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Role of Glycopeptides and Peptides in Inhibition of Crystallization of Water in Polar Fishes [and Discussion]

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Role of glycopeptides and peptides in inhibition of crystallization of water in polar fishes

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In the ice-laden polar oceans, water temperatures of −2 °C are common. This temperature is 1.1 °C below the equilibrium freezing point (−0.9 °C) of the fishes' body fluids. Avoidance of freezing in these environments has been linked to the presence of unusual blood peptides and glycopeptides. These molecules have molecular masses ranging from 2.5 to 20 kDa and are viewed as having antifreeze properties because they lower the freezing point of water by a non-colligative process. A 2% solution of antifreeze has a freezing point of −1.2 °C and ice formed in their presence melts at −0.02 °C. Measurements of antifreeze concentrations in ice indicate that these molecules, unlike other proteins of similar size and conformation, are incorporated into the solid phase during freezing and adsorb to it. Adsorption of the antifreezes to ice appears to inhibit growth along the preferred axes (*a*-axes) by raising the curvature of the growth steps on the basal plane. At temperatures below −1.2 °C, crystal growth occurs in the form of long spicules whose axes are parallel to the *c*-axis, the non-preferred axis of growth.

Introduction

During the winter season the polar oceans and the near shore waters of the north temperate oceans are at the freezing point of seawater (−1.9 °C), a temperature well below the freezing point of a typical marine teleost (−0.8 °C) (Black 1951). This one degree difference between the freezing point of the body fluids and the environment results in freezing because supercooling or undercooling in the presence of ice is impossible (Scholander *et al.* 1957). Partial freezing of either the body fluids or tissues of fishes has been shown in all cases to lead to death, although several hours may elapse between thawing and death (Scholander *et al.* 1953). In the absence of ice, some fishes can undercool by 1 °C and exist in this metastable state for the duration of their lifetime (Scholander *et al.* 1957).

In the shallow ice-laden waters of the polar oceans many fishes spend their entire lives at the freezing point of sea water and frequently come into contact with ice (DeVries & Wohlschlag 1969; DeVries 1970, 1971*a*, *b*, 1974; Hargens 1972), yet do not appear to freeze. In fact some even use ice crystal formations associated with icy environments as a habitat to escape predators and to forage for food, but do not freeze under these conditions (Andriashev 1970; DeVries & Lin 1977*a*). Freezing in these fishes does not occur until the temperature is lowered below −2 °C in the presence of ice, and results in death (Scholander *et al.* 1957; DeVries & Lin 1977*a*).

In most temperate marine fishes, sodium chloride is the principle electrolyte present in the blood and is responsible for 85% of the freezing point depression (Gordon *et al.* 1962). The remainder of the freezing point depression is due to small amounts of potassium, calcium, urea,

glucose, and the free amino acids (Potts & Parry 1964). In fishes inhabiting freezing environments, concentrations of sodium chloride in the body fluids are elevated relative to temperate forms (Fletcher 1977, 1981; O'Grady & DeVries 1982). However, they are in fact responsible for only 40–50 % of the observed freezing point depression (DeVries 1980; Fletcher 1977, 1981; O'Grady & DeVries 1982). Concentrations of other ions and small organic solutes are similar to those found in temperate water fishes (Dobbs & DeVries 1975; DeVries 1980). In these cold-water fishes the low freezing points have been shown to be associated with solutes in the colloidal fraction of the blood, indicated by the observation that over half of the serum freezing point depression is retained by a dialysis membrane with a molecular mass cut off of 3000 Da (DeVries 1980, 1982). The large freezing point depression associated with the colloidal fraction suggests that relatively large solutes are involved and also implies that these solutes exert their effect by a non-colligative mechanism. In most Antarctic fishes and many north-temperate fishes these solutes are relatively large glycopeptides, while in some Arctic and north-temperate fishes they are peptides. The glycopeptides appear in many sizes with molecular masses ranging between 2600 and 34000 Da (DeVries 1974; Feeney 1978, 1982), whereas the peptides range between 3200 and 14000 Da (DeVries 1982; Slaughter & Hew 1981). On a mass basis both are as effective as sodium chloride in depressing the freezing point of water (DeVries & Wohlschlag 1969). On a molal basis, however, they depress the freezing point by 200 to 300 times more than that expected on the basis of colligative relations alone (DeVries 1971). These glycopeptides and peptides appear to lower only the freezing point in a non-colligative manner but show the expected colligative effect on the melting point of the solid phase. The non-colligative lowering of the freezing point has been referred to as an antifreeze effect and these molecules are referred to as 'antifreezes'.

Biological antifreeze agents

Glycopeptides

The glycopeptide antifreezes were first isolated from the blood of Antarctic nototheniid fishes (DeVries & Wohlschlag 1969). They make up approximately $3\frac{1}{2}$ % (by mass) of most of the body fluids except the cytosol and urine (DeVries 1974; Duman & DeVries 1975). Biochemical analyses show the presence of eight separate glycopeptides in the blood and most of the extracellular fluids (DeVries *et al.* 1970) except the bile and intestinal fluid, which contain only the two low molecular mass forms (O'Grady *et al.* 1982*a*, *b*, 1983). Their molecular masses range from 2400 to 3400 Da. Glycopeptides 1 to 5 are composed of repeating units of glycotripeptides in which the disaccharide β-D-galactopyranosyl-(1–3)-acetamido-2-deoxy-α-D-galactopyranose is linked to the threonine residue of the tripeptide, alanyl-threonyl-alanine (Komatsu *et al.* 1970; DeVries *et al.* 1971; Shier *et al.* 1972, 1975). Glycopeptides 6, 7 and 8 differ from 1 to 5 in that the amino acid proline replaces some of the alanines beginning at position seven and appears at every third position until the C-terminal is reached (Lin *et al.* 1972; Morris *et al.* 1978). Glycopeptide 8 appears to be a heterogeneous mixture in that it is composed of three identically sized molecules in which the prolines occupy different positions in the polypeptide (Lin *et al.* 1972; Morris *et al.* 1978). The same eight glycopeptides have been isolated from three northern gadids (Van Voorhies *et al.* 1978; Osuga & Feeney 1978; Hew *et al.* 1981). The positions occupied by proline in antifreeze glycopeptide 8 in some of these species differ from those reported for the Antarctic nototheniids (Hew *et al.* 1981; Fletcher *et*

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al. 1982; O'Grady *et al.* 1982*b*). Other northern cods have glycopeptides that differ substantially in size, but other than containing arginine they have the same composition as that described (Raymond *et al.* 1975; Fletcher *et al.* 1982).

Peptides

Peptide antifreezes have been found in several north-temperate and Arctic fishes. They vary in size and composition. Only those found in the winter flounder *Pseudopleuronectes americanus* have been characterized. Three separate peptides have been isolated from the blood of this fish and they are composed of 8 amino acids in which alanine accounts for 60% (by mass) of the residues (Duman & DeVries 1976; Hew 1981). Most of the remainder are polar residues such as aspartate, glutamate, lysine, serine and threonine. Preliminary sequence studies of each of the three peptides shows a repeat pattern of the two polar residues threonine and aspartate (asparagine) separated by two alanines, and each of these polar sequences is separated by 7 non-polar residues, which are usually 6 alanines followed by a leucine residue (DeVries & Lin 1977*b*). The Alaskan plaice *Pseudopleuronectes quadritaberulatus* has evolved peptide antifreezes that have a sequence similar to those of the winter flounder except that leucine is absent (DeVries 1980). Antifreeze peptides have also been isolated from the Bering Sea and the Atlantic sculpins, *Myoxocephalus verrucosus* and *M. scorpius*, respectively, which appear to be similar in size and composition to those of the flounder, except that they contain a few residues of the non-polar amino acids isoleucine, glycine, methionine and proline (Raymond *et al.* 1975; Hew *et al.* 1980; Fletcher *et al.* 1982*a*). The sea raven *Hemitripterus americanus* has a peptide antifreeze that differs substantially from the other antifreezes in that it contains relatively large amounts of glycine, some of the aromatic amino acids and reduced amounts of alanine (Slaughter *et al.* 1981). The presence of the aromatic amino acids and large amounts of glycine is unusual as none of the other peptide antifreezes contain them. The only Antarctic fish that possesses a peptide antifreeze is the eel pout *Rhigophila dearborni* and it has twelve amino acid residues, most of which are alanine, and in addition to the non-polar residues mentioned above in the sculpin antifreeze peptides, it also contains valine (DeVries 1980).

Biochemical studies of fish antifreezes indicate that they are either glycopeptides or peptides, and that in some related species they are the same while in others they are quite different. In some cases, fishes belonging to unrelated families and inhabiting opposite hemispheres have evolved the same antifreeze glycopeptides (Van Voorhies *et al.* 1978), while others that are sympatric species of the same family, and therefore apparently more closely related, have evolved glycopeptide antifreezes that show considerable variation in their amino acid compositions.

Physicochemical properties

A variety of physical studies have shown that both the glycopeptide and peptide antifreezes are expanded molecules (DeVries *et al.* 1970; Raymond *et al.* 1977; Ananthanaryanan & Hew 1977; Franks & Morris 1978). Although X-ray diffraction, detailed circular dichroism studies and natural abundance ^{13}C nuclear magnetic resonance studies have been done on the glycopeptides, little can be concluded about their secondary structure in solution except that they are extended structures (Raymond 1976; Ahmed *et al.* 1975; Berman *et al.* 1980; Bush *et al.* 1981). With the peptide antifreezes, however, circular dichroism studies and viscosity measurements indicate that all except one are helical rods (Raymond *et al.* 1977;

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Ananthanaryanan & Hew 1977; Slaughter *et al.* 1981). In this conformation, the polar residues aspartate and threonine are generally separated by two alanine residues equivalent to a distance of 4.5 Å† (DeVries & Lin 1977*b*). The existence of this repeat spacing between threonine and aspartate in the peptide appears to be of importance in the recognition of the ice lattice and the binding to the oxygen and hydrogen atoms in it (DeVries & Lin 1977*b*).

Non-colligative freezing points

The fact that relatively large molecules in the blood of polar fishes produce a substantial depression of the freezing point indicates that the depression is effected via a non-colligative mechanism. Investigations of freezing and melting of the blood and solutions of the purified

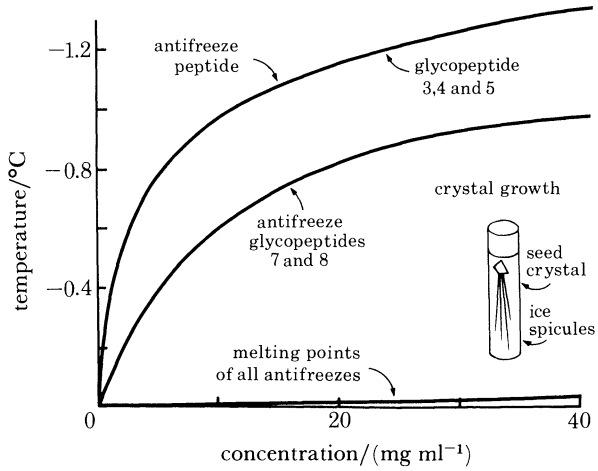

FIGURE 1. Freezing and melting points of aqueous solutions of the peptide and glycopeptide antifreezes at several concentrations. The large molecular mass glycopeptides and peptides have essentially the same antifreeze activity, while the low molecular mass glycopeptides have much less. The melting points are the same for all sizes. Inset: ice growth is in the form of spicules parallel to the *c*-axis.

antifreezes have revealed a most unusual freezing–melting behaviour. The melting point of the solid phase (a seed ice crystal of approximately 100 μm diameter) occurs at a temperature predicted by colligative relations; that is, the melting point is in accord with what is expected from the Raoult law, where by definition the melting point would be the equilibrium freezing point. At this temperature the vapour pressure over the solid and liquid phase are equal (Pauling 1953). The freezing point of blood and antifreeze solutions (temperature at which growth of the 100 μm seed crystal begins), however, is 1 to 1.5 °C lower than the melting point (DeVries 1971; Feeney & Hofman 1973; Scholander & Maggert 1971; Raymond & DeVries 1977; Tomimatsu *et al.* 1976). In the blood of Antarctic notothenid fishes, a seed crystal melts at −1 °C while ice propagates rapidly from the face of the seed at −2.2 °C (DeVries 1971, 1982). In a 2% (by mass) aqueous solution of glycopeptide or peptide antifreeze, a 100 μm diameter seed crystal melts at approximately −0.02 °C, but will not increase in size until the temperature is lowered to −1.2 °C (figure 1). In between these temperatures neither growth nor melting is observed, even if the crystal is observed for several weeks (DeVries & Lin 1977*a*; Raymond 1976; Raymond & DeVries 1977). Below −1.2 °C growth occurs and, as in the

† 1Å = 10^{-10} m.

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blood, is very rapid and in the form of long thin spicules with the direction of growth parallel to the *c*-axis (DeVries 1971; Raymond & DeVries 1977).

Most of the glycopeptide and peptide antifreezes exhibit the same depression of the freezing point of water on a mass basis except for some of the smaller glycopeptides isolated from the Antarctic nototheniids (Duman & DeVries 1972; Lin *et al.* 1972) and northern cods (Fletcher *et al.* 1982; O'Grady *et al.* 1982*b*). Solutions of the low molecular mass glycopeptides produce only about half of the non-colligative lowering of the freezing point that the larger glycopeptides and peptides do when compared on a weight basis. When compared on a molar basis this antifreeze effect is less pronounced in the smallest glycopeptide (glycopeptide 8) and increases with molecular size (Schrag *et al.* 1982). Below a certain molecular mass the antifreeze effect diminishes rapidly (Schrag *et al.* 1982). The antifreezes also affect the crystal habit in that when growth of the seed crystal occurs it is in the form of long spicules in which most of the growth is parallel to the *c*-axis (Raymond & DeVries 1977).

Freezing point estimates of polar fish fluids and antifreeze solutions have been obtained under conditions where substantial undercooling (4–6 °C) exists before freezing. In this manner freezing takes place rapidly, and the freezing points obtained are much higher (Raymond & DeVries 1972; Osuga *et al.* 1978; Slaughter *et al.* 1981; Schrag & DeVries 1982*a*), and with glycopeptides 7 and 8 they approximate values expected on the basis of colligative relations. However, substantial undercooling does not approximate conditions experienced by polar fish and the freezing points thus obtained are therefore viewed as non-physiological (DeVries 1982, 1983). The observed freezing rate dependence is consistent with a freezing point depression mechanism involving inhibition of growth at the ice–water interface.

Possible mechanisms of antifreeze activity

Immobilization of water

In the past it has often been suggested that the antifreezes cause the large freezing point depression of water through structuring or immobilizing water in a manner that would reduce the amount of water available for ice formation (DeVries *et al.* 1970; Haschemeyer *et al.* 1977). The expanded structures of the antifreeze and the abundance of side chains rich in hydroxyls for the glycopeptides, and in polar groups for the peptides suggest that they may indeed be good candidates for structuring water in their vicinity. Recent studies employing nuclear magnetic resonance techniques (Haschemeyer *et al.* 1977) indicate that the amount of bound water is small. Isopiestic determinations of water binding under equilibrium conditions indicate that antifreezes bind only slightly more water than other proteins of a similar size when in solution (Duman *et al.* 1980). So, the antifreeze effect cannot be explained by water structuring or immobilization, in view of the low antifreeze concentrations required to produce the effect, and the small number of water molecules associated with the antifreeze in solution.

Adsorption inhibition of crystallization

In the field of crystal growth and inhibition of crystal growth there is a considerable amount of information indicating that the presence of small amounts of impurities can inhibit the crystallization of solutes or the growth of small crystals (Buckley 1952). Such inhibitors are usually characterized by a specificity for a particular kind of solute or crystal, and large polymers composed of repeating units appear to be more effective than small non-repetitive

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ones. In the inhibition of crystallization, inhibitors are thought to bind to embryonic crystal nuclei, thereby preventing further growth. Because of their small size these molecular nuclei or clusters have high surface free energies, and therefore are unstable and will redissolve into solution. With crystals that are large enough to be stable because of low surface free energies, adsorbed impurities are thought to inhibit or slow growth by interfering with the propagation of steps across the face of the crystal. In many cases, adsorption of impurities also causes a change in the type of crystal growth, or habit, observed when the supersaturation point is exceeded (Butchard & Whetstone 1949). The inhibition of the freezing of water, or the inhibition of the growth of ice crystals in the presence of the antifreezes at temperatures below the equilibrium freezing point, appears to be an example of this adsorption–inhibition phenomenon.

Adsorption

Studies of the freezing behaviour of solutions of the glycopeptide and peptide antifreezes indicate that they interact with and adsorb to ice during freezing (Duman & DeVries 1972; Lin *et al.* 1972; Raymond & DeVries 1977). Antifreezes do not concentrate in brine pockets when antifreeze solutions containing small amounts of sodium chloride are frozen at $-2\,^{\circ}C$, but preferentially interact with ice (Raymond & DeVries 1977). This affinity for the solid phase varies with molecular mass; the small glycopeptide molecules appear to adsorb less than the larger ones, in correlation with their lower antifreeze activity. The affinity for ice, as well as the antifreeze activity, is lost if the chemical structures of the antifreeze are altered (Shier *et al.* 1972; Duman & DeVries 1972). For glycopeptides, alteration of the hydroxyls of the carbohydrate moiety (Lin *et al.* 1976), or shortening of the molecule by limited cleavage of the polypeptide backbone (Komatsu *et al.* 1970) leads to loss of activity. Reduction in the size of the glycopeptides by sequential degradation results in a decrease in antifreeze activity and below a molecular mass of 2000 Da, a rapid decline of activity occurs (Schrag *et al.* 1982). For the peptides, modifications of the carboxyl groups of the regularly spaced aspartic and glutamic acid residues result in loss of activity (Duman & DeVries 1976). Specific modification of the sculpin peptide antifreeze by attachment of fluorocene to its 4 lysine residues results in complete loss of activity (Schrag & DeVries 1982*b*). These observations are consistent with the idea that large polymers made up of repeating subunits are better inhibitors of crystallization than the subunit alone. At very low antifreeze concentrations with slight undercooling, ice growth occurs with expression of faces that correspond to $(1\,0\,\bar{1}\,0)$ faces. These faces are rarely observed when ice is grown from the melt (Knight *et al.* 1983); but are common in ice grown from the vapour phase (Knight 1967). Low concentrations of the antifreezes also prevent recrystallization (Knight 1982). These observations are consistent with an adsorption–inhibition mechanism involving adsorption of the antifreeze at the ice–water interface.

The loss or reduction in potential hydrogen bonding groups upon chemical modification of the polar side chains suggests that hydrogen bonding may be involved in the attachment of antifreezes to ice. For the antifreeze to be strongly adsorbed to ice, potential hydrogen-bonding residues would need to occupy positions in the antifreeze molecule to allow them to align with the opposite oxygens and hydrogens in the ice lattice in a regular pattern. Although there are uncertainties about the secondary structure of the glycopeptide antifreeze, examination of space filling models reveal that such alignments are possible. Many of the hydroxyls of the disaccharide side chains are separated by a distance of 4.5 Å, a distance that also separates the oxygens parallel to the *a*-axes in the ice lattice. A 7.36 Å repeat spacing of the oxygens in

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the ice lattice also exists, but it is parallel to the *c*-axis (Fletcher 1970). In a completely extended conformation, alternate carbonyl groups are separated by 7.3 Å (Pauling *et al.* 1951), which is very close to the 7.36 Å oxygen repeat spacing in ice (DeVries 1980). There is some evidence for the existence of a completely extended conformation (Franks & Morris 1978), but it is not overwhelming.

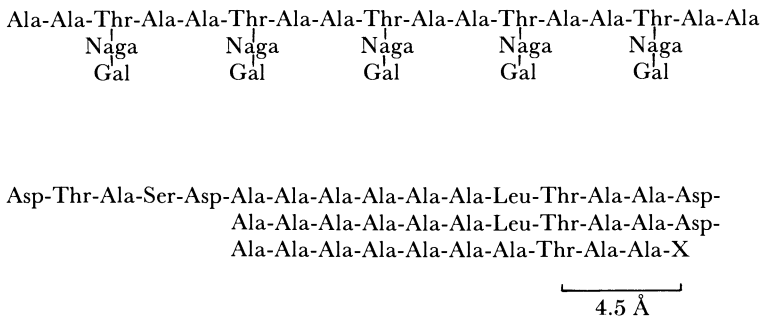

FIGURE 2. The primary structure of glycopeptides 1–5 isolated from the Antarctic notothenid, *Dissostichus mawsoni*. The basic glycotripeptide is repeated in the different sizes except glycopeptides 6, 7 and 8, where prolines replace some of the alanines. The lower panel shows the structure of one of the antifreeze peptides isolated from the blood of the winter flounder, *Pseudopleuronectes americanus*. In the conformation of a helical rod, the aspartic and threonine residues are separated by 4.5 Å, a distance that also separates the oxygens on the prism faces parallel to the *a*-axes in hexagonal ice.

Studies of the antifreeze peptides from the winter flounder have provided much clearer evidence for a lattice match. Structural studies of the flounder antifreeze peptides indicate the presence of clusters of polar amino acids separated by long sequences of non-polar alanine residues followed by a leucine (figure 2). The polar clusters usually contain threonine and aspartate separated by two alanines (DeVries & Lin 1977*a*; Hew & Yip 1976). In the Alaskan plaice peptide, a similar polar, non-polar arrangement is also present (DeVries 1982). Physical and chemical studies indicate that these peptides are in the form of an α-helix and resemble a rigid rod (Ananthanaryanan & Hew 1977; Raymond & DeVries 1974). The polar side chains of this helical rod are located on one side of the molecule while the other side has many non-polar methyl groups of alanine projecting from it (DeVries & Lin 1977*a*). The distances between the aspartate and threonine residues in such a molecule is found to be 4.5 Å, a repeat distance that also separates adjacent oxygens in the basal plane and in the prism faces that are parallel to the *a*-axes in hexagonal ice. This lattice match between the polar residues and the oxygens in the ice lattice strongly suggests that the peptides orient themselves on the lattice and adsorb to it through hydrogen bonding. Figure 3 illustrates how the flounder peptide might be aligned parallel to one of the *a*-axes of the lattice and hydrogen bond to the prism face of hexagonal ice. In such a model every third and possibly fourth row of oxygens does not participate in hydrogen bonding. This is important because an uninterrupted 4.5 Å repeat spacing of the polar residues may result in nucleation activity.

Similar polar, non-polar sequences have been found in an antifreeze peptide isolated from the Alaskan plaice (DeVries 1982, 1983), in which the repeat spacing of the polar threonines and aspartates are conserved. Preliminary sequence studies of fragments of the sculpin and eel pout antifreeze peptides also show the presence of some threonine and serine residues separated

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from either aspartate or glutamate residues by 4.5 Å. Lysine and arginine residues separated by 4.5 Å have also been identified (Schrag 1983; unpublished work).

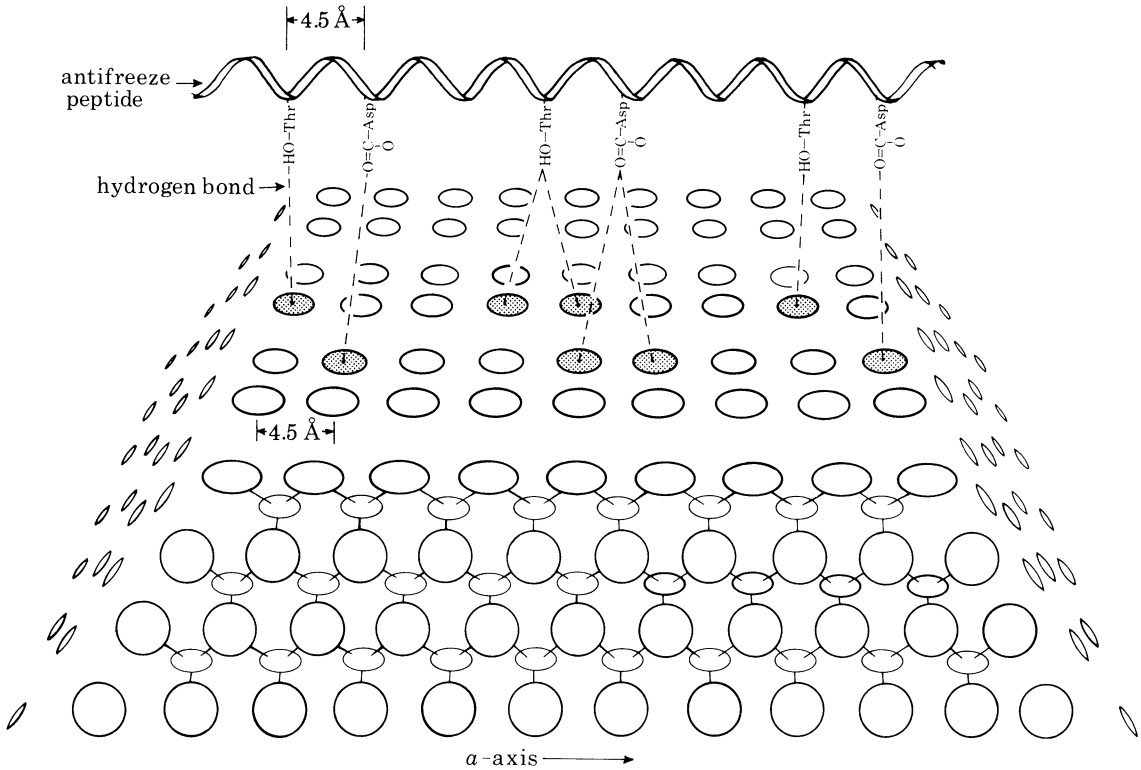

FIGURE 3. Model of flounder antifreeze hydrogens bonded to prism face of hexagonal ice, parallel to one of the *a*-axes. Stippled circles represent oxygens in the ice lattice that participate in hydrogen bond formation with the hydroxyl of the threonine residue and the carboxyl of the aspartic acid residue. These two residues are separated by 4.5 Å, a distance that also separates the oxygens in the prism face parallel to the *a*-axes of hexagnoal ice.

The conservation of the threonine and aspartate repeat distance in antifreeze peptides from unrelated fishes suggests to us that a 4.5 Å repeat spacing of polar residues is necessary for lattice binding and antifreeze function. However, further sequence studies are needed to verify this point. This 4.5 Å repeat spacing is absent in some antifreezes. An antifreeze peptide isolated from the sea raven has a very different composition, and appears to lack a helical conformation (Slaughter *et al.* 1981). The difference in composition and the lack of a helical structure, and therefore presumably the 4.5 Å repeat distance as well, suggests that a lattice match based on a 4.5 Å spacing is absent. It is possible that for the sea raven peptide and the glycopeptides, binding does not require a regular repeat spacing, but only the presence of hydrogen bonding groups at widely separated sites, which can hydrogen bond to three or four oxygens that are not necessarily arranged in any specific regular repeating pattern in the ice lattice. In support of this hypothesis, within the disaccharide sidechain of the glycopeptide, there are 3 or 4 hydroxyl groups projecting from the same side of the disaccharide, some of which are separated by distances that closely approximate the 4.5 Å spacing between the oxygens of the water molecules in the ice lattice, and others that are separated by approximately 7 Å.

Examination of space filling models shows that these hydroxyl groups do not appear to align with any particular array of oxygens in the ice lattice, but that they could form hydrogen bonds

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with 3 or 4 oxygens in a given area of the ice lattice. If the location of the disaccharides alternate on opposite sides of the polypeptide chain, there is sufficient orientational flexibility to preclude assignment of a specific repeat pattern for binding.

Inhibition of crystallization and crystal growth

The transition of water from the liquid into the solid phase occurs by either homogeneous nucleation or heterogeneous nucleation (Franks 1982). With only one to two degrees of undercooling it is unlikely that homogeneous nucleation would be involved in the freezing of polar fishes. Heterogeneous nucleation is most likely the mechanism by which ice formation occurs in polar fishes when they occasionally freeze. For particles within the fish to act as a nucleation site at a temperature of −2 °C, they must be 300–400 Å in diameter. Exogenous ice crystals penetrating the integument, or food particles within the intestine, are probably some of the most important potential sources for nucleation of the aqueous body fluids of these fishes. The intestinal fluid, however, is protected by the presence of high levels of glycopeptides, which apparently are not digested (O'Grady *et al.* 1983). These glycopeptides either inactivate potential nucleators or inhibit the growth of the ice that they nucleate. In any case, there is no evidence for visible ice formations in the body fluids, and research is presently being done in several laboratories to explain the anomalously low freezing points in these fishes. To more fully understand the nature of the problem it is helpful to examine other examples of this phenomenon.

In some gels and tissues, anomalously low freezing points have been reported. In these systems the low freezing points of water have been examined on the basis of increased surface free energy, which results from the high ratio of surface area to volume of the ice crystals formed. Physical constraints imposed by compartmentalization in gels and tissues result in the formation of microcrystals that have high surface free energies (Block *et al.* 1963; Kuhn 1956), and because of their high surface free energies these microcrystals are stable only at lower temperatures and therefore have lower equilibrium freezing points.

The adsorption of fish antifreezes to microscopic ice crystals could result in a relatively large increase in surface area with respect to the increase in volume, with a concomittant increase in surface free energy and a consequent requirement of a lower temperature for freezing to occur. In other words, the freezing point is depressed. The manner in which this may take place is now described. Ice crystal growth occurs when water molecules join the crystal at growth steps on the basal plane (figures 4 and 5). Growth of hexagonal ice is primarily on the basal plane of the crystal along the *a*-axes (Knight 1967; Franks 1982). Adsorption of antifreeze molecules at steps on the basal plane appears to block growth in the region where the antifreeze covers the step because the diameter of the antifreeze molecule is approximately twice the height of the step, and therefore ice cannot propagate over it. Most antifreeze molecules are approximately 100 Å long, and when bound to ice the non-polar region probably projects into the aqueous environment. Adsorption of several antifreeze molecules on a single long uninterrupted step will divide it into many steps of short lengths. Further growth is possible only between the adsorbed molecules, and assumes a curved front (Raymond & DeVries 1977). It is these highly irregular fronts that contribute to a large rate of increase of surface area to volume.

The magnitude of freezing point depression is dependent on the rate of change of surface area with respect to that of the volume, which, for ice growing in a circular plate, is inversely

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proportional to the radius (Raymond 1976). Therefore, for a particular amount of undercooling, there is a corresponding critical radius of curvature that determines the extent of growth. But given sufficient undercooling, the radius of curvature of the small circular fronts between adsorbed molecules will decrease from infinity (straight front) to a minimum value of half the distance between two adjacent molecules. It follows that the smaller the spacing between the adsorbed molecules, the greater is the undercooling required to allow the step to propagate

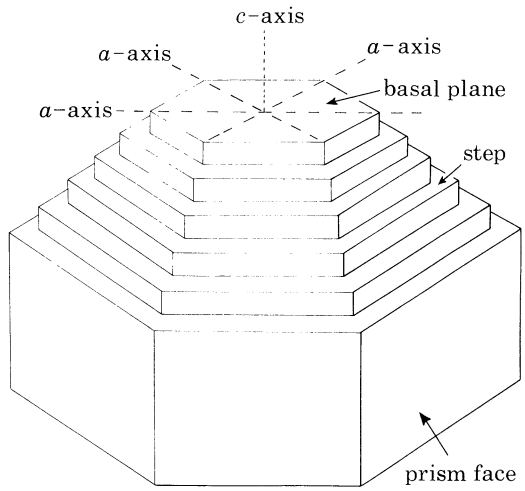

FIGURE 4. Diagrammatic illustration of hexagonal ice crystal showing prism faces and steps between basal planes. The *a*-axes are parallel to prism faces and perpendicular to the *c*-axis.

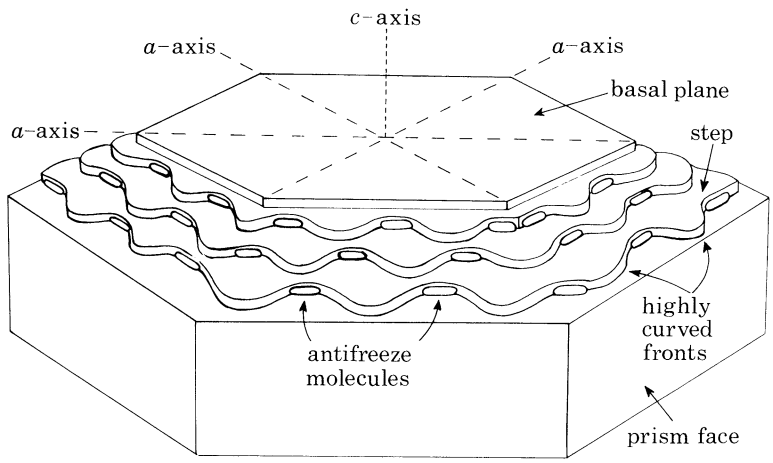

FIGURE 5. Model illustrating adsorption inhibition as a mechanism of non-colligative lowering of the freezing point of water. Antifreeze molecules adsorbed on the basal plane interfere with the propagation of steps across the basal plane because the steps become highly curved (see text for details).

through. If the undercooling is increased so that the radius of curvature reaches its minimum, then one will have a row of semicircles tangential to one another. If the antifreeze molecule is short, adjacent semicircles can easily fuse around it to form 'cusps'. Such a 'cusp' is an unstable shape due to edge tension and becomes a favourable site for further growth. Once the adsorbed molecule becomes surrounded, the step straightens and propagates until it is retarded or stopped by the next row of impurities, where the process repeats itself unless the undercooling is lessened or the joining of the fronts is prevented. If the distance between adjacent semicircular

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fronts is increased due to the adsorption of larger molecules, the joining of individual fronts around the adsorbed molecule is prevented even with extensive undercooling (Raymond 1976).

The spacing between the antifreeze molecules on the ice crystals appears to be a function of their concentration in the liquid phase, and of their size (Raymond 1976). By assuming a single surface density of antifreezes, and a square array distribution of the antifreeze molecules on the crystal faces, the undercooling necessary for growth (freezing point depression) is proportional to the square root of the concentration of adsorbed molecules. By using this relation it has been shown that there is good agreement between the predicted and experimental curves relating freezing point depression to antifreeze concentration (Raymond & DeVries 1977) for the antifreeze peptides and large molecular mass glycopeptides. For small glycopeptides the predicted freezing point depressions are approximately twice the experimental values. The cause of this discrepancy is unknown, but may be related to the relatively small size of these molecules. If completely extended, glycopeptide 8, which has a molecular mass of 2400 Da is approximately 45 Å long. This distance may be insufficient to prevent adjacent semicircular fronts from fusing at moderate undercooling. With the larger molecular mass glycopeptides the extended lengths would be of the order of 300–400 Å and so the fronts would be widely separated with little chance for fusion.

At high antifreeze concentrations ice growth observed at temperatures below the predicted values is extremely fast and is parallel to the *c*-axis, the non-preferred direction of growth. Of course some growth must occur along the *a*-axes for a crystal to be three-dimensional, but it is small relative to the *c*-axis growth. By contrast, at very low antifreeze concentration, the expression of prism faces occurs. Conceivably, growth on the prism faces is only slowed to the extent that there is still enough time for the edges to completely fill out to produce well defined hexagonal crystals. These observations are consistent with a mechanism involving inhibition or slowing of growth at the ice–water interface at the steps on the basal plane.

Avoidance of freezing in fishes

Many polar fishes spend their life time (6–30 years) at −2 °C, yet no evidence for the presence of microscopic or macroscopic ice crystals in their body fluids has been found even though they often come into contact with ice (DeVries 1982). If small crystals enter the fish or form from internal nucleators, they will not melt because the melting point of ice in their blood is 1 °C above the ambient water temperature. Thermal sites where melting could occur have not been found in any polar fishes (DeVries & Lin 1976*b*). If ice crystals of such a size (over 10 μm) were to accumulate, they could obstruct the circulation of the fish. So at present it is unclear at what site the antifreezes prevent ice formation in fishes. They could function at the level of inhibition of heterogeneous nucleation, or inhibition of propagation of crystals from the environment across the integument into the body fluids. Another possibility is that small crystals do enter, or form within the fluids, and that the antifreeze inhibits their growth before they reach sizes (800–1000 Å) that are stable at −2 °C. Inhibition of the growth of small crystals (100 Å diameter) into stable crystals would perhaps provide time for these structures to melt. So it is likely that the antifreezes function at the level of inhibition of nucleation or inhibition of growth of crystals of subcritical radii at −2 °C. Inhibition of the conversion of embryos or ice nuclei into stable crystals will perhaps provide time for these unstable structures to dissociate, and for the resulting water molecules to disperse. However, experimental demonstration of this hypothesis has yet to be devised and performed.

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Discussion

T. J. PRICE (*Unilever Research, Port Sunlight Laboratory, U.K.*). It is well known that the nucleation of ice is a heterogeneous process above about −40 °C. It therefore seems likely that the inhibition of freezing *in vivo* involves adsorption of the polymer on the most favourable nucleation sites on foreign solid surfaces present, rather than on either well formed or embryo ice crystals.

A. L. DEVRIES. Some of the fluids of polar fishes living at −2 °C lack antifreeze and these fluids (urine and cytosol) remain supercooled by 1 °C throughout the life of the fish. Ice formation does not occur in the urine and cytosol of these fishes, which suggests to us that at this temperature 'foreign solid surfaces' and cellular structural components that act as nucleation sites are unimportant or absent. In addition, the fact that all fishes can remain supercooled by 2 or 3 °C in ice-free water indefinitely would appear to support this conclusion. Exposure of fishes lacking biological antifreeze systems to seawater that contains ice crystals at −2 °C results in ice propagation across the body wall into the fluids. With fishes possessing antifreeze systems, ice does not appear to propagate across the body wall into the fluids. This is so even in injured ones where the interstitial fluid of the muscle mass is exposed to the ice-laden environments. Such observations would appear to be consistent with the theory that *in vivo* avoidance of freezing involves inhibition of growth, or propagation, of 'well formed' ice crystals by the adsorption–inhibition mechanism described in this paper.

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