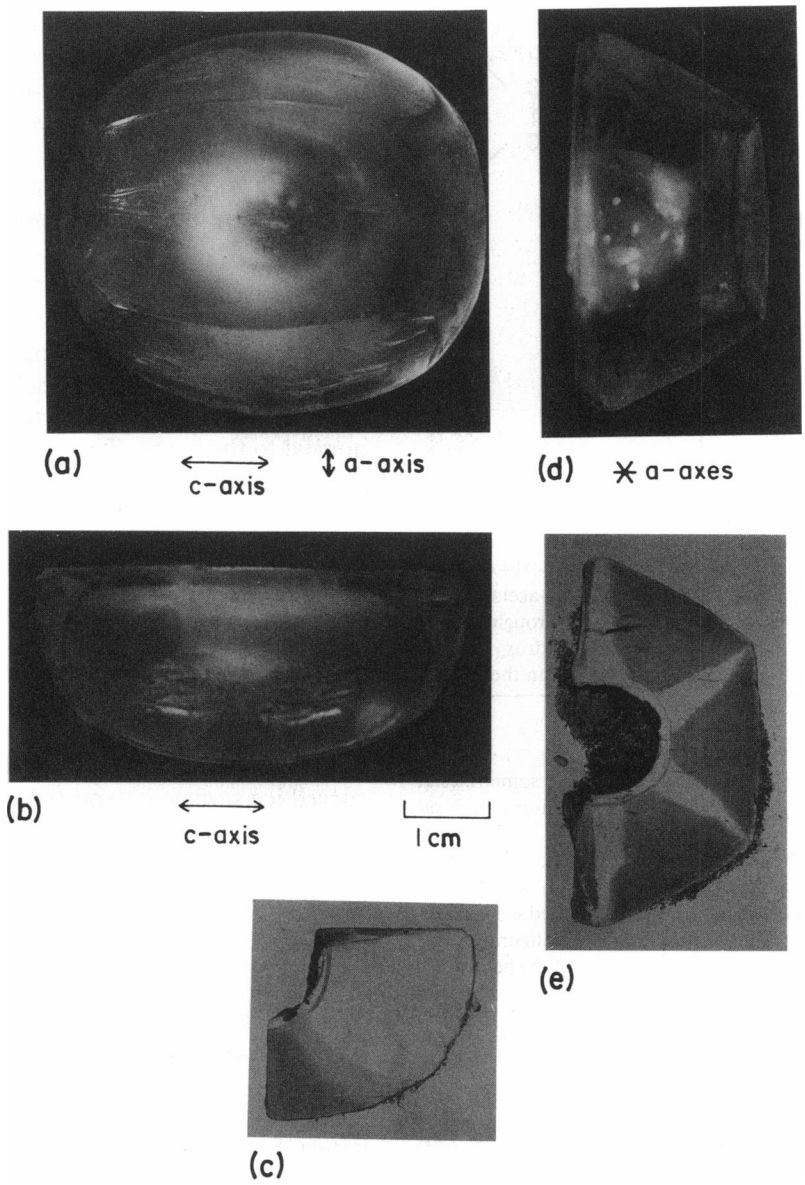

FIGURE 2 Ice "hemisphere" grown from 2 mg/ml solution of AFGP 7–8, showing curved prism facets. *a* Is an end view, the *c*-axis from left to right; *b* is a side view looking normal to the *c*-axis; and *d* is a side view looking down the *c*-axis. *e* Is an etched cross-section through the middle, seen with bright-field, diffuse illumination, in the plane of the view in *d*, showing the correspondence of the etched areas (*dark*) with the facets. Note the border of the seed crystal. The black material in the hole left by the end of the cold finger is ice scrapings. *c* Is an etched half-section through the middle in the plane of the view of *b*.

interface itself. This interface is probably not correctly represented as an abrupt transition. In fact, the best numerical representation shows the loss of the ice structure at the interface to be fairly gradual, occurring over two or three lattice parameters: say 10 Å or somewhat less (Karim and Haymet, 1988). This is difficult to deal with, because there is no way to take it into account in a static model, and dynamic models are yet to be applied to this kind of adsorption problem.

We proceed to use a static model in spite of these difficulties, with two justifications: first, that the adsorption is virtually permanent (otherwise the growth would not be stopped completely), and second, that it is exquisitely orientation sensitive, as the experimental results (Fig. 2, for instance) show. The AF molecules do "sense" the permanent, static, oriented ice structure. Thus, a match and a permanent bonding scheme between antifreeze molecules and their adsorption planes, viewed as static, abrupt, "perfect" interfaces, is expected. The model will represent the junction between antifreeze and ice, but the ice-water interface itself cannot be modeled realistically in this way.

2. The individual bonds

DeVries (1984), Chou (1992), and others have suggested that the binding of AFs to ice is probably via hy-

drogen bonds between polar groups and the ice surface. However, a simple application of this scheme quickly encounters problems. When an antifreeze molecule is in water solution, its polar groups (the hydrophilic groups) are hydrogen-bonded to water molecules, probably to two or three at once. (See Brady, 1989, and Van Eijck et al., 1990, for models of solvation structure relevant to the disaccharides of AFGP.) According to current understanding of hydrophobic interactions, these bonds are fairly strong but also rather temporary, as are the hydrogen bonds within water itself. It is hydrophobic groups that structure the neighboring water molecules in a relatively permanent way, as indicated by their large effect upon entropy, though their bonding to water is relatively weak. The water assumes its structure so as to minimize the number of its own broken hydrogen bonds (e.g., Tanford, 1980; Rossky, 1985). This decreases the entropy of the water, increases the free energy, and results in hydrophobic molecules or groups being quite insoluble. In terms of this conceptual framework, the lack of permanence of the hydrogen bonds to hydrophilic groups would make it difficult to understand the permanence of the bonding of AF to ice if the polar groups are hydrogen-bonded to the ice surface in the manner suggested.

Another possibility that has not been raised before, however, is that the AF molecules are not hydrogen-bonded to the ice surface, but that parts of them actually reside within the ice surface. Hydrogen bonds are still involved, because ice itself is completely hydrogen bonded; but since ice is tetrahedrally coordinated, there are now three hydrogen bonds for each polar group of an AF molecule that is involved in the binding, rather than one. For a single attachment point to release, three hydrogen bonds must break at once, giving a much greater permanence. The model of bonding to the ice surface is illustrated in Fig. 3 *a*, and that of bonding in the surface in Fig. 3 *b*. The latter is much the more restrictive, because the polar group involved must fit within the ice lattice at the surface and it must bond tetrahedrally. An hydroxyl group obviously would fit quite well.

Another argument for Fig. 3 *b* over Fig. 3 *a* is that the only real difference between the two is the location of the ice-solution interface. In either case the hydroxyl group that is involved is surrounded by water molecules, but if most of these were considered to be in the liquid phase, then their bonds would be breaking and reforming as happens in liquid water. Then the same, presumably, would be happening with the hydrogen bond to the ice. If, however, they are essentially fixed in place, then for the adsorption to be energetically favorable their positions should conform to the ice crystal, and the interface in Fig. 3 *a* is in the wrong place; that gives Fig. 3 *b*.

The hydrogen atoms in pure ice are well known to be disordered, two being close to each oxygen on the average but fluctuating between the two allowable locations along each oxygen-oxygen line. As drawn in Fig. 3, these

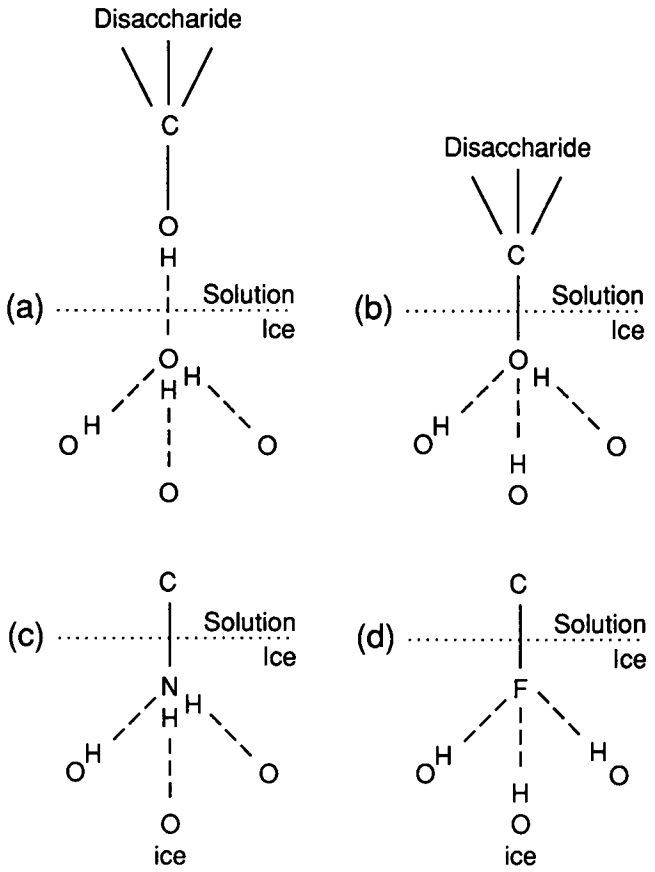

FIGURE 3 One broken bond at the ice-solution interface can be satisfied by an hydroxyl group of the AFGP molecule in two conceptual ways, illustrated here. In *a*, one draws a single hydrogen bond from the hydroxyl group, with the AF molecule completely in solution. In *b*, the hydroxyl is fully shared with the ice crystal. *c* and *d* show how NH_3 and F might function similarly.

sharings of polar groups between AF molecules and ice must influence the local hydrogen "balance." The hydroxyl in Fig. 3 *b* is the donor of one hydrogen bond to and the acceptor of two from the ice; the amide in Fig. 3 *c* is the donor of two and the acceptor of one; and the fluoride of Fig. 3 *d* is the acceptor of three. Since both NH_4OH and HF are soluble within ice crystals to the extent of a few percent, with N and F in the oxygen positions, these substitutions appear well justified. Amide involvement in binding some AFPs to ice is suggested below, and fluoride has no natural application but would be a logical extension. The AFGPs that are the main concern here have only hydroxyl groups to fill this kind of binding role.

3. The specific binding structure

Ball-and-stick models of the AFGP and the ice surface were constructed to the same scale, with the object of finding how they might bind together. The molecular models made by Maruzen Co. ("H G S biochemistry molecular models") proved adaptable to this purpose,

though all the O-O connectors for the ice had to be shortened and drilled out, to build the ice to the 1 cm to 1 Å scale of the antifreeze molecule models. This kind of modeling has inherent problems that lend it a degree of subjectivity. The model bond lengths and angles are unrealistically rigid, but the bonds themselves allow free rotation except when the connectedness of the structure prevents it. The bonds along the backbone of the polyproline helix had to be cemented into their proper angles to allow the helix to be handled without its bonds rotating, while the α-helical model is very rigid already because of its internal hydrogen bonds. The model is not space filling, and so it allows some atoms to be placed unrealistically close together unless they are bonded to each other. Because of this combination of properties, some latitude is allowed in the model structures for atoms being too close together, when it appears that a little extra flexibility would allow them to be far enough apart. Thus, this kind of modeling can only suggest possibilities for specific configurations. In fact two possible configurations were found, and the more likely looking is illustrated.

The procedure was first to look for a way for a disaccharide to have at least two hydroxyls shared with the ice, on the prism face. Then there must be room along the a-axis direction for another one, two lattice repeats (9.038 Å) away. Finally, the helical peptide backbone must be able to connect the disaccharides so situated.

Requiring at least two shared hydroxyls is a strong restriction, and the reason for it is the fact that an adsorption plane exists. One bond per disaccharide would provide fits to the ice, but there would be little requirement for the binding to be on the prism plane: it could be on any plane that contains the a-axis, and therefore has the proper spacing. The bonding must define an adsorption plane, so it probably involves a planar array of bonds, not a linear one. (It is worth remembering, in considering other AFs, that adsorption inhibition may not necessarily require an adsorption plane, though the AFGP studied here and the α-helical AFPs described previously do appear to have them. One can imagine an adsorption alignment of linear molecules without a well defined adsorption plane.)

No binding configuration with more than two shared hydroxyls per disaccharide could be found. Figs. 4 and 5 illustrate one of the two arrangements with two hydroxyls per disaccharide that may satisfy these requirements. Fig. 4 gives plan and side views of an ice prism surface with the oxygens that have one bond unsatisfied shown as circles. Those represented by Xs are tetrahedrally bonded within the ice. The envelopes of the disaccharides are shown as dashed lines, the locations of the glycosidic linkages to the threonines of the peptide backbone by triangles, and A and B are the shared hydroxyl groups, also identified in Fig. 1. The two pyranose rings in this scheme are not in the same plane, but the hydrophobic side of each disaccharide is disposed toward the

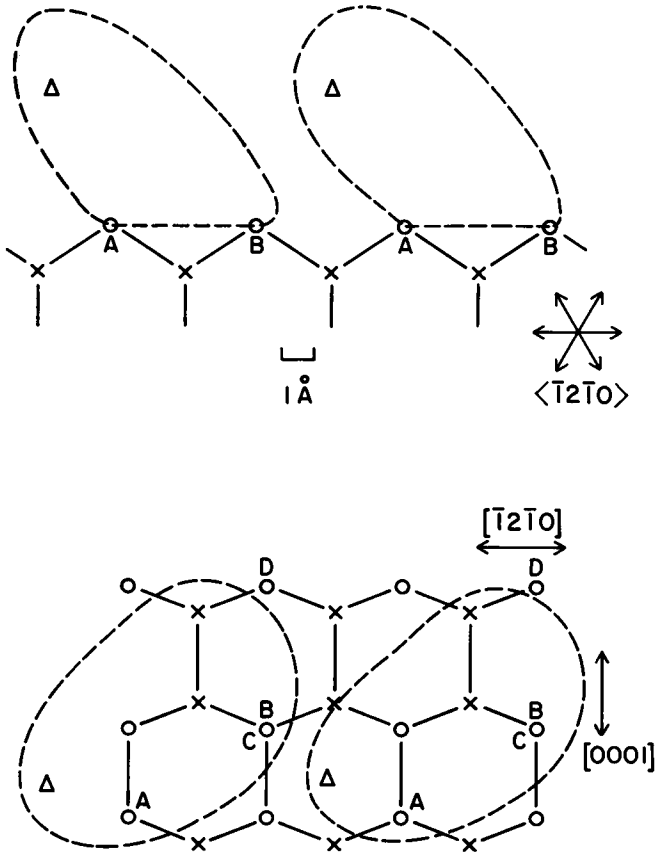

FIGURE 4 The prism surface of ice, viewed along the c-axis at the top, and in plan at the bottom, with the oxygen atoms that have one unsatisfied bond shown as circles and the next layer as Xs. A and B are the suggested locations where OHs of the disaccharides fit, the ones labeled A and B in Fig. 2. (C and D are the locations for hydroxyls C and D in Fig. 1, for an alternate bonding structure.) The dashed outlines are the disaccharides, and the triangles the locations of the glycosidic linkages to the threonines of the peptide backbone.

ice, and toward the hydrophilic side of an adjacent disaccharide. Fig. 5 is a photograph of the model of the proposed arrangement, including the peptide backbone. The disposition of the disaccharide groups with respect to the peptide backbone suggested for the solution structure by Bush and Feeney (1986) is not that of Figs. 4 and 5, and in fact their structure cannot fit onto the ice with the bonding and alignment schemes adopted here. The other potential fit is not shown, but for it the hydroxyls labeled C and D in Fig. 1 replace the surface oxygens so labeled in Fig. 4.

The binding scheme with polar groups in the ice surface provides a remarkably good accommodation of the α-helical, winter flounder AFP to the ice. The hydroxyl groups of the four threonines (positions 2, 13, 24, and 35) can fit in the ice surface, along with the amide groups of asparagine in positions 16 and 27 and the hydroxyl of aspartic acid in position 5 as well, keeping bond angles and lengths quite close to their ideal values. The model itself is not quite flexible enough to accomplish this all at

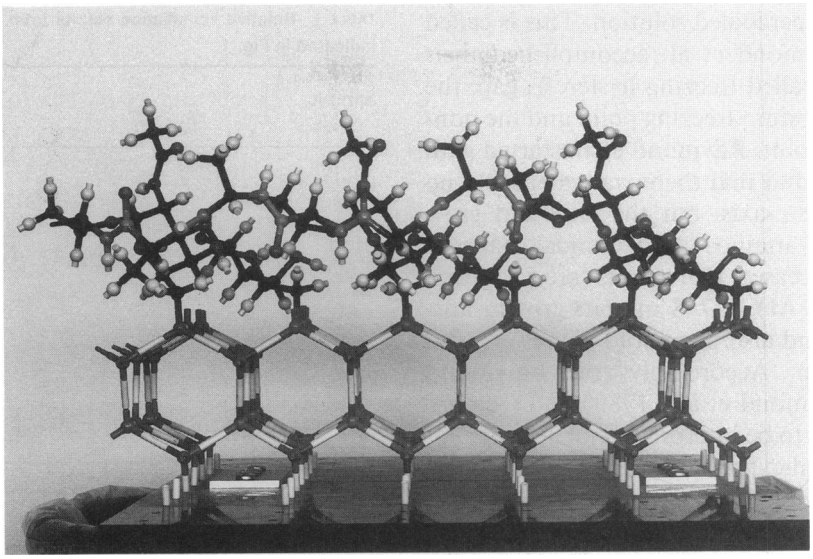

FIGURE 5 Photo of the AFGP model on the ice, in the arrangement shown in Fig. 4. Three disaccharides are shown with the helical peptide backbone, on the prism face of the ice, looking down the *c*-axis.

once, but the bond angles are very close to those needed, and small displacements would allow it. In fact the fit in this case is much more convincing than that of either possibility for AFGP 7–8, because the AFP molecule is more complicated, and virtually all of the polar groups except those involved in the salt bridge (which projects into the liquid) are involved. Also, it is encouraging that the winter flounder AFP only fits well in the deduced orientation, and not in the orientation that would be equivalent as far as the ice symmetry is concerned, but not equivalent for the chiral α-helix (see Knight et al., 1991). Furthermore, it only fits well in one direction. Referring to Fig. 3 *a* of the reference, considering the two oblong etched regions shown in the center of the circle, the amino end of the helix extends toward the top right for the upper region and toward the bottom left for the lower.

Because of the periodically-spaced threonines, there is little choice in locating the winter flounder α-helix onto the ice. However, the situation is different with the α-helical AFP derived from sculpin. Most of the polar groups available are the amines of lysine and arginine, and they have great freedom of location with respect to the helix backbone. Furthermore, the sequence is not periodic, though the resulting helix is decidedly amphiphilic (Hew et al., 1985). A fit has been found in about the deduced alignment on the deduced adsorption plane, but there is much less confidence in it being a unique result or in its being used as evidence of correctness for the bonding hypothesis advanced herein. Note that when the antifreeze molecule is not periodic, as in this case, there is no need for it to be aligned in a rational crystallographic direction. Indeed, the sculpin AFP probably is not. Thus, the grouping of its alignment direction with that of winter flounder (Knight et al., 1991) probably was incorrect, and the fact that the actual alignment probably is close to it appears now to be a coincidence.

DISCUSSION

One appealing feature of this binding interpretation is the number of hydrogen bonds it provides between even the smallest antifreeze molecule and the ice. AFGP 8 has 24 hydrogen bonds (6 for each of the four disaccharides) and AFGP 7 has 30. The winter flounder AFP has at least 21. This is chemical, not physical adsorption, and this many bonds makes it easier to understand why the adsorption is so permanent.

This paper has ignored the longer AFGPs, which have from 6 to ~55 repeats of the basic sequence, because they present a much more complicated picture. Their adsorption plane has higher indices, which we interpret as resulting from the 3% misfit onto the ice that forces the longer AFGPs into a slightly different accommodation with the ice surface, giving a surprisingly high index adsorption plane. Also, these longer polymers may adsorb to ice in segments, with "loops and tails" extending into the liquid. Their effects upon ice crystal growth are different in important but complex and problematical ways from those of AFGP 7–8.

Others have studied the effects of AFGP 7–8 on ice growth morphology using experimental arrangements different from that used here (Raymond et al., 1989; Chakrabartty et al., 1989). It is worth noting the differences. In this study the heat of fusion is withdrawn through the growing crystal, and in the absence of growth anisotropy (and of any temperature stratification in the solution), the growth shape would be hemispherical. In the other studies, the growth is accomplished by withdrawing heat very slowly from the solution, so that

the ice grows into a supercooled solution. This is called free growth, and Raymond et al. accomplished their growth within the so-called freezing hysteresis gap, the gap between the equilibrium freezing point and the non-equilibrium freezing point. Raymond et al. started with a single crystal and found that there was essentially no growth normal to the c-axis, but the ends did grow slowly until the crystal attained a bipyramidal shape, at which time all growth ceased. Our results (see Fig. 2 and Discussion) show that AFGP 7–8 inhibits growth normal to prism planes and also normal to planes close to the prism orientation. Accordingly, the bipyramid "faces" found by Raymond et al. (1989) and Chakrabartty et al. (1989) are to be interpreted not as crystallographic faces with specific, rational indices, but as representing the greatest deviation from the prism orientation that still supports adsorption and inhibition at the experimental supercooling. These orientations then define the final shape at which growth stops: at which all crystal surfaces in contact with the solution are inhibited.

APPENDIX

The initial experiments demonstrating orientation-sensitive incorporation of AFGP used tritium-labeled material. Essentially, the same ice growth technique was employed, but the ice was sawed into small pieces that were melted and their tritium content assayed by scintillation counting.

Fig. 6 and Table 1 give one set of results. The figure shows how an ice slab normal to the c-axis was cut into pieces, and the Table gives the results in terms of scintillation counts per 100 μl per minute. The best resolution is obtained by comparing Nos. 4, 5, and 6: 5 at the prism plane orientation, 4 and 6 adjacent. The partition coefficient is at least as high as a few tenths when the growth interface is in the prism orientation, and it is obviously much lower otherwise, but the orientation resolution is not very good. Duman and DeVries (1972) reported equipartitioning of antifreeze between ice and water during freezing, but this was in the fibrous growth mode of the longer AFGPs and may represent physical inclusion of solution between fibers, rather than single-molecule incorporation within ice, as happens here according to our interpretation. The polycrystalline ice near the flat side of the hemisphere should be ignored, since orientations are unknown and incorporation occurs in the grain boundaries. The contrasts in scintillation counts at the other two prism orientations of the ice sample are less clear because the growth shape of the ice is not strictly hemispherical, and the locations of the prism orientation at these two places change as the ice grows.

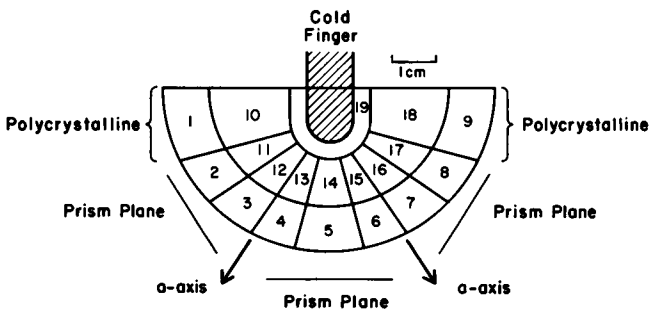

FIGURE 6 Ice slab normal to the c-axis, grown from a low concentration solution of tritiated AFGP ($\sim 10^{-3}$ mg/ml), was cut into samples as indicated for the scintillation-counting analysis results of Table 1. Samples 1, 9, 10, and 18 were polycrystalline, the rest from a single crystal as shown.

TABLE 1 **Relative scintillation counts from the ice samples indicated in Fig. 1**

Sample no.	Counts	Sample no.	Counts
1	2,720	11	1,980
2	2,660	12	5,900
3	90	13	260
4	115	14	4,770
5	2,440	15	850
6	72	16	6,450
7	790	17	2,070
8	2,800	18	1,160
9	4,600	19	1,130
10	2,020	liquid, start	19,500
		liquid, end	25,800

Autoradiography was used to get better orientation resolution, and was found to work well. A flat ice surface was scraped smooth, allowed to evaporate for 24 to 48 h to accumulate the antifreeze molecules at the surface. Then 24- to 48-h exposures of autoradiograph film pressed emulsion side to the ice surface gave excellent results. However, the surface etching after the evaporation corresponded with the radiographic patterns, and so etching became the method of choice.

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