Antifreeze proteins: Effective Adaptation to Low Temperatures

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Key words: ice structuring protein, antifreeze protein/glycoprotein, freeze avoidance, freeze tolerance, low temperature adaptation

Introduction

Adaptation to low temperature presents manifold challenges. In particular, extreme environments, such as the polar regions, require organisms to make sophisticated adjustments at the molecular, cellular and organismal levels (Rogers *et al*., 2007). Chilling slows cellular function, alters protein-protein interactions, and reduces membrane fluidity, among other effects (Verde *et al*., 2006). Further, freezing temperatures induce ice crystal growth, which disrupts cellular processes, and is often lethal when formed intracellularly (Carpenter and Hansen, 1992; Knight *et al*., 1984). If species are unfit to cope, high mortality rates can result.

Through the process of natural selection, some basic strategies have evolved for survival of cold temperatures, and according to Marchand, fall into three main categories: migration, hibernation and resistance (1996). The first two work well for seasonal climates. However, both come with risks – migration can involve high mortality rates, and hibernation requires the energetically expensive process of rewarming, especially for young and small-sized animals (Bale, 2002). In polar regions that are consistently low temperature, a preferred approach is resistance.

An effective strategy for cold resistance is the expression of antifreeze proteins (AFPs) (a type of ice structuring protein – ISP), a class of polypeptides that allow survival in cold environments. AFPs and AFGPs (antifreeze glycoproteins) are relatively high molecular mass molecules that have the ability to stabilize membranes during chilling and control ice crystal growth during freezing of cells and tissues (Davies *et al*., 1988; DeVries, 1971; Fletcher *et al*., 2001). One class of AFGPs and six classes of AFPs (AFP types I-V, and plant) have been identified in a wide variety of species. These proteins confer efficient, highly effective protection against low-temperature damage. The majority of early studies on AFPs focused on polar fish species (AFGP and AFP types I-IV), but have more recently expanded to include other vertebrates, insects, plants, fungi, and bacteria.

The aim of this review is to discuss low-temperature adaptation, AFP evolution and mechanisms of function, as well as applications in research and medicine.

Cold temperature adaptation strategies

Damage due to chilling, sometimes referred to as 'cold shock', is due mainly to cell membrane failure. Loss of membrane integrity includes lateral lipid rearrangement and leakage of solutes and ions across the membrane as the lipids pass through their phase transition temperatures (Arav *et al*., 2000; Drobnis *et al*., 1993; Marchand, 1996; Prosser, 1990; Quinn, 1985; Ricker *et al*., 2005; Tsvetkova *et al*., 2000; Wolkers *et al*., 2002). Such events result in damage that compromises function and eventually survival. Freezing of tissues adds another dimension of challenge, due to formation of ice crystals. Growing ice crystals cause mechanical disruption of cellular structure, particularly that of membranes, and are usually lethal when formed intracellularly. As extracellular ice crystals advance, they exclude dissolved solutes, causing osmotic shock and progressive cell dehydration (Marchand, 1996). This causes protein denaturation, and membrane disruption including membrane leakage and fusion. In addition, an increase in viscosity of interstitial fluid can alter metabolic processes, causing further damage (Verde *et al*., 2006). Perhaps one of the most damaging consequences of freezing tissues is the possibility of recrystallization – fusion of small ice crystals into larger ones in order to minimize their surface area – which leads to physically damaging accumulation of ice (Knight *et al*., 1995; Marchand, 1996).

Resistance strategies to cooling damage can be classified in two general categories: freeze avoidance and freeze tolerance. Organisms that cannot tolerate ice formation in their tissues will activate supercooling upon exposure to cold temperatures (Block *et al*., 1990; Dautel and Knülle, 1996; Duman, 1977; Ishikawa, 1984; Lowe *et al*., 1971; Mazur, 1984; Neuner, 2014; Somero and DeVries, 1967; Storey, 2006; Zachariassen and Kristiansen, 2000). Supercooling is the ability of an organism to delay phase change from fluid to solid to temperatures well below the normal freezing point of water. In this way, ice crystal formation is altogether avoided. Supercooling can be achieved by various methods such as minimization of cellular water content, evacuation of gut contents (typical for insects such as the stag beetle *Ceruchus piceus*), and production of large amounts of cryoprotectants (low molecular weight compounds such as salts, sugars, and polyols) (Marchand, 1996; Prosser, 1990; Margesin and Schinner, 2013; and references listed above). The goldenrod gall moth larva *Epiblema scudderiana* decreases its cellular water content from 60% to 25% of fresh weight during cold hardening (Rickards *et al*., 1987). An example of supercooling by cryoprotectant occurs in the Atlantic cod *Gadus morhua*, where plasma salt and sugar contents are increased in response to decreasing water temperatures (Verde *et al*., 2006). Combinations of these strategies are often used, as in the arctic willow insect

Rhabdophaga spp., which removes gut contents and produces glycerol levels up to 20% of its weight, allowing supercooling to –66 °C – one of the lowest yet recorded (Ring and Tesar, 1981).

Supercooling is an effective short-term mechanism, but creates a state where any slight disturbance can cause ice nucleation. In contrast to freeze avoidant species, freeze *tolerant* species are able to withstand ice crystal formation by promoting early, gradual freezing in their tissues. They are also able to increase the amount of intracellular water bound to proteins and membranes at low temperatures. These organisms use ice nucleating proteins, or INPs, which induce formation of extracellular ice at high subzero temperatures. While supercooling is found mostly in smaller-bodied organisms like insects (less water in tissues to freeze), ice nucleation is found across many phyla, including fish, amphibians, reptiles and plants and bacteria (Duman, 2001; Johnston, 1990; Lee and Costanzo, 1998; Storey and Storey, 2013; Warren and Wolber, 1991). The mechanism of action involves reduction of energetic barriers to ice formation by providing adsorption sites that orient water molecules, thus facilitating ice lattice formation. Ice forms slowly around INPs extracellularly, and can take up to two days to reach maximum accumulation (Bale, 2002; Marchand, 1996). This minimizes mechanical and osmotic stress, as solution movement out of the cell gradually reaches equilibrium with the freeze-concentrated extracellular fluid. Often both freeze avoidance and tolerance are used in concert by organisms. The overwintering beetle larva *Pytho deplanatus* is freeze-tolerant, but also produces large amounts of glycerol to supercool to –54 °C (Ring and Tesar, 1981). Some Dendroides species alternate approaches from one year to the next (Prosser, 1990).

If allowed to grow unchecked, however, extracellular ice can lead to serious sub-lethal, and eventually lethal, damage. In order to limit the growth of ice crystals, both avoidant and tolerant organisms synthesize AFPs to stabilize membranes during chilling, and control ice crystal growth during freezing (Bale, 2002; Davies *et al.,* 2002; DeVries, 1971; Griffith and Yaish, 2004; Harding et al., 2003). Due to their non-colligative properties, AFPs are protective at concentrations 300-500 times lower than low molecular weight cryoprotectants. During chilling, it has been shown that AFPs interact with cell membranes to stabilize them and prevent leakage of cell contents (Hays *et al.,* 1996*;* Rubinsky *et al*., 1991; Tomczak *et al*., 2002). AFPs also block ice formation, which decreases the freezing point of solutions without affecting the melting point value, an effect known as freezing hysteresis (Davies and Hew, 1990; DeVries *et al*., 1970; Hew and Yang, 1992). Once freezing has

occurred, AFPs further stabilize tissues by preventing recrystallization of ice crystals. As mentioned earlier, AFPs have been identified and characterized in a wide range of species (Bale, 2002; Basu *et al*., 2015; Biggar *et al*., 2013; Cheung *et al*., 2016; Davies *et al*., 2002; Ding *et al*., 2015; Do *et al*., 2014; Griffith and Yaish, 2004; Hanada *et al*., 2014; Hashim *et al*., 2013; Kondo *et al*., 2012; Leinala *et al*., 2002; Wen *et al*., 2016). Representative examples are shown in Table 1.

Table 1. Select species that produce antifreeze proteins

Category	Organisms
Fish – present in blood, liver, skin AFGP, AFP types I-IV and others	Antarctic notothenioids and toothfish, Arctic and Atlantic cod, right-eyed flounders, sculpins, sea raven, smelt, herring, eelpout, wolffish, cunner wrasse, longsnout poacher
Vertebrates -	Adelie penguin (egg), Mexican salamander, pig, wood frog
Insect – present in hemolymph AFP type V (hyperactive) and others	spruce budworm, fire-colored beetle, mealworm beetle, darkling beetles, ribbed pine borer, deer tick, desert beetle, midges
Plant – present in apoplast	carrot, winter rye, bittersweet nightshade, peach, perennial ryegrass, Antarctic hairgrass and pearlwort, *Ammopiptanthus sp.*
Bacteria, protists, and fungi-	sea ice bacteria (*M. primoryensis*, *Colwellia sp*.), polar diatoms, Antarctic algae, Arctic and Antarctic yeast, Grey snow mold

Origin and evolution of AFPs

Antifreeze protein structural diversity and the variety of species that express AFPs indicate that many distinct proteins have adopted a role in chilling and freezing resistance. The majority of evolutionary studies have centered on fish antifreeze proteins, and the current view is that the non-homologous forms of AFPs and AFGPs evolved in response to climate change. Data suggest that some antifreeze protein genes arose via lateral gene transfer, such as AFP type II in herring, smelt and sea raven (Graham *et al*., 2008; Graham *et al*., 2012; Sorhannus, 2012). Others appear to have arisen via gene duplication/amplification, such as AFP type III in pouts and wolfish, as well as AFPs in polar diatom species (Deng *et al*., 2010; Desjardins *et al*., 2012; Sorhannus, 2011) Antifreeze proteins in some lineages appear to have evolved independently after species diverged in time, through the process of convergent evolution, such as AFP type I in flounder, sculpin and cunner, AFPs in spruce budworm and mealworm beetle, as well as AFGPs in polar fish species (Chen *et al*., 1997; Davies *et al*., 1988; Davies *et al.,* 2002; Graham, 2013; Leinala *et al*., 2002; Verde *et al*., 2006). An extensively studied example of the latter concerns AFGP evolution in Antarctic teleost fish (order Notothenioidei) and Arctic cod (family Gadidae) (Chen *et al*., 1997; Cheng and Chen, 1999; DeVries *et al*., 1970; Fletcher *et al*., 2001; Harding *et al*., 2003; Petricorena and Somero, 2007; Verde *et al*., 2006). Despite protein sequence and 3-D structure similarities between the two groups, detailed analyses of gene sequences and substructures show strong evidence that the AFGPs in these polar fishes evolved independently.

The evolution and expansion of teleosts began 175 ma, during the middle Jurassic period. At this time in history, the oceans were consistently warmer, and the polar environments were temperate, nearing modern subtropical temperatures (Davies *et al*., 2002; Verde *et al*., 2006). (Fossils of alligators and flying lemurs have been found on Ellesmere Island, west of Greenland (Buchdahl, 1999)). Fossil studies estimate that divergence of notothenioids and Gadidae occurred at least 40 ma. After this time, during the late Eocene and early Oligocene epochs (40–25 ma), progressive cooling began, which lead to Antarctic glaciation, as indicated by ice-rafted debris in the Southern Ocean and $\delta^{18}O$ records in equatorial Pacific fossils. Like other poikilotherms, fish have little control over body temperature, and as a result of their close association with the aquatic environment, have heightened sensitivity to environmental conditions (Marchand, 1996). Sequence divergence data show that evolution of a novel ice structuring gene in

notothenioid fish species occurred about 15–5 ma (Chen *et al*., 1997; Cheng and Chen, 1999; Harding *et al*., 2003; Scott *et al*., 1986). The gene evolved from a trypsinogen-like protease gene. It is likely that the AFGP sequence was a part of the ancestral gene and was selected for by environmental conditions. Data for Arctic cod suggest gene sequence divergence of AFGPs from progenitors occurred at about 2.5 ma – all of which correlates with paleoclimatic data before and during those time periods (Chen *et al*., 1997). Taken together, these studies demonstrate a striking example of convergent evolution of protein structure and function from distinct organisms in spatially distant environments.

Properties of AFPs and mechanisms of activity

In temperate climates, exposure to low temperatures stimulates synthesis of antifreeze proteins in large amounts. In polar climates, AFPs are constitutively expressed. The major function of AFPs in cold temperature environments are: inhibition of ice crystal growth, prevention of ice recrystallization after freezing, and protection of cell membranes and membrane components.

Initial comparisons of antifreeze proteins reveal broad differences in tertiary structure. Sizes range from 2.6 kDa to 67 kDa, with structures varying from predominantly random to complex arrangements of α–helical and β–sheet conformation (Davies *et al*., 2002; Griffith and Yaish, 2004; Harding *et al*., 2003). For instance, AFGPs are mostly unstructured in solution, and consist entirely of a glycotripeptide repeat (Ala-Ala-Thr), with each threonine unit linked to a disaccharide (Harding *et al*., 2003; Hew and Yang, 1992). In contrast, AFP type I is a relatively simple alanine-rich α-helix, with four essential threonine residues that are thought to play a major role in binding ice (Hew and Yang, 1992; Davies *et al*., 2002; Tomczak *et al*., 2002).

Such variation in AFP structure might suggest drastically different mechanisms of binding to ice. However, the general theme is consistent across categories. AFPs bind to ice crystal fronts, via combinations of hydrogen bonding, hydrophobic, and van der Waals interactions between amino acid side chains and surface water molecules (Davies *et al*., 2002; DeVries *et al*., 1970; Duman, 2015; Hew and Yang, 1992; Kuiper *et al*., 2001; Leinala *et al*., 2002). On an atomic scale, periodic positioning of protein side chains (polar or nonpolar) align with the ice crystal lattice, maximizing

complementarity. Ice crystals present many faces with different atomic geometries, and data suggest that this may account for the variety of AFP structures, as each individual AFP may have complementarity with a different crystal face (Davies *et al*., 2002; Griffith and Yaish, 2004). On a larger scale, binding of AFPs to ice fronts has a so called 'pinning' effect, analogous to rivets on an airplane wing. The anchoring of proteins at distinct locations along the ice front forces the crystal through these pinning points into a highly curved shape. This curved shape has a high free energy, the growth of which is a less favorable process, requiring a lower temperature to proceed (Davies *et al*., 2002; Hew and Yang, 1992; Knight et al., 1991). In this way, the advancement of the ice crystal in a specific range of temperatures is severely impeded or blocked altogether. In addition to direct ice binding activity, recent data suggest that AFP and AFGP may function in a long-range fashion, by perturbing hydrogen bonding of the bulk water surrounding the protein (Ebbinghaus et al., 2012). This may explain why these molecules are so effective at such low concentrations, as compared to other cryoprotectants.

Inhibition of ice crystal growth results in a decrease in solution freezing point temperature relative to the melting point temperature, referred to as thermal hysteresis. For AFGP and AFPs I-IV, maximum hysteresis reached is approximately 1.5 °C, and for plant AFPs it is about 0.5 °C (Davies *et al*., 2002; Griffith and Yaish, 2004). Data suggest that the relatively weaker hysteresis activity of plant AFPs is due to a decrease in matching between the proteins and ice lattice. Insect AFPs, which are termed hyperactive, have a much greater hysteresis effect, with a maximum value of 4-5 °C. This observation has been attributed to the ice lattice geometry that forms in these organisms, which is energetically more difficult to constrain than those found in fish, and would require a much higher level of protein activity (Davies *et al*., 2002). Taken together, the ability of AFPs to effectively bind ice and lower the freezing point of biological fluids contributes powerful cold resistance in freezing environments.

Another threat to organisms comes with the possibility of ice recrystallization. After freezing and during re-warming, small ice crystals may fuse to form large aggregates of ice in tissues (Duman, 2015; Griffith and Yaish, 2004; Knight *et al*., 1984; Marchand, 1996). Even at constant subzero temperatures, ice structures are in flux: small crystals will disappear while larger ones grow, and crystals can undergo migration, also known as Ostwald ripening. This process is driven by energetics, with the goal of minimizing high-energy surface area by combination of smaller crystals, as well as changing surface morphology to eliminate costly sharp edges.

Recrystallization of ice disrupts cell structure, and can induce dehydration and osmotic stress. AFPs prevent recrystallization with the same mechanism used to block ice crystal growth – direct adsorption onto the ice crystal front (Worrall *et al*., 1998; Yu and Griffith, 1999; Yu *et al*., 2010). In order to prevent coalescence of crystals, then, the previously described 'pinning points' must exist between adjacent ice crystals. Many AFPs have multiple ice-binding domains, often on opposite sides of the 3-D structure (Davies *et al*., 2002; Griffith and Yaish, 2004). Some AFPs with single ice-binding domains will form oligomers, which are then able to bind multiple crystal sites. In addition, most organisms synthesize several AFPs, each binding to a different face of the ice lattice, and work in concert to inhibit growth. One can imagine a collection of single proteins or polymers of AFP providing physical barriers between ice crystals. Plant and insect antifreeze proteins appear to be the most effective at preventing recrystallization, this compared to fish AFPs, whose principal function is blocking ice crystal growth (Davies et al., 2002; Griffith and Yaish, 2004; Duman, 2015). For all of the protective abilities of antifreeze proteins during cooling and at subzero temperatures, it appears that there is a tradeoff during rewarming (Knight and DeVries, 1989). Recent work has shown that the ice stabilizing activity of AFPs during cooling – by binding directly to the ice surface – subsequently prevents melting of ice during warming (Celik *et al.*, 2010; Cziko *et al*., 2014). This 'superheating' of ice above the equilibrium freezing/melting point is termed melting hysteresis. For organisms that live in temperatures consistently close to freezing, this presents a problem because the stabilized ice may accumulate in their tissues over time. These potentially lethal aggregates of ice could trigger damaging inflammatory responses, as well as cause blockages in circulatory vessels (Cziko *et al*., 2014). It has been suggested that there must be mechanisms in place to sequester these ice crystals, as no elimination process has yet been identified.

Yet another role for antifreeze proteins arises during chilling – prevention of cold shock. As tissues are cooled, cell membranes become 'leaky' due to rearrangement of bilayer lipids, and membrane proteins aggregate and subsequently lose function (Arav *et al*., 2000; Drobnis *et al*., 1993; Marchand, 1996; Prosser, 1990; Quinn, 1985; Ricker *et al*., 2005; Tsvetkova *et al*., 2000; Wolkers *et al*., 2002). During chilling, it has been shown that both AFGP and AFP type I interact with membranes to stabilize them and prevent leakage of cell contents (Hays *et al*., 1996; Rubinsky *et al*., 1991; Tablin *et al*., 1996; Tomczak *et al*., 2002). When membrane lipids are cooled through their phase transition temperature (T_m), they undergo a

change from liquid to solid (or gel) phase. Each lipid type has a unique T_m value, which means that, at a given temperature, some lipid populations will have changed to gel phase, while others remain in liquid phase. The borders of the lipid 'domains' are analogous to cracks in a dam – they are weak points through which fluid and solutes can pass into or out of the cell. AFGPs from Antarctic toothfish (*Dissostichus mawsoni*) and bald rock cod (*Trematomus borchgrevinki*) inhibited this leakage in model membranes, but the mechanism of protection is still unclear (Hays *et al*., 1996). In this case, a nonspecific direct interaction between protein and membrane was proposed. Later studies with AFP type I from winter flounder revealed that the protein inhibited leakage in galactolipid membranes (Tomczak *et al*., 2002). The flounder AFP interacted with the hydrophobic core of the membrane, changing lipid packing order and increasing T_m. By making the membrane more homogenously 'solid', the protein reduced membrane permeability during chilling and rewarming.

The three-fold combination of protection during chilling, freezing, and thawing makes antifreeze proteins a highly efficient and effective tool for cold adaptation. It is important to remember that these molecules do not exist in a vacuum, however. The presence of other cryoprotectants such as polyols, sugars and ice nucleating proteins (INPs) contribute significantly to cold resistance. It is not surprising, then, to find that these cryoprotectants often work in concert with AFPs, producing stable supercooled states and/or preventing recrystallization of ice that was seeded by INPs during freezing (Bale, 2002; Duman, 1982; Johnston, 1990; Warren and Wolber, 1991; Zachariassen and Kristiansen, 2000). Little is wasted, energetically speaking – the components are used for multiple purposes and recycled to be used again later. Every fish species examined so far that produces AFPs lacks kidney glomeruli, structures which would normally draw molecules of this size into the urine (Eastman and DeVries, 1986; Marchand, 1996). Thus, the AFPs are left in circulation. So, while synthesis of antifreeze proteins is energetically expensive, especially in the relatively large amounts that are necessary, mechanisms are in place to conserve these precious molecules.

Final thoughts

In this review, we have discussed some of the remarkable adaptations exhibited by cold hardy organisms, such as supercooling, ice nucleation and antifreeze activity. Such strategies, often used in combination, are sophisticated biochemical and biophysical means for surviving the lowest of

environmental temperatures. Production of antifreeze proteins appears to be a relatively recent occurrence in evolutionary terms, with data from teleost fishes showing appearance of a novel AFGP gene around 15-5 mya in the Antarctic and 2.5 mya in the Arctic. These events correlate with sea-level glaciation in the polar regions.

Antifreeze proteins, despite wide structural and species diversity, employ similar mechanisms for protection during chilling and freezing. The convergent evolution of functionally unrelated genes into AFP genes in different species demonstrates a profound response to environmental pressure. Many organisms that have adapted AFPs have also lost expression of hemoglobins and heat protection proteins, called heat shock proteins, such as hsp 70 (Rogers *et al*., 2007). An interesting question is raised in the literature (see Beers and Jayasundara) - considering this tradeoff, what implications will it have for the ability of a species to survive current trends in global climate change (2015)? Does specialization for cold tolerance put limits on organisms in warmer environments?

Stemming from the research into protective effects of AFPs is their application to areas such as cryostorage and cryosurgery, transgenics, and food additives (Costanzo et al., 1995; Griffith and Yaish, 2004; Harding *et al*., 2003; Hew and Yang, 1992). AFGPs and AFPs have been used to cryogenically store various cell types including oocytes, sperm, human platelets and thylakoid membranes. Transformation of plants with AFP genes has led to improved supercooling ability by 1-3 °C (Griffith and Yaish, 2004). As an additive in ice cream, AFPs yield a creamy, reduced fat product, and control ice crystal growth that occurs during thawing (Harding *et al*., 2003).

The integration of molecular and global mechanisms, from genome to biosphere level, is perhaps the most interesting aspect of the antifreeze protein work and related studies. It serves as a reminder that no physical or biological system operates in a vacuum, and that a unifying approach is both illuminating and necessary to comprehend the details and interrelationships of the phenomena.

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