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Role of Antifreezing Proteins in Fishes

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Abstract: Antifreeze proteins (AFP) and antifreeze glycoproteins (AFGP) comprise a structurally diverse class of proteins that have in common the ability to bind to ice and inhibit its growth. This action at the surface of ice in contact with a solution of AFP leads to a lowering of the freezing point (non-equilibrium) below the melting point, referred to as thermal hysteresis (in °C). AFPs are incorporated within ice when it freezes because of their affinity for its surface. There, even in the frozen state, they inhibit the growth of large ice crystals at the expense of small ones (recrystallization inhibition), particularly when ice approaches the melt temperature and becomes more fluid. As a result of these beneficial properties, AFPs and AFGPs have been incorporated into the freeze-resistance or freeze-tolerance strategies of many organisms such as marine fishes, insects, plants and bacteria.

Keywords: Antifreeze proteins, antifreeze glycoproteins, freezing point

1. Introduction

Birds, fishes, amphibians, insects and plants have evolved several strategies to cope with extreme low temperature in their natural habitat. Mechanisms involved in their cold tolerance provide valuable clues to the studies on bacterial cold tolerance. One of the major strategies adopted by the cold-tolerant fishes, insects and plants is the production of specific proteins, which help them to maintain their body fluid in the liquid state at sub-zero temperatures. In general, they are called antifreeze proteins (AFPs), though other terms such as antifreeze glycoproteins, antifreeze polypeptides are also used to denote the structural features of these antifreeze proteins.AFPs protect the organisms (Fig 1) from freezing at temperature below 1°C by binding with ice crystals and modify their growth through an adsorptioninhibition mechanism [1]. Marine teleosts living in polar waters have a body temperature of about $\pm 1.9^{\circ}$ C, the same as the ambient temperature of the surrounding seawater. However, their body fluids are hypoosmotic to sea water and have a melting point of approximately $\pm 0.7^{\circ}$ C. Thus, the polar teleosts are super-cooled by more than 1°C[2]-[3].One could therefore predict that the fish sooner or later would experience a lethal freezing. Polar fish are protected against freezing by the presence in their body fluids of antifreeze proteins, which display a capacity to inhibit ice growth when partly frozen fluid samples are cooled.



Figure 1: The antifreeze molecules allow ice fish to live in subfreezing water by plugging gaps in existing small ice crystals and preventing the attachment of more ice molecules. Ice crystal growth is thus effectively stopped [4].

Through this unique technique, they protect themselves from cell membrane damage and some other harmful physical and chemical changes. Though, AFPs were first identified in fishes [5], they also have been found in plants [6], fungi [7] and bacterial species [8]-[6]-[9]. Beside their diversified sources various structurally distinct AFPs have evolved independently [10]. A total of 5 structurally distinct antifreeze proteins are identified in fish so far and classified as Antifreeze glycoprotein (AFGP) and antifreeze protein type I, type II, type III, and type IV based on their distinct physicochemical and structural features [11]. Antifreeze activity of AFPs attracts a lot of attention due to their wide potential commercial applications including preservation, transgenic production [12] and cryosurgery. AFPs have potential applications in fisheries for the production of economically valuable fishes against low temperature.

2. History

In the 1950s, Canadian scientist Scholander set out to explain how Arctic fish can survive in water colder than the freezing point of their blood. His experiments led him to believe there was "antifreeze" in the blood of Arctic fish. Then in the late 1960s, animal biologist Arthur DeVries was able to isolate the antifreeze protein through his investigation of Antarctic fish [13]. These proteins were later called antifreeze glycoproteins (AFGPs) or antifreeze glycopeptides to distinguish them from newly discovered non-glycoprotein biological antifreeze agents (AFPs). DeVries worked with Robert Feeney [14] to characterize the chemical and physical properties of antifreeze proteins. Duman and Olsen [15] noted AFPs had also been discovered in over 23 species of angiosperms, including ones eaten by humans. As well, they reported their presence in fungi and bacteria.

3. Diversity and origins of fish antifreeze proteins

There are two categories of fish antifreeze proteins - the AFGPs (antifreeze glycoproteins) and the AFPs (Table 1). AFGPs of the unrelated antarcticnotothenioids and northern cods occur as family of size isoforms composed of various number of a simple tri-peptide repeat, Ala-Ala-Thr, with Thr linked to a disaccharide, galactose-Neach acetylgalactosamine. The cod AFGPs differ only in an Thr ->Arg substitution [16]. occasional Through comparative analyses of the sequence and structure of AFGP gents from the two fishes, a common ancestry was ruled out. Thenotothenioid AFGP gene was derived from a trypsinogen-like serine protease gene [17]. The cod AFGP gene is not homologous with trypsinogen and thus must arise from different genomic origin[18].

AFPs are sequentially numbered type I, II, III and IV in the order of their discovery. Type IAFPs of flat fishes (pleuronectids) and unrelated sculpins (cottidae) are small α -helical molecules comprising three or four of an 11 residue repeat. Type II AFPs are Cys-rich folded proteins identified in three very divergent fishes - searaven, smelt and herring-and the homologous to the carbohydrate recognition domain of calcium-dependent (C-type) lectins[19]. Type III AFPs are small globular proteins with unbiased amino acids from eel pouts and wolf-fish (zoarcoids). The 12.3 kDa typeIV AFP of the longhorn sculpin is the newly discovered fish AFP and it shares around 20% sequence identity with members of the exchangeable apolipo protein super family [20].

				Evolutionary
	Protein sequence	Mr	Protein structure	precursor/homolog
Fish antifreeze proteins				
AFGP	3-residue repeats AAT-disaccharide	2.7 - 34 kDa	Amphipathic Extended	Trypsinogen-type
			polyproline II-type helix	serine protease
Type I AFP	1 1 -residue repeats Txx 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?	Apolipoprotein
			4-helix bundle?	
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

Table 1: Structures and origins of antifreeze proteins

4. Sub-classification of AFPs

Type I AFPs are long, single α helices found in several close relatives of the longhorn sculpin (including the shorthorn sculpin, *Myoxocephalusscorpius*) and in the unrelated righteye flounders. They exist in solution as 3-5 kDa monomers and are unrelated to type IV AFPs. Their helicity can be attributed to a high content of alanine (60%), stabilizing N- and C-terminal cap structures [22] a reinforcement of the helix dipole by complementary charged groups at both termini, and internal salt bridges [23]. The main difference between the flounder and sculpin subtypes is that the former is built up of a clearly defined repeat of 11

amino acids (TxxNxxxxx, where x is principally alanine, and N is sometimes aspartate or threonine [24], and the latter is non-repetitive and more amphipathic, with several lysine and arginine sidechains projecting from the same face of the helix. The two subtypes also bind to different planes of ice [25] and, if their high amino acid identity is attributed to the need for alanine for helix stabilization, there is some question whether the subtypes are in fact homologous.

Interestingly, Gong *et al.* [26] have now discovered two subtypes within the winter flounder, *Pleuronectesamericanus.* The repetitive type is only expressed in the liver and is exported to the circulation by

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means of a conventional signal polypeptide. The other subtype, which is expressed in peripheral tissues such as skin, scales, fin and gills, as well as intestine and stomach, lacks a signal sequence and is remarkably similar to the sculpin subtype. Although it is still not clear whether the flounder and sculpin AFPs are homologs, the flounder skin and liver subtypes are clearly related because their genes are found side by side in the genome and they share significant nucleic acid sequence identity [27].

Type II AFPs are 14-24 kDa homologs of the carbohydraterecognition domain of calcium-dependent lectins[28]. Homology modelling based on the lectin domains indicates that these proteins share the same characteristic fold which includes disulfide bridges, and some α helix and β sheet, but also a relatively large proportion of coil structure [29]-[30]. On the basis of their Ca^{2+} requirement for activity, these AFPs can also be classified into two subtypes. The herring and smelt type II AFPs are completely dependent on Ca^{2+} for activity. Substitution with other divalent metal ions $(Ba^{2+}, Zn^{2+}, and$ $Mg2^+$) not only decreases the activity of herring type II AFP, but also leads to marked alterations in ice crystal morphology[30]. The simplest interpretation is that Ca^{2+} is directly involved in binding to ice, in the same way that it mediates sugar binding to C-type lectins [31]. In line with this suggestion, most of the calcium-ligand amino acids are conserved between those homologs; however, Ca²⁺ also appears to stabilize herring type II AFP against proteolysis and alter its fluorescence properties, and therefore one cannot rule out that the metal ion may act at a distance via a conformational change to the AFE In contrast, searaven type II AFP is completely calcium-independent and lacks the calcium-liganding amino acids. It is rather moreactive than its herring and smelt counterparts and produces significantly different ice crystal morphology [30]-[32].

Antifreeze glycoproteins or AFGPs are found in Antarctic notothenioids and northern cod. They are 2.6-3.3 kD[33]

• Type I AFP (Fig 2) is found in winter flounder, longhorn sculpin and shorthorn sculpin. It is the best documented AFP because it was the first to have its three dimensional

structure determined [34]. Type I AFP consists of a single, long, amphipathic alpha helix, about 3.3-4.5 kD in size. There are three faces to the 3D structure: the hydrophobic, hydrophilic and Thr-Asx face [34].



Figure 2: Three faces of the Type 1 AFP [35]

- Type I-hyp AFP (where hyp stands for hyperactive) is found in several right eye flounders. It is approximately 32 kD (two 17 kDdimeric molecules). The protein was isolated from the blood plasma of winter flounder. It is considerably better at depressing freezing temperature than most fish AFPs [36].
- Type II AFPs are found in sea raven, smelt and herring. They are cysteine-rich globular proteins containing five disulfide bonds[37].
- Type III AFPs are found in Antarctic eelpout. They exhibit similar overall hydrophobicity at ice binding surfaces to type I AFPs. They are approximately 6kD in size [33].
- Type IV AFPs are found in longhorn sculpins. They are alpha helical proteins rich in glutamate and glutamine [38]. This protein is approximately 12KDa in size and consists of a 4-helix bundle [38]. Its only posttranslational modification is a pyroglutamate residue, a cyclized glutamineresidue at its N-terminus [38]. Scientists at the University of Guelph in Canada are currently examining the role of this pyroglutamate residue in the antifreeze activity of type IV AFP from the longhorn sculpin (Fig 3).

	Insects			
AFP (Type 1)	AFP (Type 2)	AFP (Type 3)	AFGP	Hyperactive
Alanine-rich α-helical	Cystein-rich globular	Globular	(Alanine- alanine- threonine plus dissaccharide groups) repeats	β-helical

Figure 3: Structure of different AFPs [39]-[5]

5. Mode of Action

Sea water in cold regions freezes not at 0° C but at -1.9° C due to sodium chloride and other substances dissolved in it. In order to resist freezing (Fig 4) of their blood, fishes inhabiting these harsh environments (e.g., Antarctic cod, Antarctic and Arctic eel pout, Arctic polar cod, and Winter flounder) must have the freezing point of their blood below this temperature. However, low molecular weight dissolved solutes in the fish's blood cannot account for depression of freezing point by more than 1.4^{9} C. It is the AFPs which cause a further depression of freezing point of the blood by about 0.5^{9} C or more. In addition to lowering of the freezing point of the body fluid and preventing damage caused by ice recrystallization, AFPs in some cases are believed to protect membranes from cold-induced damages by inhibiting thermotropic phase transitions and preventing leakage by blocking ion channels.

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Figure 4: Schematic of the mechanism of ice growth inhibition by AFPs [39]

Normally, ice crystals grown in solution only exhibit the basal (0001) and prism faces (1010), and appearsas round and flat discs [40]. However, it appears the presence of AFPs exposes other faces. It now appears the ice surface 2021 is the preferred binding surface, at least for AFP type I [41]. Through studies on type I AFP, ice and AFP were initially thought to interact through hydrogen bonding [42]. However, when parts of the protein thought to facilitate this hydrogen bonding were mutated, the hypothesized decrease in antifreeze activity was not observed. Recent data suggest hydrophobic interactions could be the main contributor [42]. It is difficult to discern the exact mechanism of binding because of the complex water-ice interface. Currently, attempts to uncover the precise mechanism are being made through use of molecular modelling programs (molecular dynamics or the Monte Carlo method) [40].

Biological proteins usually have hydrophobic amino acids in the core (away from water molecules in the solvent) and hydrophilic amino acids on the surface. Unusually, AFPs have many hydrophobic amino acids on their surface. These form part of the special binding surface that only sticks to ice nuclei, i.e. to solid but not liquid H₂O. There have been a number of theories about how this works, but up till now no experimental observation of the molecular mechanism because hydrogen is almost invisible to most imaging techniques. Neutrons see hydrogen very well, so neutron scattering was the only way that are able to locate the positions of H₂O molecules at the protein's binding surface. As identified an 'ice-type' tetrahedral cluster of waters at the binding surface. These mimic the arrangement of water molecules in ice, so we used these positions as the starting point to build up the rest of the ice crystal and model the ice-AFP interaction. The ice model has six rings of four H₂O molecules, which leaves a gap, or hole, in the middle. The hydrophobic regions of the type-III AFPs fit into these holes and bind with the ice's surface. These 'holes' are what distinguish the structure of ice crystal nuclei from water, and explain how AFPs can be present in a solvent without attaching to the water molecules until they start to freeze.

Interms of the energetics of binding, there are several components. These include enthalpic contributions from the

van der Waals interactions that come from ideal surfacesurface complementarity, together with adventitious hydrogen bonds that happen to be formed within the icebinding site contact (Fig 5).



Figure 5: Contributions to the energetics of AFP binding to ice

According to the structure and function study on the antifreeze protein from the fish winter flounder, the antifreeze mechanism of the type-I AFP molecule was shown to be due to the binding to an ice nucleation structure in a zipper-like fashion through hydrogen bonding of the hydroxyl groups of its four Thr residues to the oxygen along the direction in ice lattice, subsequently stopping or retarding the growth of ice pyramidal planes so as to depress the freeze point [43]. The above mechanism can be used to elucidate the structure-function relationship of other antifreeze proteins with the following two common features:

- 1. Recurrence of a Thr residue (or any other polar amino acid residue whose side-chain can form a hydrogen bond with water) in an 11-amino-acid period along the sequence concerned, and
- 2. A high percentage of an Ala residue component therein [43].

6. Commercial Applications

Commercially, there appear to be countless applications for antifreeze proteins [44]. Numerous fields would be able to benefit from the protection of tissue damage by freezing. Businesses are currently investigating the use of these proteins in:

- Increasing freeze tolerance of crop plants and extending the harvest season in cooler climates
- Improving farm fish production in cooler climates
- Lengthening shelf life of frozen foods
- Improving cryosurgery
- Enhancing preservation of tissues for transplant or transfusion in medicine[45]
- Therapy for hypothermia

7. Fish and insect AFPs: differences and similarities

We have extended structure function studies to the newly characterized insect AFPs [46, 47, 48] to see if there is some common theme for their ice-binding mechanism. It should

be noted that insect antifreezes are considerably more active than those from fishes. At a concentration of 20 µ M, AFP from the spruce budworm Choristoneurafumiferana (CfAFP) has roughly four times the thermal hysteresis activity of a 400 µ M solution of type I AFP from winter flounder [46]. We suspect that this difference in species activity relates more to the ice crystal morphology than to the AFP's affinity for ice. CfAFP produce a hexagonal ice crystal that bursts along the a-axes, whereas all fishAFPs shape ice into a hexagonal bipyramid that typically bursts out of the tips along the c-axis. We suggest that the tips of the hexagonal bipyramid are the weak spots for containment of growth of the crystal. Thus, ice crystal shape may account for the lower species activity of fish AFPs compared with insect AFPs. One explanation for the difference in ice crystal morphology is that we findCfAFP binds to the basal plane as well as the primary prism planes. This was demonstrated by ice etching studies [46].

8. Conclusion

Various organisms inhabiting cold environments produce a certain type of protein in their bodies during winter. The proteins are known as antifreeze proteins (AFPs) or antifreeze glycoproteins (AFGPs). Thus far, these proteins have been found in various species of fish, insects, bacteria and plants (hereafter, AFPs and AFGPs are collectively referred to as AFPs). AFPs function as inhibitors of ice growth. Consequently, liquid water does not completely freeze if it contains dissolved AFPs, even at temperatures lower than the melting point Tm of ice. AFPs have received considerable attention with regards to freeze tolerance and freeze avoidance in organisms. food processing, cryopreservation and ice slurries. AFPs are of interest with respect to not only ice crystals, but also other types of crystals. For example, elucidating the mechanism whereby AFPs control ice growth might aid the understanding of the control of bio-mineral crystal growth by organic molecules, and might also be useful for developing crystal growth technologies such as crystal morphology engineering, as well as for designing novel composite materials. Waters of the southern ocean are so cold that temperate and tropical fish would freeze if they were placed in this environment. The presence of salt in sea water allows it to remain liquid until about -1.9°C, almost 2 degrees below the freezing temperature of freshwater. The antifreeze proteins, along with normal body salts, depress the freezing point of blood and body fluids to 2.5° C, slightly below the freezing point of sea water. These proteins bind to and inhibit growth of ice crystals within body fluids through an absorption-inhibition process. The proteins attach to small ice crystals, stemming their growth. This mechanism that inhibits further growth of the ice crystal remains under study, but apparently Antarctic fish are able to survive with very small ice crystals present in their body fluids.

There may be several commercial applications of these antifreeze proteins. These compounds are about 300 times more effective in preventing freezing than conventional chemical antifreezes at the same concentrations. The effectiveness of the fish antifreeze proteins in inhibiting ice growth suggests that they could be used to prevent freezing of food and freezing injury in several applications. For example, they could be used in the cryopreservation of foods that normally are rendered inedible due to ice crystal damage or to engineer cold resistance in living plants, as well as for the cryopreservation of tissues and organs. The study of the mechanism of how antifreezes bind to ice and inhibit its growth also provides insights into how other biomolecules affect growth of such pathogenic (harmful) bio-crystals as those associated with gout, kidney, and gall stones. Lastly, these proteins may have applications as nonpolluting de-icing agents. To date, NSF-funded investigators have successfully introduced two of the four different types of fish antifreeze proteins into yeast and bacteria through recombinant DNA technology. Using these cloned genes and molecular technology, researchers can produce large quantities of antifreeze proteins through large-scale fermentation.

9. Acknowledgements

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10. Future scope of the study

Antifreezing proteins play a very important role in protecting the fishes from freezing of their blood and it also helps in maintenance of normal homeostasis. So, further work is needed to identify proteins or compound which might be playing an important role in the survival of cold water fishes.

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