THE ROLE OF ANTIFREEZE PROTEINS IN THE ANTARCTIC FISH, NOTOTHENIOIDEI

Trematamus bernaccii

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ABSTRACT

The changing decrease in temperature of the southern ocean in geological history has lead to the extensive radiation of a group of fish, the suborder Notothenioidei. These fish have evolved an adaptive characteristic, antifreeze proteins, to help them survive the \(-1.8^\circ\text{C}\) freezing waters of the southern ocean.

These antifreeze proteins are large repeating units of glycotripeptide structures with a linked disaccharide unit that adsorbs to an ice crystal lattice and lowers the freezing point of the water so as to prevent the ice from growing inside the animals tissues, which is potentially lethal. It is the presence of these antifreeze proteins that allow these fish to dominate the fish fauna in the freezing waters of the southern ocean today.
INTRODUCTION

In the late Paleozoic there was one gigantic continent called Pangaea, and a single world ocean called Panthalassa (Eastman, 1993). The modern cycle of plate tectonics, continental drift and seafloor spreading, caused Pangaea to separate into two super continents known as Laurasia in the northern hemisphere, and Gondwana land in the southern hemisphere. Antarctica was once a part of Gondwana land occupying a central position in the super continent surrounded by other present day southern continents. To Antarctica’s east was Australia and India, and to the west was Africa and South America. Gondwana land began to break up less than 200 million years ago and Antarctica became isolated from all other southern continents about 90 million years ago, when Australia began drifting northwards. An ocean between the two continents did not form until 53 million years ago (Eastman, 1993). Once Antarctica was fully isolated, the Antarctic circumpolar current began to develop around the entire continent. By removing the warm subtropical currents from the continent, this circumpolar current served as a barrier to heat flow and thermally isolated Antarctica (Eastman, 1993).

A general cooling trend of the Antarctic began in the late Paleocene /early Eocene and has continued until present. Exactly when the Antarctic continent became covered with a permanent ice sheet is still being debated, but recent land and ocean based drilling projects have extended the record of the Antarctic ice sheet back to at least the early Oligocene (Eastman, 1993). In the last 50 million years, ocean water temperatures have dropped from about 20 °C to 0 °C. The changing water temperatures of the southern ocean forced the fish fauna there to evolve adaptive characteristics or become extinct. The fish that have evolved, radiated to form the species which are present now.
One group of fish, the Notothenioidei, a suborder of the order Perciformes, have become adapted and dominate the fish fauna in the southern ocean, as they have specially adapted to the freezing waters by acquiring antifreeze proteins in their blood, which stops their body fluids from freezing. This adaptation allows these fish to survive in an environment where many other species would die.

**EVOLUTION**

There is estimated to be c. 21,723 species of fish inhabiting the waters of the earth (Eastman, 1993). The Antarctic fish fauna comprises of about 274 of these species. This is slightly over 1% of all species in the world, and is fairly limited considering the southern ocean between the continent and the Antarctic convergence is over 35 million km², which is about 10% of the world's ocean (Eastman, 1993). Of the 274 Antarctic fish species, 95 are from the suborder Notothenioidei (34.5%) and are the major component of the fauna. These numbers are changing all the time as new species are continually being discovered. Endemism is high within this group, 97% for species and 85% for genera (Eastman, 1993). They are also extremely important economically because 90% of all fish collected in the southern ocean are Notothenioids.

The suborder is divided into six families: 1) Bovichtidae (thornfish), 2) Nototheniidae (notothens), 3) Harpagiferidae (spiny plunderfish), 4) Artedidraconidae (plunderfish), 5) Bathydraconidae (dragonfish), and 6) Channichthyidae (icefish).

The exact evolutionary relationship of species between these six families is still being debated as new analytical techniques are continuing to be used, contradicting previous work.
There was little fossilization in the Antarctic since the Eocene, during the period, which the Notothenioidei are thought to have arisen. The absence of fossils suggests the need to extrapolate evolutionary evidence from extant species by cladistic analysis of morphological characteristics to determine the evolutionary relationship.

A lot of work has been carried out on determining the phylogenetic relationship of the species in the Notothenioidei such as Eastman (1993), who constructed a family tree of the Notothenioidei families that is generally accepted and used as the best working hypothesis (Figure 1).

Figure 1: The phylogenetic tree of the Notothenioidei families (from Kock, 1992).

Mitochondrial and nuclear molecular phylogeny techniques by Bargelloni et al., (1994) agree with the morphological and physiological cladistic analysis of Eastman (1993), but suggest that the species arrangement within families was in need of revision. Continual molecular analysis (Ritchie et al., 1997) still suggests revision of species relationships, as a problem of a sister group still has not been resolved. To
discover Notothenioid phylogenetic intra relationships, it is better for character polarization to use its sister group, rather than a more distant Perciform suborder (Ritchie et al, 1997). Some authors have suggested the Blennioidei or Zoarceodei suborder as a sister group to the Perciforms in molecular analysis (Kock, 1982). However, there is no ambiguous morphological or physiological character that can clearly establish a Perciform subgroup as the sister group to the Notothenioidei (Eastman, 1993). Even within the suborder notothenioidei, there is not a unique character that distinguishes it from other groups. Therefore, the evolutionary relationship of the group still remains to be determined.

The Notothenioidei are a benthic shallow water species that inhabit the continental shelf and slope of the Antarctic and are surrounded by deep water. They are thought to have radiated so extensively in the area due to a decrease in the temperature of the ocean waters, but also due to isolation, habitat loss and climatic cycles, which have played a role in their evolution (Clarke et al., 1996).

The overall decrease in climatic temperature may be related to Milankovitch cycles, which are variations in the orbit of the earth on periods ranging from 23,000 to $10^5$ years (Clarke et al., 1996). The ancestor fish of the Antarctic were thought to have had limited habitats. There were no estuaries or shallow embayments except on the Antarctic Peninsula. When exactly the switch in glacial and interglacial conditions occurred, they may have been rapid, and the present fish were forced to adapt or become extinct, because being benthic and surrounded by deep water, they had nowhere to go. We are unsure whether the Antarctic ice sheet extended over the entire continental shelf, but suggestions are that when the ice sheet retreated, it left empty habitats for the Notothenioid diversification as ancestor fish were forced out by the ice and made extinct. The result has been the striking adaptive radiation of Notothenioid
fish, which dominate the present Antarctic fish fauna (Clarke et al., 1996). This radiation was probably fuelled by the lack of competition from non-Notothienoiid fish, availability of a new habitat, and climatic variability.

The success of the Notothienoidei radiation can be attributed to the development of the antifreeze proteins. Though not the only reason the group is successful, it is the aim of this paper to review the antifreeze proteins. A variety of other biochemical, physiological and morphological specializations have also occurred to enable the fish to function at low temperatures, such as in some families the loss of haemoglobin or a swim bladder.

**WATER PROPERTIES**

The properties of water will changes at different temperatures. In temperatures above 100 °C, water is changed from the liquid state to the gaseous state. Similarly, if water is lowered to a temperature of 0 °C, it changes from a liquid phase to a solid phase, ice. Ice is a problem for biological life as the formation of ice crystals within a cell is usually lethal, as the crystals grow in size they rupture and destroy the cells by breaking cell membranes (Randal et al, 1997). Animals exposed to long periods of winter temperatures have developed two survival strategies. To be either, freeze tolerant where they can survive extensive freezing and ice formation in the body or freeze intolerant where they die if there is internal ice formation (Schmidt-Nielson, 1997). The freezing point of fresh water is 0 °C. However, if you lower the temperature of water below 0 °C, freezing does not necessarily take place. This water is said to be super cooled. The probability of water becoming super cooled depends on the temperature, the presence of nuclei for ice formation and time (Schmidt-Nielson, 1997). In the absence of foreign nucleating materials, a sample of very pure water is
readily cooled to $-20 \, ^\circ C$ before it freezes. The moment an initial ice nucleus is formed, freezing progresses rapidly throughout the sample.

Salt water differs somewhat to freshwater. It has many dissolved substances in it. These solutes lower the freezing point and also the extent of super cooling that can occur before freezing takes place. When ice forms, it is less dense than water and therefore floats at the surface in both fresh and salt water. Fresh water is most dense at $4 \, ^\circ C$. When ice forms at the surface, the water underneath may still be $4 \, ^\circ C$. Salt water does not exhibit this property. When cooling of the salt water forms ice, the solute concentration in the remaining liquid increases, thus gradually lowering the temperature at which more ice can form. This is why the temperature of the southern ocean remains around $-1.8 \, ^\circ C$ under the ice.

This is a problem for marine fish in Antarctica. $-1.8 \, ^\circ C$ is colder than their body fluid temperature and they risk freezing. Teleost fish are not freeze tolerant. The formation of ice leads to the destruction of the frozen areas and if freezing extends to the vital organs, the fish rapidly dies.

These fish cannot remain super cooled as there are ice crystals in the water column where they live which serve as nuclei for ice formation. Although the osmotic concentration (ion concentration), of an average teleosts body fluids is about 300-400 mOsmol (milli osmoles), compared with seawater at 1000 mOsmol, this only depresses the freezing point of water to between $-0.6 \, ^\circ C$ to $-0.8 \, ^\circ C$, as there are not enough electrolytes in the body to prevent the extent of freezing that occurs in seawater. The Notothenioidei have thus evolved antifreezes in their blood to prevent ice crystals from forming in their tissues.
ANTIFREEZES

Antifreezes were first discovered and isolated from Antarctic fish in 1969 by A. DeVries and D. Wohlschlag (DeVries, 1984). They were isolated from the three species *Trematomus borghrevinki*, *T. bernacchii* and *T. hansonii*. The experiment aimed to determine how these fish, which are found in McMurdo Sound, prevented their body fluids from freezing. In most temperate marine fish, the main electrolyte in the blood, sodium chloride (NaCl), depresses the freezing point of the blood by 85%. Other electrolytes such as potassium, calcium, urea, glucose and amino acids also contribute, but not to the extent of NaCl. However, in polar species NaCl concentrations are elevated comparatively, but account for less than half, about 40-50%, of the depressed freezing point (DeVries, 1984: Duman et al., 1975). Biochemical analysis of the blood filtrate by DeVries & Wohlschlag (1969) lead to the isolation of a large protein found to account for over half of the freezing point depression.

These large molecules are now known as glycopeptides. Glycopeptides are found in the Antarctic fish and some northern temperate fish, however most northern temperate fish and Arctic fish generally possess peptides. Glycopeptides are larger than peptides with molecular masses between 2,600-34,000 Da (Daltons) compared to 3,200-14,000 Da respectively. On a concentration basis, glycopeptides depress the freezing point 200 to 300 times more than NaCl (DeVries, 1984; DeVries, 1971). This means that a smaller concentration of glycopeptides or peptides is much more effective at lowering the freezing point than NaCl in increasing concentrations, Figure 2.
Figure 2: Molar concentration comparison of the freezing point depression of glycopeptides, sodium chloride and glucose (from Schmidt-Nielsen, 1997).

All glycopeptides and peptides appear to lower the freezing point in a non-colligative manner. In other words, they don’t lower the freezing depression point by having a higher concentration. This is referred to as an antifreeze effect and these molecules are now referred to as antifreezes. They do however have a colligative effect on the melting point of the solution, Figure 3.

Figure 3: Freezing and melting points of solutions of peptides and glycopeptides in increasing concentrations (from DeVries, 1984).

The glycopeptides are large molecules and thus make up 3.5 % by mass of the body fluids (DeVries, 1984). Biochemical analysis has found the molecules to be expanded but no knowledge is known of their secondary structures. Eight separate glycopeptides have been identified (Table 1), each ranging in different molecular
mass. They are found mainly in the blood and most of the extra cellular fluid of fish, and only two are found in the intestinal fluid to prevent food particles from freezing.

<table>
<thead>
<tr>
<th>Anitfreeze glycopeptide</th>
<th>Molecular Weight (Daltons)</th>
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<tr>
<td>1</td>
<td>33,700</td>
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<tr>
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<td>3,500</td>
</tr>
<tr>
<td>8</td>
<td>2,600</td>
</tr>
</tbody>
</table>

Table 1: Molecular weights of the eight antifreeze glycopeptides (from Eastman, 1993).

The glycopeptides are composed of repeating units of glycotripeptides in which the disaccharide β-D-galactopyranose-(1-3)-2-acetamido-2-deoxy-α-D-galactopyranose is linked to the threonine residue of the tripeptide alanyl-threonyl-alanine (DeVries, 1982), or simply repeating units of the amino acids alanine, threonine, alanine, with a disaccharide derived from galactose attached to each threonine (Figure 4).

![Figure 4: The repeating structural unit of the antifreeze glycopeptides, alanine, alanine, threonine, with the attached disaccharide joined to the threonine amino acid (from Eastman, 1993).](image)

This structure differs between glycopeptides. Glycopeptides 1-5 are this structure, whereas glycopeptides 6-8 differ in that the amino acid proline replaces some of the alanines. The structural difference changes even more when the glycopeptides of northern temperate fish are compared.
It has been found that below a certain molecular mass, the antifreeze effect diminishes rapidly, and if there is any change in the structure of the glycopeptide, it becomes less effective. This is explained by how it attaches to ice crystals.

The Notothenioids live in the water column where there are ice crystals present. Some even actively live close to the pack ice and ice platelets that form under the ice to escape predation. Therefore, exogenous ice crystals can penetrate the fishes integument and cause freezing as there are nuclei present for ice formation. The antifreeze glycopeptides act by preventing the addition of water molecules to the crystal lattice of ice and therefore the growth of ice crystals. They do this by adsorbing to an ice crystal in such a way as to lower the freezing point of the water crystal and thus prevent growth.

The way an antifreeze adsorbs to an ice crystal was found to depend on its chemical structure. If they are modified by heat or other means, their effect is lost. Alteration of the glycopeptide structure resulting in the loss or reduction in potential hydrogen bonding groups of the disaccharide side chains suggests hydrogen bonding is involved in the joining of antifreezes to ice (DeVries, 1984). ‘For the antifreeze to be strongly adsorbed to ice, potential hydrogen bonding residues would need to occupy positions in the antifreeze molecule to allow them to align with the opposite oxygen and hydrogen in the ice lattice in a regular pattern’ (DeVries, 1984). Even though the secondary structure of the glycopeptides is not known, models reveal that such an alignment is possible. The spacing between the hydroxyl components of the disaccharide side chain was found to have a distance that corresponds to the spacing of the parallel oxygen molecules in the ice crystals a-axis. Also the spacing between the oxygen molecules in the c-axis of the ice crystal is very similar to the spacing between the alternate carbonyl groups on the corresponding glycopeptide chain.
However, for this spacing to match the molecule is fully extended and not how it occurs naturally. This theory needs much work as glycopeptides differ in structural arrangement and this cannot account for all antifreeze adsorptions. This does not explain with certainty that this is how antifreezes adsorb, but rather offers a hypothesis as to how they may adsorb to ice. We do however know for certain that the chemical arrangement and structure of the molecule is what binds the glycopeptide as an alteration removes the glycopeptide antifreeze properties. We are therefore certain that it is the structure of the molecule that adsorbs it to the ice.

Once the glycopeptide molecule has adsorbed to the ice crystal, the crystal increases in surface area relative to volume. It is thought ice crystals grow by the joining of water molecules to the ice crystal on the basal plane steps of the crystal. Glycopeptides adsorb to the steps, and therefore the ice cannot grow here and is forced to grow in the spaces between molecules. This causes the steps to develop a large surface area because of their curvature on the steps where the ice has grown between glycopeptide molecules (Figure 5A and B).

A)
Figure 5: Diagrammatic illustration of how the antifreeze molecules force the ice crystal to grow in surface area at the step structure of the ice crystal between antifreeze molecules. A) DeVries, 1984, B) Schmidt-Nielsen, 1997.

Growth stops when the surface area to volume ratio reaches a critical point. If the spacing between the antifreeze molecules is reduced, as it is when the molecules adsorb to the ice and cause the steps to grow between, then the cooling required to allow the step to keep growing through the spacing must be increased (DeVries, 1988). In other words, the freezing point of water is reduced because the temperature needs to be lowered for extra growth of the ice crystal at the steps.

**DISCUSSION / CONCLUSION**

Since the discovery of antifreeze proteins in Antarctic fish in 1969, the amount of papers published on the subject was the greatest during the 1970’s and 1980’s. Few authors have worked on the subject and most papers therefore are repetitive as the same papers are referred to in all papers related to the same subject.
The apparent knowledge of how glycopeptides work in Antarctic fish seems well known. In Eastman’s 1993 book, *Antarctic fish: Evolution in a unique environment*, the then up-to-date information of glycopeptides, had not advanced much since the 1970’s.

During the 1990’s to present, the development of molecular techniques has moved the interest of finding out how glycopeptides work to using them in working towards a suitable evolutionary relationship of the Notothenioidei suborder. However, the problem of a unknown/undefined sister suborder to the Notothenioidei group has left an ongoing debate as to which species are related more closely to one another.

Generally, the only unknown fact left to be resolved aside from the evolutionary relationships, is to break the puzzle as to how exactly the molecules of the glycopeptide adsorb and adhere to the parallel oxygen molecule in the crystal lattice they prevent from growing and how structurally different glycopeptide molecules do the same thing, if not completed already.

Future advances may look towards the discovery of glycopeptide use in other physiological functions and how they may be used for the human advantage in industry, medicine or pharmaceuticals.
REFERENCES:


