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Article

Biochemical Adaptation to the Freezing Environment - the Biology of Fish Antifreeze Proteins

Zhengjun Li[1], Qingsong Lin[1], Woon-Kai Low[2], Megan Miao[3], and Choy L. Hew[1]*

[1]*Department of Biological Sciences, National University of Singapore Singapore, 119260*
[2]*Department of Pharmacology and Molecular Sciences, Medical Institutions Johns Hopkins University, Baltimore, MD, USA, 21205*
[3]*Division of Structural Biology, Hospital for Sick Children Toronto, Canada, M5G 1X8*

Abstract : Many organisms are known to survive in icy environments. These include both over wintering terrestrial insects and plants as well the marine fish inhabiting high latitudes. The adaptation of these organisms is both a fascinating and important topic in biology. Marine teleosts in particular, can encounter ice-laden seawater that is approximately 1°C colder than the colligative freezing point of their body fluids. These animals produce a unique group of proteins, the antifreeze proteins (AFPs) or antifreeze glycoproteins (AFGPs) that absorb the ice nuclei and prevent ice crystal growth. Presently, there are at least four different AFP types and one AFGP type that are isolated from a wide variety of fish. Despite their functional similarity, there is no apparent common protein homology or ice-binding motifs among these proteins, except that the surface-surface complementarity between the protein and ice are important for binding. The remarkable diversity of these proteins and their odd phylogenetic distribution would suggest that these proteins might have evolved recently in response to sea level glaciations just 1-2 million years ago in the northern hemisphere and 10-30 million years ago around Antarctica. Winter flounder, Pleuronectes americanus, has been used as a popular model to study the regulation of AFP gene expression. It has a built-in annual cycle of AFP expression controlled negatively by the growth hormone. The signal transduction pathways, transcription factors and promoter elements involved in this process have been studied in our laboratory and these studies will be presented.

Key words : low temperature adaptation, antifreeze glycoproteins, antifreeze proteins

1. Antifreeze proteins - adaptation to the freezing environment

Many organisms in ice-laden environments often encounter low temperatures below the equilibrium freezing point of their body fluid. Physiological and biochemical adaptations of these organisms to survive at low temperatures are fascinating and important topics in biology. One important strategy is the production of antifreeze glycoproteins (AFGPs) or antifreeze proteins (AFPs) (Davies and Sykes 1997; Fletcher *et al.* 2001). These macromolecular antifreezes depress the freezing-point of body fluids and protect the organisms from freezing to death during periods of $<0°C$. AF(G)Ps (including both AFGPs and AFPs) are currently being identified in many organisms exposed to freezing stress, including marine fish, insects, plants, fungi and bacteria (Davies and Hew 1990). The most striking characteristic of Cys-rich insect AFPs is their potent antifreeze activity as they are at least ten times more effective than fish AF(G)Ps on a molar basis. Interestingly, AFPs isolated from winter rye (*Secale cereale L.*) are recognized as homologues of plant pathogenesis-related proteins. These AFPs contain two class I endochitinase, two β-1,3-endoglucanase and two thaumatin-like proteins (Hon *et al.*

*Corresponding author. E-mail : dbshead@nus.edu.sg

1995). Normally, these three proteins are associated with pathogenesis response, but there is no thermal hysteresis activity of the corresponding enzymes from non-freeze tolerant plants. It is speculated that AFPs in winter rye are oligomeric complexes that increase the effectiveness in freeze tolerance (Yu and Griffith 1999).

One of remarkable characteristics of AF(G)Ps is that it can depress the freezing point of solutions in a noncolligative manner without significantly altering the melting point (Fletcher *et al.* 2001). The resulting difference between the melting and freezing points is termed thermal hysteresis, which is widely used as an indicator of antifreeze activity. Since AF(G)Ps function without affecting the colligative properties of the system, they do not affect the osmotic pressure, which could be lethal for organisms.

Another feature of AF(G)Ps is that it can alter ice crystal morphology. In the absence of AF(G)Ps, normal ice growth from the liquid phase at mild undercooling occurs faster along the prism face than the basal plane and forms a circular disc-like crystal (Chao *et al.* 1995). AF(G)Ps can bind to the prism face and the ice crystal can be modified from a circular plate to a hexagonal bipyramidal ice crystal or, in extreme cases, long ice spicule, depending on the AF(G)P concentration and the source of AF(G)Ps (Fig. 1). The exact shape of ice crystal is dependent upon the type of AF(G)P bound and reflects the affinity of the AF(G)P for different ice crystal surfaces. Therefore, ice crystal morphology can be used to compare the relative antifreeze activity when no measurable thermal hysteresis activity is available.

Inhibition of ice recrystallization is another important function of AF(G)Ps. Ice recrystallization is a process in which large crystals grow at the expense of small ones during frozen storage and thawing (Fig. 2) (Knight and Duman 1986; Griffith and Ewart 1995). The low thermal hysteresis activity of plant AFPs has led to the speculation that the major role of AFPs in plants is to inhibit ice recrystallization of extracellular ice formed when plants are exposed to the subzero temperatures.

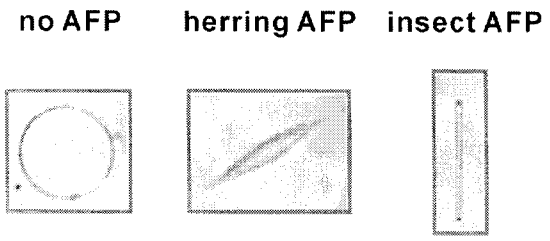

Fig. 1. Ice crystal morphology in the presence of AFPs.

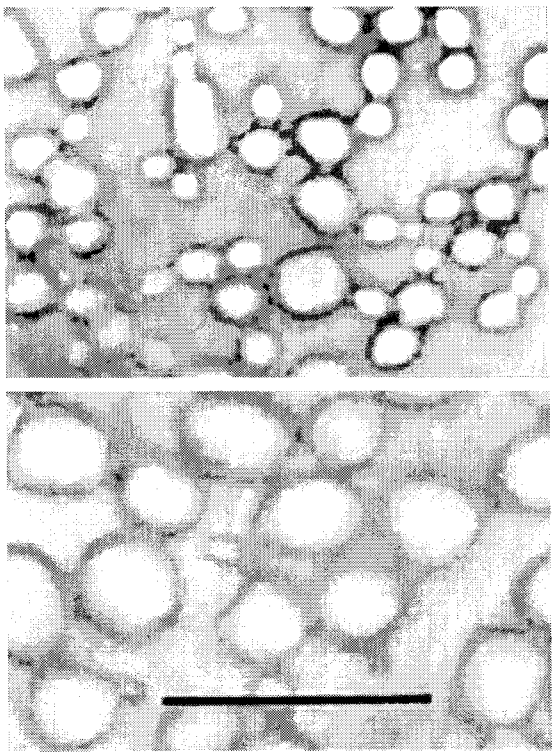

Fig. 2. Recrystallization inhibition activity of purified carrot AFP. The protein concentration was 1.5 μg/ml. Ice crystals remained small in the sample containing purified carrot AFP (a) compared with ice crystals in a control sample (b). Scale bar, 100 μm (Worrall *et al.* 1998).

Besides the ability of ice binding and freezing point depression, it has been shown that AF(G)Ps appear to protect mammalian cell membranes from damage at temperatures above 0°C (Wu and Fletcher 2000).

2. Fish AF(G)Ps

Although the equilibrium freezing (melting) point of fish body fluids is typically −0.7°C, many marine teleosts from polar oceans and north temperate seas survive in ice-laden seawater that is frequently as low as −1.9°C. Since fish AF(G)Ps at physiological concentrations can lower the freezing point by about 1°C, thermal hysteresis activity can, at least partially, explain why these antifreeze-producing fish do not freeze. To date, fish AF(G)Ps are most widely known and they are extensively studied for their function, mechanism of action, and control of gene expression. Five biochemical classes of AF(G)Ps are derived from fish, including AFGPs which contain carbohydrates, and four types of non-glycosylated AFPs defined as Type I to IV

Table 1. Characteristics of fish AF(G)Ps.

	AFGP	Type I	Type II	Type III	Type IV
Carbohydrate	yes	no	no	no	no
Molecular weight	2.6-34 kDa	3-7 kDa	14-24 kDa	6-7 kDa	12.3 kDa
Structure	Ala-Ala-Thr polymer with disaccharide on each Thr	Ala-rich, amphipathic α-helix	Cys-rich, globular	globular, 8 β-strands form β-sandwich	Gln-rich, four-helix bundle
Homologues or antecedents	trypsinogen gene for notothenioids	not found	C-type lectin	sialic acid synthase	apolipo-protein E3
Fish species	Antarctic notothenioids, Northern cod	righteye flounder, sculpin	herring, smelt, sea raven	eel pout, wolffish	longhorn sculpin

based on their diverse structures (Davies and Sykes 1997). Characteristics of these AF(G)Ps are summarized in Table 1. They are biochemically extremely divergent, which include their primary, secondary, and tertiary structures. Despite their fundamental differences, they converged functionally and have been shown to similarly bind to ice crystals.

AFGPs

About thirty years ago, DeVries, Feeney, and their colleagues discovered the first macromolecular antifreeze AFGPs (DeVries and Wohlschlag 1969; DeVries *et al.* 1970). These proteins are present in the blood plasma of Notothenioid fish in the Antarctic Ocean and cods from Arctic and subarctic waters. The most studied glycoproteins are from two Antarctic fish species, *Trematomas borgrevinki* and *Dissostichus mawsoni*, and from a northern fish, *Boreogadus saida*.

Eight isomers of AFGPs with molecular mass varying from 2,600 to 34,000 Da, have been isolated. These isomers differ primarily depending on the number of the glycotripeptide repeats, which varies from 4 to 50 (Davies and Sykes 1997). The peptide backbones of AFGPs are made up of a tripeptide repeat $(Ala\text{-}Ala\text{-}Thr)_n$, with a disaccharide β-D-galactosyl-(1→3)-α-D-N-acetyl-galactosamine attached via a glycoside linkage to the hydroxyl side chain of the Thr of each repeat. In some smaller AFGPs, the first Ala is replaced by Pro.

Nuclear Magnetic Resonance (NMR), solution Raman spectroscopy, and circular dichroism spectroscopy have been used to characterize the secondary structure of the AFGPs (Barrett 2001). The experimental evidence suggests that AFGPs exist in solutions as extended left-handed threefold helices with the carbohydrate moiety oriented at one side of the molecule and the hydrophobic amino acid backbone on the other. A γ-turn motif has also been proposed based on the data from Raman and infrared spectrometry. Both conformations allow all disaccharides to be positioned on one side of the molecule to interact with the ice surface. Nevertheless, the compact structures of AFGPs for ice binding have been questioned by recent NMR studies. It has been observed that larger AFGPs are essentially random coils in solutions, even though smaller AFGP analogues exhibit some local structures.

Type I AFPs

Type I AFPs are Ala-rich (>60%) proteins found in many righteye flounders (*Pleuronectes*) and sculpins (*Myoxocephalus*) (Davies and Sykes 1997; Fletcher *et al.* 2001). The most widely studied is from the winter flounder. Type I AFPs can be classified into two subtypes, liver type and skin type, based on their different cellular localizations. Liver-type AFPs are produced primarily in the liver as preproAFPs and secreted into the circulatory system, where they undergo protease processing to become functional AFPs. Skin-type AFPs are widely distributed, and abundant in skin and other exterior tissues. They are synthesized as mature polypeptides without signal sequences. This implies that they might function intracellularly. Immunohistochemistry results confirmed that in winter flounder, skin-type AFPs were restricted to the cytoplasm of the gill epithelial cells. However, they could be located only in the interstitial space of the skin, suggesting an alternative secretion pathway that bypasses the Golgi apparatus (Fletcher *et al.* 2001).

These AFPs are α-helical polypeptides with molecular mass ranging from approximately 3 to 7 kDa. Eleven-amino-acid-repeats $Thr\text{-}X_{10}$, where X is primarily Ala, are basic components in AFPs of all flounders and some sculpins (Fletcher *et al.* 2001). The three-dimensional structure of wflAFP-6 (previously named as HPLC-6), a major AFP in winter flounder, revealed a single α-helix. It further indicated that polar residues of the AFP are located mostly on one side of the helix, whereas the nonpolar Ala and other hydrophobic residues reside primarily on the

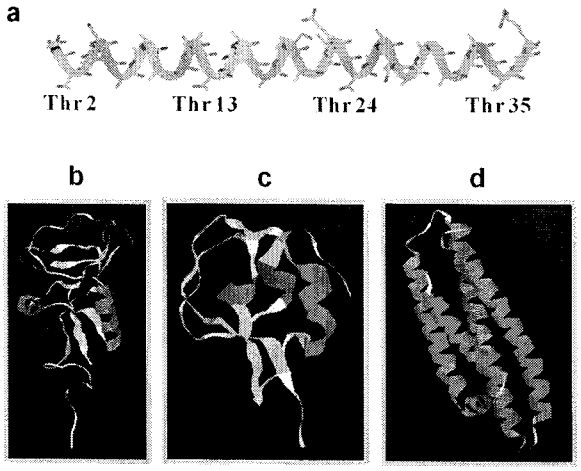

Fig. 3. Structure of fish AFPs. (a) X-ray crystal structure of wflAFP-6 in winter flounder. (b) 3D NMR structure of type II AFP in sea raven. (c) X-ray crystal structure of type III AFP in ocean pout. (d) Structure of apolipoprotein E3, type IV AFP homologue (Fletcher *et al.* 2001).

other side with some exceptions (Fig. 3a). Sicheri *et al.* further demonstrated the existence of internally hydrogen-bonded N- and C-terminal cap structures, which are known to increase stability of α-helical peptides (Sicheri and Yang 1995). These cap structures combined with the intra-helical hydrogen bonds, the salt bridge and the dipole interactions at the N- and C- termini, all contribute towards stabilization of the helices.

Type II AFPs

Type II AFPs are Cys-rich globular proteins with a molecular mass ranging from 14 to 24 kDa, which are found in herring (*Clupea harengus harengus*), smelt (*Osmerus mordax*), and sea raven (*Hemitripterus americanus*) (Davies and Sykes 1997; Fletcher *et al.* 2001). These AFPs show no homology to other AFPs, but they are homologous to the carbohydrate-recognition domain (CRD) of the long-form Ca^{2+}-dependent (C-Type) lectin-like domain based on sequence identity and on a similar fold found in the solution structure of sea raven AFP (Fig. 3b). Determination of the solution structure of sea raven AFP revealed a single globular domain that consisted of two helices and nine β-strands forming two β-sheets.

All type II AFPs contain five disulphide bridges that are necessary for activity (Davies and Sykes 1997). A further requirement for herring and smelt AFP activities is the need for Ca^{2+}. Furthermore, examination of herring AFP by site-directed mutagenesis demonstrated that the ice-binding surface corresponded to the carbohydrate-binding site of C-type lectin CRDs. In contrast, sea raven AFP binds to ice in the absence of Ca^{2+}, and possesses only two of the five amino acids required for Ca^{2+}-binding present in lectins. Mutational analysis has demonstrated that the Ca^{2+}-binding region is not involved in ice binding. Genomic comparisons suggested that the sea raven AFP is more closely related to pancreatic stone protein than to CRD, and further mutational analysis suggested that sea raven AFP ice-binding activity resides in the site corresponding to the $CaCO_3$-binding surface of pancreatic stone protein.

Type III AFPs

Type III AFPs, isolated from zoarcid fish such as ocean pout (*Macrozoarces americanus*), eel pouts (*Rigophila dearborni*), as well as wolffish (*Anarhichas lupus*), are small globular proteins with no obvious bias in terms of amino acid composition (Fletcher *et al.* 2001). The molecular mass of type III AFPs is between 6,000 and 7,000 Da. These AFPs can be divided into two homology groups, QAE and SP. The pIs of the QAE forms are just below neutrality, whereas the SP forms have basic pIs.

X-ray structures of both QAE and SP type III AFPs have been determined (Fletcher *et al.* 2001). In addition, the NMR structure of QAE type III AFP is also available (Sönnichsen *et al.* 1996). These data indicate that type III AFP has a compact, angular structure, in which the general fold comprises many short, imperfect β-strands, and one turn of α-helix in the loop region together with numerous β-structure-like interstrand main chain hydrogen bonds that give the protein a rigid, globular fold (Fig. 3c). Combined with the extensive mutagenic studies, a flat surface, on which most of the hydrophilic residues are clustered together, is regarded as the ice-binding surface. However, understanding the binding mechanism of type III AFPs is complicated by the fact that they appear to make energetically favourable interactions with several ice surfaces (Antson *et al.* 2001).

Type IV AFPs

The recently isolated type IV AFP from longhorn sculpin (*Myoxocephalus octodecimspinosis*) is 12.3 kDa (Deng *et al.* 1997). Comparison of the primary structures reveals that this AFP is related to the low-density lipoprotein receptor-binding domain of human apolipoprotein E3. X-ray crystallography of apolipoprotein E3 indicates that it forms a helix bundle as shown in Fig. 3d. The secondary structure of this AFP contains a highly α-helical conformation, suggesting that the higher order structure of this

protein may be a helix bundle.

3. Ice-binding mechanism

AF(G)Ps, particularly fish AF(G)Ps, have contributed a great deal to the understanding of basic principles underlying protein-ice recognition. A widely accepted ice-binding mechanism of AF(G)Ps is adsorption inhibition (Hew and Yang 1992). According to this mechanism, AF(G)P is able to generate thermal hysteresis activity by: (i) adsorption to the prism planes; (ii) prevention of addition of water molecules to the ice lattice; (iii) efficient inhibition of the energetically favorable *a*-axis growth; and (iv) subsequent inhibition of further ice crystal growth. This postulated mechanism of noncolligative freezing point depression is caused by the Kelvin effect (Hew and Yang 1992; Fletcher *et al.* 2001). The Kelvin (or Gibbs-Thompson) effect results in the lowering of the local freezing point of ice contributed by a curved growth front generated by the bound AF(G)Ps. This curved front increases surface free energy, and subsequently inhibits further water addition and crystal growth until a critical temperature is reached (Fig. 4).

However, an understanding of the driving forces of AF(G)Ps to inhibit ice crystal growth at the molecular level is still under investigation (Davies *et al.* 2002). It has been proposed that binding of AF(G)Ps to ice surfaces likely involves hydrogen bonding between the polar residues and the ice surface. However, mutagenesis studies on examining the role of Thr residues in type I AFP has led to a reevaluation of the important role of hydrogen bonds in protein-ice interaction. Substitution of two Thr residues with Val was generated to conserve the methyl group of Thr, but not its hydroxyl group. The activity of the Val variant was equal to that of the wild type AFP, demonstrating the key role of the Thr methyl group in ice binding in type I AFP. These results suggest that van der Waals interactions between protein and ice are the major enthalpic contribution to ice binding, with entropic contribution from the release of surface associated water being the driving force.

Recent studies indicate a basic requirement for AF(G)Ps to inhibit ice crystal growth is the surface-surface complementarity between the AF(G)Ps and ice (Liou *et al.* 2000; Davies *et al.* 2002; Jia and Davies 2002). Close contacts derived from this surface match suggest a prominent role for van der Waals packing interactions and result in the irreversible ice-binding of AF(G)Ps. X-ray crystallography of wflAFP-6 demonstrates regular spacing of Thr, Leu, and Asx side chains along the helix (Fig. 5). The *i*, *i* + 11 spacing of the repeating residues places them 16.5 Å apart on the same surface of the helix. This distance matches the 16.7-Å spacing between equivalent ice lattice oxygen atoms. Similar to

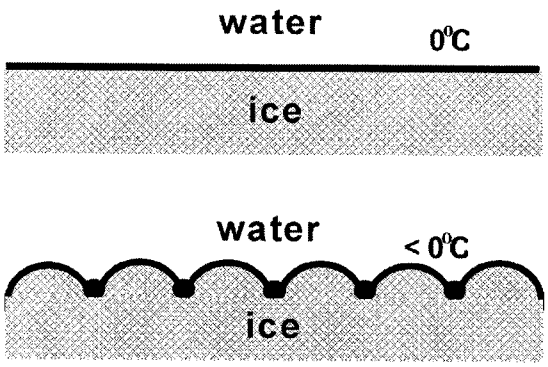

Fig. 4. Kelvin effect of ice growth inhibition by AF(G)Ps. A raised curvature on the surface of ice between bound AF(G)Ps lowers the local freezing point.

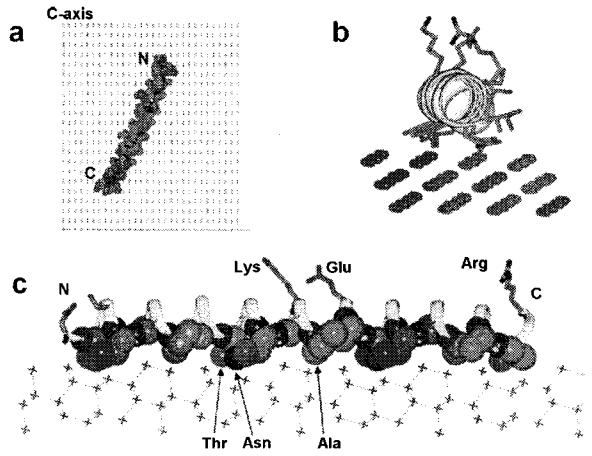

Fig. 5. Model of wflAFP-6 being adsorbed in (20$\bar{2}$1) ice plane. The protein binds in [0$\bar{1}$12] direction. (a) Top view normal to the ice plane. The N-terminus is located at the top (toward the *c*-axis). (b) Helical view looking directly at the C-terminus of the protein. Overall helicity of the protein is maintained in this final conformation. The spheres indicate oxygen atoms of water in the ice crystal. (c) Space filling model, showing structural fit between the ice plane and the protein. Shorter side chains of Ala sit on the ridge and Thr and Asn side chains fit into the valley, whereas the bulkier side chains (Lys, Glu and Arg) face away from the ice (Dalal and Sönnichsen 2000).

type I AFPs, analyses of the globular type III AFP of eel pout X-ray structure together with site-directed mutagenesis further support the importance of the complementary ice-binding site (Jia *et al.* 1996). However, the possibility that hydrogen bonding may play a role in other AF(G)Ps, such as AFGP and type II AFPs to dock on the ice surface could not be ruled out completely. Additional specificity and stability of binding can be provided through hydrogen bonding as well. Therefore, a definitive mechanism, which involves hydrophobic and/or hydrophilic interactions combined with the role they play in adsorption of the AF(G)Ps to the ice surface, needs further exploration.

So far, only the static models are available to elucidate the molecular mechanism of action for AF(G)Ps due to the fact that the ice-water interface has not been well characterized. Recent evidence shows that loss of organized ice structure at the interface is fairly gradual. So, the interface seems unlikely to be an abrupt transition state as typically represented in the static models. Further complications have arisen from the complexity of ice structure and the fact that different AF(G)Ps are able to bind to specific surfaces of ice (Davies and Sykes 1997). Computational simulations and dynamic modeling studies on interactions between AF(G)Ps and the transition state of water-ice will help to gain a molecular insight into the ice-binding mechanism of AF(G)Ps at the water-ice interface.

4. Evolution of fish AF(G)Ps

It is generally suggested that evolution of AF(G)Ps occurred very recently in geological time, as a direct response to the appearance of ice in seawater. The thermal isolation of Antarctica and its surrounding Southern Ocean was estimated to have been initiated approximately 22 million years ago (mya) (Cheng 1998), and the first geological evidence for ice in seawater dates back to approximately 10-30 mya (Fletcher *et al.* 2001). It has been estimated that the notothenioid AFGPs were converted from their progenitor gene approximately 5-14 mya, which correlates well with the estimated time for the chilling of the Southern Ocean (10-14 mya), and correlates with the burst of radiation in the five notothenioid families. The onset of Arctic glaciation is believed to have occurred much more recently, approximately 2.5 mya, with geological evidence of seawater ice dating back to 1-2 mya. Furthermore, it is believed that the initial glaciation was followed by cyclical glacial advances and retreats. The more recent, and cyclical nature of glacial advances and retreats in the north may in part be the cause of the vast structural diversity of AF(G)Ps. The northerly fish AF(G)Ps may have evolved independently in distinct species on several occasions due to changing environmental pressures, compared with the greater uniformity of the Antarctic

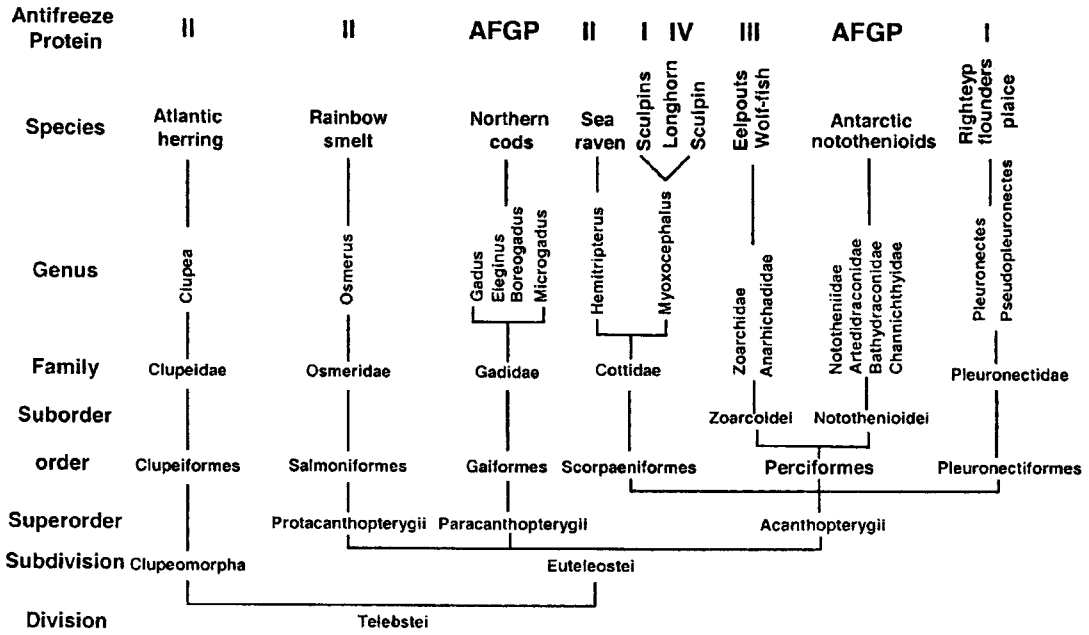

Fig. 6. Evolutionary relationship of AF(G)P-producing teleost fish. The tree is not to scale (Cheng 1998).

AFGPs, where conditions have been more constant, and cooling occurred much earlier.

It is apparent that the distribution of AF(G)P types in teleosts does not match taxonomic relationships (Fletcher *et al.* 2001). Different types of AF(G)Ps can occur in closely related species, while the same type can occur in rather distant species (Fig. 6). Since different types of AF(G)Ps have dramatically different structures, they are not likely to have evolved from a common origin. As ice can present many different faces, any proteins that have an affinity to certain ice surface(s) might have a chance to evolve into AF(G)P. Thus, occurrences of different types of AF(G)Ps result from convergent evolution. Occurrences of the same types of AF(G)Ps in distant species, as addressed below, are also believed to be the results of convergent evolution or convergence in parallel.

AFGPs are found in both Antarctic Nototheniids and northern cods. Despite the high structural similarity at the protein level, their genes appeared to be unrelated (Cheng and Chen 1999). They have different intron-exon structures and different signal sequences, use different codon sets for the Ala-Ala-Thr repeats, and the AFGP units in the polyprotein precursor are flanked by different sequences. It appears that the Notothenid AFGP gene may have evolved from a trypsinogen gene (Fletcher *et al.* 2001), while the precursor of the cod AFP gene has not been identified. Thus, the occurrence of AFGPs appears to be a result of recent convergent evolution. Type II AFPs are found in herring, smelt, and more distantly related sea raven. All three AFPs show sequence and structural similarities to CRD of C-type lectins (Fletcher *et al.* 2001). It is unlikely that these three type II AFPs are related by direct descent from a common progenitor AFP, which was converted from a C-type lectin before all three fish species diverged. Otherwise, type II AFP would pre-exist in a large section of the teleost radiation. A more plausible explanation is that these type II AFPs have evolved from C-type lectins on separate occasions, so-called convergence in parallel. Type I AFPs from flounders and sculpins and other fish species are also likely to be derived from a common lineage on more than one occasion, although no ancestors have been identified so far. Type III AFPs are unique to zoarcid species and the similarities in some isoforms shared by northern and southern hemisphere zoarcids is greater than the most diverse isoforms within one species (Fletcher *et al.* 2001). Thus, it is believed that type III AFPs occurred earlier than the dispersion of zoarcid species. Sequence comparison studies suggested that type III AFPs are homologous to the C-terminal region of sialic acid synthase (Baardsnes and Davies 2001). Similarly, sequence comparison implied that the type IV AFP from longhorn sculpin may share a common origin with the low-density lipoprotein receptor-binding domain of human apolipoprotein E3 (Deng *et al.* 1997).

5. AF(G)P production and gene regulation

Most fish AF(G)Ps are produced in large amounts and as multiple isoforms due to the existence of multiple copies of AF(G)P genes, most of which are arrayed in tandem repeats. For example, physiological concentration of AFP in winter flounder serum can be as high as 10 mg/ml. There are about 30-40 copies of genes encoding with at least 9 different AFP isoforms.

The production of most AF(G)Ps shows a distinct seasonal cycle, with considerable variation in the timing of production among species. As AF(G)Ps are such a diverse group of proteins, it is not unlikely that their genes are regulated in different ways. However, so far only the production of winter flounder AFPs has been extensively studied (Fletcher *et al.* 2001) (Fig. 7). Winter flounder has both liver- (wflAFP) and skin-type (wfsAFP) AFPs. The wflAFP level in the serum can be a thousand times higher in winter than in summer, while the wfsAFP level only shows minor seasonal variation (about five to ten times

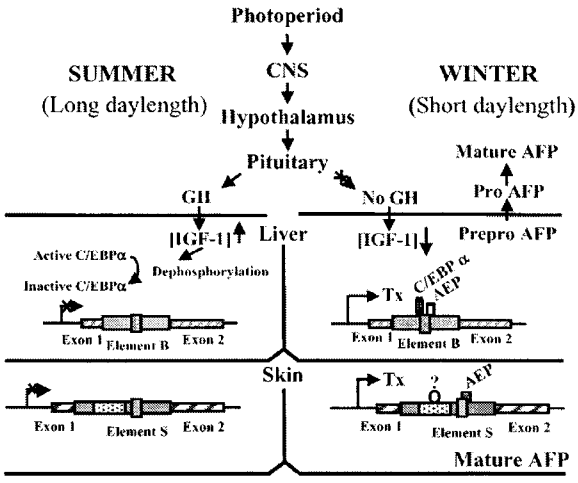

Fig. 7. Model for hormonal and tissue-specific regulations of the wflAFP and wfsAFP genes. Abbreviations: CNS, central nervous system; GHRH, growth hormone-releasing hormone; GH, growth hormone; IGF-1, insulin-like growth factor-1; C/EBPα, CCAAT/enhancer-binding protein α; AEP, antifreeze enhancer-binding protein, Tx, transcription (Miao *et al.* 1998).

higher in winter than in summer). Therefore, the mechanism of gene regulation for these two types of flounder AFPs should be different.

Photoperiod has been shown to be a major determinant for the seasonal variation of wflAFPs. It acts through the central nervous system to control the release of the growth hormone (GH) from the pituitary gland (Fletcher *et al.* 2001). During the summer months, release of GH stimulates synthesis of an insulin-like growth factor (IGF-1) from the liver, which inhibits liver-type AFP gene enhancer activity. The enhancer is located within the only intron of the AFP gene and has been defined as a 19 bp fragment called Element B. It contains presumptive DNA-binding motifs for the C/EBPα transcription factor and an AP-1 like protein termed an antifreeze enhancer-binding protein (AEP). IGF-1 causes dephosphorylation and deactivation of C/EBPα and/or alters the level of AEP expression, resulting in transcriptional inhibition of liver-type AFP genes. With loss of long daylength in autumn, production of GH is inhibited. Hence, the transcriptional repression is released. C/EBPα and AEP become active and/or their levels elevated, and their interaction with the core enhancer Element B activates wflAFP gene expression.

WfsAFP genes also have an enhancer sequence in their intron called Element S. It only differs from Element B by the central insertion of a TA dinucleotide, which results in loss of C/EBPα binding ability. This might be responsible for the more ubiquitous expression of wfsAFP genes in different tissues. It may also be the reason why the expression of wfsAFP genes is largely not affected by seasonal or hormonal changes.

6. Summary

AF(G)Ps in fish demonstrate an interesting adaptation to low temperatures. Although these proteins have a common ability to inhibit ice crystal growth, they are structurally diverse in binding to specific ice surfaces and evolve from various evolutionary precursors to adapt to the freezing environment.

References

Antson, A.A., D.J. Smith, D.I. Roper, S. Lewis, L.S. Caves, C.S. Verma, S.L. Buckley, P.J. Lillford, and R.E. Hubbard. 2001. Understanding the mechanism of ice binding by type III antifreeze proteins. *J. Mol. Biol.*, 305, 875-889.

Baardsnes, J. and P.L. Davies. 2001. Sialic acid synthase: the origin of fish type III antifreeze protein? *Trends Biochem. Sci.*, 26, 468-469.

Barrett, J. 2001. Thermal hysteresis proteins. *Int. J. Biochem. Cell Biol.*, 33, 105-117.

Chao, H., C.I. DeLuca, and P.L. Davies. 1995. Mixing antifreeze protein types changes ice crystal morphology without affecting antifreeze activity. *FEBS Lett.*, 357, 183-186.

Cheng, C.H. 1998. Evolution of the diverse antifreeze proteins. *Curr. Opin. Genet. Dev.*, 8, 715-720.

Cheng, C.H. and L. Chen. 1999. Evolution of an antifreeze glycoprotein. *Nature*, 401, 443-444.

Dalal, P. and F.D. Sönnichsen. 2000. Source of the ice-binding specificity of antifreeze protein type I. *J. Chem. Inf. Comput. Sci.*, 40, 1276-1284.

Davies, P.L. and C.L. Hew. 1990. Biochemistry of fish antifreeze proteins. *FASEB J.*, 4, 2460-2468.

Davies, P.L. and B.D. Sykes. 1997. Antifreeze proteins. *Curr. Opin. Struct. Biol.*, 7, 828-834.

Davies, P.L., J. Baardsnes, M.J. Kuiper, and V.K. Walker. 2002. Structure and function of antifreeze proteins. *Phil. Trans. R. Soc.* Lond. B, 357, 927-935.

Deng, G., D.W. Andrews, and R.A. Laursen. 1997. Amino acid sequence of a new type of antifreeze protein, from the longhorn sculpin *Myoxocephalus octodecimspinosis*. *FEBS Lett.*, 402, 17-20.

Fletcher, G.L., C.L. Hew, and P.L. Davies. 2001. Antifreeze proteins of teleost fishes. *Annu. Rev. Physiol.*, 63, 359-390.

Griffith, M. and K.V. Ewart. 1995. Antifreeze proteins and their potential use in frozen foods. *Biotechnol. Adv.*, 13, 375-402.

Hew, C.L. and D.S. Yang. 1992. Protein interaction with ice. *Eur. J. Biochem.*, 203, 33-42.

Jia, Z., C.I. DeLuca, H. Chao, and P.L. Davies. 1996. Structural basis for the binding of a globular antifreeze protein to ice [published erratum appears in Nature 1997 Feb 6; 385(6616):555]. *Nature*, 384, 285-288.

Jia, Z. and P.L. Davies. 2002. Antifreeze proteins: an unusual receptor-ligand interaction. *Trends Biochem. Sci.*, 27, 101-106.

Knight, C.A. and J.G. Duman. 1986. Inhibitor of recrystallization of ice by insect thermal hysteresis proteins: A possible cryoprotective role. *Cryobiology*, 23, 256-262.

Liou, Y.C., A. Tocilj, P.L. Davies, and Z. Jia. 2000. Mimicry of ice structure by surface hydroxyls and water of a beta-helix antifreeze protein. *Nature*, 406, 322-324.

Miao, M., S.L. Chan, C.L. Hew, and G.L. Fletcher. 1998. Identification of nuclear proteins interacting with the liver-specific enhancer B element of the antifreeze protein gene in winter flounder. *Mol. Mar. Biol. Biotechnol.*, 7, 197-203.

Sicheri, F. and D.S. Yang. 1995. Ice-binding structure and mechanism of an antifreeze protein from winter flounder. *Nature*, 375, 427-431.

Sönnichsen, F.D., C.I. DeLuca, P.L. Davies, and B.D. Sykes. 1996. Refined solution structure of type III antifreeze protein: hydrophobic groups may be involved in the

energetics of the protein-ice interaction. *Structure*, 4, 1325-1337.

Worrall, D., L. Elias, D. Ashford, M. Smallwood, C. Sidebottom, P. Lillford, J. Telford, C. Holt, and D. Bowles. 1998. A carrot leucine-rich-repeat protein that inhibits ice recrystallization. *Science*, 282, 115-117.

Wu, Y. and G.L. Fletcher. 2000. Efficacy of antifreeze protein types in protecting liposome membrane integrity depends on phospholipid class. *Biochim Biophys Acta*, 1524, 11-16.

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