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Review

Structure, function and evolution of antifreeze proteins

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Abstract. Antifreeze proteins bind to ice crystals and modify their growth. These proteins show great diversity in structure, and they have been found in a variety of organisms. The ice-binding mechanisms of antifreeze proteins are not completely understood. Recent findings on the evolution of antifreeze proteins and on their structures and mechanisms of action have provided new understanding of these proteins in different contexts. The purpose of this review is to present the developments in contrasting research areas and unite them in order to gain further insight into the structure and function of the antifreeze proteins.

Key words. Antifreeze; ice; protein structure; protein function; evolution; cell membranes.

Introduction

The antifreeze proteins and glycoproteins [AF(G)P] were first identified in Antarctic teleost fishes as the causative agents of serum freezing point depression [1]. In Antarctic fish species, the antifreeze glycoproteins (AFGP) were found to lower the freezing point by more than 1 °C to match that of seawater, and their name reflects this biological function. The antifreeze glycoproteins were immediately recognised as unusual because they caused a freezing point depression far greater than would be predicted from colligative properties alone [1]. Later studies demonstrated that the antifreezes lower the freezing point by interacting directly with the ice surface and causing a thermal hysteresis [2], and this would account for their highly efficient noncolligative freezing point depression. Excellent progress has been made in the study of antifreeze-ice interactions. However, a precise definition of the interactions between AF(G)Ps and ice that would give rise to antifreeze activity has not yet been obtained. It would seem obvious that functional groups positioned to match the ice lattice on a particular plane would lead to ice binding and antifreeze activity. Yet in this context we have not succeeded in defining what, exactly, distinguishes an antifreeze protein from a non-antifreeze protein. This is a fascinating question in protein structure and function and in protein evolution. It is also of interest in biotechnology because the design of new peptides or of peptidomimetics with antifreeze activity will require a priori knowledge of the requisite structures for ice binding and growth inhibition.

Among teleost fishes, five unrelated types of AF(G)Ps have been characterised, and each has a very narrow taxonomic distribution [3]. Since their discovery in fish, numerous different AFPs have been found in plants,

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Table 1. Overview of antifreeze protein characteristics.

Protein	Species	Structural type	Protein homology	Ice binding site	References
Fish type I AFP	righteye flounders, sculpins	single α-helix, many with sequence repeat	undetected	Thr and residues associated	[19, 20, 25, 79, 80]
Fish type II AFP	sea raven, rainbow smelt, Atlantic herring	globular; smelt and herring are Ca^{2+}-dependent	galactose-binding; C-type lectins in herring	with it corresponds to Ca^{2+} binding site	[31, 33, 81]
Fish type III AFP	eel pouts such as ocean pout (*Macrozoarces americanus*)	globular with one flattened surface	undetected	residues on and flanking flat surface	[82, 83]
Fish type IV AFP	longhorn sculpin (*Myoxocephalus octodecimspinosis*)	antiparallel helix bundle	low-density lipoprotein receptor-binding domain of apolipoprotein E3	unknown	[84]
AFGP	cods and Antarctic nototheniids	polymer of Ala-Ala-Thr and variants, disaccharide on each Thr	none – de novo evolution	Gal-GalNAc	reviewed in [4, 49]
Chitinase AFP	winter rye	endochitinase	endochitinase	unknown	[58]
Glucanase AFP	winter rye	endoglucanase	endoglucanase	unknown	[58]
Thaumatin AFP	winter rye	thaumatin-like	thaumatin	unknown	[58]
Budworm AFP	spruce budworm (*Choristoneura fumiferana*)	Thr- and Cys-rich (non-repeating)	undetected	unknown	[61]
Beetle AFP	*Dendroides canadensis*, mealworm beetle	Thr- and Cys-rich with repeating structure	undetected	unknown	[62, 63]

animals, fungi and bacteria [4]. However, their distribution appears strictly limited to species that are exposed to cold or freezing temperatures, and in many cases their occurrence within those species is limited to periods of cold exposure. The diversity and specificity of the AF(G)Ps is complicated by the recent discovery of activities in these proteins that are distinct from ice binding and freezing point depression [5]. They appear to protect mammalian cell membranes from damage at temperatures above 0 °C [6, 7]. Thus, our perspectives on the distribution, diversity and roles of AF(G)Ps are in transition.

This review will focus on some of the current developments in our knowledge of AF(G)Ps. Recent reviews have addressed many aspects of AF(G)Ps [3, 5, 8–10]. However, in various areas of study, our understanding of AF(G)Ps is progressing rapidly. Therefore, in this review, recent progress in our understanding of AF(G)P evolution will be meshed with information on their structure and function. By integrating different areas of antifreeze research, we intend to gain new insights into their activity and their role in nature.

Overview of AFP diversity

AFPs from several species have been isolated and characterised. The proteins for which sequence or structural information has been obtained include the five types of AFPs found in fish, two types from insects and the three AFPs of a plant, winter rye. Characteristics of these proteins are summarised in table 1. The diversity of the antifreezes has been covered in recent reviews [3, 4, 10]. Evidence for even greater variety among antifreezes is accumulating. Antifreeze activity is found in many species of plants, but with the exception of the winter rye (*Secale cereale*) AFPs, the active proteins remain to be characterised. The antifreezes of bacteria are also not yet well understood, although investigation is progressing. For example, the AFP of *Pseudomonas putida*, a common soil bacterium, has been purified, and it has an amino acid composition and other compositional properties similar to those of the bacterial ice nucleating proteins. This protein differs from other known antifreezes in having both ice nucleating and antifreeze activities [11]. Sequencing and structural studies on this protein will be of great interest.

The different AF(G)Ps have two unusual and contrasting origins. Some are rogue proteins that have emerged from protein families with completely unrelated functions. Others are genuinely novel proteins whose genes came to exist through relatively recent genetic events. The surprising diversity of the AF(G)Ps together with their very narrow distributions has led to the hypothesis that they have each evolved recently and independently, well after present animal and plant orders or species had attained their current forms [12]. During geologically recent cooling and glaciation events, it is likely that species underwent tremendous selective pressure to avoid freezing under progressively cooler conditions [12]. This situation would have favoured any means of lowering the freezing point that would not cause physi-

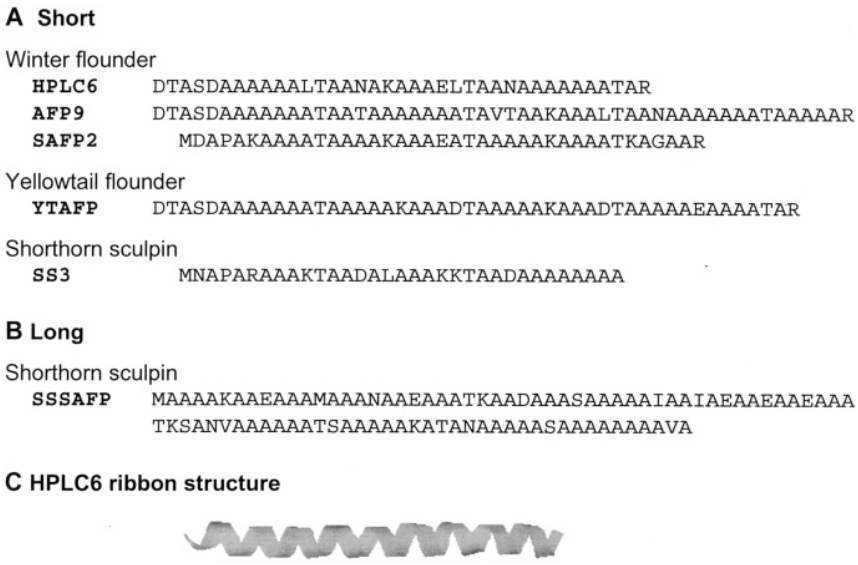

Figure 1. Protein sequences and a structure of representative fish type I AFPs. The sequences are aligned by eye for ease of comparison. The ribbon diagram of HPLC 6 image was generated using RasMol. References are: winter flounder serum HPLC6 [79], winter flounder serum AFP9 [19], winter flounder skin SAFP2 [27], yellowtail flounder YTAFP [20], shorthorn sculpin serum SS3 [25], shorthorn sculpin skin SSAFP [28], and HPLC6 structure [13].

ological disturbances, and it could well have led to the present multitude of noncolligative antifreezes.

An alanine theme with variations

The type I fish AFPs are alanine-rich α-helical proteins, and they are found in the righteye flounders and in certain sculpins (fig. 1). Most are small proteins that form single amphiphilic or partly amphiphilic α-helices [13]. The prototype and most carefully studied type I AFP (isoform HPLC6) is the major serum isoform of winter flounder (*Pleuronectes americanus*) [13, 14]. This AFP and another abundant isoform (HPLC8) found in flounder serum are both 37-residue proteins that contain 11-residue sequence repeats consisting of Thr-X_{10} [15]. The Thr residues of winter flounder type I AFP (HPLC6) are regularly spaced along one side of the helix. The distance between Thr OH oxygens is 16.5 ± 0.5 Å, which corresponds to water molecule spacing on the ice surface in the direction of HPLC6 binding. The major serum antifreezes of winter flounder are encoded by a multigene family and expressed primarily in liver [16]. Expression of these genes is under environmental control through growth hormone, and serum AFP levels are severely depressed in summer [17, 18].

Winter flounder also have a four-repeat serum antifreeze [19] that is more active than the more common three-repeat proteins. In yellowtail flounder (*P. fer-rugineus*), the primary serum antifreeze has four repeats, although the sequence is distinct from those of flounder, and this protein has lower activity than the three- or four-repeat winter flounder AFPs [19, 20]. The lower activity of the yellowtail flounder AFP appears to result from its lack of certain ice-binding residues [13, 20]. The yellowtail flounder AFP is also encoded by a multigene family [20]. It seems likely that the flounder have evolved antifreezes with different repeat numbers by unequal crossing over [21]. It is interesting that no two-repeat or five-repeat AFPs have been identified in flounder. Studies have suggested structural and functional constraints on repeat numbers in these antifreezes. Analyses of synthetic or chimeric antifreeze variants have suggested that two-repeat antifreezes are unstable and/or inactive, and the five-repeat form may have limited solubility in aqueous solutions [22–24].

Shorthorn sculpin (*Myoxocephalus scorpius*) have an α-helical serum antifreeze that is similar to that of winter flounder in size and also contains about 60% Ala (fig. 1). However, in spite of these similarities, the proteins do not have very similar sequences, and gene hybridization experiments did not reveal any relationship (P. L. Davies et al., personal communication). The obvious repeating elements present in the flounder serum AFPs were not evident in the sculpin protein [25]. The quasi-similarity between the sculpin and flounder proteins prompted great interest in obtaining a sculpin

AFP clone. However, no AFP clone could be isolated from sculpin liver complementary DNA (cDNA) libraries. This led to a logical hypothesis that the similar sculpin and flounder serum AFPs were the products of convergent evolution from different genetic elements [12].

The possibility of a connection between the sculpin and flounder AFPs emerged from an investigation unrelated to sculpin AFPs. In a study of winter flounder AFP gene expression, Gong et al. [26] observed that AFP genes in flounder skin are not environmentally controlled through growth hormone, like the genes expressed in liver. This prompted further examination of the AFPs of flounder skin. Skin AFPs distinct from those of serum were discovered, and they were shown to be encoded by a separate subset of genes from those in liver [27]. The AFPs encoded by the skin-expressed genes were Ala-rich and contained the Thr-X_{10} repeat typical of the serum AFP. However, these proteins began with the sequence MDAPA, like the sculpin serum proteins [27]. This provided weak but suggestive evidence for a relationship between the sculpin serum and flounder skin AFPs.

The slight but promising similarity between the flounder skin AFP and the sculpin serum AFP led to an attempt to clone AFP from sculpin skin instead of liver. A sculpin skin cDNA library was screened using a flounder skin AFP cDNA probe. The positive clone encoded a 92-residue Ala-rich α-helical type I AFP [28]. The protein is much larger than any other type I AFP (fig. 1). Moreover, it contains no obvious internal sequence repeats, and aside from sequence identities resulting from overlapping Ala residues, it shows no similarity to the sculpin or flounder AFP sequences. Expression of the protein in *Escherichia coli* and subsequent activity analysis revealed that this unusual protein is a genuine AFP [28]. However, this AFP from sculpin skin bears no similarity to the sculpin serum AFP.

In addition to their sequences suggesting a relationship with sculpin AFP, flounder skin AFPs have another surprising feature. These AFPs lack signal and prosequences that are present in flounder serum AFP [27]. The absence of pre- and prosequences suggest that the protein may be intracellular [27], which runs counter to the classical definition of AFPs as proteins that halt extracellular ice growth. However, the actual cellular location of skin AFPs in vivo has not yet been determined. The possible functions of an intracellular AFP are unclear at present, although a number of potential roles have been advanced [5, 27]. There are instances in which proteins with no signal sequences are exported from cells via a pathway independent of the endoplasmic reticulum [29, 30]. Therefore, before we can define the role of skin AFPs, it will be important to determine experimentally whether they are actually retained in cells.

Among type I AFPs, there are many levels of variation. In blood serum of a single species, winter flounder, there are multiple sequence isoforms. In skin of the same species, there are multiple isoforms distinct from those in blood. The blood of shorthorn sculpin contains yet another AFP, unrelated to that of the flounder blood, and sculpin skin contains an AFP that is much larger than all the others and distinct in other ways. Compounding this diversity is the possibility that specific AFPs might be intra- or extracellular and consequently have different biological roles. The questions of evolutionary relationships among these AFPs and their evolutionary origins remain to be answered. Sequence analysis and Southern hybridisations have demonstrated that the skin and liver-expressed AFP genes in winter flounder are indeed related [27]. However, the sculpin skin AFP that was isolated by screening with a flounder cDNA shows no DNA sequence similarity to any of the flounder genes apart from the abundant Ala codons. Substantial percentages of protein sequence identity are sometimes observed among type I AFPs. However, these are largely due to multiple Ala alignments as a consequence of their Ala richness. Sequence analysis algorithms that rely on statistical matrices, such as BLAST or FASTA, are not very useful in the analysis of these proteins. The repeated Ala residues in the proteins and Ala-specific codons in DNA encoding type I AFPs can lead to spurious statistical results, and the lack of highly conserved residues such as Cys or Trp could result in failure to detect weak but important similarities. The elusive gene for the shorthorn sculpin serum AFP will be even more interesting to study in light of the recent developments and current limitations in our understanding of flounder and sculpin type I AFPs.

From carbohydrates to ice

Type II AFPs are 130- to 150-residue globular proteins containing five disulfide bonds [31–33]. They are found in three species: Atlantic herring (*Clupea harengus harengus*), rainbow smelt (*Osmerus mordax*) and sea raven (*Hemitripterus americanus*) [33]. Type II AFPs evolved from preexisting proteins with a completely different recognition activity. They are homologous to the carbohydrate recognition domains (CRDs) of Ca^{2+}-dependent (C-type) lectins [31–33]. They show up to 30% sequence identity with CRDs of C-type lectins, and their folds are very similar [31–36]. C-type CRDs are found in a number of different proteins with structural, immunological and metabolic roles [37]. The soluble C-type lectins of vertebrates consist primarily of man-

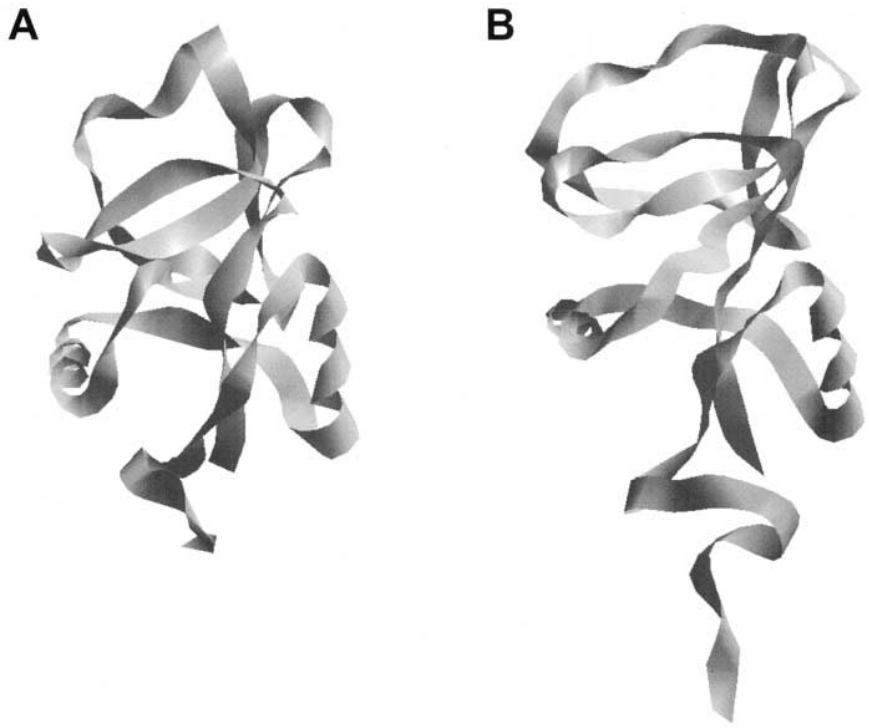

Figure 2. Type II AFPs shown as ribbon diagrams. (*A*) Structural model of herring AFP obtained by homology modelling [35]. (*B*) Solution structure of sea raven AFP determined by NMR [36]. Images were generated using Hyperchem.

nose-binding lectins and other collectins that play roles in innate immunity involving recognition of pathogen surface carbohydrates [37]. Soluble galactose-binding C-type lectins are purported to play a similar role in invertebrates [38].

C-type lectins comprise a large protein superfamily. It is not clear which of these lectins gave rise to type II AFPs. Statistical sequence alignments suggest that the AFPs are most closely related to particular subsets of these lectins, including invertebrate galactose-binding lectins, hepatic lectins (asialoglycoprotein receptors) and lithostathine (pancreatic stone proteins) [33]. However, the domain structure shared by the C-type lectins is highly conserved within the superfamily. Models of the AFPs derived from crystal structures of the mannose-binding protein and E-selectin reveal a remarkable similarity between their folds and those of the AFPs [34, 35] (fig. 2). The solution structure of sea raven AFP was recently determined by nuclear magnetic resonance (NMR) [36], and it further confirms the similarity of type II AFPs to C-type lectins (fig. 2).

Interesting comparisons can be made between smelt, herring and sea raven AFPs. Smelt and herring AFPs are approximately 80% identical. Both bind Ca^{2+} and require it for activity [31, 35]. Smelt and herring AFPs also contain precise residues corresponding to a galactose-binding C-type site (see below) [33]. Moreover, these two species may not be very distantly related. Smelt belong to the order Osmeriformes and herring to the Clupeiformes [39], which, in an older classification, were grouped into a single order [40]. Taken together, these factors suggest that smelt and herring AFPs could have emerged from a single C-type lectin. Whether the AFP evolved prior to the divergence of lineages that gave rise to smelt and herring remains a question. The hypothesis that AFPs evolved recently would contradict that idea. However, a single lectin AFP with a biological function unrelated to ice binding might have existed in smelt and herring and then, through minor convergent changes, gone on to become an AFP in the two groups. In contrast, sea raven AFP is only about 40% identical to the AFPs of herring and smelt. It is not Ca^{2+}-dependent and does not contain the conserved residues forming a galactose-binding site [31, 33]. In addition, sea raven is very distantly related to smelt or herring [39]. Thus, it appears more likely that the sea raven AFP evolved separately from AFPs

of smelt and herring, and it is possible that it evolved from a different C-type lectin.

The ice binding site of a type II AFP from Atlantic herring was located using site-directed mutagenesis and found to correspond precisely to the carbohydrate binding site of C-type lectins [41]. Thus, ice binding and consequent antifreeze function evolved in this protein by a change in ligand specificity at the carbohydrate binding site of a preexisting C-type lectin. The molecular changes that lead to this new binding specificity are not known at present. However, they are likely to be very subtle. Proteins in the C-type lectin superfamily have a number of conserved residues that correspond to a Ca^{2+} binding site and also represent the primary carbohydrate ligands as shown in a crystal structure [42]. A three-residue-sequence 'EPN' is diagnostic of a mannose or fucose binding site, whereas a different sequence in the same position 'QPD' is typically found in lectins that bind galactose [43]. Substitution of EPN with QPD in mannose-binding lectins brings about galactose binding [43–45]. Other proteins in the superfamily that do not bind carbohydrate such as the mammalian pancreatic stone proteins and Habu snake X/Xi binding factor do not contain either of these sequences [46, 47]. The type II AFP of sea raven is missing these sequences and, logically, does not bind carbohydrate [48]. However, the type II AFPs of herring and smelt are unusual members of the group because they do have the QPD galactose binding motif but without the expected carbohydrate binding activity [48] (K. V. Ewart, unpublished results). The absence of other residues that stabilise galactose on the lectins may contribute to the lack of lectin activity in the AFPs [41]. The mutagenesis experiment that demonstrated the location of the ice binding site in herring AFP consisted of substitution of the QPD motif in herring AFP with EPN [41]. The loss of antifreeze activity in the EPN mutant showed that ice surfaces are bound at a site that corresponds precisely to the carbohydrate binding site. It is appealing to consider that the ice may be recognised as a derivative of galactose at the molecular level by the lectin-like herring AFP. This concept might also be useful to consider in seeking to understand protein-ice interactions in general.

Further insight into the evolution and mechanism of ice binding in type II AFPs will emerge from two sources. A crystal structure of the AFP will be indispensable in determining precisely which residues to alter in further mapping of the ice binding site by mutagenesis. In addition, identification and cloning of fish C-type lectin or multiple lectins that gave rise to the AFPs would be helpful in tracing the evolution of ice recognition in these AFPs from carbohydrate recognition function in lectins.

Recent emergence of antifreeze genes

The antifreeze glycoproteins (AFGPs) are mucinlike polymers of Ala-Ala-Thr with minor sequence variations. Their relative molecular masses range from about 2000 to 32,000, depending on the number of tripeptide repeats. The AFGPs are found exclusively in certain Antarctic families and in the north temperate cods [49, 50]. They are remarkably similar in these two disparate fish groups, having generally the same sequences and closely related polymer size distributions [1, 51, 52]. The AFGPs are O-glycosylated on each Thr residue. The disaccharides on the Thr residues were identified as N-acetyl-D-galactosamine ($\beta 1-3$) galactose in the Antarctic notothenioid AFGPs [53]. The carbohydrate composition of the cod AFPs suggested a similar structure [51, 54], and NMR analysis of AFGP from saffron cod (*Eleginus gracilis*) has confirmed the presence of the corresponding terminal galactose on the disaccharide [54].

A gene encoding a large polyprotein containing multiple AFGPs was first isolated from the notothenioid, *Notothenia coriceps neglecta* [55]. Cloning of another AFGP gene from another notothenioid, *Dissostichus mawsoni*, revealed 5′ and 3′ ends corresponding to portions of the trypsinogen gene [56]. Sequence comparisons showed that the region encoding the AFGP polyprotein had evolved by repeated duplication and rearrangement of a 9-bp segment crossing the first exon-intron boundary of the trypsinogen gene [56]. The AFGP gene of notothenioids did not arise by divergence from another protein as the type II AFPs did. Instead, this was a new gene, elaborated from a very small preexisting DNA segment, which was partially noncoding DNA in the original trypsinogen gene that it came from [10, 56].

A common evolutionary origin was initially inferred for the cod and notothenioid AFGPs based on their extensive similarity [12], but cloning of an AFGP gene from cod revealed a completely different relationship between these glycoproteins. Chen et al. [57] screened a genomic library from Greenland cod (*Boreogadus saida*) using a sequence from the notothenioid AFGP. The cod AFGP gene sequence revealed the basis for these AFGP repeats and the similarity with the notothenioid AFGPs, but flanking regions of this gene were completely distinct from those of the notothenioid AFGP and bore no similarity to trypsinogen [57]. Moreover, the spacers dividing the AFGP units in the polyprotein translation product were Arg residues in the cod, whereas they were tripeptides consisting of Leu-Ile-Phe and derivatives in the notothenioids [55–57] (fig. 3). Thus, evolution of the cod and of notothenioid AFGPs were independent events in which novel genes encoding the same glycoprotein were formed by repeated duplication of small gene fragments with no related coding function.

The similarity of the AFGPs encoded by unrelated genes in cod and nototheniids is reminiscent of the relationship among type I AFPs of sculpin skin and of flounders. In the case of both type I AFPs and of AFGPs, DNA sequences are similar enough to those of other species to be isolated by hybridisation with them and yet do not appear to share a common evolutionary origin. It will be interesting to obtain the cDNA and gene sequences for the nonrepeating type I sculpin blood AFP that is similar to the flounder skin AFPs but does not hybridise with them (Z. Hu and C. L. Hew, unpublished). It will also be useful to sequence the entire gene for the larger sculpin skin AFP. This would provide the sequence information necessary to determine whether these different type I AFPs arose by convergent evolution like the AFGPs.

The emergence of active AFGPs from small gene fragments may have implications in our understanding of the minimal functional unit for antifreeze activity. To determine the turning point at which duplication of a small fragment of DNA was no longer spurious but was an event undergoing selection, it would be helpful to know the size of the minimum unit of an AFGP that interacts with ice. Conversely, defining the smallest unit of DNA encoding an AFGP that would have been the object of selection might help to guide us in defining the minimum unit is that has genuine ice-recognition activity.

Multifunctional antifreeze proteins

The AFPs of winter rye comprise three different classes of proteins, and they are all similar to plant pathogenesis-related (PR) proteins. Winter rye contains AFPs with N-terminal sequences nearly identical to glucanases, chitinases and thaumatins [58]. Further study of the chitanase AFP led to an interesting insight into AFP evolution. Chitinase from cold-acclimated rye plants was affinity-purified on colloidal chitin, and this preparation showed antifreeze activity [58]. However, chitinases do not generally have this activity. Chitinase purified from tobacco plants by the same method showed no antifreeze activity. This implies that the rye contains a protein that, while still able to interact with chitin, can also bind to ice. It is not clear whether chitinase AFP simply binds chitin or actually retains chitinase activity. Chitinase enzyme activity was not tested specifically in the chitinase AFP but only in the whole affinity-purified chitinase preparation, which may have contained regular chitinase as well as the chitinase AFP.

One of the means by which new proteins (or, more precisely, new protein functions) are thought to develop is by gene duplication and divergence, whereby a gene is duplicated and the second copy, which is presumed to be redundant, subsequently acquires a new function [59]. However, in the case of the rye chitinase AFP, the chitin recognition function was not lost prior to the emergence of antifreeze activity. An interesting alternative to the duplication and divergence model is a hypothesis whereby the emergence of homologous genes with different functions begins with the development of a second function within a gene, followed by the possibilities of duplication and loss of the initial function [60]. The presence of conventional chitinases in most plants and of a novel chitinase AFP in winter rye would support the latter hypothesis. In the chitinase AFP, chitin binding was not lost prior to the appearance of ice binding.

Glucanase AFPs and chitinase AFPs of winter rye appear to be subsets of these PR proteins that are produced exclusively at lower temperatures [58]. Interesting considerations regarding these proteins are (i) whether they evolved ice binding at their existing carbohydrate recognition sites, as in the type II AFP of herring, or elsewhere on the molecules, and (ii) whether the cold induction of chitinase AFP is an example of differential gene regulation within a family of proteins analogous to that of flounder skin and blood AFPs.

A ***Notothenia coriiceps neglecta*** **polyprotein**
most common sequence repeats

AATAATAATPATAA***LNF***
LHF
FNF
CNF
AATAATPATAATPA***LIF***

B **Arctic cod polyprotein**
typical sequence repeats

AATPATAATPATAATPATAATAATAATSATAATAA***R***
AATPATAATPATPATAA***RAAR***

Figure 3. Sequences of representative AFGP repeats contained in polyprotein sequences. Predicted mature protein sequences are in regular type, and predicted linker and cleavage sites are in italics. (*A*) Representative sequences found in an Antarctic notothenid [55]. (*B*) Representative sequence found in the Arctic cod [57].

Insect antifreezes

Antifreezes have recently been identified in insects, and these proteins are at least 10 times more effective than those in fish, as measured by activity in vitro (fig. 4). Insect AFPs bear no similarity to those in fish or plants. The spruce budworm (*Choristoneura fumiferana*) AFP is a 9-kDa Cys- and Thr-rich protein that is hydrophilic

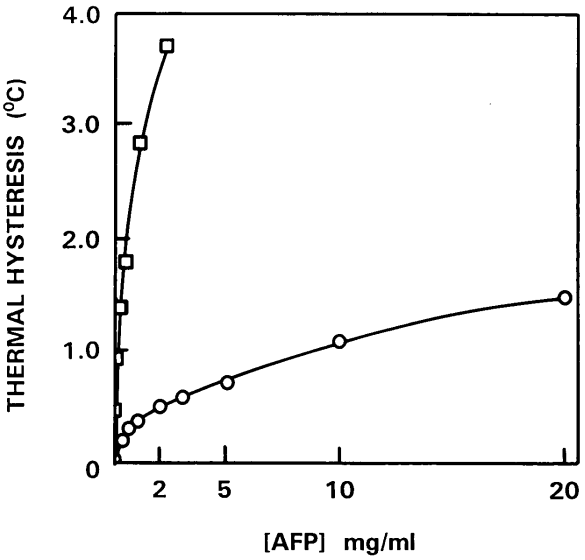

Figure 4. Comparison of activities of recombinant spruce budworm (□) and fish type III AFPs (○) as a function of protein concentration. Activity is measured as thermal hysteresis. Reprinted with permission from [61].

and very basic (pI = 9.5) [61]. The AFP of mealworm beetle (*Tenebrio molitor*) is close to 9 kDa, and it is also Cys- and Thr-rich [62]. However, the mealworm beetle protein is composed predominantly of a series of 12-amino acid repeats, and the sequence bears no similarity to that of budworm AFP [62]. An AFP homologous to that of mealworm has been identified in another beetle, *Dendroides canadensis* [63].

The folding structures of insect AFPs are unknown. Given the unusually high antifreeze activities of these proteins, there will be considerable interest in determining their structures and mechanisms of ice binding. None of the insect AFPs are homologous to other proteins in sequence databases, which precludes homology modelling. However, initial structural study of these proteins is progressing rapidly. The disulfide bonds in the *Dendroides* AFP have been mapped, and they reveal a pattern of repeated bridges consistent with the Cys repeats in the protein sequence [64] (fig. 5).

Functions of antifreezes

Ice-binding mechanisms

Ice crystal surface recognition is the feature that distinguishes the AF(G)Ps from non-AF(G)Ps. It is widely accepted that the AF(G)Ps generate a freezing point depression by adsorption to ice surfaces and consequent inhibition of further growth (absorption inhibition model) [2]. However, beyond the ability to depress the freezing point, there are many interesting similarities and differences in function among the AF(G)Ps. Knight et al. [65–67] showed that different type I AFPs and AFGPs bind to distinct ice crystal surfaces. The smallest AFGPs binds to prism planes of ice (10–10), whereas the type I AFP of flounder and sculpin bind to distinct pyramidal planes, 20–21 and 2–10, respectively (65–67). Although these proteins cause the same unusual ice crystal morphologies and inhibit ice crystal growth, they must recognise different patterns at the molecular level. A feature that unites most of the AF(G)Ps is the lack of appreciable binding to the basal planes of ice crystals [69]. Thus, AF(G)Ps may be defined most simply as proteins that adsorb primarily to nonbasal planes of ice.

The molecular interactions between AF(G)Ps and ice are not well resolved. Ice-binding residues or sugars of the various AF(G)Ps have been identified by the study of mutants or synthetic analogues with residue substitutions and by chemical modification of sugars (reviewed in [3, 49]). However, throughout most of this work, it was assumed that the ice-binding residues of AF(G)Ps must form hydrogen bonds with ice. Contrasting models were developed, one in which functional groups might sit rigidly on an ice surface and another in which they are incorporated into the ice lattice [13, 66]. But none questioned the assumption that hydrogen bonding was solely responsible for interaction with ice. Initial suggestions that van der Waals interactions might contribute to ice binding by type I AFPs were based on the role of Leu in type I AFPs [69]. A structural study of type III AFPs suggested that hydrophobic groups might also contribute to their binding to ice [70]. Unequivocal evidence for a role of van der Waals forces in protein-ice interactions was obtained in an elegant study of Thr residues in type I AFPs [71]. Substitution of two Thr residues with Val was undertaken to conserve the methyl group of Thr but not its hydroxyl group. The activity of the Val variant was equal to the unmodified AFP. In a similar investigation, an AFP analogue with all four Thr residues replaced by Val was shown to retain activity [72]. These studies specifically demon-

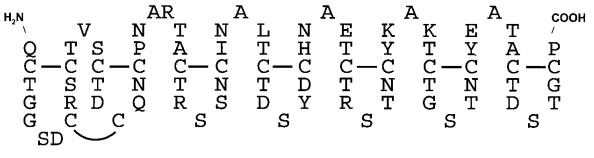

Figure 5. Beetle (*Dendroides canadensis*) sequence showing disulfide bridges. The disulfides are indicated by lines connecting the Cys residues [64].

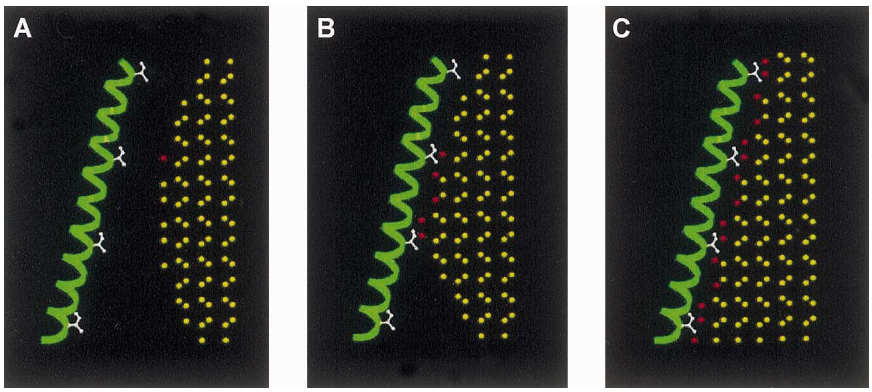

Figure 6. Graduated binding of type I AFP to ice. Minimal contact required for expression of an ice crystal surface plane is illustrated here. (*A*) Type I AFP, represented by a green ribbon with white Thr residues, is close to the ice crystal surface, which is indicated by yellow O atoms. (*B*) Addition of water to the ice lattice creates a surface with the specific surface plane that is large enough to bind one repeat (Thr-Thr) of the AFP helix. The length of this section of ice is 16.7 Å and it corresponds to the Thr-Thr distance in the AFP. (*C*) Residency of the single AFP repeat on the ice surface leads to the extension of the ice surface plane and further AFP stability on the ice. Reprinted with permission from [73].

strated the key role of the Thr methyl group in ice binding by type I AFPs. In a larger sense, this work has also generated a valuable paradigm shift in showing conclusively that ice binding can be mediated by van der Waals interactions.

Understanding of AF(G)P action must be sought at a scale intermediate between the effects of AF(G)Ps on ice crystal morphology and the precise molecular interactions between amino acid residues (or carbohydrates) and ice surfaces. The aim is to define the number and the constellation of interactions necessary for antifreeze activity. In other words, what is the minimum structural requirement and number of binding units for a protein to bind to ice? Short (15-residue) analogues of the 37-residue type I AFPs were found to be inactive [22]. However, the activities of the analogues may have been impaired by compromised folding and stability rather than by a lack of necessary ice binding sites. A minimised 15-residue AFP analogue containing appropriate helix-capping residues and stabilised with a lactam bridge caused ice crystals to express the planes typically observed in the presence of full-length AFP [73]. This analogue did not show antifreeze activity as assayed by thermal hysteresis. However, ice crystals grown in solutions of the analogue were typical 12-sided bipyramids with c:a axis ratios of approximately 3.3 [73]. Modelling studies showed that the analogue would cover only five layers of water molecules on the ice lattice. Consequently, the five-molecule layer in ice is the minimum area of coverage known to be needed for a type I AFP to affect ice growth (fig. 6). In terms of antifreeze structure and function, this is significant because it defines the minimum structure required to cause expression of a distinct ice plane on the ice surface [73]. Moreover, this finding suggests that ice binding by type I AFPs is not an all-or-nothing event, but may occur gradually over the length of the AFP once the minimal contact unit is formed [73]. This finding is equally relevant in understanding AFP evolution. A 15-residue stretch of α-helix with properly spaced ice-binding residues is sufficient to modify ice growth (fig. 6). Therefore, it represents the smallest functional unit for type I AFP selection. Although this unit is not stable in solution without the lactam bridge [74], it might be stable in the context of a larger protein. If a protein were to have this short sequence or a variant of it with the appropriate ice-binding elements properly oriented on one surface, it would be an AFP. This information may become valuable in tracing the origins of type I AFPs, which remain unknown. An even more exciting application of this finding is in the design of crystal-modifying proteins. As we work towards precise definition of the minimal unit needed to effect changes in a crystal morphology, it will become possible to begin designing the building blocks needed in a peptidomimetic. Because a 15-residue length is within the range of peptide lengths expressed in display libraries, it should also be possible to select more effective antifreezes and other crystal-binding proteins from these libraries.

Interestingly, the size of the minimised type I AFP [73] coincides with that of the smallest AFGPs. The glycoproteins occurring naturally in fish can be as short as 14 residues (for example Atlantic cod AFGP-8), and they have measurable effects on ice crystal morphology and solution freezing points [66, 74]. Moreover, AFGP 8 from *B. saida* retains 20% of its activity when the 2

N-terminal Ala residues are removed, leaving a 12-residue glycopeptide [75]. The structure-function relationship in AFGPs is not as well understood as in type I AFPs, and it is complicated by the fact that ice interaction is sugar-mediated [49]. Moreover, comparison of type I AFPs and AFGPs is compromised by their binding to different ice surface planes [65, 66]. It is possible that the minimal active AFGP would be even smaller than 14 residues. However, this would be a difficult hypothesis to test, because glycoproteins cannot be easily synthesised. Therefore, the identification of the small ice-active type I AFP peptide unit may provide the best starting material for work towards rational design and improvement of existing AF(G)Ps for biotechnology applications.

Properties and biological roles

Ice binding by AF(G)Ps has effects other than thermal hysteresis. A different property of AF(G)Ps is their ability to inhibit ice recrystallisation. This inhibition differs from freezing point depression in that it requires 100–500 times less AFP. The AF(G)Ps are believed to inhibit recrystallisation by their adsorption onto ice crystals [76]. The molecular events that generate ice recrystallisation inhibition have not been determined. However, the biological value of this effect is significant.

The antifreezes of different organisms have divergent functions in their survival. In freeze-avoiding fish and insects, the AF(G)Ps must depress the freezing point substantially in order to be effective. In contrast, the AFPs of freeze-tolerant plants are believed to act during freezing and thawing to modulate ice crystal growth and/or prevent ice recrystallisation. Although they may do essentially the same thing at the ice surface, their functions in survival are very different. The distinct types, localisation and regulation of AF(G)Ps might reflect this. The discovery of potentially intracellular AFPs in winter flounder has led to new questions about their role in fish. It was believed that the role of AFPs in fish required only an extracellular presence. Now, the potentially intracellular AFPs have prompted a reconsideration of the simple freeze-avoidance role of AFP in fish. Freezing is not expected to occur readily within cells. If these proteins are indeed intracellular, they might play other roles.

The AFGPs have a membrane-binding function in vitro that is in complete contrast to their apparent in vivo ice-binding activities. Experiments have suggested that the AFGPs were able to protect cell membranes from damage during transition to low but nonfreezing (i.e. 2 °C) temperatures (fig. 7) [6, 7]. The mechanism for this effect is not evident. Investigation using liposomes revealed that AFGPs interact directly with lipid bilayers and prevent damage during cooling and rewarming through the lipid phase transition temperature [6]. A similar effect was detected using platelets: cold-induced platelet activation was prevented by the addition of AFGP to the medium [7]. It is not yet clear how the AFGPs bind to and protect membranes, nor is it known whether the different AFP types share this ability. The AFGP concentration range over which membrane protection is evident corresponds to the concentrations at which freezing point depression takes place. In addition, Knight et al. [66] suggested that the similar repeating nature of membranes and of ice crystal surfaces is a relevant consideration in the study of AFP-ice interactions. If the AFGP binds to the membrane surface, it might recognise on it a pattern that is congruent with the prism planes of ice crystals. However, the orientation of AFGPs in the membrane bilayers and the precise mode of recognition and binding will need to be deciphered before more information can be drawn from this work.

The discovery of membrane interactions generates further questions regarding the evolution of AF(G)Ps. Might the AF(G)Ps have first evolved as a means of cold protection for membrane lipids and then have become ice binding when temperatures dropped further

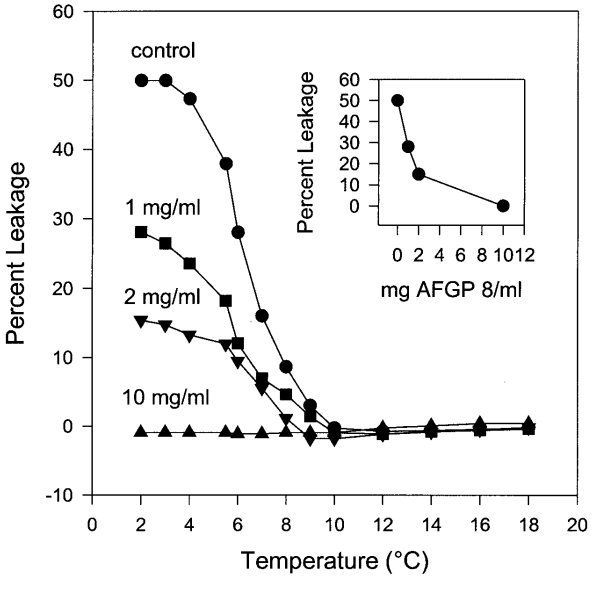

Figure 7. Percentage leakage of a dye, carboxyfluorescein, from dielaidoylphosphatidylcholine liposomes upon cooling. A range of concentrations of AFGP 8 (a small AFGP) were added to the sample at 18 °C. The control contains no AFGP. The inset graph shows the variation in dye leakage from the liposomes at 2 °C as a function of AFGP concentration. Reprinted with permission from [6].

to subzero levels? Or, is the interaction of some AF(G)Ps with membranes a fortuitous consequence of their ability to recognise specific surfaces on ice crystals that have a repeating structure similar to that of some membranes? Further study of the structure and function of the AF(G)Ps may provide an answer.

Perspectives

Ice binding is a property of many different proteins. However, the proteins that share this property are extremely diverse. Unlike most proteins with a particular function that comprise clear-cut evolutionary families, the AF(G)Ps can occur as functionally distinct exceptions in otherwise largely homogeneous protein families. Moreover, the origins of some of the proteins such as fish type I or the insect AFPs are unknown. The fact that a very small sequence unit (14–15 residues) is sufficient to generate ice-binding activity [66, 73] may offer an explanation for the evolution of the repeating AF(G)Ps. The AFGPs of nototheniids arose by duplication of a 9-bp repeat. Studies on the short AFGPs and the type I AFPs would suggest that only a very short unit would be required for a selectable activity. From there, continuing selection would lead to the development of much larger repeated antifreezes in a species or group. Perhaps type I AFPs and repeating insect AFPs are the result of repeated duplication similar to that of the AF(G)Ps.

The emergence of AFGPs in Antarctic fish by repetition of a small gene fragment, which constitutes the new creation of a whole gene, has been named 'macro-adaptation', as opposed to 'micro-adaption' that occurs through smaller changes in existing genes [10]. Because very short (15-residue) peptides are sufficient for this activity of AFGPs and type I AFPs, they may be ideal candidates for macro-adaptation. In this case, a selectable activity is present on a very small piece of DNA that can be repeatedly duplicated, giving rise to progressively stronger ligand binding and a novel gene.

The relationship between function and evolution is equally interesting in the case of C-type lectin AFPs (fish type II). These proteins did not arise from small sequence repeats but emerged as a result of small changes (micro-adaptation) in preexisting C-type lectins [10, 77]. At the molecular level, it is not yet known what the ice ligands on this protein are, only that they correspond to a carbohydrate binding site. Moreover, the functions of type II AFPs and the C-type lectins that gave rise to them are not clearly defined. The possibility of overlaps in function and/or regulation has not been investigated. These are promising research areas.

Study of AF(G)Ps and their various effects on ice suggest that their most basic activity should be considered to be ice surface modification rather than measurable hysteresis. This has two implications in antifreeze research. (i) Ice crystal morphology observations should reveal many more antifreezes than have been previously identified. (ii) Engineering of superactive antifreeze proteins should be more straightforward than equivalent work on more conventional proteins. The identification of a minimal type I AFP showed that ice binding and modification can be generated in a relatively small linear peptide unit. Extrapolating from the small AFP analogue to full-length AFPs, it is obvious that simply repeating the unit will result in greater antifreeze activity. In the type I AFPs, further repeats may lead to limitations in solubility [24]. However, solubility is not a limiting factor for AFGPs. A similar relationship between unit repetition and activity is observed in short and long AFGPs, and the long ones are soluble [49]. The situation is more complex for the globular AFPs, which are unlikely to have linear ice-binding repeats. Type III AFPs, for example, are known to have nonlinear sites in that they comprise nonsuperimposable parts of the globular protein and bind to ice in a nonlinear pattern [78]. Type II AFPs may be similar in both respects. The disulfide bonding pattern of the highly active *Dendroides* AFP [64] suggests that it, too, may have a nonlinear ice binding site (fig. 5). Thus, study of these nonlinear sites should lead to equally interesting developments in our understanding of AFPs.

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