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Corresponding Author	Family Name	**Kristiansen**
	Particle	
	Given Name	**Erlend**
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	Organization	NTNU University Library
	Address	Trondheim, Norway
	Email	erlendkr@ntnu.no
Abstract	Antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs) have evolved independently in a variety of life forms and are characterized as a group only by their common ability to prevent existing ice crystals from growing in supercooled solutions. This chapter attempts to give a broad overview of some of the shared and unique characteristics of AF(G)Ps found in polar fish and freeze-avoiding arthropods. These include structural, evolutionary, regulatory and operational characteristics. Structurally similar AF(G)Ps are found within distantly related groups, and different forms are found in more related groups. Today's phylogenetic patterns of distribution are the results of several evolutionary processes. Expression of AF(G)Ps in fish and in insects are often influenced by environmental cues that signal the onset of winter, that act on hormonal control mechanisms of gene expression. Within species, there are large number of genes coding for AF(G)Ps, and these are often arranged in tandem. In many species of fish and insects, the genes themselves are constructed by multiple repeats in sequence, resulting in many isoforms of mature AF(G)Ps, some constructed from a varying number of repeat segments. Several similar helical secondary structures are found in unrelated mature AF(G)Ps, implying that these structures are effective general scaffolds for ice binding. The ice-binding surface sites of these proteins are located at planar regions of their surface and apparently structure water in an ice-like manner to secure effective binding to the ice lattice. Thus, AF(G)Ps comprise a diverse group that have many general characteristics in common, but also others that set them apart.	
Keywords (separated by '-')	Antifreeze protein - Antifreeze glycoprotein - Structure - Ice binding - Ice-binding site - Protein structure - Isoforms	

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Chapter 2
Characteristics of Antifreeze Proteins

Erlend Kristiansen

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2.1 Introduction

Antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs) are characterized as a group only by their common ability to prevent existing ice crystals from growing in supercooled solutions. They are found in many different life forms inhabiting cold, and often ice-laden, habitats, acting as protective means against a hostile thermal environment. Some polar unicellular organisms, including diatoms, fungi and bacteria, excrete AFPs to modify their external icy environment (Hoshino et al. 2003; Janech et al. 2006; Hanada et al. 2014), and an Antarctic bacterium use a membrane-bound AFP to adhere onto floating ice, allowing it to reside in the nutrient-rich upper part of the water column (Bar Dolev et al. 2016). Many freeze-tolerant organisms, that adaptively allow their extracellular body fluids to freeze, produce proteins that are classified as AFPs, since they cause a separation of the melting and freezing temperatures of ice in vitro. Such organisms include many plants (Urrutia et al. 1992; Duman and Olsen 1993; Worrall et al. 1998) and arthropods (Tursman and Duman 1995; Duman et al. 2004; Wharton et al. 2009; Walters et al. 2009). These proteins presumably function to control the shape and distribution of the endogen extracellular ice mass.

AF(G)Ps act as antifreeze agents in freeze-avoiding organisms, i.e. animals that die if endogenous ice is formed and that consequently rely on supercooling of their body fluids to survive. They have been shown to stabilize the supercooled state by inactivating structures within the body fluids that could initiate freezing and by preventing ice from penetrating through the body wall of the animal (Olsen and Duman 1997a, b; Olsen et al. 1998; Duman 2002). They enable hypoosmotic bony fish to occupy the cold polar waters, where these fishes may spend their entire lives

E. Kristiansen (✉)
NTNU University Library, Trondheim, Norway
e-mail: erlendkr@ntnu.no

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27 in a supercooled state, often in contact with external ice (DeVries 1982). The
28 evolution of the AF(G)Ps of polar fish has been driven by the cooling of the Arctic
29 and Antarctic waters, processes that resulted in subfreezing water temperatures being
30 reached some 5–14 million years ago in the Antarctic, and 13–18 million years ago
31 in the Arctic (Kennett 1977; Eastman 1993).
32 They are also found in many freeze-avoiding terrestrial arthropods, including
33 insects and spiders (Husby and Zachariassen 1980; Duman et al. 2004) and collem-
34 bolans (Graham and Davies 2005; Hawes et al. 2014). Even in these terrestrial life
35 forms, they may provide protection against lethal freezing throughout the
36 supercooling range of the animal, on occasion down to –30 °C or below
37 (Zachariassen and Husby 1982). Thus, these structures have common functions in
38 diverse organisms associated with life in a cold environment.
39 AF(G)Ps are categorized as hyperactive or moderately active, based on their
40 potency to cause antifreeze activity at equimolar concentrations. In addition to the
41 distinct differences in antifreeze potency, the shape of the ice crystals that forms in
42 the presence of moderately active and hyperactive AF(G)Ps are also characteristic:
43 hexagonal bipyramids (e.g. Baardsnes et al. 2001; Loewen et al. 1998; Ewart et al.
44 1998) and flattened hexagonal discs, respectively (e.g. Liou et al. 2000; Graether
45 et al. 2000). The underlying structural cause of the differences between these two
46 activity groups appears to be differences in their ice-binding sites (IBS).
47 The intention of this chapter is to point to some structural, physiological and
48 evolutionary characteristics of the AF(G)Ps found in freeze-avoiding polar fish and
49 arthropods. It is by no means exhaustive, and it is referred to Chaps. 5 and 6 of Vol.
50 1 for further discussion of fish and insect AF(G)Ps and Chaps. 7 and 8 of Vol. 1 for
51 AFPs in plants and other species. Chapter 9 of Vol. 1 and Chap. 4 of this volume
52 give more in-depth analysis of evolutionary aspects and the interaction between AF
53 (G)Ps and ice, respectively, and Chap. 6 of this volume focuses on the antifreeze
54 mechanism.

55 2.2 Structure

56 The independent evolution of AF(G)Ps in various taxa has resulted in structural
57 diversity within this functionally defined group (Graether et al. 2000; Fletcher et al.
58 2001; Graham and Davies 2005; Graham et al. 2007; Kiko 2010; Lin et al. 2011;
59 Hawes et al. 2014). However, structural similarities are also abundant.

60 *2.2.1 Polar Fish*

61 There are currently reported five distinct kinds of antifreeze proteins in polar fish:
62 AFGP and AFP type I–IV. However, the categorization of AFP type IV as a
63 functional AFP has recently been questioned (see below). Table 2.1 shows the

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Table 2.1 Taxonomic listing of the AF(G)Ps of polar fish t1.1

Subdivision Teleostei	Family	Genus/species	Type	
Infradivision Clupeomorpha	Clupeidae	Herring	II (+ Ca^{2+})	t1.3
Infradivision Euteleostei				t1.4
Superorder Protocanthopterygii	Osmeridae	Smelt	II (+ Ca^{2+})	t1.5
Superorder Paracanthopterygii	Gadidae	Northern cods	AFGP	t1.6
Superorder Acanthopterygii				t1.7
Order Scorpaeniformes				t1.8
Suborder Cottoidei				t1.9
Superfamily Cottoidea	Cottidae	Sculpins	I/IV	t1.10
	Hemitripteridae	Sea raven	II (– Ca^{2+})	t1.11
	Agonidae	Longsnout poacher	II (– Ca^{2+})	t1.12
Superfamily Cyclopteroidea	Cyclopteridae	Snailfish	I	t1.13
Order Perciformes				t1.14
Suborder Labridae	Labridae	Cunner	I	t1.15
Suborder Zoarcoidei	Zoarcidae	Eelpouts	III	t1.16
	Anarhichadidae	Wolf fish	III	t1.17
Suborder Notothenioidei	5 families		AFGP/IV	t1.18
Order Pleuronectiformes	Pleuronectidae	Right-eyed flounders	I	t1.19

taxonomic occurrence of the AF(G)Ps, and their structures are illustrated in Fig. 2.1. 64
As can be seen from the table, similar types of AF(G)Ps are scattered among 65
distantly related groups of teleosts. These patterns of distribution have for the 66
different kinds been attributed to convergent evolution (Chen et al. 1997a, b; 67
Graham et al. 2013), to lateral gene transfer (Graham et al. 2008a, 2012) and to 68
development from a common ancestor (Graham et al. 2013). Most fish AF(G)Ps are 69
reportedly moderately active, with the exception of some large variants that are 70
hyperactive. 71

2.2.1.1 Type I 72

The type I AFPs are α-helical proteins (Yang et al. 1988), see Fig. 2.1a. There are 73
three kinds of AFP type I, based on their genetics and the size of the mature proteins. 74
The overall structure is amphipathic, with the ice-binding side somewhat hydropho- 75
bic (Baardsnes et al. 2001). They are widely distributed among bony fishes, having 76
been identified in members of four superfamilies in three different orders, namely the 77
Pleuronectiformes (in flounders), Perciformes (in cunners) and Scorpaeniformes 78
(in snailfish and sculpins) (Hew et al. 1980; Evans and Fletcher 2001; Hobbs et al. 79
2011), see Table 2.1. 80

There are two subsets of type I AFP within each species examined, coded by two 81
different gene families; the liver-type AFPs have signal peptides, and these isoforms 82
are secreted into the blood stream (Gourlie et al. 1984). The skin-type, in contrast, 83
lack such signal peptides and are mostly located within skin and other peripheral 84

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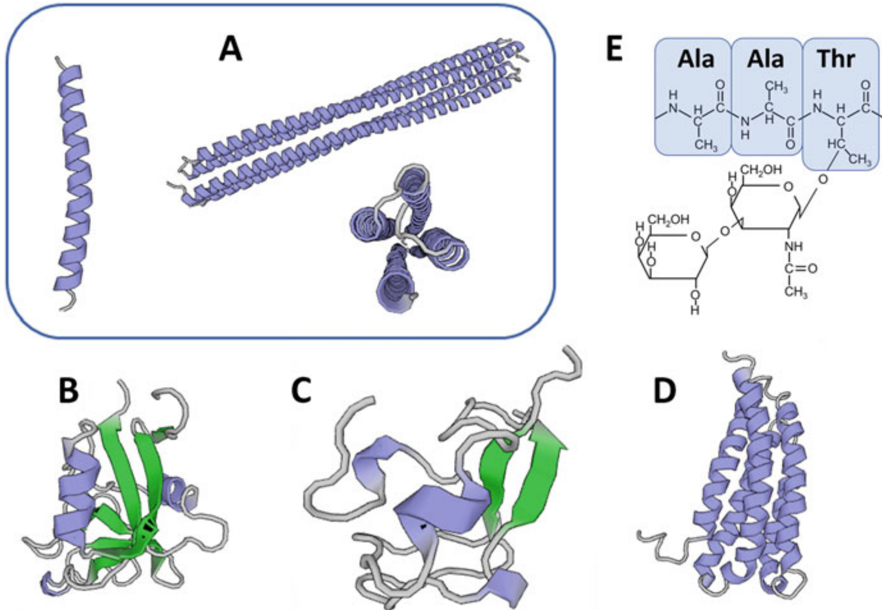

Fig. 2.1 The five different kinds of AF(G)Ps in polar fish. (**a**) Type I (PDB 1WFA) together with the hyperactive AFP maxi (PDB 4KE2). (**b**) Type II (PDB 2PY2). (**c**) Type III (PDB 1HG7). (**d**) Type IV, the illustration is of Apolipophorin III, a structural homologue of AFP type IV (PDB 1LS4). (**e**) The principal AAT repeat unit of AFGPs showing its o-link to its disaccharide. The different illustrations do not show correct proportions to each other. Colour codes: *Grey*: peptide backbone. *Blue*: α-helix. *Green*: β-strands

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tissues (Gong et al. 1996; Low et al. 1998; Evans and Fletcher 2006). Both these kinds of isoforms are small peptides with masses of about 3.3–4.5 kDa. The circulating liver-type AFPs of the flounders (Gourlie et al. 1984; Graham et al. 2008a) and the cunner (Hobbs et al. 2011) are constructed from 3–4 repeats of an 11-amino acid sequence TxxD/Nxxxxxxx, where x is usually Ala (Chao et al. 1996), whereas the circulating liver-type in snailfish lacks such a basic repeat (Evans and Fletcher 2005a). The skin-type of flounders, longhorn sculpins and cunner are very similar to each other and constructed from the same 11-amino acid repeat seen in the liver-type of flounder and cunner (Low et al. 2001). In addition, shorthorn sculpin has a larger 95 amino acid skin-type isoform that lacks repeat pattern (Low et al. 1998), and the skin-types of snailfish, as is the case of its liver-type, lack the 11-amino acid repeat (Evans and Fletcher 2005a).

A third kind of AFP type I is found in several Pleuronectiformes and is characterized by being much larger than the other skin- and liver-types. In addition, this kind is hyperactive. Winter flounder (*Pseudopleuronectes americanus*), yellowtail flounder (*Limanda ferruginea*) and American plaice (*Hippoglossoides platessoides*) each contains a large hyperactive isoform of type I (Gauthier et al. 2005; Graham et al. 2008b). The best studied of these is that of the winter flounder, and this variant

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is denoted Maxi, see Fig. 2.1a. Such a large type I AFP is the sole AFP known from the blood of American plaice (Gauthier et al. 2005). These 17 kDa molecules are constructed from similar 11 residue repeats seen in many of the smaller forms (Graham et al. 2008b). They are dimers in solution of mass 34 kDa, and each monomer folds back onto itself, resulting in a four-helix bundle (Sun et al. 2014). Interestingly, comparable folding patterns have also been proposed for an AFP from a fungus (Badet et al. 2015) and from a Hymenopteran insect (Xu et al. 2018), hinting to an effective configuration for ice binding.

Graham et al. (2013) proposed that the wide phylogenetic distribution of type I AFP is the result of independent evolution of these proteins within each of the four superfamilies they are found. This proposal was based on studies of their genetic sequences, that revealed differences in both codon usage and non-coding regions, strongly suggesting different progenitors in the four groups. Gauthier et al. (2005) suggested that the smaller isoforms of flounders may have evolved from the larger AFP I types in this group. This was based on the observation that American plaice only contain a single large isoform. Evans and Fletcher (2005b) suggested that the AFPs of snailfish may have resulted from a shift in the reading frame of genes coding for eggshell proteins or keratin.

2.2.1.2 Type II

Type II AFPs are homologue to the carbohydrate recognition domain of Ca^{2+}-dependent (C-type) lectins (Ewart et al. 1998; Loewen et al. 1998). They are found in species from four different families from three distantly related groups of teleosts (see Table 2.1). Herring (Clupeidae) is from the infradivision Clupeomorpha, whereas smelt (Osmeridae), sea raven (Hemitripteridae) and poacher (Agonidae) are from different groups within the infradivision Euteleostei. The latter two are from the same superfamily, whereas smelt is from a different superorder.

Type II AFPs have masses varying from 14 to 24 kDa and an overall globular structure consisting of two α-helixes and nine β-strands in two β-sheets (Gronwald et al. 1998, see also Fig. 2.1b). The observed three-dimensional folding pattern is very similar to rat mannose-binding protein, a member of the family of C-type lectins from which they are likely derived. Type II AFPs are unique in having five internal SS bonds rather than 2–4 such bonds found in C-type lectins.

There are two distinct kinds of Type II AFPs; those isolated from smelt (Osmeridae) and herring (Clupeidae) require Ca^{2+} as a cofactor for activity, whereas those isolated from sea raven (Hemitripteridae) and poacher (Agonidae) are fully active in the absence of this cofactor. The IBS of these Ca^{2+}-dependent and Ca^{2+}-independent forms are located at different parts of their surfaces. Those that require Ca^{2+} for activity have IBS corresponding to the carbohydrate-binding site of C-type lectins (Ewart et al. 1998), whereas the IBS of the Ca^{2+}-independent variants are located outside this region (Loewen et al. 1998).

All AFP II have a unique SS-bond pattern not seen in related proteins and they also share great (>85%) identity in both amino acid sequence and conserved genetic

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145 sequences, including intron and exon regions. Due to this great similarity among the
146 AFP type II, Graham et al. (2008a) and Sorhannus (2012) proposed that their
147 scattered phylogenetic pattern of distribution is unlikely to be the result of conver-
148 gent evolution, as in the case of type I AFPs. Instead, it is probably the result of a
149 transfer of genes between the different groups of AFP type II-producing fish. Such
150 so-called lateral gene transfer may have occurred during events of mass spawning. In
151 the case of the Ca^{2+}-dependent AFP type II, Graham et al. (2012) found evidence to
152 suggest that smelt was the recipient of genetic material from herring.

153 #### 2.2.1.3 Type III

154 Type III AFPs are 7 kDa globular proteins only found in the two closely related
155 families Zoarcidae (eelpouts) and Anarhichadidae (wolf fish) in the suborder
156 Zoarcoidei, see Fig. 2.1c. The primary sequence has no obvious repeats and the
157 folding pattern is complex, involving several short strands paired in two antiparallel
158 β-sheets, in addition to several helixes.

159 Type III AFPs are found in two structural variants that are categorized by their
160 isoelectric points (Chao et al. 1993). One group, the QAE forms, has pI below 7 and
161 are consequently anionic at physiological pH, whereas the other group, the SP forms,
162 has pI above 7 and are therefore cationic at physiological pH. Both QAE and SP
163 forms are present in the animal. The SP forms reportedly have a lower activity than
164 the QAE forms (Nishimiya et al. 2005). Takamichi et al. (2009) reported that the
165 addition of minute amounts of a fully active QAE form to an inactive SP form
166 isolated from the Japanese fish *Zoarces elongatus Kner* resulted in the SP form
167 obtaining the same activity as the QAE form. These findings suggest that these two
168 forms may cooperate in vivo. A natural 14 kDa intramolecular dimer has been
169 identified, where two monomeric AFP III are linked by a short strand (Miura et al.
170 2001).

171 Since the occurrence of AFP type III is confined only to two closely related
172 families of fishes, these forms presumably originated in a common ancestor (Graham
173 et al. 2013). Baardsnes and Davies (2001) reported that the protein sequence of a
174 type III AFP showed about 40% identity and 50% similarity to parts of the
175 C-terminal domain of sialic acid synthase, an enzyme that binds carbohydrate as
176 part of its function. Deng et al. (2010) elaborated on the evolutionary events that
177 presumably preceded the development of today's type III AFP. Apparently, the
178 N-terminal part of a functional sialic acid synthase molecule, that showed rudimen-
179 tary antifreeze activity associated with its C-terminal, was replaced by a signal
180 peptide. This caused the AFP-precursor to be secreted from the cells, and this
181 molecular de-coupling of the enzymatic and antifreeze functions allowed selective
182 pressure to act solely towards the antifreeze function.

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2.2.1.4 Type IV

Type IV AFP is a 12 kDa lipoprotein-like protein with about 60% α-helix content, see Fig. 2.1d. Its proposed structure consists of four amphipathic α-helixes of similar length folded in a four-helix bundle (Deng and Laursen 1998). Type IV AFP has been found in many species, including Arctic longhorn sculpin (*Myoxocephalus octodecemspinosus*) and shorthorn sculpin (*M. scorpius*) (Deng and Laursen 1998; Gauthier et al. 2008) and two Antarctic nototheniids, *Pleuragramma antarcticum* and *Notothenia coriiceps* (Lee et al. 2011; Lee and Kim 2016). However, its role as a functional AFP has been questioned, since it is a very weak AFP, causing only 0.07 °C thermal hysteresis at a concentration of 0.5 mg/mL, and is present in blood in concentrations less than 100 μg/mL, far too low to protect these fishes against freezing in icy waters (Gauthier et al. 2008; Lee and Kim 2016). Its ability to cause thermal hysteresis could therefore be incidental. Gauthier et al. (2008) proposed that, although type IV has the potential to develop into a functional AFP, it has not been selected for this purpose due to the presence of other functional AFPs. This is supported by the presence of type IV AFP in temperate, subtropical and tropical species, including species living in fresh water (Liu et al. 2009; Xiao et al. 2014; Lee et al. 2011; Lee and Kim 2016). These species have no need for any freeze protection, and type IV AFP may instead be involved in embryogenesis, since several of its homologues are essential in this process.

2.2.1.5 AFGPs

AFGPs are found in two distantly related and geographically separate groups of teleost fish, the Arctic cods (family Gadidae of the superorder Paracanthopterygii) and the Antarctic Nototheniids, (suborder Notothenioidei of the superorder Acanthopterygii). They contain a varying number of the tripeptide AAT, where the hydroxyl group of each Thr is O-linked to a disaccharide (β-D-galactosyl-(1,3)-α-D-*N*-acetylgalactosamine), see Fig. 2.1e for an illustration of the basic unit. In this unit, the carbohydrate moiety makes up about 60% of the mass. The smallest variants contain only 4 of these repeat units and have a mass of about 2.6 kDa and the largest contain about 50 repeat units with a mass of 33 kDa. The differently sized AF(G)Ps are arranged into eight distinct size groups (DeVries 1982), and each group contains a number of isoforms (Wu et al. 2001).

The secondary structure of AFGPs has been difficult to elucidate. There is mounting evidence to suggest that they obtain a type II polyproline helix, but only at low temperatures (Franks and Morris 1978; Bush et al. 1984; Mimura et al. 1992; Tachibana et al. 2004). In this configuration, each triplet AAT makes one turn in the coil, resulting in the carbohydrate units being in a regular arrangement on one side of the molecule. Such an arrangement gives the molecule and overall amphipathic character, where the carbohydrate side is more hydrophilic, and the protein backbone with the methyl group of Ala, is more hydrophobic. The shape of the ice crystals that

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223 form in the presence of AFGPs also suggests a regular configuration; these ice
224 crystals are hexagonal bipyramids, exposing only a single crystal plane to the
225 surrounding solution onto which the AFGPs are adsorbed. Such crystal plane
226 specificity likely requires that all adsorbed molecules have the same configuration.
227 Wöhrmann (1996) reported that an exceptionally large 150 kDa AFGP from the
228 nototheniid *Pleuragramma antarcticum* was hyperactive. No other AFGP is known
229 to be hyperactive.
230 The AF(G)Ps found in Gadoids and nototheniids, members of different superor-
231 ders of teleosts, have evolved independently (Chen et al. 1997a). Those of the
232 Antarctic nototheniids apparently evolved from a trypsinogen gene (Chen et al.
233 1997b) some 5–14 million years ago, whereas those of the Arctic gadoids evolved
234 from a non-coding part of their DNA some 13–18 million years ago (Baalsrud et al.
235 2018). The timing of their independent emergence coincides well with the reported
236 time the Antarctic and Arctic waters reached subfreezing temperatures (Kennett
237 1977; Eastman 1993).

238 *2.2.2 Arthropods*

239 Table 2.2 shows a taxonomic listing of known or tentative arthropod AFPs with
240 some structural features indicated. The table suggests that AFPs in closely related
241 species are homologue structures with a common progenitor. Almost all arthropod
242 AFPs are constructed as shorter repetitive segments in series and almost all contain
243 variations of the tripeptide pattern TxT within the repeats. The table also shows the
244 high prevalence of the β-helical folding pattern, a feature that undoubtedly has
245 evolved by convergent evolution in distantly related groups (Liou et al. 2000;
246 Graether et al. 2000; Graether and Sykes 2004). Some of the variants of AFPs
247 found in arthropods are illustrated in Fig. 2.2.

248 2.2.2.1 Insects

249 There is structural information available on AFPs or putative AFPs from five orders
250 of insects, Coleoptera, Hymenoptera, Lepidoptera, Diptera and Hemiptera.

251 **Coleoptera** The beetles within the superfamily Tenebrionidea all have AFPs with
252 very similar sequences that most likely are homologue structures (Table 2.2). These
253 AFPs are constructed of 5–7 tandem repeats of the 12 or 13-mer consensus amino
254 acid sequence TCTxSxxCxxAx. Notably, the Thr in position 1 and 3 and the Cys in
255 position 2 and 8 in the repeat are highly conserved in isoforms within and between
256 species.
257 The conserved positions of the Cys within the 12-mer repeat structure observed in
258 the AFPs identified from species within the superfamily Tenebrionidea results in
259 every sixth residue in the sequence being occupied by a Cys. The two Cys within

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AU2 t2.1 **Table 2.2** Taxonomic listing and structural features of known and putative AFPs from arthropods

Phylum Arthropoda	Family	Species	Code	MW (kDa)	Primary repeat	Secondary
Class Entognatha						(D) Antiparallel L-h PPII helixes,
Order Collembola	Hypogastruridae	*Hypogastrura harveyi*[1,2]	sfAFP	6.5 and 15.7	Gxx	stacked in two sets.
		Gomphiocephalus hodgsoni[21]	GomphyAFP	9	? Rich in Gly and Cys	?
Class Insecta						
Order Coleoptera						
Intra order Cucujiformia						
Superfamily Tenebrionoidea	Tenebrionidae	*Tenebrio molitor*[6,7,19]	TmAFP	8.3–12	TCTxSxxCxxAx (x)	(D) R-h β-helix
		Dendroides canadensis[8]	DAFP	7.3–12.4	”	(A) ” (sim. to TmAFP)
		Microdera punctipennis[9]	MpAFP	12.7	”	(A) ” (”)
		Pterocoma loczyi[c,10]	PLAFP	~ 12	”	(A) ” (”)
		Anatolica polita[c,11]	ApAFP	10.9 and 11.4	”	(A) ” (”)
Superfamily Cucujoidea	Cucujidae	*Cucujus clavipes*[a]			”	(A) ” (”)
Superfamily Chrysomeloidea	Cerambycidae	*Rhagium inquisitor*[12,13]	RiAFP	13	TxTxTxT + $x_{9–15}$	(D) Flattened β-helix
		R. mordax[14]	RmAFP	13	”	(A) ” (sim. to RiAFP)
Infra order Scarabaeiformia						
Superfamily Scarabeaoidea	Lucanidae	*Dorcus curvidens*[b]		11.4–14.3	TCTxSxxCxxAx (x)	(A) R-h β-helix (sim. to TmAFP)

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t2.19 **Table 2.2** (continued)

Phylum Arthropoda	Family	Species	Code	MW (kDa)	Primary repeat	Secondary
Order Hymenoptera						
Sub order Apocrita	Apidae	*Apis cerena cerena*[22]	AcerAFP	60		(M) 3 α-helixes looped together
Order Diptera	Chironomidae	Sp. "Lake Ontario midge"[16]		5.7–10.4	xxCxGxYCxG. Glyco.	(M) L-h solenoid coil
Order Hemiptera						
Suborder Heteroptera	Scutelleridae	*Eurygaster maura*[c,15]	EmAFP	10.2	TxT + x_{10}	(M) L-h β-helix
Order Lepidoptera						
Superfamily Tortricoidea	Tortricidae	*Choristoneura fumiferana*[3,4]	CfAFP	9–12	TCT + x_{12}	(D) L-h β-helix
		and sister species				
Superfamily Geometroidea	Geometridae	*Campaea perlata*[5,6]	iwAFP	3.5 & 8.3	TxTxTxTxTxxx	(M) R-h flattened β-helix
Class Arachnida						
Order Ixodida	Ixodidae	*Ixodes scapularis*[c,17]	IAFGP	~ 23	TAA Probably Glyco.	?
Order Trombidiformes	Tetranychidae	*Tetranychus urticae*[c,18]		10–21	NCTxCxxCxNCx	(M) β-helix
Class Maxillopoda						
Order Calanoida	Stephidae	*Stephos longipes*[20]		26 kDa	No apparent repeat	β-helix with a parallel α-helix

Abbreviations: (A): assumed by this author based on sequence similarity. (D): determined. (M): modelled. L-h: Left-handed. R-h: right-handed. Sim. to: similar to. Glyco.: Glycosylated. [a]Mentioned in Duman (2015). [b]Sequence only published in NCBI. [c]Only assumed to be an AF(G)P, as no hysteresis activity is reported. (1) Graham and Davies (2005); (2) Pentelute et al. (2008); (3) Tyshenko et al. (2005); (4) Graether et al. (2000); (5) Lin et al. (2011); (6) Graham et al. (2007); (7) Liou et al. (2000); (8) Andorfer and Duman (2000); (9) Qiu et al. (2010); (10) Ma et al. (2008); (11) Ma et al. (2012); (12) Kristiansen et al. (2011); (13) Hakim et al. (2013); (14) Kristiansen et al. (2012); (15) Guz et al. (2014); (16) Basu et al. (2015); (17) Neelakanta et al. (2010); (18) Bryon et al. (2013); (19) Liou et al. (1999); (20) Kiko (2010); (21) Hawes et al. (2014); (22) Xu et al. (2018)

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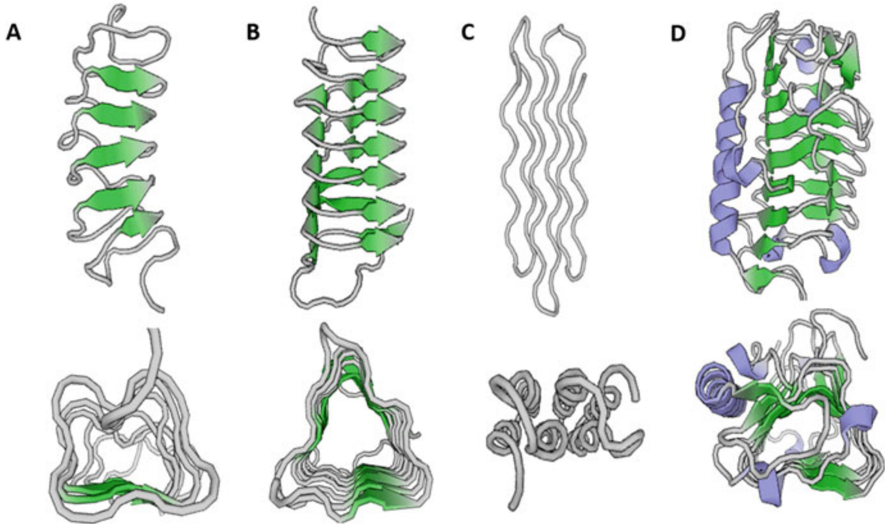

Fig. 2.2 Some different kinds of AFPs from Arthropods. (**a**) TmAFP from the coleopteran *T. molitor* (PDB 1L1I). (**b**) CfAFP from the lepidopteran *C. fumiferana* (PDB 1M8N). (**c**) An AFP from the collembolan *Hypogastrura harvey*i (PDB 2PNE). (**e**) A crustacean AFP from *Stephos longipes.* The illustration is of the AFP from *Colwellia* sp., a structural homologue (PDB 3WP9). The upper illustrations are frontal views, the lower illustrations are views from the top. The different illustrations do not show correct proportions to each other. Colour codes: *Grey*: peptide backbone. *Blue*: α-helix. *Green*: β-strands

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each repeat form an SS bond (Li et al. 1998a; Liou et al. 2000). Liou et al. (2000) showed that the AFPs of *Tenebrio molitor*, TmAFP, fold as a tight regular right-handed solenoid, where each 12-mer repeat segment form one full turn in the coil. Each segment forms β-strands and the strands form β-sheets. This folding pattern results in a β-helix where the Thr residues in position 1 and 3 in each repeat are stacked on one side of the structure and form a highly regular ladder of 5–7 TCT motifs. The side chains of the Thr residues within each motif point outward from the structure, whereas the SS bonds between position 2 and 8 within each repeat cross the coil in a regular manner, contributing to the tightness and stability of the structure. Li et al. (1998a) found that the disulphide pattern in AFPs from the closely related *Dendroides canadensis*, DAFP, is similar to that of TmAFP. Li et al. (1998b) reported high content of β-sheet also in DAFP, and Jia and Davies (2002) and Wang et al. (2009) modelled DAFP according to the folding pattern of TmAFP. Other tenebrionid species that reportedly have the same consensus sequence as *T. molitor* and *D. canadensis* are *Microdera punctipennis* (Qiu et al. 2010), *Pterocoma loczyi* (Ma et al. 2008) and *Anatolica polita* (Ma et al. 2012). Given the degree of sequence similarity between AFPs of different species within Tenebrionidea (Table 2.2), there is little doubt that they fold into the same configuration as TmAFP. An illustration of the folding pattern of TmAFP is shown in Fig. 2.2a.

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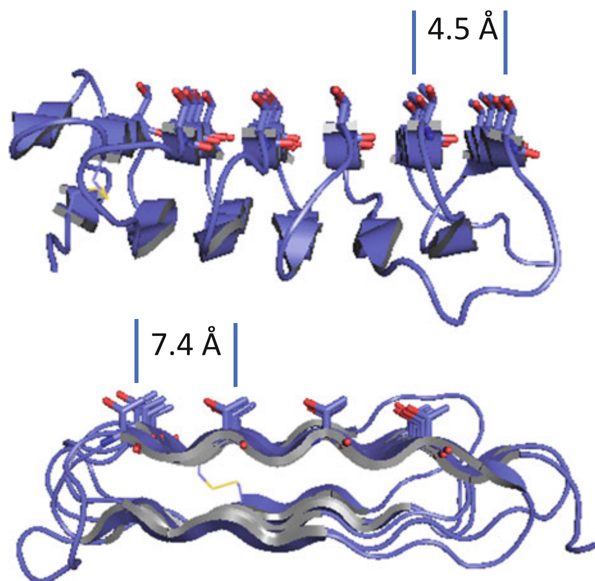

Fig. 2.3 The flatness and regularity of IBSs. RiAFP from the cerambycid beetle *Rhagium inquisitor* (PDB 4DT5) oriented to depict the flatness and regularity of the IBS and the distances between Thr residues in the TxTxTxT motifs within and between the β-stands in the IBS. The side chains of the Thr residues are protruding upwards from the β-sheet

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279 The two closely related species of longhorn beetles, *Rhagium inquisitor* and
280 *R. mordax*, express AFPs, RiAFP and RmAFP, respectively, which contain an
281 expanded version of the TxT motif seen in the Tenebrionidea AFPs. The consensus
282 sequence of RiAFP and RmAFP is the repeat TxTxTxT interrupted by stretches of
283 13–20 residues that do not have any obvious pattern (Kristiansen et al. 2011, 2012).
284 Six of these segments fold into a flattened β-helical configuration with the TxTxTxT
285 motifs stacked on one side in a regular ladder (Kristiansen et al. 2012; Hakim et al.
286 2013). In the case of the longhorn beetles, there are only two cysteines present
287 (Kristiansen et al. 2011), and these form a single SS bond at the N-terminal of the
288 molecule (Hakim et al. 2013). An illustration of RiAFP is given in Fig. 2.3.
289 The beetle *Dorcus curvidens* belongs to the family Lucanidae in the intraorder
290 Scarabaeiformia. Nevertheless, its reported nucleotide sequences coding for AFPs
291 (Nishimiya et al. 2007) is very similar to those of the tenebrionids of the intraorder
292 Cucujiformia. A BLAST search of one of these sequences (AB264320.1) showed
293 86% identity to a nucleotide sequence coding an isoform of *Tenebrio molitor*
294 (AF159114.1), and a BLASTp showed that the identity was 75% at the amino
295 acid level, higher than that between several of the *D. curvidens* isoforms. This is
296 quite noteworthy, given the fact that these species are more distantly related than the
297 tenebrionid and cerambycid beetles, that share no sequence similarity between
298 their AFPs.

299 **Hymenoptera** Xu et al. (2018) reported on an AFP from the Chinese honeybee,
300 *Apis cerena cerena*, denoted AcerAFP. This 60 kDa AFP consists of 365 amino
301 acids, is rich in alanine and contains 11 repeats of the four residues AAxA. The
302 recombinant protein expressed a 0,5 °C antifreeze activity and was found to have

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63–96% sequence similarity to gene sequences from 9 other species spanning several suborders of Hymenoptera, reported in the NCBI database (Xu et al. 2018), suggesting a wide hymenopteran distribution of AcerAFP. Some 96.4% of the protein consists of α-helixes and the remainder is loops, and the proposed tertiary structure consists of three α-helical regions of the protein that is folded onto each other. Interestingly, this tertiary structure is quite similar to that of the hyperactive Maxi fish type I AFP found in winter flounder (Sun et al. 2014).

Lepidoptera The repetitive occurrence of two Thr residues spaced one residue apart seen in the coleopteran AFPs is also found starting at every 15th position throughout the sequence of CfAFP, the AFPs found in the lepidopteran genus, *Choristoneura*. There is no apparent consensus repeat pattern in CfAFP beyond the TxT motif. This is analogue to the situation with RiAFP from the beetle *R. inquisitor*, were the wider TxT motif is separated by stretches devoid of any clear consensus sequence. Nevertheless, these AFPs have been shown to fold into a β-helix configuration in a manner similar to that of the coleopteran TmAFP (Graether et al. 2000). Each turn in the helix is composed of 15 residues, resulting in the repetitive TxT motifs being stacked on one side of the helix to form a ladder of TxT motifs, as seen in TmAFP. In the case of CfAFP, the helix is left handed rather than right handed, and although these AFPs are also stabilized by many internal SS bonds crossing the helix, these do not form the highly regular pattern seen in TmAFP (Gauthier et al. 1998; Graether et al. 2000). Figure 2.2b shows an illustration of the folding pattern of CfAFP. Tyshenko et al. (2005) suggested that isoforms found in *Choristoneura fumiferana* and closely related species in the same genus emerged from a common progenitor prior to species divergence, about 3.2–3.7 million years ago. This time frame corresponds to the cold period preceding the Pleistocene ice ages that started some 3 million years ago.

Lin et al. (2011) reported that AFPs from the lepidopteran inchworm *Campaea perlata*, CpAFP, are constructed of a series of the basic consensus repeat TxTxTxTxTxxx. Different isoforms were identified that formed two subsets, four small isoforms of ~ 3.5 kDa and five isoforms with masses of ~ 8.3 kDa. One of the larger isoforms was modelled as a flattened β-helix, where four motifs of the wider TxTxTxTxT repeat is stacked into a ladder on one side of the flattened helix (Lin et al. 2011), analogue to the structure determined in the coleopteran RiAFP (Hakim et al. 2013).

Diptera Basu et al. (2015) reported that a midge from the family Chironomidae produces an AFP consisting of repeats of the consensus 10 residue sequence xxCxGxYCxG. This 9.1 kDa protein has an even higher content of cysteine than TmAFP, DAFP and CfAFP. An energy-stabilized model was constructed based on the helical configuration, where each of the eight turns in the construction consists of only 10 residues. The two cysteines within each 10-residue repeat form an internal SS bond and these bonds cross the coil in a regular manner akin to the pattern seen in the coleopteran TmAFP. In this construction, one side of the molecule consists of a regular ladder of stacked YCx motifs. The position x is usually occupied by Thr or Val. The side chains of the residues flanking the Cys in the motif point outward and

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347 are the suspected ice-binding site. The coiled structure is not likely to form β-sheets,
348 and its configuration was therefore described as a solenoid (Basu et al. 2015).
349 Several isoforms appear to be present in the species, ranging from 5.7 to 10 kDa.

350 **Hemiptera** Guz et al. (2014) identified a putative AFP, EmAFP, in the sun pest
351 *Eurygaster maura*. Although antifreeze activity was not explicitly reported, it was
352 interpreted as being an AFP based on sequence features and its association with the
353 overwintering stage. The 10 kDa protein shows 52% similarity with the Lepidop-
354 teran CfAFP and has a repetitive pattern of TxT spaced 12–13 residues throughout
355 the sequence. It contains four Cys resides suspected of forming two internal SS
356 bonds. It was proposed to fold as a left-handed helix, leaving the TxT motif as a
357 regular ladder on one flat side of the protein, as reported for TmAFP and CfAFP.

358 #### 2.2.2.2 Collembola

359 Graham and Davies (2005) discovered a glycine-rich hyperactive AFP, sfAFP, from
360 the collembolan snow flea, *Hypogastrura harveyi*. The primary sequence is a repeat
361 of the triplet Gxx, where the first x-position is often also a Gly. The protein exists as
362 two isoforms, a small 6.5 kDa variant and a 15.7 kDa variant. The smaller form has
363 two internal SS bonds whereas the larger has only one. Their sequences are not very
364 similar, suggesting that their separation is ancient. The smaller isoform has been
365 shown to fold into six short polyproline helixes, where each triplet makes one turn in
366 the helix (Lin et al. 2007; Pentelute et al. 2008). Interestingly, the type II polyproline
367 helix fold is also the likely configuration of AFGPs of polar fish. The overall
368 arrangement of these helixes in sfAFP is a structure consisting of two flat sheets,
369 where each sheet consists of three parallel type II polyproline helixes and the three
370 helixes in each of the two sheets run antiparallel to each other. This folding pattern
371 results in the overall structure having two flat sides, one more hydrophobic than the
372 other. Mok et al. (2010) modelled the larger isoform according to the same folding
373 pattern. In this form, there are 13 type II polyproline helixes where 12 of these form
374 two flat sheets, each made up of six helixes. An illustration of the folding pattern of
375 the smaller isoform of sfAFP is given in Fig. 2.2c.
376 Hawes et al. (2014) reported on the amino acid composition of a 9 kDa AFP from
377 the Antarctic springtail, *Gomphiocephalus hodgsoni*, denoted GomphyAFP. Even
378 though *G. hodgsoni* and *H. harvey* belong to the same family of springtails, the
379 composition of these collembolan AFPs is distinctively different. GomphyAFP
380 contains far less glycine than sfAFP (~12%, vs. ~50%) and far more cysteine than
381 sfAFP (~14% vs. 1–5%). The content of glycine is high compared to the known
382 non-collembolan AFPs, whereas the high content of cysteine suggests a structure
383 stabilized by many disulphide bonds, as seen in most of the known insect AFPs.

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2.2.2.3 Arachnida 384

Neelakanta et al. (2010) reported on a putative antifreeze protein in the tick *Ixodes* 385
scapularis, of the order Ixodida. The protein has about 70% sequence identity to the 386
protein scaffold of AFGPs of polar fish, consisting of long stretches of the triplet 387
AAT, and was subsequently named IAFGP. No information was provided to show 388
that this protein is an AF(G)P or if it is glycosylated in a manner akin to that seen in 389
the AFGPs of polar fish. Expression of IAFGP in *I. scapularis* is upregulated by the 390
presence of the bacterium *Anaplasma phagocytophilum*, a human pathogen to which 391
the tick is a host and vector. This was interpreted as reflecting a symbiotic relation- 392
ship, since it implies that the bacteria induce increased cold tolerance in its host. 393

Bryon et al. (2013) reported upregulation of genes that code for putative AFPs in 394
diapausing individuals of the mite *Tetranychus urticae*, from the order 395
Trombidiformes. These proteins were examined only in silico, and identity as 396
AFPs was only inferred, based on comparison to structural features of known 397
AFPs from insects. The predicted AFPs consist of 92–210 residues with the identi- 398
fiable consensus 12-residue repeat pattern NCTxCxxCxNCx. This pattern contains 399
two more Cys residues than those of the tenebrionid beetles and the lepidopteran 400
C. fumiferana. Automatic generation of 3D configuration suggests that they fold in a 401
manner similar to the AFPs of *T. molitor*, where a stack of the tripeptide motif NCT 402
forms a β-sheet that comprises the tentative IBS of the protein. In this proposed 403
configuration, two of the Cys residues of each repeat form a disulphide pattern 404
similar to that seen in TmAFP, whereas the two additional Cys residues in the repeat 405
is directed inwardly and may also form SS bonds. 406

2.2.2.4 Crustacea 407

Kiko (2010) reported that the copepod *Stephos longipes* expresses two isoforms of a 408
hyperactive AFP that shows strong homology to AFPs identified in several diatoms, 409
bacteria and a snow mold. This wide phylogenetic distribution of an apparent 410
homologue structure in both prokaryotes and eukaryotes is by all accounts the result 411
of lateral gene transfer, as is apparently also the case for the type II AFPs from fish. 412
Hanada et al. (2014) described a homologue found in the Antarctic sea ice bacterium 413
Colwellia sp.; the structure consists of a β-helical domain and an α-helix aligned 414
parallel to the β-helix. The β-helical domain folds into a left-handed helix with a 415
triangular cross section and three parallel β-sheets. The IBS of the protein is located 416
on one of the flat sides of the β-helix. An illustration of the folding pattern of this 417
protein is given in Fig. 2.2d. 418

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419 ## 2.3 Isoform Diversity

420 As mentioned in the previous section, the phylogenetic occurrence of the various fish
421 type AF(G)Ps are proposedly the results of independent convergent evolution (type I
422 and AFGPs), lateral gene transfer (type II) and development from a common
423 ancestor (type III). Among arthropods, a common progenitor is implied for many,
424 and common secondary structural features have evolved by convergent evolution
425 among distantly related species.

426 At the organismal level, there are many different isoforms of AFPs present in the
427 body fluids, and they result from a high number of genes. These genes are generally
428 arranged in tandem, suggesting extensive gene duplication (Scott et al. 1985; Hew
429 et al. 1988). The AFGPs of both Antarctic notothenids and Arctic cods are coded by
430 polyprotein genes, where the polyprotein is post-translationally cleaved to produce
431 the mature AFGPs (Chen et al. 1997a, b; Hsiao et al. 1990; Baalsrud et al. 2018).
432 One such gene found in *Notothenia coriiceps neglecta* codes for 46 mature proteins
433 (Hsiao et al. 1990). In *Dissostichus mawsoni*, Chen et al. (1997b) found 41 copies of
434 polyprotein sequences, coding isoforms belonging to four of the eight known size
435 groups of isoforms, and Baalsrud et al. (2018) found that the number of copies of
436 genes in Arctic cods varied with the species according to their thermal environment.
437 Scott et al. (1985) reported that winter flounder has about 40 genes coding for AFP I,
438 and Hew et al. (1988) found 150 genes coding for AFP type III in ocean pout. There
439 is a similar situation in insects; in the coleopteran *T. molitor*, there are some 30–50
440 gene copies (Liou et al. 1999), and some 27 isoforms of TmAFP have been described
441 to date (Graham et al. 2007). Some 30 isoforms have been described in the related
442 *D. canadensis* (Nickell et al. 2013). The CfAFP of the lepidopteran *C. fumiferana* is
443 coded by about 17 different genes, each found in 2–5 copies tandemly arranged
444 within the genome (Doucet et al. 2002). Thus, AF(G)P expression is augmented by
445 high gene dosage caused by gene duplication in both insects and fish.

446 Many AF(G)Ps are constructed as repeat segments in series, and some of the
447 variation among isoforms is caused by a varying number of repeat segments. As
448 mentioned, the unrelated AFGPs of Antarctic notothenids and Arctic cods have
449 from 4 to 50 segments of the basic AAT unit. Several of the AFP type I contain three
450 or four segments of its 11-residue repeat unit (Chao et al. 1996; Gourlie et al. 1984;
451 Low et al. 2001; Graham et al. 2008b; Hobbs et al. 2011). The isoforms of the
452 coleopterans *T. molitor* and *D. canadensis* vary from five to eight copies of a repeat
453 pattern (Liou et al. 1999; Andorfer and Duman 2000), whereas those of the lepidop-
454 teran *C. fumiferana* have either five or seven segments of the repeat (Doucet et al.
455 2000). Thus, in both fish and insects the genes themselves coding these functional
456 proteins apparently evolved by similar mechanisms; duplication of internal repeat
457 patterns, resulting in groups of isoforms within the organism that differ in their
458 number of repeats, analogous to the apparent process by which the high gene dosage
459 evolved. In the case of the large fish type I variants found in flounders, Gauthier et al.
460 (2005) proposed that smaller isoforms may be derived from larger precursors.

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Gene duplication results in certain isoforms within the organism being more closely related to a common original gene than to others, causing isoforms to form subsets based on structural similarity. For instance, the QAE and the SP forms of AFP type III share about 50% identity whereas the similarity is about 75–90% within each group (Chao et al. 1993). As mentioned, the AFP type I found in right-eyed flounders, sculpins, snailfish and cunner are coded by two gene families; one group codes for proteins with signal peptides and are produced in the liver and secreted to the blood stream, while another group, the skin-type, mostly lacks coding for signal peptides and are produced and located in other tissues (Gong et al. 1996; Low et al. 1998; Evans and Fletcher 2006). The isoforms of the coleopteran *D. canadensis* are divided into three subsets, group I, II and III, based on sequence similarity (Andorfer and Duman 2000). In the lepidopteran *C. fumiferana*, they are also classified into three subsets, based on the length of the 3′untranslated region (UTR) of their mRNAs: those with short UTRs (9 kDa), those with intermediate UTR (12 kDa) and those with long UTRs (9 kDa). Members of each group are more structurally similar to other members of that group than to members of the other two groups of isoforms (Doucet et al. 2000).

The isoforms of closely related species of insects and of fish are homologue structures, as they most likely evolved in a common ancestor prior to species divergence. Tyshenko et al. (2005) characterized isoforms homologue to those of the lepidopteran *C. fumiferana* in three other species of *Choristoneura*; phylogenetic comparison of the sequences found in these four sister-species showed that the isoforms formed two subsets. Each subset contained isoforms from all four species. The similarities within each subset were greater than between subsets, showing that sequence similarity between some of the isoforms was greater between species than within. This is in contrast to the situation when comparing homologue isoforms from the two more distantly related tenebrionid beetles *Tenebrio molitor* and *Dendroides canadensis* (Graham et al. 2007), where the isoforms are more similar within each species.

It is not clear if the evolutionary drive towards this high number of isoforms has been a selection towards some unknown specific isoform functionality or a selection towards augmenting protein production. Scott et al. (1985) pointed out that the ~40 genes coding for AFP type I in winter flounder seems very high, since protein production could be improved by other mechanisms than gene dosage, i.e. by enhanced transcription or translation rates or increased mRNA stability. The flounders produce their AFPs over periods of several weeks, and the high gene-number appears somewhat excessive. Swanson and Aquadro (2002) suggested that isoform diversity in the coleopteran *T. molitor* is the result of functional selection at the amino acid level, suggesting specific functionality. Graham et al. (2007) did not find support for this contention and suggested that selection instead has operated on the nucleotide level towards greater AT content at the third codon position. This nucleotide selection presumably facilitates transcription at low temperature and is functionally neutral at the protein level. Thus, the selection may have been towards a more effective expression rather than specific function. This is supported by the observations that populations of polar fish inhabiting warmer waters have lower gene

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506 dosage coding AF(G)Ps (Hew et al. 1988; Desjardins et al. 2012; Baalsrud et al.
507 2018; Yamazaki et al. 2019). On the other hand, Duman et al. (2002) found a specific
508 pattern of expression of different isoforms in the coleopteran *D. canadensis*, Ma
509 et al. (2012) found differential expression of two AFP isoforms from the coleopteran
510 *A. polita* and Doucet et al. (2000, 2002) found expression of some isoforms to be life
511 stage specific in the lepidopteran *C. fumiferana*, hinting to differentiation in isoform
512 function.

513 2.4 Synthesis and Distribution

514 Low temperature and short day-length are environmental cues of winter, and both
515 conditions have been shown to stimulate production of AFPs in insects (Duman
516 1977; Patterson and Duman 1978; Horwath and Duman 1983a; Ma et al. 2012), a
517 collembolan (Meier and Zettel 1997), and fish (Duman and DeVries 1974; Fourney
518 et al. 1984; Fletcher et al. 1989a). In addition, dry conditions and starvation also
519 stimulate AFP production in several insects (Duman 1977; Patterson and Duman
520 1978; Graham et al. 2000).

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521 Short day-length seem to act by affecting hormonal control of expression. In
522 winter flounder, expression of the liver type is strongly influenced by photoperiod,
523 acting through the central nervous system on the pituitary gland (Fourney et al. 1984;
524 Fletcher et al. 1989a). During the summer, long day-length causes release of growth
525 hormone from the pituitary that blocks transcription of AFP genes. As the day-length
526 shortens during fall, the level of growth hormone decreases, and transcription of
527 AFP genes in the liver ensues. Removal of the pituitary in individuals during
528 summer caused strong production of liver-type AFPs (Fourney et al. 1984; Fletcher
529 et al. 1989a). However, such removal does not affect the levels of skin-type AFPs,
530 suggesting that these genes are not under pituitary control (Gong et al. 1995). Since
531 the expression of skin-type AFPs are temperature sensitive, their regulation may be
532 post-transcriptional, with the half-life of their mRNAs being increased by low
533 temperature (Gong et al. 1995).

534 In the coleopterans *D. canadensis* and *T. molitor*, short day-length apparently
535 affects AFP production by affecting the level of juvenile hormone (Horwath and
536 Duman 1983b; Xu and Duman 1991; Xu et al. 1992), a hormone primarily released
537 from the corpus allatum. Individuals treated with juvenile hormone and kept under
538 long day-length conditions and room temperature produced high levels of AFPs,
539 while control individuals did not. In *D. canadensis*, addition of the anti-juvenile
540 hormone Precocene II prevented AFPs from being expressed under short photope-
541 riod at room temperature, while the untreated controls expressed AFPs. Precocene II
542 also prevented expression of AFPs in individuals kept under winter conditions
543 (Xu and Duman 1991). In isolated fat body cells, juvenile hormone induces tran-
544 scription in both *T. molitor* and *D. canadensis*, but only if the individuals had been
545 previously exposed to juvenile hormone (Xu and Duman 1991; Xu et al. 1992),

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suggesting that some factor(s) other than juvenile hormone is needed to induce AFP production.

In contrast to the environmental sensitivity of AFP expression seen in many species, that of the lepidopteran *C. fumiferana* seems to be strictly developmentally controlled. Individuals from different life stages expressed different levels of AFPs and these levels were quite insensitive to changing light conditions and temperatures (Doucet et al. 2002), and transcription levels are negatively affected by hormones in vitro (Qin et al. 2007).

2.4.1 Sites of Synthesis and Distribution in Polar Fish

Several sites of synthesis of AF(G)Ps have been identified. In Arctic species, a major source is the liver. These liver-type variants are exported directly into the blood stream. Contrary to longstanding belief, Cheng et al. (2006) showed that Antarctic notothenioids do not synthesize any of their AFGPs in the liver but uses the pancreas and associated tissues. Following synthesis, the AFGPs are released into the intestinal fluid via the pancreatic duct. Since the pancreas is the only identified site of production of AFGPs in Antarctic notothenioids, their circulating AFGPs have apparently entered their blood by uptake from the intestine. Cheng et al. (2006) also discovered that the pancreas was a second major site of synthesis in Arctic species producing all known types of AF(G)Ps. Since the intestinal fluid of polar fish expresses antifreeze activity (O'Grady et al. 1982; Præbel and Ramløv 2005; Cheng et al. 2006), a similar circulatory pattern relying on uptake of AF(G)Ps from the intestine may well be a second source of AF(G)Ps in the blood stream of non-notothenioid fishes, in addition to those secreted directly into their blood steam from the liver.

This indirect route from the site of synthesis via the intestinal fluid to the blood stream in Antarctic notothenioids probably reflects the importance of preventing ingested ice crystals from inoculating the intestinal fluid (Cheng et al. 2006); since the polar fishes are hypoosmotic to their environment they ingest seawater as part of their obligate osmoregulation. This potentially exposes them to ice crystals in the ingested water. In addition to the danger of direct inoculation of body fluids through the intestinal wall, such ingested ice crystals may potentially grow as salts are removed during the process of water uptake and the intestinal fluid becomes progressively hypoosmotic to seawater along the length of the intestine (O'Grady et al. 1983). The need to combat this danger has apparently caused the pancreas, with its direct connection to the intestinal fluid via the pancreatic duct, to become a major site of synthesis in diverse taxa of polar fishes and the only such site in Antarctic notothenioids.

Why do Arctic fishes rely on two major sites of synthesis of their blood-borne AF(G)Ps and the Antarctic notothenioids have only one? Perhaps it is due to differences in the need to rapidly augment the circulating levels of AF(G)Ps. The water temperatures of the Antarctic are permanently below freezing. Fishes living in

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587 these waters would have no need to rapidly augment the circulating amounts of AF
588 (G)Ps in response to environmental changes, i.e. have hepatic synthesis with a direct
589 excretion to the blood. Arctic fishes, on the other hand, may well need to augment
590 their antifreeze protection due to seasonal variations or because of migration into
591 colder waters, and the direct route from the site of synthesis in the liver to the blood
592 may be relevant.

593 The skin-type isoforms of type I AFP are synthesized in tissues that are exposed
594 to the exterior icy environment. These tissues include skin, gill filaments and dorsal
595 fins, in addition to intestine and brain (Gong et al. 1996; Low et al. 1998; Evans and
596 Fletcher 2006). In sculpin, there is no expression of skin-type genes in the liver (Low
597 et al. 1998), whereas co-expression of skin-type isoforms in liver does occur in
598 winter flounder (Gong et al. 1996).

599 Although all AF(G)P-producing polar fish contain AF(G)Ps in their blood, less is
600 known about their distribution in other body fluid compartments. The Antarctic
601 nototheniids produces AFGPs of eight distinct size groups. Ahlgren et al. (1988)
602 reported that all size groups of AFGPs are distributed passively throughout the
603 extracellular body fluids of two species of Antarctic nototheniids but they were not
604 present intracellularly. No AFGPs were found in the brain or urine, attributable to the
605 blood–brain barrier and the aglomerular kidneys of these fishes (see below). Bile
606 contains AFGPs, and O'Grady et al. (1983) argued that this is a route for transfer of
607 blood-borne AFGPs to enter the intestine. Evans et al. (2011) also observed injected
608 fluorescently tagged AFGPs in most extracellular fluids, except urine and brain.

609 For the Arctic winter flounder and shorthorn sculpin, the genes for their skin-type
610 AFPs lack coding regions for signal peptides, indicating that they are not excreted
611 from the cells but function intracellularly (Gong et al. 1996; Low et al. 1998). In
612 snailfish, however, the skin-type AFP I is identical to those circulating in blood,
613 suggesting excretion into the blood stream after synthesis (Evans and Fletcher
614 2005a). Also, liver-type AFP II from sea raven, *H. americanus,* is located in skin
615 tissue (Evans and Fletcher 2006), suggesting uptake of liver-type AFP II from the
616 blood or synthesis of similar AFPs in skin and liver. Low et al. (1998) also found
617 expression of skin-type AFPs in the brain of shorthorn sculpin. Thus, contrasting the
618 findings from the Antarctic nototheniids, several Arctic non-nototheniid species
619 have been shown to have AFPs in their cells and brain tissue.

620 **Preventing Urinary Loss of AF(G)Ps in Polar Fish** Loss of AF(G)Ps represents
621 an energetic cost to the organism. The apparent absorption of AFGPs from the
622 intestine in nototheniids (Cheng et al. 2006) probably reduces their loss during
623 evacuation of the gut. AF(G)Ps circulating in the blood, however, may potentially
624 be lost via the urine. Molecules with sizes below 68 kDa are filtered out in the
625 glomeruli (Eastman 1993), suggesting that AF(G)Ps may become filtered out of the
626 plasma during urine formation. Such filtration could be countered by energetically
627 costly reabsorption of AF(G)Ps from the filtrate. In Antarctic nototheniids, this
628 potential problem is effectively avoided by evolutionary degeneration of their
629 glomeruli (Eastman and DeVries 1986). Formation of urine in such aglomerular

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species is based on secretion rather than filtration, and the loss of AFGPs is effectively avoided (Dobbs and DeVries 1975; Eastman 1993).

Eastman et al. (1987) did not find aglomerular kidneys when examining diverse taxa of Arctic teleosts that produce AF(G)Ps. Instead, Arctic fishes have an anionic repulsion barrier in the basement membrane of the nephron. This repulsion barrier operates in the same manner as the mammalian anionic repulsion barrier (Kenwar et al. 1980), where carboxyl-rich glycoproteins in the basement membrane restrict filtration of anionic molecules, including anionic AF(G)Ps (Petzel and DeVries 1980; Boyd and DeVries 1983, 1986). The type I AFPs are reportedly repelled at the basement membrane by this mechanism (Petzel and DeVries 1980; Boyd and DeVries 1983). As mentioned above, the QAE and SP variants of AFP type III have opposite charges at physiological pH and both are present in the animal. Boyd and DeVries (1986) found that the AFP type III-producing northern eelpouts have glomerular kidneys and an anionic repulsion mechanism. Thus, although retention of the anionic QAE forms may be similar to that seen for the winter flounder type I AFPs, the cationic SP forms would be expected to filter out. Many of the Arctic fishes only express AF(G)Ps during parts of the year (Scott et al. 1985; Reisman et al. 1987). In these species, a means of reducing urinary loss may be to lower their glomerular filtration during winter (Hickman 1968). Interestingly, Eastman et al. (1979) found that, contrary to the northern eelpouts, the AFP III-producing Antarctic eelpout has non-functional glomeruli. In this case, there would be no problem with potential loss of the SP variants of the AFP type III, and the urine did not contain any AFP type III (Eastman et al. 1979).

Contrary to the earlier findings (Petzel and DeVries 1980; Boyd and DeVries 1986; Eastman et al. 1987), Fletcher et al. (1989b) did find AF(G)Ps in the urine of several Arctic species. These included type I AFP in the urine of winter flounder (*Pseudopleuronectes americanus*), type II AFP in the urine of sea raven (*Hemitripterus americanus*), type III AFP in the urine of ocean pout (*Macrozoarces americanus*) and AFGPs in the urine of Atlantic cod (*Gadus morhua*). There was no AFP type I in the urine of shorthorn sculpin (*Myoxocephalus scorpius*). The levels in the urine varied substantially, and the presence of relatively high concentrations of AFPs in the urine may be a consequence of concentrating small amounts of AFP from a large volume of urine by water reabsorption (DeVries and Cheng 2005). The presence of AF(G)Ps in the urine may be functional, as they presumably afford the same freeze protection to the urine as to other fluid compartments (Fletcher et al. 1989b).

2.4.2 Sites of Synthesis and Distribution in Insects

Only a few studies provide information on the site of synthesis and/or distribution of AFPs in insects. Taken together, these studies report the presence of AFPs in one or several of the different body fluid compartments hemolymph, gut fluid, pre-urine, muscular tissue and epidermal tissue (Duman et al. 2002; Nickell et al. 2013;

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671 Ramsay 1964; Graham et al. 2000; Kristiansen et al. 1999, 2005; Buch and Ramløv
672 2017; Guz et al. 2014). The fat body is the major site of protein synthesis in insects
673 (Arrese and Soulages 2010), and all species examined have shown synthesis of AFPs
674 in this organ. Other tissues shown to transcribe AFP genes are gut tissue, Malpighian
675 tubules and epidermis. All species examined have several isoforms of the AFPs, and
676 evidence exists of specific distribution of isoforms in body fluids and between life
677 stages.

678 Duman et al. (2002) reported on the expression and distribution of 12 isoforms in
679 the beetle *D. Canadensis*. These are divided into three groups, I, II and III, based on
680 their sequence similarity. Mature isoforms belonging to group I are only located in
681 the hemolymph whereas those of group II and III are located in the gut fluid. The
682 genes of all isoforms are transcribed in the fat body, whereas group II and III are also
683 transcribed in the gut tissue. In addition, there is expression of several of the isoforms
684 belonging to group I and II, but not III, in epidermal tissue. Nickell et al. (2013)
685 reported that 24 isoforms from *D. canadensis*, of which 18 were previously
686 unknown, were transcribed in the Malpighian tubules. Representatives of all groups
687 (I, II, III) were transcribed in the Malpighian tissue. Hysteresis activity in this species
688 has been reported from Malpighian tubule fluid, excreted rectal fluid (Nickell et al.
689 2013), gut fluid and hemolymph (Duman et al. 2002).

690 Ramsay (1964) observed hysteresis activity in all extracellular fluid compart-
691 ments of the closely related beetle *Tenebrio molitor*, except the fluid of the Malpi-
692 ghian tubules. The individuals tested by Ramsay were reared at room temperature.
693 These extracellular compartments included gut fluid, hemolymph and perirectal
694 fluids. Graham et al. (2000) reported transcription of AFPs in *T. molitor* in fat
695 body, midgut and hindgut but not in ovaries or the male reproductive tract.

696 Kristiansen et al. (1999) studied the hysteresis activity in different body fluid
697 compartments of the beetle *Rhagium inquisitor* and found activity in both gut fluid
698 and hemolymph. In addition, extracts of larval tissue, where hemolymph had been
699 washed away and fat body and gut removed by dissection, showed considerable
700 activity. These findings strongly suggested the presence of substantial amounts of
701 intracellular AFPs in the muscular tissues. In addition, extracts from the fat body also
702 showed high activity. Although the complete amino acid sequence of only a single
703 13 kDa isoform is known from *R. inquisitor*, Kristiansen et al. (2005) observed at
704 least six additional distinct activity peaks during ion exchange chromatography of its
705 hemolymph, suggesting that multiple isoforms are present in the hemolymph. Buch
706 and Ramløv (2017) used fluorescently tagged monoclonal antibodies raised against a
707 homologue single isoform of the closely related *R. mordax* and found that the protein
708 was present in gut tissue, gut fluid and cuticle. The pattern of fluorescence in summer
709 individuals was indicative of cellular storage of these AFPs during summer.

710 Guz et al. (2014) reported that the tentative AFP, EmAFP, from the hemipteran
711 *Eurygaster maura* only showed significant transcription levels for this protein in the
712 gut tissue. Only trace amounts of mRNA were detected in the fat body, ovary,
713 Malpighian tubules, trachea, heart, flight muscle or the nervous system.

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2.5 Characteristics of Ice-Binding Sites 714

The ice-binding sites (IBS) of AFPs are reportedly very planar and more hydrophobic than the rest of the structure (Yang et al. 1988; Sönnichsen et al. 1996; Haymet et al. 1998; Yang et al. 1998; Graether et al. 2000; Liou et al. 2000). The hydrophobic character of the IBS presumably causes the protein to orient away from the solution and towards the ice surface, whereas the flatness of the IBS is probably to obtain a good structural fit to the crystal plane. The planar character of the IBS of RiAFP is illustrated in Fig. 2.3. 715 716 717 718 719 720 721

The residues making up the ice-binding sites of AF(G)Ps are generally organized in a repetitive manner, resulting in repetitive distances between the residues. For instance, in the helical type II polyproline helix configuration proposed for the moderately active AFGPs (Franks and Morris 1978; Bush et al. 1984; Mimura et al. 1992; Tachibana et al. 2004), the repeat distance between hydroxyl groups of the disaccharide units is about 9.31 Å (Knight et al. 1993). This distance is very close to that between oxygen atoms in the ice lattice in the primary crystal plane oriented along the *a*-axis, the experimentally determined adsorption plane and orientation of these AFGPs (Knight et al. 1993). Similarly, for the moderately active AFP Type I, the 11-residue spacing between hydroxyl groups in the side chains of Thr residues in the α-helix is 16.5 Å, matching very closely the 16.7 Å spacing of oxygen atoms along a single direction on the crystal plane they are known to adsorb (Knight et al. 1991). In the β-helical AFPs, the width between hydroxyl groups of outwardly projecting Thr residues in the TxT motifs is about 7.4 Å within each β-strand. The length between strands is about 4.5 Å (Liou et al. 2000). These distances in the IBS of RiAFP are illustrated in Fig. 2.3 and occur between water molecules in multiple orientations on several crystal planes. 722 723 724 725 726 727 728 729 730 731 732 733 734 735 736 737 738

Exactly how AF(G)Ps adsorb onto ice crystals has been a topic of debate (Garnham et al. 2011). A number of studies have shown that AF(G)Ps have bound water molecules arranged in an ice-like lattice at their ice-binding sites (Liou et al. 2000; Leinala et al. 2002; Garnham et al. 2011; Hakim et al. 2013; Sun et al. 2014). In all likelihood these water molecules fuse with the solidifying ice surface at temperatures below the melting point and de-couple from the ice surface as the temperature is raised above the melting point. Essentially, the AF(G)Ps “freeze” onto and “melt” off the ice, depending on the temperature (Kristiansen and Zachariassen 2005). Thus, the functionality of the specific arrangement of residues in the IBS may well be to structure the hydration water at the ice-binding site rather than interacting directly with specific oxygens in ice (Sun et al. 2014; Chakraborty and Jana 2019). 739 740 741 742 743 744 745 746 747 748 749

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2.5.1 Moderately Active AF(G)Ps 750

In the moderately active fish AF(G)Ps, the ice-binding sites consist of residues organized in ways that restrict the AF(G)Ps to adsorb onto a single specific ice 751 752

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753 crystal plane and in a specific orientation on that plane. The specificity in absorption
754 orientation was documented by Laursen et al. (1994) who showed that chiral L and D
755 variants of AFP type I adsorbs at mirror image orientations at the same crystal plane.
756 Due to their single plane-specific adsorption, ice crystals in the presence of
757 moderately active fish AF(G)Ps obtain the shape of a hexagonal bipyramid
758 (e.g. Baardsnes et al. 2001; Loewen et al. 1998; Ewart et al. 1998). This shape is
759 the only possible shape that exposes a single protected plane towards the surround-
760 ing solution. Characteristically, such hexagonal bipyramid crystals freeze from their
761 apex at the hysteresis freezing point. Apparently, moderately active AF(G)Ps only
762 weakly protect the apexes of the bipyramidal crystals, which is the probable cause of
763 their moderate activity (Jia and Davies 2002).

764 ### *2.5.2 Hyperactive AF(G)Ps*

765 The IBS of the hyperactive AFPs, such as the β-helical forms found in many insects
766 (Table 2.2) have both a width and a length, enabling them to adsorb onto multiple
767 planes and in multiple orientations. The high occurrence of the β-helix folding
768 pattern among hyperactive AFPs may reflect the good 2D-fit between internal
769 residue-to-residue distances within the β-sheet and distances between oxygen
770 atoms in ice (Graether and Sykes 2004). This may have been the driving force that
771 caused today's abundance of this structural scaffold in unrelated AFPs (Table 2.2).
772 Interestingly, both the large hyperactive Maxi variant of fish AFP type I and the
773 hyperactive AFP from the collembolan snow flea obtain width and length of their
774 ice-binding sites by having several helixes side by side.
775 Crystals that form in the presence of hyperactive AFPs express several crystal
776 planes towards the surrounding solution. It is likely that their hyperactivity is caused
777 by their ability to adsorb onto multiple crystal planes and thereby effectively protect
778 the entire surface. The ability to adsorb onto the basal plane has been proposed as the
779 root cause of their hyperactivity (Liou et al. 2000; Graether et al. 2000; Pertaya et al.
780 2008).

781 ## 2.6 Conclusions

782 AF(G)Ps have independently evolved in many different groups of fish and arthro-
783 pods inhabiting cold regions. Their present-day taxonomic distribution reflects
784 complex evolutionary processes, where convergent evolution and lateral gene trans-
785 fer have led to both analogue and homologue structures being found in distantly
786 related species. The simple repetitive construction of the AFGPs, type I AFPs and
787 many AFPs found in arthropods, as a series of shorter repeat sequences, is presum-
788 ably the result of internal duplication of repeats that has resulted in functional genes.
789 The more complex structures (AFP type II and III) are apparently derived from

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functional proteins originally involved in binding of carbohydrates. In the case of the repetitive structures, they all fold into helical configurations with their IBS composed of regularly spaced residues located on one side of the coil.

Both fish and insects have a high gene dosage of AF(G)Ps that apparently is the result of gene duplication. All species examined have high numbers of isoforms, and it is unclear if this is due to a selective pressure towards divergence in isoform function or exclusively towards augmenting protein production. Several sites of synthesis have been identified in both fish and insects, and isoform-specific location of expression is prevalent. In many species, expression is regulated by environmental cues acting through hormonal mechanisms, but some species appear to be insensitive to such cues and expression may be linked to developmental stage.

In polar fish, both the site(s) of synthesis and mechanism(s) to prevent urinary loss of AF(G)Ps seem to be related to the permanence of their thermal environment; The Antarctic waters are permanently cold and thermally stable, whereas the temperature of Arctic waters vary with location and season. The AFGPs of Antarctic notothenioids take an indirect ("slow") route from their pancreatic site of synthesis to the blood via the intestine, whereas Arctic AF(G)P-producing species also have hepatic synthesis, affording them an additional direct ("fast") secretion from the liver to the blood. In Antarctic species, prevention of urinary loss of AF(G)Ps is primarily achieved by degeneration of the kidney-glomeruli, a permanent physiological adaptation to a constant environment. In Arctic species, on the other hand, a charge-based repulsion mechanism in the basement membrane of the nephron prevents urinary loss of AF(G)Ps, affording these species functional kidneys year-round.

The functionality of AF(G)Ps arises from the ability of their IBS to irreversibly adsorb onto the surface of ice crystals. The IBS is reportedly more hydrophobic than the rest of the protein surface, presumably orienting the IBS towards the ice. In the presence of moderately active AF(G)Ps, bipyramidal crystals are formed that exposes only a single protected crystal plane to the surrounding solution. In the presence of hyperactive AF(G)Ps, ice crystals expose several protected planes to the solution. These crystal habits must arise from features of the IBS. In moderately active fish AFGPs and AFP type I, the helical folding results in the IBS consisting of a single row of ice-binding residues, apparently affording these proteins the ability to only adsorb onto a single plane. In the hyperactive helical arthropod AFPs the IBS is made up of several parallel such rows of residues that cause the IBS to fit several planes and orientations. In some hyperactive AFPs the IBS is formed by several inter- or intramolecular helixes side by side. This organization of the helixes results in several parallel rows of ice-binding residues and consequently provide the necessary ability of the AF(G)P to adsorb onto multiple planes and orientations similar to other hyperactive AFPs.

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Query Refs.	Details Required	Author's response
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