

FREEZING AVOIDANCE IN POLAR FISHES

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Summary

The field of fish antifreeze research started in the late 1950s with the simple observation and question: why do shallow-water polar marine bony fishes, which have a higher colligative freezing point than the icy seawater they inhabit, not freeze? The identification in 1968 of the macromolecular antifreeze compound and how it interacts with ice crystals to stop them from growing leading to freezing avoidance launched a new, exciting area of research into this novel adaptation to extreme cold, which continues to expand in many directions today. The major questions are what are these molecules, how do they work, where do they come from, and how do they evolve. Diverse types of antifreeze proteins are now found in different fishes, and new types continue to be discovered in fish and other organisms. Sophisticated physical and structural studies have led to an understanding of how some antifreeze proteins bind to ice, but the commonality that unites such disparate molecules in achieving the same function remains elusive. The structural diversity also means diverse evolutionary origins and molecular studies to decipher where these novel proteins come from have uncovered remarkable convergent evolutionary pathway and mechanism. What began as investigations into how cold-water teleost fish meet the

freezing challenge is now a multifaceted research area that encompasses ecophysiology, organismal physiology, protein biochemistry, ice physics, structural biology, molecular evolution, organismal evolution, and polar paleogeography. In the end, what these investigations collectively strive to achieve is to provide a thorough picture and understanding of how polar fishes avoid freezing. This paper presents a perspective on some of these related major aspects of fish antifreeze research.

1. Introduction

The polar seas hover closely around $-1.9\text{ }^{\circ}\text{C}$, the freezing point of seawater. Although this is much milder when compared to terrestrial temperatures, which can reach the cold extreme of $-80\text{ }^{\circ}\text{C}$ in the Antarctic winter, for marine bony fishes, it presents a daunting challenge to their survival, especially if ice is present. Seawater temperature is determined by the colligative property of solutions; full strength seawater contains about 1000 mOsm of solutes (mostly salts) which depress its freezing point (fp) to about $-1.86\text{ }^{\circ}\text{C}$. The waters of the high polar latitudes are almost constantly at freezing and ice covered. The inshore shallow water of some of the northern cool temperate coasts could reach similar frigid conditions in the winter. Bony fishes are hyposmotic to seawater, that is, the colligative solutes in their body fluids are at much lower concentration than seawater, generally $\sim 400\text{--}600\text{ mOsm}$ for cold-adapted species. This means they would freeze at about $-0.75\text{ }^{\circ}\text{C}$ to $-1.3\text{ }^{\circ}\text{C}$, substantially above the fp of seawater. Unlike organisms that can survive freezing and thawing (see *Freeze Tolerance*), even partial freezing in teleost fish is always lethal.

Thus, the only viable survival strategy for cold-water marine teleost fish is to avoid freezing. The simplest one is behavioral. Some leave for warmer offshore water or seek out nonfreezing water layers during winter. Others live in ice-free deep water in a metastable supercooled state. Ice formation generally does not occur below 30 m of water due to the effect of hydrostatic pressure on the *in situ* freezing temperature, which becomes lower than the colligative fp of seawater at $\sim 30\text{ m}$ and below. In the late 1950s, Scholander and coworkers described fishes that inhabit the $-1.7\text{ }^{\circ}\text{C}$ deep (200–300 m) water of the northern Labrador fjord. These fish are hyposmotic to seawater, with a colligative blood fp of $-0.8\text{ }^{\circ}\text{C}$, and thus are supercooled by $0.9\text{ }^{\circ}\text{C}$ with respect to ambient temperature. However, their ice-free deep water habitat allows them to remain supercooled and not freeze for the duration of their lives.

More recently, colligative depression of freezing point by seasonal elevation in blood osmolyte concentration has been found to be another strategy for freezing avoidance. The rainbow smelt, *Osmerus mordax*, produces high serum levels of glycerol in the winter, reaching over 400 mOsM. This, in conjunction with serum electrolytes and other osmolytes renders the smelt almost isosmotic with seawater, which enables it to avoid freezing in frigid marine environments. Highly augmented colligative fp depression as a mechanism to avoid freezing is common among terrestrial insects, but may be rare among teleosts, as it has only been observed in the rainbow smelt thus far.

For marine teleosts that are confined to frigid shallow marine environments because of life histories or geographical constraints, freezing death would seem inescapable based on physical colligative considerations alone. Shallow-water habitats of the polar regions, and of the high-latitude coasts in the winter, are laden with ice crystals in the column. The

hyposmotic teleost fishes could not avoid freezing by supercooling in these habitats, because the environmental ice will quickly propagate and freeze the body fluids of the fish upon contact. Paradoxically, thriving fish faunas are found in the freezing waters of both the Arctic and Antarctic, as well as the northern coastal winter waters, and the search for why these endemic fish do not freeze began in the early 1960s.

The prevailing physical principle that freezing point depression of solutions is effected by dissolved small colligative solutes set the early research on identifying the agent responsible for fish freezing avoidance on that path. Scholander and coworkers tallied all identifiable serum ions and osmolytes from the Arctic sculpin *Myoxocephalus scorpius* and fjord cod *Gadus ogac* collected at the Labrador coast, and collectively these could only account for 60% of observed serum freezing point depression in the winter. In the fjord cod, after precipitation of the serum proteins by strong oxidizing acid such as trichloroacetic acid (TCA), the acid-soluble fraction of the serum was found to contain high levels of nitrogen, in excess of circulating levels of nitrogen-containing molecules such as free amino acids and nucleotides. This high non-protein nitrogen level was thought to be somehow associated with the fjord cod's ability to avoid freezing, but the identity of the actual antifreeze compound remained unidentified for almost another decade.

In his doctoral work on freezing avoidance in Antarctic nototheniid fishes in the 1960s, DeVries discovered that when the soluble fraction of heat- or TCA-treated serum was dialyzed to remove all small ions and osmolytes, substantial freezing-point depressing activity was retained within the dialysis tubing. This seminal work and observation clinched the macromolecular nature of the antifreeze agent in these fish, and led to the isolation and characterization of the first fish antifreeze, a heat- and acid-resistant carbohydrate-containing protein, termed antifreeze glycoprotein (AFGP), from the Antarctic nototheniid *Pagothenia borchgrevinki*.

The macromolecular paradigm of fish antifreeze and its role in freezing avoidance established with the nototheniid AFGP discovery launched a new field of research that continued to expand in many facets in the subsequent three decades through today. Among these are the discovery of new types of antifreeze proteins in different fishes and other organisms (insects, plants and microbes), and investigations of the physical mechanism of how they interact with ice and prevent ice growth, their *in vivo* functional role or roles, the regulation of their expression, the ancestral origin and evolutionary pathway of these novel proteins, the paleoenvironmental driving force leading to their creation, as well as the potential use of these ice-growth inhibiting proteins in real world applications.

2. Diversity of Fish Antifreeze Proteins

The two decades following the discovery of AFGP in the Antarctic nototheniid fish envisaged a flurry of studies in uncovering the antifreeze agents in the northern teleost fishes. Members of the cod fish family Gadidae were found to have a very similar AFGP as the Antarctic nototheniid fish. Three other antifreeze proteins, unglycosylated and structurally distinct were found in other taxa; these were named type I, II, and III AFPs based on the structural difference and chronology of their discovery. The latest addition is type IV AFP described in 1997. Figure 1 shows the types of currently known fish antifreeze proteins and some of the fish taxa that possess them.

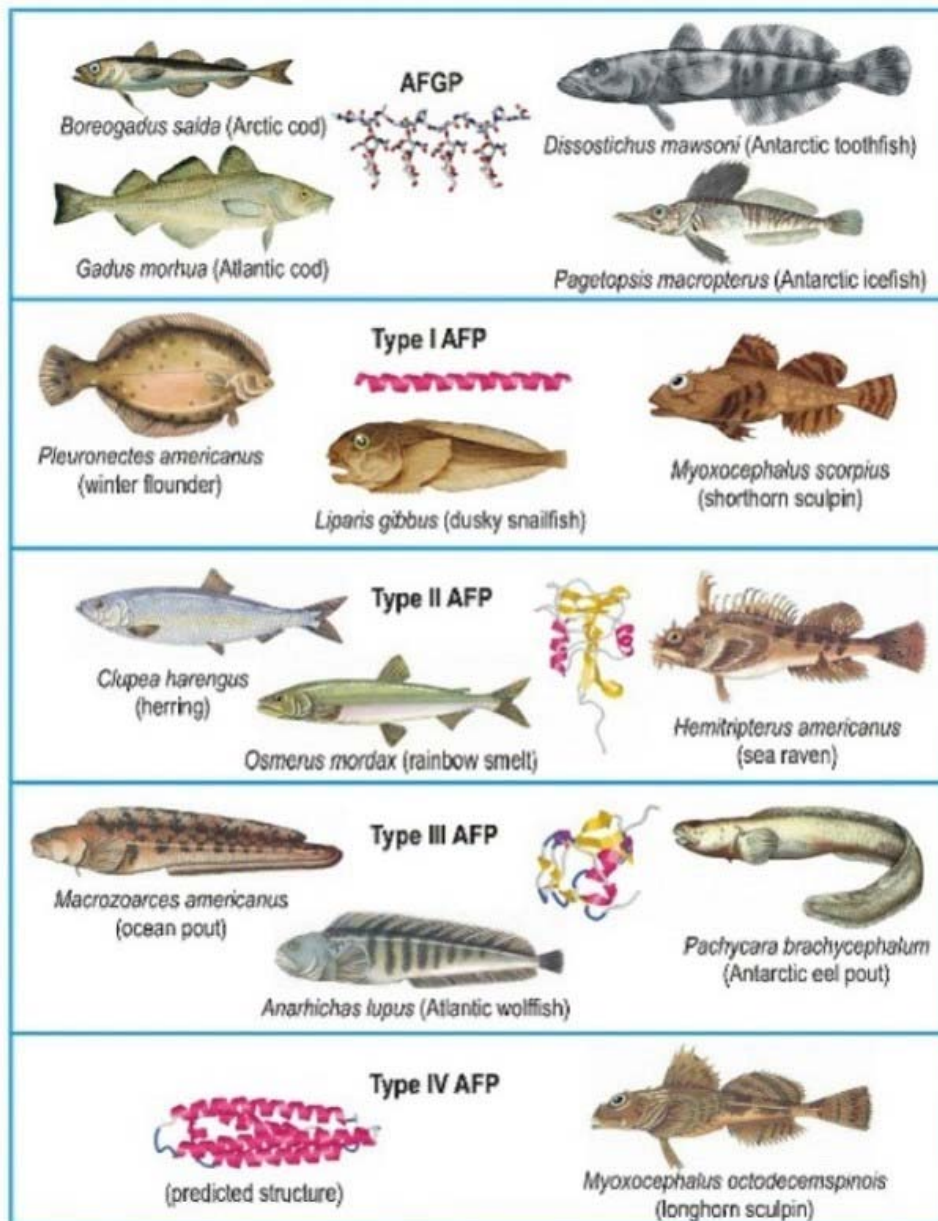


Figure 1. Types of antifreeze protein, their structure, and some of the fish associated with each type

2.1. Antifreeze Glycoprotein

The AFGP in the Antarctic nototheniid fish is present as a family of size isoforms, all composed of repeating units of a simple glycotripeptide monomer, (Thr-Ala/Pro-Ala-)_n with a disaccharide galactose--acetylgalactosamine attached to each Thr. Based on electrophoretic mobility, eight sizes named AFGP 1–8 were first identified from the Antarctic nototheniid *P. borchgrevinki*. With four glycotripeptide repeats, AFGP8 is the smallest (molecular weight = 2600 daltons) and AFGP1 is the largest (MW=36,000 daltons). Some larger sizes and many more intermediate sizes based on protein and gene analyses have now been identified in various taxa of the five related endemic Antarctic

families (Nototheniidae, Artedidraconidae, Harpagiferidae, Bathydraconidae, and Channichthyidae) comprising the suborder Notothenioidei. Notothenioid fishes are modern bony fishes belonging to the order Perciformes in the superorder Acanthopterygii.

The AFGPs of Arctic and northern cod fishes are found in members of the genus *Microgadus*, *Eleginus*, *Boreogadus*, *Gadus*, and *Arctogadus* in the family Gadidae. Cod fishes belong to the order Gadiformes and superorder Paracanthopterygii, and are presumed to have evolved long before the Antarctic notothenioids, and thus the two groups of fishes are unrelated. However cod AFGPs have a very similar glycotriptide repeat as the notothenioid version, except for an occasional Arg for Thr replacement in some species. The size heterogeneity is also similar except the largest size isoform is usually smaller than its counterpart in the notothenioid fish.

The three-dimensional structure of AFGPs has not been determined but a number of evidence indicate that they have an expanded polyproline type II helical structure. The carbohydrate side-chains comprise as much as 60% of the glycoprotein, and impart the observed heat and acid resistance. The high level of nonprotein nitrogen in *G. ogac* observed by Scholander and coworkers in the early 1960s was in fact the denaturation-resistant proteinaceous AFGP. In contrast, antifreeze peptides (section 2.2) are acid-labile. Arctic sculpin has an antifreeze peptide, which would be precipitated by TCA, and explains why Scholander and co-workers did not observe elevated non-protein nitrogen level in sculpin serum, as opposed to the case of the fjord cod.

2.2. Type I, II, III, and IV Antifreeze Peptides (AFPs)

The four types of AFPs are small peptides or proteins without attached sugars. They were first classified based on distinctive differences in their protein sequences, which was corroborated by higher order structural differences determined subsequently (Figure 1). A great deal of antifreeze research effort in the 1990s through today has been devoted to solving the three-dimensional structures of antifreeze proteins which are essential for elucidating structure-function relationships in the interaction of antifreeze with ice to bring about the observed noncolligative freezing point depression (Section 3).

Type I AFPs are found in three groups of fish, the right-eyed flat fishes (flounders and plaice, order Pleuronectiformes), the cottid sculpins, and the Arctic liparid snail fishes (order Scorpaeniformes). They are 4–7 kDa, Ala-rich (>50%) peptides, some of which have an 11-residue repeat ending with a Thr. High resolution X-ray crystal structures of the winter flounder AFP show it to be a near-perfect α -helix. Type II AFPs are 11–24 kDa, cystine-rich proteins found in three highly divergent fish—rainbow smelt (order Salmoniformes), herring (order Clupeiformes), and sea raven (order Scorpaeniformes). NMR (Nuclear Magnetic Resonance) structural determinations showed type II to be a β -structured proteins with a few disulfide bridges. Type III AFPs are 7 kDa and 14 kDa globular proteins with unbiased amino acid composition found in the closely related zoarcid eel pouts and anarhichadid wolffishes (order Perciformes). Its structure has been determined to very high resolution by X-ray crystallography and NMR. Type IV AFP of long-horn sculpin (order Scorpaeniformes) is 12.3 kDa and is predicted to have a helix bundle structure.

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Biographical Sketch

Chi-Hing C. Cheng received her BS degree in Zoology in 1975 from Texas A & M University, and both her MS degree (1977) and PhD degree (1989) in Molecular and Integrative Physiology from the University of Illinois, Urbana-Champaign. She did postdoctoral research at the University of Illinois characterizing antifreeze proteins from polar fishes for two years after obtaining her doctoral degree, and subsequently as Senior Research Scientist expanding her research emphases into antifreeze gene families and molecular evolution of these novel proteins. In 2000, she was appointed as Assistant Professor in the Department of Animal Biology at the University of Illinois and continued her research on the structure-function and evolution of polar fish antifreeze systems. She has traveled regularly to Antarctica for the field aspects of her research since 1984, and more recently to north Norway and the Barents Sea for her work on Arctic fish.