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REVIEW

Animal ice-binding (antifreeze) proteins and glycolipids: an overview with emphasis on physiological function

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ABSTRACT

Ice-binding proteins (IBPs) assist in subzero tolerance of multiple cold-tolerant organisms: animals, plants, fungi, bacteria etc. IBPs include: (1) antifreeze proteins (AFPs) with high thermal hysteresis antifreeze activity; (2) low thermal hysteresis IBPs; and (3) ice-nucleating proteins (INPs). Several structurally different IBPs have evolved, even within related taxa. Proteins that produce thermal hysteresis inhibit freezing by a non-colligative mechanism, whereby they adsorb onto ice crystals or ice-nucleating surfaces and prevent further growth. This lowers the so-called hysteretic freezing point below the normal equilibrium freezing/melting point, producing a difference between the two, termed thermal hysteresis. True AFPs with high thermal hysteresis are found in freeze-avoiding animals (those that must prevent freezing, as they die if frozen) especially marine fish, insects and other terrestrial arthropods where they function to prevent freezing at temperatures below those commonly experienced by the organism. Low thermal hysteresis IBPs are found in freeze-tolerant organisms (those able to survive extracellular freezing), and function to inhibit recrystallization – a potentially damaging process whereby larger ice crystals grow at the expense of smaller ones – and in some cases, prevent lethal propagation of extracellular ice into the cytoplasm. Ice-nucleator proteins inhibit supercooling and induce freezing in the extracellular fluid at high subzero temperatures in many freeze-tolerant species, thereby allowing them to control the location and temperature of ice nucleation, and the rate of ice growth. Numerous nuances to these functions have evolved. Antifreeze glycolipids with significant thermal hysteresis activity were recently identified in insects, frogs and plants.

KEY WORDS: Antifreeze proteins, Ice-binding proteins, Ice-nucleating proteins, Cold tolerance

Introduction

Subzero survival of ectotherms requires extensions of adaptations required for the survival of cool, above freezing temperatures (i.e. metabolic, membrane, developmental, behavioral etc.). In addition, two primary physiological modalities have evolved to permit subzero tolerance. Either the organisms seasonally become (1) 'freeze tolerant', meaning they can survive freezing of body fluids, generally only extracellular, or (2) 'freeze avoiding' by evolving adaptations that prevent freezing beyond the lowest temperatures normally experienced in their habitat (Zachariassen et al., 2004). It is not surprising, given the number of diverse species inhabiting areas with potentially lethal freezing temperatures, that numerous adaptations, and suites of adaptations, are used to achieve freeze tolerance or avoidance (for reviews, see Lee and Denlinger, 1991; Denlinger and Lee, 2010; Lee, 2010; Carrasco et al., 2011a,b; Storey and Storey, 2012, 2013). However, certain adaptations have evolved in widely divergent organisms, for example, antifreeze proteins (AFPs) and other ice-binding proteins (IBPs), as well as antifreeze glycolipids (AFGLs).

AFPs were first discovered by DeVries in Antarctic marine fish (DeVries and Wohlschlag, 1969; DeVries, 1971), where, as their name implies, they function to prevent freezing. Since then, AFPs have been identified in numerous other organisms, such as freeze-avoiding insects and other terrestrial arthropods. However, proteins with similar, but considerably lesser, antifreeze (thermal hysteresis or TH) activities have been found in freeze-tolerant species, where they do not prevent organismal freezing, but instead function to control the site and temperature of ice formation, ice crystal structure and rate of freezing. Other proteins, ice-nucleating proteins (INPs), actually promote ice formation by limiting supercooling. Adaptive INPs are present in many freeze-tolerant organisms, functioning to initiate freezing at high subzero temperatures in the extracellular fluid. Incidental, non-adaptive, INPs are sometimes present in freeze-avoiding species as well, where they must be either removed or masked by AFPs in winter. The term 'ice-binding proteins' (IBPs) is now used to include both true AFPs in freeze-avoiding species and the proteins in freeze-tolerant species (low TH activity proteins and IBPs) (Wharton et al., 2009). The physiological functions of these proteins in animals, along with the more recently identified AFGLs, are the subjects of this review.

Marine teleost fishes

Physiologists had long recognized that, because marine teleost fish are freeze avoiding and hypo-osmoregulators, with body fluid equilibrium freezing/melting points (eqFMP) of –0.6 to –0.9°C, they should be susceptible to lethal freezing in ice-laden seawater at ~–1.9°C, especially when in contact with ice. Yet these high latitude waters were known to sustain significant fish populations. In spite of efforts by excellent biologists (i.e. Scholander et al., 1957; Gordon et al., 1962) to identify the adaptation(s) underlying this apparent paradox it was not until the late 1960s that DeVries discovered AFGPs in Antarctic notothenioid fish (DeVries and Wohlschlag, 1969; DeVries, 1971). Large molecular mass molecules, such as proteins, had not previously been investigated as potential antifreezes because the eqFMP of water is dependent on the concentration of solute, and sufficient protein to depress the eqFMP to protect the fish from freezing based on colligative properties is not possible. However, DeVries demonstrated that AFGPs lowered the freezing point of water by a non-colligative mechanism whereby the AFGPs adsorb to the ice surface and prevent water molecules from joining the crystal lattice in the normal fashion, thereby lowering the non-equilibrium hysteretic freezing point (hFP) below the eqFMP and creating the distinctive TH, the difference between the hFP and the eqFMP (DeVries, 1986). This preserves the hypo-osmotic condition of the fish, but prevents freezing.

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List of abbreviations

AFGL	antifreeze glycolipid
AF(G)P	antifreeze glycoprotein or protein
AFGP	antifreeze glycoprotein
AFP	antifreeze protein
DAFP	*Dendroides canadensis* antifreeze protein
eqFMP	equilibrium freezing/melting point
hFP	hysteretic freezing point
hMP	hysteretic melting point
IBP	ice-binding protein
INP	ice-nucleating point
SCP	supercooling point (nucleating or crystallization temperature)
TH	thermal hysteresis

Structures and evolution of fish antifreeze proteins

The sequence of the helical AFGPs consists of repeating units of the tri-peptide A-A-T- with the threonine hydroxyls glycosylated to a disaccharide, β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-α-D-galactopyranose (DeVries, 1971; Shier et al., 1975). The AFGPs are present in multiple isoforms with M_r ranging from ∼3.5×10^3 to 32.0×10^3 and occasional slight variations in amino acid sequence. Thermal hysteresis was later identified in a number of northern fish (Duman and DeVries, 1974, 1975). Functionally similar antifreeze proteins and glycoproteins, AF(G)Ps, were subsequently identified in numerous fishes inhabiting subzero marine waters.

AFGPs evolved independently in the northern gadid (cod) fishes (Raymond et al., 1975; VanVoorhies et al., 1978), and thus offer an excellent example of convergent evolution (Chen et al., 1997a). In notothenioids, the A-A-T repeating sequence evolved from a trypsin-like serine protease progenitor by *de novo* duplications of a coding element at the junction of intron one and exon two of the trypsin gene, followed by numerous duplications (Chen et al., 1997b; Cheng and Chen, 1999).

Four structurally distinct types of AFPs, lacking carbohydrate, are currently known from fishes (Fletcher et al., 2001; Jia and Davies, 2002; DeVries and Cheng, 2005; Davies, 2014). Type-I AFPs, initially identified in winter flounder, *Pleuronectes americanus*, (Duman and DeVries, 1976; DeVries and Lin, 1977) are α-helical coils where ∼two-thirds of the residues are alanine and regularly spaced threonine and other more hydrophilic residues project from one side of the amphiphilic protein (Knight et al., 1991; Sicheri and Yang, 1995; Harding et al., 1999). Type-I AFPs are found in other flounder and certain cottids (sculpins), indicating they have also evolved independently on multiple occasions (Graham et al., 2013). In contrast, type-II AFPs present in smelt, herring and some cottids (sea raven, *Hemitripterus americanus*) are globular, cysteine-rich and have apparently evolved from C-type lectins (Slaughter et al., 1981; Ng and Hew, 1992; Ewart et al., 1998). While some type-II AFPs require Ca^{2+} for TH activity, others do not, once again suggesting evolution from different sources. Type-III fish AFPs form β-sheets, and are present in zooarcids (Wang et al., 1995), both in antarctic (Cheng and DeVries, 1989) and northern waters (Sorensen et al., 2006; Albers et al., 2007). The AFP of the Antarctic zooarcid, *Lycodicthys dearborni*, has homology with a sialic acid synthase (Cheng and DeVries, 1989; Baardsnes and Davies, 2001), apparently exhibiting a case of evolutionary escape from adaptive conflict where an ancestor sialic acid synthase gene with AFP activity was selected for prior to gene duplication (Deng et al., 2010). Two forms of the type-III AFPs have been identified, one of which lacks TH activity. However, mixing the two isoforms results in enhanced activity of the active form (Nishimiya et al., 2005). Recently a TH-inactive form from *Zoarces viviparous* was shown to dimerize (Wilkens et al., 2014).

The globular type-IV AFP was described from blood serum of long-horned sculpin, *Myoxocephalus octodecimspinosus* (Deng et al., 1997). However, its functional status as an AFP is questionable, as TH is not measureable in this species and levels of the protein are too low in this and other species to result in functional antifreeze activity, although the expressed protein has TH activity (Gauthier et al., 2008). Two AFP-IV transcripts were recently identified in two freshwater cyprinid fish, carp (*Carassius auratus*) and zebrafish (*Danio rerio*), and one of the two proteins has essential developmental functions (Xiao et al., 2014). Note that AFP is not necessary for antifreeze purposes in freshwater fish, especially in the warm water zebrafish.

In addition to the AFGPs, many Antarctic notothenioids produce a 15 kDa AFP lacking carbohydrate and a biased amino acid sequence (Jin, 2003; DeVries, 2004). Although the TH activity of this AFP is low, when combined with the AFGPs the thermal hysteresis is enhanced twofold. Consequently, the protein was termed an antifreeze-potentiating protein (AFPP). Both the AFPP and the AFGPs are required to achieve full TH activity in the fish, because the AFGPs bind to the basal plane of ice whereas the AFPP binds to the prism plane.

Thus, there are multiple, structurally distinct AF(G)Ps currently known in fish. They have evolved independently multiple times, even within one AF(G)P type. In addition, a single family of fishes (i.e. cottids), even single species, can exhibit multiple AF(G)P types.

Thermal hysteresis and the ice-binding mechanism

Although the structures of AF(G)Ps vary widely, the one common characteristic is TH, which identifies their presence (DeVries, 1971, 1986). Growth of an ice crystal occurs as water molecules add to the surface in a broad low radius of curvature, low surface free energy, front. TH is dependent on the abilities of the AF(G)Ps to adsorb to the surface of ice at specific preferred growth sites, usually on one of the prism planes. This 'adsorption–inhibition' mechanism restricts growth to regions between the AFPs with high radius of curvature and high surface free energy. Consequently, according to the Kelvin effect, significant growth (usually in the non-preferred *c*-axis) does not occur until the hFP is reached (Raymond and DeVries, 1977; Raymond et al., 1989; Knight et al., 1991).

The unique repeat structures of the AFPs, especially obvious in the AFGPs and type-I AFPs, suggested strongly that hydrogen bonding through regularly spaced hydroxyl groups of the AF(G)Ps to oxygen in the ice lattice provided the mechanism for binding (DeVries, 1971; DeVries and Cheng, 1992; Sicheri and Yang, 1995). Considerable recent evidence, based on TH- producing proteins from various sources, indicates that the ability of both AFPs and low-TH IBPs to organize water molecules in an ice-like fashion around more hydrophobic residues is involved. This organized water then freezes into the semi-liquid region at the surface of the solid ice, thereby attaching the AF(G)P (Jia and Davies, 2002; Graether and Sykes, 2004; Garnham et al., 2011; Hakim et al., 2013; Sun et al., 2014; Davies, 2014). It is likely that both mechanisms are involved to greater or lesser extents, depending on the AF(G)P (Ebbinghaus et al., 2012; Meister et al., 2013, 2014).

Nuances of antifreeze protein function in fishes

Fish AF(G)Ps evolved primarily to prevent freezing, but there are nuances in achieving this function. For example, AF(G)Ps are typically present in the blood serum and presumably in the interstitial fluid, but some species also have AF(G)Ps distributed

in the ocular fluid, gills, skin and surface mucus (Ahlgren et al., 1988; Valerio et al., 1992; Gong et al., 1996; Evans et al., 2011) to better guard against inoculation from external ice contacted by the fish. Interestingly, some of these AFPs are produced locally and lack signal peptides, indicating they are intracellular in the skin, gill epithelia, etc. Some species also have gut AF(G)Ps (Evans et al., 2012) to inhibit initiation of freezing by ice crystals swallowed by the fish, a high probability because the hypo-osmotic marine fish drink constantly to replace body water. In fact, Antarctic notothenioids produce the majority of their AFGPs in specialized cells in the stomach and exocrine pancreas (recall that the AFGPs evolved from a pancreatic trypsin gene), excrete them into the gut via the pancreatic duct and subsequently take them into the blood across the rectal epithelia, although some is lost in the feces (Evans et al., 2012). The liver absorbs some of the serum AFGPs and secretes them back into the gut via the gall bladder. Although the evolution of the AFGPs is different in the arctic gadids, they also synthesize AFGPs in the pancreas, along with the liver.

In spite of the presence of AF(G)Ps, Antarctic notothenioids can have small ice crystals in the blood (Tien, 1995; Praebel et al., 2009). Thus, they may technically be freeze tolerant, because they have ice in their body fluids, even though the percentage of water as ice is small. The ice crystals, with adsorbed AFPs, are accumulated in the spleen as foreign particulates, apparently by sphlenic macrophages (DeVries and Cheng, 2005; Evans et al., 2011). In Antarctic notothenioids, these ice crystals may never melt over the lifespan of the fish (which can be 20 years or longer). In fact, the AF(G)Ps in combination with the AFPP may exacerbate this situation (Cziko et al., 2014), because not only do AF(G)Ps lower the hFP below the eqFMP, but they also slightly raise the temperature at which ice crystals melt (Knight and DeVries, 1989; Celik et al., 2010). Thus, the AF(G)Ps induce a hysteretic melting point (hMP) that is higher than the eqFMP and this may limit melting of AFGP-coated ice crystals. Although the melting hysteresis is smaller than the freezing TH (never more than 20% of the freezing TH), this may cause a problem because long-term temperature recordings in McMurdo Sound, Antarctica showed water temperatures rarely exceed the hFP of the fish, and only in the upper 100 m of the water column. These crystals have the potential to cause damage to the fish by clogging small blood vessels. However, sequestration of AFGP-coated ice crystals in the spleen may minimize this problem. Also, the ability of AF(G)Ps to inhibit recrystallization (discussed later) should keep the ice crystals small.

The AF(G)Ps are small proteins and therefore should be filtered by the kidneys and lost in the urine; however, this does not occur (Dobbs and DeVries, 1975a). The notothenioids are the only family of fish in which all species are aglomerular, relying on secretion for entry of solute into the urine (Dobbs and Devries, 1975b). Even those species of AF(G)P-producing fish that are not anatomically aglomerular are functionally so (Eastman et al., 1979).

In addition to their antifreeze function, some fish AF(G)Ps can stabilize membranes at low temperature, in particular AFP types I (Rubinsky et al., 1990; Tomczak et al., 2002) and III (Hirano et al., 2008). These AFPs prevent the usual increase in permeability as membranes are cooled through their phase transition temperature. In artificial membranes, the lipid composition of the membrane affects whether protection is provided (Tomczak et al., 2001). Presumably, this is also the case with natural membranes.

Terrestrial arthropods

TH has been identified in numerous freeze-avoiding and freeze-tolerant insects, as well as other terrestrial arthropods, including centipedes, spiders, ticks, mites and collembola. In a study of insects in Alaska, 26% of 75 species tested had hemolymph TH (Duman et al., 2004). Compared with fish, the hemolymph TH in freeze-avoiding terrestrial arthropods is higher, ranging from 2 to 13°C, while that of freeze-tolerant species is usually only a few tenths of a degree. Consequently, the IBPs of the freeze-avoiding species are properly labeled AFPs, because their specific activity is high and their primary function is to prevent freezing. These AFPs will be treated first in this section of the review.

Freeze avoidance

Freeze-avoiding terrestrial arthropods must generally promote supercooling to survive winter temperatures. While most freeze-avoiding insects accumulate high concentrations of colligative antifreezes such as glycerol and other polyols in winter (Lee, 2010), a one molar concentration is required to lower the eqFMP by just 1.86°C. Even addition of AFPs to further lower the freezing temperature to the hFP will generally not suffice, especially if winter temperatures are extreme. Supercooling refers to the ability of water to cool below the eqFMP, or hFP if AFPs are present, without freezing. Small volumes of extremely pure water can actually supercool to ~–40°C before homogeneous nucleation induces freezing as the water structure becomes progressively more ice-like and the size of developing 'embryo crystals' eventually become large enough to seed the water. Such high levels of supercooling do not often occur in biological systems because various surfaces, including some proteins, act as heterogeneous ice-nucleating sites by organizing water into critical-sized embryo crystals at higher temperatures than would occur in their absence. These ice nucleators can be either endogenous (i.e. certain proteins) or exogenous (i.e. certain ingested bacteria), and they may be adaptive (as in the extracellular fluid of freeze-tolerant organisms) or incidental and problematic in freeze-avoiding species (Zachariassen et al., 2010).

Polyols and other solutes that lower the eqFMP by colligative effects also lower the supercooling point (SCP), the temperature where spontaneous ice nucleation occurs. However, SCPs are only lowered on a one-to-one, or at best two-to-one, basis relative to depression of the eqFMP (reviewed in Duman et al., 1995). Therefore, while polyols are often important, additional means may be needed to further lower the SCP. Consequently, most freeze-avoiding insects stop feeding and clear the gut in autumn to remove microbial ice nucleators (Lee, 2010). Some species also remove incidental INPs from hemolymph (Neven et al., 1986), but this is not always possible as these may be essential proteins. However, AFPs can bind to and inhibit ice nucleators and/or embryo crystals (Parody-Morreale et al., 1988; Olsen and Duman, 1997a,b; Nickell et al., 2013), thereby extending supercooling. Terrestrial organisms can also be inoculated by external ice across the body surface, but AFPs can also inhibit this (Olsen et al., 1998).

Thermal hysteresis was first identified in hemolymph, and certain other fluid compartments, of *Tenebrio molitor* beetle larvae by Ramsay in his studies of their cryptonephridial rectal complex (Ramsay, 1964). At that time, the connection between these proteins and cold tolerance had not been made and Ramsay indicated that the responsible proteins were perhaps involved in the ability of the rectal complex to reabsorb water from the hindgut, because they were concentrated in the perirectal space (Grimstone et al., 1968). There is evidence to support this view (Patterson and Duman, 1978). However, hemolymph TH was later identified in several insects. While low levels of TH are sometimes present in summer, activity is increased considerably in winter (Duman, 1977a,b, 1979a; Husby and Zachariassen, 1980; Zachariassen and Husby, 1982), including

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in cold-acclimated *T. molitor* (Patterson and Duman, 1978), when these insects had lower SCPs.

Hemolymph TH was also found in spiders (Duman, 1979b; Husby and Zachariassen, 1980; Zachariassen and Husby, 1982; Duman et al., 2004), mites (Block and Duman, 1989; Sjursen and Sømme, 2000) and collembola (Zettel, 1984; Meier and Zettel, 1999; Graham and Davies, 2005; Sinclair et al., 2006).

Structures of antifreeze proteins in freeze-avoiding terrestrial arthropods

Multiple types of AFPs have been described in freeze-avoiding terrestrial arthropods including insects, a collembolan and arachnids (ticks and spider mites). (For reviews of insect AFP structure, see Jia and Davies, 2002; Graether and Sykes, 2004; Davies, 2014).

Some of these are currently 'putative' AFPs because they were described only from transcripts, and neither TH of the expressed putative AFP nor TH in the organism was measured. Where the purified AFPs have been studied, they have greater specific activity than those of fish, which is probably a necessity because of the lower temperatures in terrestrial systems. While AFPs are present in fish serum at concentrations as high as 30–40 mg ml^{-1} and result in a maximum TH of ∼2°C, AFPs are present in insect hemolymph at 10-times lower concentrations yet produce greater TH, perhaps because insect AFPs bind to both the basal and prism planes of ice (Pertaya et al., 2008). It is important to realize that the level of low-temperature protection afforded to insects, etc. by AFPs is considerably greater than the measured TH because the magnitude of TH is inversely related to ice crystal size (Zachariassen and Husby, 1982; Nicodemus et al., 2006) and the size of embryo crystals or ice penetrating the cuticle is considerably smaller than the crystals used in TH measurements.

Coleoptera (beetle) antifreeze proteins

The first described insect AFPs were from beetle larvae, *Tenebrio molitor* (Graham, et al., 1997; Liou et al., 1999) and *Dendroides canadensis* (Duman et al., 1998; Andorfer and Duman, 2000). The similar AFPs are present as multiple 6–13 kDa isoforms, consisting of 12- or 13-mer repeating units where every 6th residue is cysteine. Certain other amino acids are highly conserved, especially threonines, such that T-C-T units appear regularly and form a right handed β-helix that comprises the ice-binding sites on one side of the somewhat flattened β-barrel proteins. The cysteines form disulfide bridges across the interior of the barrel (Li et al., 1998a). Similar AFPs are present in other beetles (Qiu et al., 2010; Mao et al., 2011). The inquisitor beetle *Rhagium inquisitor* (Kristiansen et al., 2011) and related *Rhagium mordax* (Kristiansen et al., 2012) have evolved AFPs different from the other beetles, some with greater specific activity. The increased activity may result from the expansion of the ice-binding motif beyond the T-C-T to include additional threonines (T-X-T-X-T-X-T) in repeats separated by residues that lack obvious repeats. *Rhagium* AFPs have just one disulfide bridge. The structural differences between *Rhagium* and other beetle AFPs is not surprising because they are from two different superfamilies of the huge Coleoptera group that contains ∼25% of all known animal species.

Lepidoptera (butterfly and moth) and Hemiptera (true bug) antifreeze proteins

Another insect AFP type is represented by larvae of the spruce budworm *Choristoneura fumiferana* (Tyshenko et al., 1997; Gauthier et al., 1998) and related *Choristoneura* species (Tyshenko et al., 2005), as well as the somewhat different inchworm *Campanea perlata* AFP (Lin et al., 2011). These insects have evolved AFPs with four disulfide bridges where 15-mer repeats produce a left-handed β-helix with T-X-T regions forming the ice-binding site on one side of the protein.

A putative AFP with structure similar to Lepidoptera AFPs is present in a hemipteran, the sunn pest *Eurygaster maura* (Guz et al., 2014), whilst wintering as diapausing adults. Whereas TH has been previously reported in hemipterans (Patterson et al., 1981; Duman et al., 2004), this is the first AFP sequence described from this important insect family. Comparison of the *E. maura* AFP with other known insect AFPs indicated the greatest sequence homology (52% identity) with *C. fumiferana* AFP, an interesting finding given the evolutionary distance between Hemiptera and Lepidoptera, suggesting convergent evolution. The *E. maura* AFP consists of 12- and 13-mer repeats containing T-C-T motifs, with four cysteines, and is predicted to fold into a left-handed β-helix.

Midge antifreeze protein

Another novel AFP was recently reported from adult midges emerging in Canada in early spring from Lake Ontario (Basu et al., 2014). It exists as a family of isomers with a repeating unit of X-X-C-X-G-X-Y-C-X-G with disulfide bonds stabilizing the left-handed solenoid and tyrosines providing the ice-binding surface. Although the immature aquatic stages do not need AFPs, the adults can encounter subzero temperatures. This is the first dipteran (fly) AFP reported.

Antifreeze proteins of other terrestrial arthropods

The arctic collembolan *Hypogastrura harveyi* produces AFPs consisting of G-X_1-X_2 repeats where X_1 is usually glycine and X_2 is alanine or valine (Graham and Davies, 2005). Six short anti-parallel polyproline type II helices form two sheets of three parallel helices oriented anti-parallel to each other (Pentelute et al., 2008).

The black-legged tick *Ixodes scapularis* produces transcripts that encode putative AFGPs similar to those of fish (Neelakanta et al., 2010). The presence of TH was not determined in the ticks, and whether the AFGP is TH-active was not tested. Also, the nature of the saccharide component, if any, is not known. Interesting details of control of tick AFGP production will be discussed later.

A family of 20 putative AFPs was identified in diapausing *Tetranychus urticae* spider mites (Bryon et al., 2013). The protein showed sequence similarities to *D. canadensis* and *T. molitor* beetle AFPs, but has significant differences. For example, the mite AFPs consist of 12- and 13-mer repeats; however, they contain additional cysteine residues, some of which may not form disulfide bonds, and the usual ice-binding motif of the beetle AFPs is not apparent in the 3D model. Transcript levels are greatly increased during diapause, a period when supercooling of the mites is enhanced, suggesting that the proteins are true AFPs, although this must be confirmed by testing expressed proteins for TH activity.

Physiological functions of antifreeze proteins in freeze-avoiding terrestrial arthropods

Like fish AF(G)Ps, AFPs of terrestrial freeze-avoiding arthropods evolved to prevent freezing, but nuances exist. A few case studies illustrate this.

Dendroides canadensis beetles

To become freeze-avoiding, *D. canadensis* larvae cease feeding in late autumn and clear the gut of microbial ice nucleators, maintain high levels of glycerol and produce AFPs (DAFPs) to lower their supercooling points from ∼–6°C in summer to –18 to –26°C in winter, depending on the severity of the winter (Duman, 2001). *D. canadensis* produces 30 known AFP isomers, and these exhibit

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tissue-specific expression (Duman et al., 1998, 2002; Andorfer and Duman, 2000; Nickell et al., 2013), suggesting that their functions vary somewhat. The 12 initially described DAFP isomers were separated into three groups based on sequence variations (Andorfer and Duman, 2000). Group I are produced in the fat body and secreted into the hemolymph whereas groups II and III are present in the mid-gut and produced both in the fat body and mid-gut epithelia (Duman et al., 2002). Hemolymph DAFPs promote supercooling by inhibiting incidental hemolymph INPs (Olsen and Duman, 1997a), and also inhibit inoculative freezing across the cuticle by external ice (Olsen et al., 1998). Gut DAFPs inhibit microbial ice nucleators ingested by the larvae (Olsen and Duman, 1997b), permitting larvae to feed later in autumn and earlier in spring, and drink during winter thaws. Combinations of certain groups I, II and III are produced in the single cell layer of epidermal cells underlying the cuticle, and are essential in inhibiting inoculative freezing (Olsen et al., 1998). Numerous isoforms are produced by the Malpighian tubule epithelium and secreted into the primary urine to inhibit ice-nucleating activity of various crystals that form in insect urine over the winter (Nickell et al., 2013). Recently, DAFPs were shown to control the size and shape of α-D-mannopyranoside crystals by binding directly to the crystals (Wang et al., 2014), suggesting that Malpighian tubule DAFPs could function to limit the size of non-ice crystals in urine.

The annual cycle of DAFP production in *D. canadensis* larvae is induced by low temperature, short photoperiods and/or short thermoperiods (Horwath and Duman, 1982, 1983a, 1984, 1986). Juvenile hormone also has a role in AFP induction, both in *D. canadensis* (Horwath and Duman, 1983b; Xu and Duman, 1991) and *T. molitor* (Xu et al., 1992).

While the increased concentration of DAFPs resulting from synthesis initiated in autumn and continuing through the winter generates higher TH, other factors also contribute. Certain hemolymph DAFPs enhance the TH activities of one another (Wang and Duman, 2005). Also, a thaumatin-like protein has similar enhancing activity (Wang and Duman, 2006), as do a number of low molecular mass solutes (Li et al., 1998b) including glycerol present in winter hemolymph at 0.5–1.0 mol l^{-1}. Enhancement is not limited to TH activity, but includes increased abilities to inhibit inoculative freezing and ice nucleation (Duman, 2002; Duman and Serianni, 2002). The mechanism of this enhancement is not clear, but anti-DAFP antibodies increase TH when added to DAFPs (Wu and Duman, 1991), probably because antibody binding to DAFP still permits the DAFP to bind to ice, but the antibody–DAFP complex, being much larger than the DAFP alone, blocks a greater surface of the ice crystal to addition of water molecules. This suggests that the various inter-protein enhancements may involve a similar mechanism. Glycerol may enhance activity by promoting formation of the protein–protein complexes. Certain sulfates that cause increased TH when added to DAFPs, increase the ability of DAFPs to affect water structure, slowing hydration bond dynamics especially around the ice-binding site, suggesting that increased ice-like structured water binds the DAFP to ice (Meister et al., 2014). The Hoffmeister effect may be responsible for enhancement of TH by monovalent salts (Wang et al., 2009a). Increasing numbers of hydroxyl groups in a series of polycarboxylates (Amornwittawat et al., 2008) and polyhydroxy compound enhancers (Amornwittawat et al., 2009) positively affected the TH of DAFP-1. Arginine is a key residue in the enhancement (Wang et al., 2009b).

Cucujus clavipes beetles

Larvae of another beetle, *Cucujus clavipes*, which produce AFPs similar to those of *T. molitor* and *D. canadensis*, exhibit extreme freeze-avoidance abilities. This species has a wide latitudinal range in North America, from North Carolina (35°N) to the tree line in arctic Alaska (68°N) where the winter temperature can reach –60°C. Overwintering Indiana larvae (42°N) have mean supercooling points of –20 to –28°C while Alaskan larvae have normal winter SCPs of ∼–40°C (Bennett et al., 2005). However, in concert with low environmental temperatures (<–20°C) Alaskan larvae 'deep supercool' and cannot be frozen, even when exposed to –150°C as the larvae vitrify (body water turns to glass) at temperatures between –58 to –78°C (Sformo et al., 2010, 2011). Unlike freezing, vitrification does not result in increased solute concentrations and volume expansion, and consequently, is not obviously lethal. Some individual larvae survive temperatures of –100°C, but mean lower lethal temperature is ∼–70°C. Both populations produce AFPs and accumulate glycerol, although glycerol concentrations are greater in Alaskan larvae. Alaskan larvae enter metabolic diapause in winter while Indiana larvae do not (Bennett et al., 2005), and proteomic studies identified numerous additional variations (Carrasco et al., 2011b, 2012). However, perhaps the most important difference between populations is that Alaskan larvae undergo cryoprotective dehydration when temperatures are low, such that body water decreases from mean summer values of ∼68% (2.1 mg H_2O mg^{-1} dry mass) to winter values of 24–45% (0.2–0.6 mg H_2O mg^{-1} dry mass) (Sformo et al., 2010, 2011). This (1) decreases the amount of water available for freezing and (2) increases AFP and glycerol concentrations. The resulting high AFP levels (TH can reach values of 13°C) contribute to supercooling and eventual vitrification by inhibiting ice nucleators, while the high glycerol concentrations also promote supercooling and increase viscosity, contributing to vitrification.

Ticks

Another interesting situation involves the black-legged tick *I. scapularis,* which is the vector of Lyme disease and granulocytic anaplasmosis caused by *Anaplasma phagoytophillum* bacteria. Other tick species exhibit TH, but the responsible factors are not known (Block and Duman, 1989; Sjursen and Sømme, 2000). Recall that *I. scapularis* produces AFGPs similar to those of fish. Transcription of *AFGP* is increased by cold exposure and there is a developmental effect as well. However, ticks infected with *A. phagocytophillum* express increased *AFGP* transcript and survive cold (–20°C) better than uninfected individuals (Neelakanta et al., 2010). Infected ticks are more active in winter, increasing their ability to transmit infection. Also, transgenic yeast expressing tick AFGP survived cold better than controls. This apparent mutualistic interaction results in increased AFGP production and improved cold tolerance for the tick and perhaps the bacteria.

Low thermal hysteresis ice-binding proteins and ice-nucleating proteins in freeze-tolerant arthropods

Freeze-tolerant species have evolved numerous and varied mechanisms that permit freezing of their body fluids. Therefore, the purpose of their TH proteins is not to prevent freezing of the whole organism. In fact, many freeze-tolerant species produce extracellular 'adaptive' ice-nucleating proteins to reduce supercooling, inducing freezing just a few degrees below the freezing point (Zachariassen and Hammel, 1976; Zachariassen, 1982; Zachariassen et al., 2010; Duman and Patterson, 1978; Duman et al., 1984, 1985, 2010). Why? Extensive supercooling followed by freezing often results in both extracellular and lethal intracellular freezing. Consequently, initiation of extracellular ice by INPs is advantageous, leading to the counter-intuitive situation whereby an extracellular INP actually promotes ice-free cytosol. Exclusion of solute from ice then results in increased

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osmotic concentration in the unfrozen fraction of the extracellular water, causing slow out-flux of intracellular water that progressively lowers the cytosolic freezing point as temperature decreases and more extracellular water freezes, thereby keeping the cytosol ice-free. INPs are considered to be IBPs because they organize water on their surface into an ice-like 'embryo crystal' that grows as temperature decreases. When the critical radius is reached, at a higher temperature than if INPs were absent, the surrounding supercooled water is seeded. The most-active INPs known are located on the outer membrane of certain bacteria (Lindow, 1983, 1995). These ubiquitous epiphytic bacteria are responsible for crop damage during mild frosts, because condensed water on the plant surface would otherwise supercool. These microorganisms can also initiate freezing in guts of animals feeding on material containing them. Consequently, freeze-avoiding insects stop feeding and clear the gut prior to winter, and some produce gut AFPs.

Whereas structural information, including considerable repeat structure, of bacterial INPs has long been known (Wolber and Warren, 1989), similar information on endogenous INPs of freeze-tolerant animals and plants is not available. However, INPs from freeze-tolerant queens of white-faced hornets, *Vespula maculate*, contain ∼20% glutamate (Duman et al., 1984). Also, hemolymph lipoprotein ice nucleators (LPINs) from larvae of the freeze-tolerant cranefly *Tipula trivittata* require two apoproteins, along with phosphatidylinositol, for activity (Neven et al., 1989). Immunological similarities indicated the presence of some level of the bacterial INP repeating units in the apoproteins (Duman et al., 1991). While not all insect hemolymph lipoproteins are INPs, those of the larvae from the freeze-avoiding stag beetle *Ceruchus piceus* are, and consequently these 'incidental' LPINs are removed prior to winter to facilitate supercooling (Neven et al., 1986). Scanning tunneling microscopy showed that the LPINs of both species organize in chains, generally side by side, suggesting cooperation in forming the critical embryo crystal size (Yeung et al., 1991; Duman et al., 1995).

Some freeze-tolerant organisms, including many insects (Duman et al., 2010) and plants (Griffith and Yaish, 2004) exhibit low hemolymph TH (a few tenths of a degree), indicating that preventing organismal freezing is not the function of these low TH IBPs. However, controlling the size and shape of ice crystals is important. When supercooled water is initially frozen, a large number of small crystals typically form. While the percentage of water molecules in the ice may not change over time, fewer, but larger, crystals result. This is recrystallization, potentially leading to tissue damage even if the ice is only extracellular (Mazur, 1984; Tursman and Duman, 1995). Recrystallization occurs because as water molecules move on and off the ice surface, the greater radius of curvature and surface free energy of smaller crystals causes a net loss of water from smaller to larger crystals. High or low TH IBPs inhibit recrystallization because of the same mechanisms that produce TH (Knight et al., 1984), even if TH is very low (Knight and Duman, 1986). Also, the shape of crystals may be altered.

Another function of low TH IBPs in freeze-tolerant organisms may be to prevent lethal inoculation of the cytoplasm by extracellular ice across cell membranes. In the freeze-tolerant centipede *Lithobius forficatus*, TH is only occasionally measureable in hemolymph, and only in autumn, although recrystallization inhibition is present throughout the winter (Tursman et al., 1994). Although the responsible IBP was not purified, antibodies against *D. canadensis* AFP reacted with protein in winter centipede hemolymph and cell membranes, but not in summer, indicating an IBP similar to that of the beetle on cell membranes (Tursman and Duman, 1995). When *D. canadensis* DAFP was incubated with summer centipede cells and the cells rinsed to remove soluble DAFP, anti-DAFP antibody reacted with the cell membrane, suggesting DAFP attached to the membranes. Also, summer cells froze in the presence of external ice at ∼–8°C, whereas winter cells (with endogenous IBP) or summer cells incubated with DAFP, froze at –12 to –15°C.

Consequently, combinations of (1) extracellular INPs that initiate freezing with minimal supercooling, (2) low TH IBPs that control recrystallization, and (3) low TH IBPs (and/or AFGLs) located on cell membranes to prevent inoculation of the cytosol can effectively control the ice initiation temperature and location, plus the rate of freezing, thereby assisting freeze tolerance.

Low TH IBPs, are present in many non-animal freeze-tolerant organisms, and structures of a number of these low-TH IBPs are known (Griffith and Yaish, 2004; Davies, 2014). Although the sequence and ice-binding properties of a TH-producing IBP have not been described in any freeze-tolerant animal, a suggested type-4 AFP was identified in the wood frog *Rana sylvatica*. Two freeze-responsive proteins, Fr10 and Li16, were shown to protect an insect (*Bombyx mori*) cell line from freeze damage at –6°C with exposure times of 1–2 h (Biggar et al., 2013). Modeling indicated that Fr10 is similar to type-4 AFP from *M. octodecimspinosus*, although there was no sequence homology between the two. A subsequent study (Sullivan et al., 2015) investigated expression of *fr10* in different tissues and organs in frogs exposed to freezing, dehydration and anoxia, and at different development times. The gene was up-regulated under these conditions in a tissue-specific fashion. AFP-Pred, a random forest software package, suggested that FR10 is a type-4 AFP. However, confirmation requires demonstration that FR10 is capable of producing TH or inhibiting recrystallization.

Other invertebrates with IBPs

Blood TH of 0.38°C in the freeze-tolerant intertidal mussel *Mytilus edulis* from the coast of Europe was reportedly due to a fish-like AFGP (Theede et al., 1976). This report has not been verified, but I could not measure TH in winter *M. edulis* from Massachusetts, USA (unpublished observation).

Hemolymph TH was identified in the Antarctic calanoid copepod *Stephos longipedes*, which inhabits brine channels in the upper layers of surface ice where temperatures can drop below the freezing point of seawater (Kiko, 2010). The responsible IBP has sequence homology to IBPs of diatoms, bacteria and snow mold. Horizontal gene transfer is suspected from bacteria or diatoms living in the same brine channels, but this has yet to be proven.

An IBP from a free-living Antarctic nematode, *Panagrolaimus davidi*, inhibits recrystallization and may contribute to freeze tolerance in this and other Antarctic nematodes (Wharton et al., 2005).

Antifreeze glycolipids

Until recently, only proteins were known to produce TH; however, AFGLs with activity similar to insect AFPs were identified in the freeze-tolerant Alaskan beetle *Upis ceramboides* (Walters et al., 2009a). *U. ceramboides* winter mainly as adults in sites ranging from thermally buffered snow-covered fallen logs to completely exposed sites. They survive temperatures down to –60°C and concentrate sorbitol and threitol (Miller and Smith, 1975; Miller, 1978; Walters et al., 2009b). The AFGL consists mainly of mannose–xylose disaccharide repeating units, β-D-manp-(1→4)-β-D-xylp (βmanpyranoside-βxylpyranoside), plus lipid (mostly phospholipid). Three groups have synthesized the disaccharide

repeats, using different protocols, and confirmed the structure (Crich and Rahaman, 2011; Ishiwata et al., 2011; Zhang et al., 2012, 2013).

Similar AFGLs were identified in additional insects, both freeze tolerant and freeze avoiding, including *D. canadensis* and *C. clavipes* beetles, two species of freeze-tolerant frogs, European *Rana lessonae* (Walters et al., 2011) and Alaskan *Lithobates (Rana) sylvatica* with the lowest recorded lower lethal temperature among frogs (Larson et al., 2014), as well as the freeze-tolerant plant *Solanum dulcamara* (Walters et al., 2011). All of these AFGLs exhibited the mannose–xylose disaccharide repeats, but freeze-tolerant nymphs of the arctic Alaskan stonefly *Nemoura arctica* wintering in small streams that freeze solid (Walters et al., 2009c) had a third saccharide.

The AFGLs are mainly associated with cell membranes, and in freeze-tolerant species apparently function to inhibit inoculation of the cytosol by extracellular ice. Also, the low levels of hemolymph AFGL inhibit recrystallization. The function of the AFGLs in the freeze-avoiding insect species is unknown, but they enhance TH of DAFPs in *D. canadensis* beetles.

Suggested future directions

Over the 40+ years since DeVries discovered AFPs in Antarctic fishes, much has been accomplished with regards the structures, physiology, evolution, etc. of these unique proteins and other IBPs, and the rate of discovery in this field has increased dramatically as multiple laboratories around the world now study these proteins. However, much remains to be learned.

Numerous new IBPs will certainly be discovered, because only a small percentage of cold-tolerant species has been investigated. Not only is structural information required, but details of the physiological functions of the proteins in these species are also essential. This is especially true for the low-TH IBPs and INPs of freeze-tolerant animals where this information is completely lacking. Investigation of function should not be limited to subzero temperature adaptations, but should include function(s) at higher temperatures as well. Why do some insects produce AFPs in summer? Evolution of the IBPs will remain a fertile area of inquiry, and may aid in understanding physiological function at both high and low temperatures. Also, applications of IBPs in agriculture, cryopreservation, etc. should certainly be investigated.

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