

Evolution of the diverse antifreeze proteins

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Different types of ice-growth-inhibiting antifreeze proteins, first recognized in fish, have now been isolated from insects and plants, and the list continues to expand. Their structures are amazingly diverse; how they attain the same function are subjects of intense research. Evolutionary precursors of several members have been identified – divergent proteins of apparently unrelated function. The hybridization of information from structural and molecular evolution studies of these molecules provides a forum in which issues of selection, gene genealogy, adaptive evolution, and invention of a novel function can be coherently addressed.

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Abbreviations

AFGP	antifreeze glycoprotein
AFP	antifreeze protein
CRD	carbohydrate-recognition domain
LS-AFP	longhorn sculpin AFP
Mya	million years ago
PR	pathogenesis related

Introduction

Biological antifreeze is an evolutionary innovation first discovered in the Antarctic notothenioid fishes almost three decades ago by DeVries [1] and classified as a family of antifreeze glycoproteins (AFGPs). Four other types of antifreeze proteins (AFPs) were later identified in various fish taxa, with the newest member (type IV AFP) discovered last year [2•]. AFPs are also found in overwintering terrestrial insects and plants – the first three insect AFP sequences being reported in the past 18 months. AFPs bind to the surface of ice crystals and deter the joining of additional water molecules, thereby depressing the temperature of macroscopic ice expansion below the colligative freezing point. In fish and insects, antifreezes confer freeze avoidance by preserving body fluids in the liquid state; in cold-hardy plants, they augment freeze tolerance by limiting the growth of small ice crystals into large damaging ones.

Antifreeze proteins have diverse structures but all recognize and bind to the same substrate – ice [3]. How this came to be intrigues both structural and evolutionary biologists. Three-dimensional structures of three fish AFPs have now been solved and structural analyses to resolve the mechanisms of how antifreezes bind to ice are being

conducted extensively [4]. The structural diversity of these proteins implies multiple evolutionary origins, confirmed by the recent identification of the progenitor of several antifreezes. Their creation was driven by a simple and clear selective force – freezing environmental temperatures. Molecular evolution of these novel, ice-binding proteins is an emerging field of research that promises to broaden the knowledge of mechanisms of new protein genesis and illuminate the biophysics and biogeography that shaped the creation of a novel protein function, which collectively is the focus of my review.

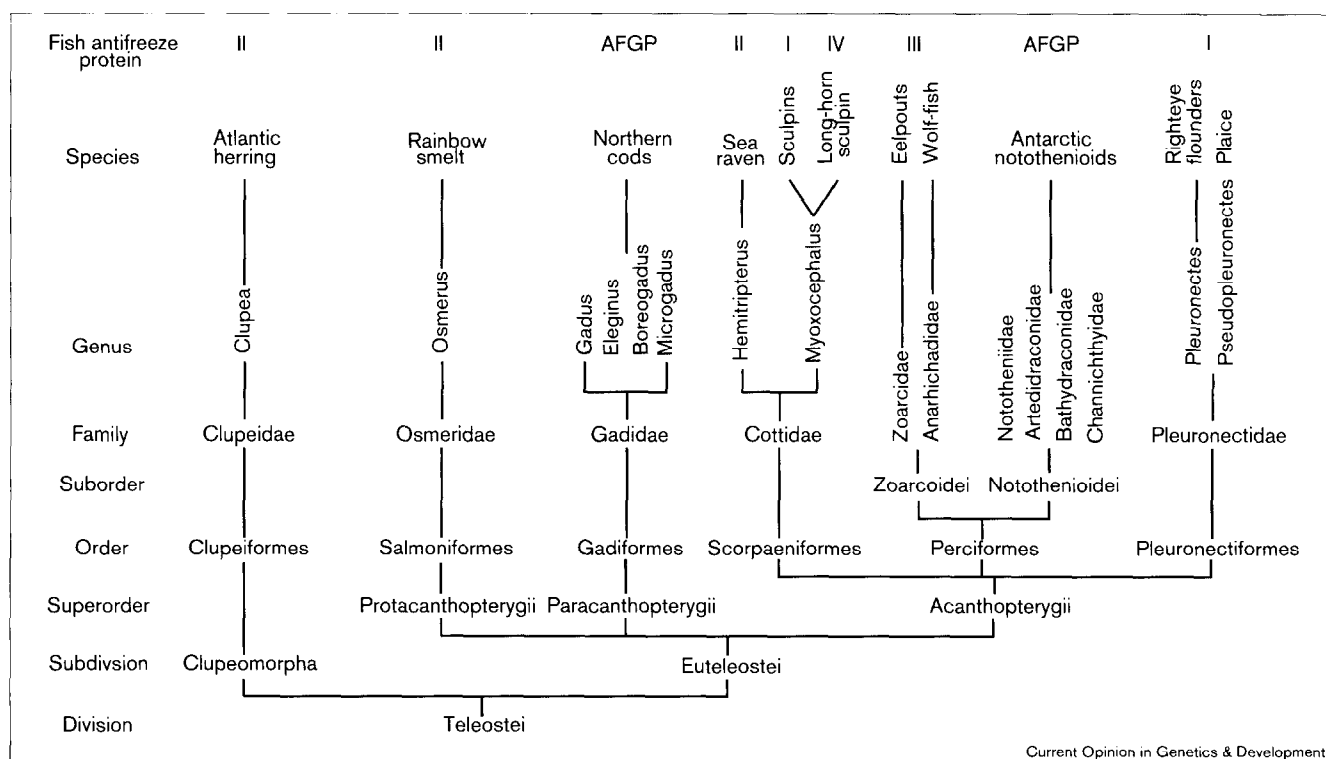
Diversity and origins of fish antifreezes

There are two categories of fish antifreeze proteins – the AFGPs (antifreeze glycoproteins) and the AFPs (Figure 1; Table 1). AFGPs of the unrelated Antarctic notothenioids and northern cods occur as a family of size isoforms composed of various numbers of a simple tripeptide repeat, Ala-Ala-Thr, with each Thr linked to a disaccharide, galactose-N-acetylgalactosamine. The cod AFGPs differ only in an occasional Thr→Arg substitution [5]. Through comparative analyses of the sequences and structures of AFGP genes from the two fishes, a common ancestry was ruled out. The notothenioid AFGP gene was derived from a trypsinogen-like serine protease gene [6•]. The cod AFGP gene is not homologous with trypsinogen and thus must arise from a different genomic origin [7•].

AFPs are sequentially numbered type I, II, III and IV in the order of their discovery (Figure 1; Table 1). Type I AFPs of flat fishes (pleuronectids) and the unrelated sculpins (cottids) are small α -helical molecules comprising three or four of an 11-residue repeat, TxxD/Nxxxxxxx (in the one-letter code, where x is any amino acid but mostly Ala). Type II AFPs are Cys-rich folded proteins identified in three very divergent fishes – sea raven, smelt and herring – and are homologous to the carbohydrate recognition domain of calcium-dependent (C-type) lectins [8]. Type III AFPs are small globular proteins with unbiased amino acids from eel pouts and wolf-fish (zoarcids). The 12.3 kDa type IV AFP of the longhorn sculpin is the newly discovered fish AFP and it shares ~20% sequence identity with members of the exchangeable apolipoprotein superfamily [2•].

Insect and plant antifreeze proteins

AFPs in overwintering insect larvae have long eluded identification because of difficulties in acquiring sufficient quantities for complete protein characterization but by cDNA cloning and sequencing, the AFP sequences of three different insect larvae were successfully obtained and reported in the past year (Table 1). All three AFPs are rich in Cys, Thr and Ser. AFPs of two beetle larvae, the common mealworm (*Tenebrio molitor*) [9•] and the

Figure 1

Evolutionary relationship (not to scale) of antifreeze-bearing fishes, based on the classification of Nelson [27]. The type of antifreeze protein is indicated at the top.

fire-colored beetle (*Dendroides canadensis*) [10[•]], are homologous (~70% protein sequence identity) and are composed of seven or more 12- or 13-residue repeats of CTxSxxCxxAxTx (x is any amino acid). The AFP sequence of the spruce budworm — larva of the moth

Choristoneura fumiferana — is not repetitive and may constitute a different type [11[•]]. These insect AFPs are much more potent antifreezes than fish ones, commensurate with the more extreme terrestrial winter temperatures that confront them.

Table 1

Structures and origins of antifreeze proteins.

	Protein sequence	Mr	Protein structure	Evolutionary precursor/homolog
Fish antifreeze proteins				
AFGP	3-residue repeats AAT-disaccharide	2.7–34 kDa	Amphipathic Extended polyproline II-type helix	Trypsinogen-type serine protease
Type I AFP	11-residue repeats Tx _x D _N xxxxxx	3–5 kDa	Amphipathic α-helix	Unknown
Type II AFP	Non-repetitive 8% cysteine	14–24 kDa	Globular	C-type lectin
Type III AFP	Non-repetitive Unbiased aa	7 kDa	Globular	Unknown
Type IV AFP	22-residue repeats	12.3 kDa	Single helix? 4-helix bundle?	Apolipoprotein
Insect antifreeze proteins				
Beetle AFP	12-, 13-residue repeats CTxSxxCxxAxTx	9 kDa	Undetermined	Unknown
Moth AFP	Non-repetitive Cys-, Thr-, Ser-rich	9 kDa	Undetermined	Unknown
Winter rye antifreeze proteins				
	Non-repetitive?	16–35 kDa	Undetermined	Pathogenesis-related proteins (glucanase, chitinase, thaumatin)

Plant AFPs are found in species that are freeze-tolerant. They occur in low concentrations and effectively inhibit recrystallization of small, extracellular ice crystals into large damaging ones. The AFP of bittersweet nightshade is a 67 kDa Gly-rich glycoprotein but its sequence has not been determined [12]. Three types of AFPs have been isolated from winter rye, and their amino-terminal sequences share >90% identity with members of three classes of plant pathogenesis-related (PR) protein — endoglucanase, endochitinase and thaumatin [13].

Viewing complexity with simplicity

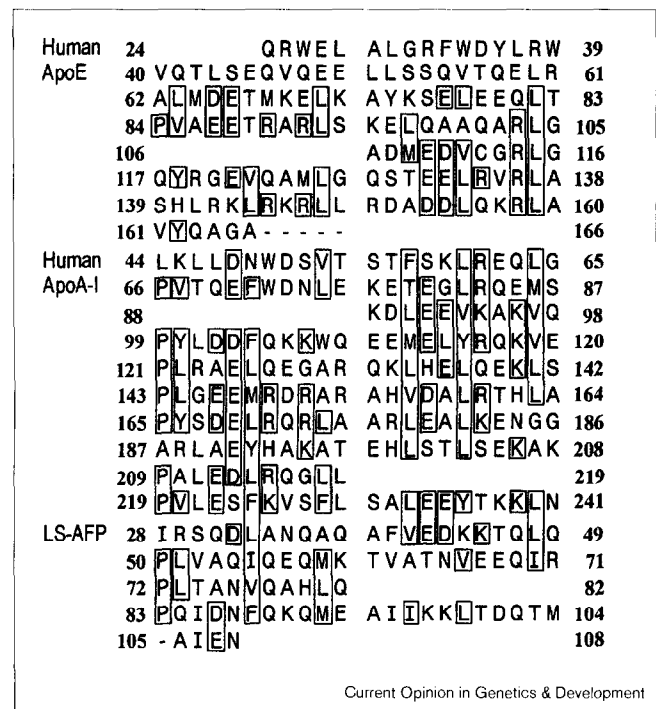
The ability of the diverse antifreezes (Table 1) to bind to ice crystals suggests that some underlying, shared structural elements or properties are responsible for the common function. What these may be are obscured by their overwhelming structural differences. The known evolutionary precursors of antifreezes, expectedly, are as divergent as the descendent AFPs are structurally varied, providing no hint of what predisposed them for selection and refinement to become an antifreeze.

By putting detailed structural differences aside, the diverse antifreezes generally fall into two broad classes: those that have a repetitive amino acid sequence and those that do not (Table 1). Their common substrate, the ice crystal, is structurally well-defined: essentially a highly ordered, geometric array of a simple monomer — water molecules. The adsorption-inhibition mechanism of antifreeze action first put forth in 1977 by Raymond and DeVries [14], which still persists as the working hypothesis of antifreeze mechanistic studies today, proposed that antifreeze proteins bind to ice through hydrogen bonding and involves a lattice match between regularly spaced hydrogen-bonding sidechains in the protein and the periodic water molecules in the ice lattice. If this model is correct, an ancestral protein molecule with hydrogen-bonding moieties positioned in appropriate regularity could be a potential candidate for recruitment to become an ice-binding antifreeze.

Repetitive sequences as archetypes for antifreeze evolution

As an endorsement of this model, about half of the known antifreezes have clear repetitive amino acid sequences: the AFGPs, type I AFPs, and the two beetle larvae AFPs (Table 1). AFGPs and type I AFP provide the clearest examples of lattice matching for ice binding. Type I AFPs of flounder and plaice adsorb on the pyramidal planes {20–21} of ice along the <01–12> direction, which has a periodicity that closely matches the lengths of the AFP's 11-residue repeats (16.5 Å); binding presumably occurs at the repeated polar Thr [15]. Notothenioid AFGPs adsorb on the prism planes along the a-axes [16]. The spacing of the tripeptide repeats (9.31 Å) is about twice the a-axes periodicity (9.038 Å) and hydrogen bonding presumably occurs at the hydroxyls of the Thr-linked disaccharides of each repeat. The amphipathy of the helical type I AFP

Figure 2



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Sequence alignment of LS-AFP and two human apolipoprotein sequence domains with known X-ray crystal structures: ApoA-I and ApoE. ApoE is the sequence of the amino-terminal LDL-receptor binding domain of apolipoprotein E [21] and ApoA-I is the sequence of the lipid-binding domain of apolipoprotein A-I [20]. The alignment follows the scheme of Li *et al.* [19] showing the 22-mer repeat structure — two 11-mer repeats in tandem — and recurring amino acid sequence pattern (boxed).

[17] and AFGPs [16] would position the putative ice-binding moieties linearly on one side of the molecule for alignment to the planar surface of ice.

The 3D structures of insect antifreezes and the spatial arrangement of their putative ice-binding residues await determination. Li *et al.* [18**] have mapped eight disulfide linkages in the AFP of the beetle *D. canadensis* and suggested the presence of seven functional domains and a possible amphipathy to the molecule, which in combination may provide a geometric fit to a particular crystallographic direction in ice akin to the helical fish antifreezes.

The evolutionary precursors of type I AFP and insect antifreezes are unknown at present; therefore the bases for their recruitment are moot. The progenitor of notothenioid AFGPs is known, a trypsinogen-like serine protease. The coding sequence for the AFGP tripeptide repeats, however, was created by *de novo* amplification of a 9 nt, partly intronic, Thr-Ala-Ala coding element which, by itself, had no functional role in the protease ancestor [6**]. It can be argued, however, that the creation of the repetitive antifreezes is consistent with a geometric-fit requirement for ice binding.

Type IV AFP is a repetitive antifreeze with known origin

The newly discovered 108-residue type IV AFP from the longhorn sculpin (*Myoxocephalus octodecimspinosus*) probably evolved from a member of the exchangeable apolipoprotein superfamily: apoA, apoC, apoE and their homologs [2**]. The longhorn sculpin AFP sequence (LS-AFP) superficially appears non-repetitive but vertebrate apolipoproteins are known to contain tandem 22-residue repeats (encoded by exon 4), each with a single conserved Pro at the first position [19]. In realigning LS-AFP with apolipoproteins, we found that residues 28–104 conform to the 22-mer repeat structure, and three of the possible four repeats carry the conserved Pro, supporting their homology (Figure 2). The 22-residue repeat region in apoA-I constitutes the lipid-binding domain, which has been shown by X-ray crystallography to be a pseudo-continuous amphipathic helix punctuated by kinks at the regularly spaced Pro [20]. ApoA-I functions to complex with otherwise water-insoluble lipids to form soluble plasma lipoproteins by simultaneously interacting with the polar head groups and hydrophobic tails of an array of phospholipids, as well as the aqueous medium. An alternate structure for LS-AFP based on the antiparallel 4-helix bundle crystal structure of the LDL-receptor-binding domain of human ApoE-I [21] has been proposed [2**,4], where the 22-mer repeat and Pro residues are less well conserved.

The actual structure of LS-AFP notwithstanding, the immediate significance of apolipoprotein as an evolutionary antecedent to type IV AFP is that we now have the first example of a repetitive antifreeze derived from a known and well characterized repetitive archetype. Comparative structural and mechanistic analyses of apolipoproteins and type IV AFP may shed light on the structural basis for the selection of a repetitive archetype for refinement into an antifreeze.

Origins of antifreeze proteins with non-repetitive sequences

Type II and III fish AFPs, the plant AFPs and possibly the moth AFP have no apparent sequence repeats (Table 1), indicating that there are alternate structural archetypes conducive towards evolution to ice-binding proteins. In this group, type II AFP and plant AFPs have known evolutionary precursors which incidentally bind or interact with sugars in some way.

The homology between type II fish AFPs and the carbohydrate-recognition domain (CRD) of C-type lectins is supported by a similar global fold between a recently obtained NMR structure of sea raven AFP and known lectin structures [22**], and the persistence of a Ca^{2+} requirement for activity in smelt and herring AFP [8,23]. In C-type lectin CRD, the carbohydrate-binding site is located in the Ca^{2+} -binding site. Directed mutation of the homologous site in herring AFP, converting it from a galactose- to a mannose-binding motif but without

affecting Ca^{2+} binding, resulted in loss of antifreeze activity. This demonstrates that the ice-binding site of herring AFP corresponds to the carbohydrate-binding site of C-type lectin CRD [24**].

The AFPs of winter rye accumulate in the apoplast during cold-acclimation. Amino-terminal sequences, serological properties, and enzymatic activities revealed them to be homologs of members of three of the five classes of plant PR (pathogenesis-related) proteins (Table 1) [13], which are defense proteins induced during the hypersensitive response to attacks of plant fungal and viral pathogens [25]. Two of the three winter rye AFP homologs, endoglucanase and endochitinase, hydrolyze long-chain polysaccharides (constituents of fungal cell wall) into oligosaccharides. Purified glucanase- and chitinase-like AFPs in fact retain this enzymatic activity and thus perform a dual function [13,26]. Thaumatococcus is a sweet proteinaceous compound first identified in a west African rainforest shrub, homologs of which were later found to be antifungal through a cell wall permeation mechanism [25]. The target again includes long-chain polysaccharides.

The selection of C-type lectin CRD in fish and the polysaccharide-hydrolyzing molecules in plant to become AFPs may have stemmed from their ability to bind or interact with carbohydrates, perhaps at the hydroxyl groups through hydrogen-bonding. Is there a structural parallel between the poly-hydroxyl groups in carbohydrates and water molecules in ice crystals? It remains to be seen if there are further examples of antifreeze evolutionary homologs that are involved in carbohydrate binding.

Novel mechanism of gene genesis

New proteins usually evolve from pre-existing proteins through gene duplication events followed by sequence divergence, and this applies to antifreeze proteins too. The Antarctic notothenioid AFPGs, in addition, exhibit new elements of evolutionary creativity. The trypsinogen-like serine protease ancestor provided only the ancillary secretory signal for the emerging AFGP and the bulk of trypsin's coding sequence was shed. The entire ice-binding protein-coding region was built from the ground up by *de novo* expansion of a rudimentary 9 nt, Thr-Ala-Ala coding element that straddled an exon-intron junction in the protease ancestor, thereby creating sense from part non-sense DNA [6**]. We have recently confirmed the occurrence of this novel process by the isolation of functional AFGP-trypsinogen evolutionary intermediates (gene and cDNA), in which complete AFGP- and protease-coding regions persist in tandem as a contiguous sequence without an intervening stop codon (CHC Cheng, L Chen, unpublished data) — a rare case of catching protein gene evolution in the act.

AFGP protein sequence convergence

The presence of near-identical AFPGs in the Antarctic notothenioids and northern cods which are phylogenetically

and geographically poles apart [27] had been a lingering, irreconcilable paradox since their discovery. The origin of notothenioid AFGPs is now known, and though that of the cod AFGPs is not, it is not trypsinogen as the two share no sequence similarity. In addition, cod AFGP genes and notothenioid AFGP genes are decidedly nonhomologous because they have different intron–exon boundaries, unrelated signal peptide sequence, and different codon bias for the tripeptide repeats [7••].

The cod AFGP gene very likely arose from *de novo* amplification of a short sequence akin to the notothenioid AFGP gene, but from a different genomic origin, when driven by similar environmental selection (freezing seawater temperatures), and shaped by the same structural and biophysical requirements for activity. These two groups of unrelated but near-identical AFGPs demonstrate that protein sequence convergence, though rare [28], can occur. Here, it is made possible by the simplicity of the 3-residue (or 9 nt) building block, which has a statistical probability of occurring at more than one genomic location, and the ease for short repeats to undergo expansion once the first duplication occurred.

Paleogeography, fish antifreeze evolution and organismal diversification

The environmental driving force for antifreeze evolution in general is freezing temperatures and for fish antifreezes in particular it is the glaciation of the polar regions and their associated bodies of marine waters. The evolution of antifreezes in fishes that did not or could not utilize other means of escape — such as migration to warmer water, or deep ice-free water — was a direct response to a clear and dire environmental stimulus. This is particularly true, and its impact most profound, for the endemic Antarctic notothenioid fish. Thermal isolation of Antarctica and its surrounding Southern Ocean is thought to begin at ~22 Mya (million years ago) with the establishment of the unrestricted, rigorous, clockwise flow of the Antarctic circumpolar current (ACC) which decoupled the warm subtropical gyres from the continent [29].

The sea-floor-reaching ACC also prevented dispersal and thus the modern Antarctic fish fauna is highly endemic and species-sparse [30,31]. It is dominated by members of a single suborder, the AFGP-bearing notothenioids (~50% of shelf species and 95% of biomass) [31], whose rise must stem from the evolution of their antifreeze capacity followed by adaptive radiation into niches vacated by unprotected fish that perished. Having the gene sequences of both the evolutionary precursor and offspring in the same fish allowed us to estimate the time of the protoase to AFGP conversion, at ~5–14 Mya [6••].

Chilling of the Southern Ocean was *terminus ante quem* the emergence of notothenioid AFGPs, thus the onset of its freezing conditions should fall within a similar time frame, which indeed was estimated to be so (at about the mid-Miocene [10–14 Mya]) by paleoceanographic

methods [29]. The AFGP emergence also correlates closely in time with the burst of radiation of the five notothenioid families estimated at ~5–15 Mya through molecular phylogenetic analyses [32], indicating that AFGP is a key evolutionary innovation that contributed greatly to the notothenioid's ecological success.

The onset of Arctic glaciation occurred much more recently, ~2.5 Mya (mid-Pliocene) [33], followed by cyclical glacial advances and retreats throughout the Quaternary epoch [34]. AFPs in northern fishes — which comprise the bulk of known fish AFPs — splendidly illustrate the multiple and independent evolution of the antifreeze function, with the most astounding example being the evolution of two entirely unrelated AFPs (type I versus type IV) in sister species of the same genus *Myoxocephalus* (sculpins) (Figure 1). How this was manifested is perplexing. One possibility is that the freezing habitat for these various fishes occurred during different glacial episodes in the northern hemisphere, resulting in the independent evolution of antifreeze at different times and selection of different precursor candidates.

Conclusion

The evolutionary origins of half of the known antifreezes remain undetermined. New antifreezes are bound to be discovered to add to the list. The recency of antifreeze evolution — unhampered by loss of information through multiple nucleotide or amino acid substitutions seen in ancient events — allows us to pinpoint the protein's ancestry, as we have demonstrated for the notothenioid AFGPs [6••]. Deciphering the origins of the diverse antifreezes and the processes that spawn them will broaden our knowledge of how novel proteins were created. Deciphering the structural bases for recruiting diverse ancestral molecules with apparently unrelated functions to become an antifreeze will enlighten us to how a novel function became invented. The two related fields are at their inception but promise to be greatly enriching by maturity.

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