Annu. Rev. Physiol. 2001. 63:359–90
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ANTIFREEZE PROTEINS OF TELEOST FISHES

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Key Words thermal hysteresis, ice, evolution, natural selection, gene expression

■ **Abstract** Marine teleosts at high latitudes can encounter ice-laden seawater that is approximately 1°C colder than the colligative freezing point of their body fluids. They avoid freezing by producing small antifreeze proteins (AFPs) that adsorb to ice and halt its growth, thereby producing an additional non-colligative lowering of the freezing point. AFPs are typically secreted by the liver into the blood. Recently, however, it has become clear that AFP isoforms are produced in the epidermis (skin, scales, fin, and gills) and may serve as a first line of defense against ice propagation into the fish. The basis for the adsorption of AFPs to ice is something of a mystery and is complicated by the extreme structural diversity of the five antifreeze types. Despite the recent acquisition of several AFP three-dimensional structures and the definition of their ice-binding sites by mutagenesis, no common ice-binding motif or even theme is apparent except that surface-surface complementarity is important for binding. The remarkable diversity of antifreeze types and their seemingly haphazard phylogenetic distribution suggest that these proteins might have evolved recently in response to sea level glaciation occurring just 1–2 million years ago in the northern hemisphere and 10–30 million years ago around Antarctica. Not surprisingly, the expression of AFP genes from different origins can also be quite dissimilar. The most intensively studied system is that of the winter flounder, which has a built-in annual cycle of antifreeze expression controlled by growth hormone (GH) release from the pituitary in tune with seasonal cues. The signal transduction pathway, transcription factors, and promoter elements involved in this process are just beginning to be characterized.

INTRODUCTION

It has been thirty years since DeVries and colleagues (1, 2) first discovered antifreeze proteins (AFPs) in the blood plasma of Antarctic Nototheniids, thus establishing the paradigm that such proteins are essential to the survival of marine teleosts inhabiting ice-laden waters. This discovery opened up a fascinating and

exciting field of research into the role such proteins play in preventing or reducing the damage caused by freezing to living organisms. AFPs have an appeal to scientists and lay-folk alike, for they can be found in many life forms—bacteria, fungi, plants, insects, and vertebrates—that encounter, or are in danger of encountering, freezing conditions in nature. AFPs are a subject of study in many disciplines; from ice physics to chemistry, from molecular biology to physiology, and from fisheries oceanography and ecology to evolutionary biology, and in recent years they have sparked the interest of the business community.

For over 20 years since their initial discovery, it was believed that AFPs were produced centrally by the liver and secreted into the blood to be distributed throughout the extracellular space. These proteins were thought to prevent the fish from freezing in ice-laden seawater, solely by reducing the freezing temperature of the extracellular fluids to safe levels. However, this line of thinking changed completely with the discovery that the winter flounder possesses two distinctly different antifreeze gene families, one of which is expressed in the liver to provide a central supply of antifreeze to the blood, and another that is expressed predominantly in gill and skin epithelia for the exclusive protection of the cells and tissues that come into direct contact with external ice (3). In addition to conferring freeze protection to the whole animal and to external epithelia, emerging evidence indicates that AFPs can interact with mammalian cell membranes and protect them from cold damage. This provocative information suggests that these proteins may also play a role in the cold acclimation process itself as reviewed by Fletcher et al (4). These recent discoveries, particularly the latter, have yet to stand the test of time. However, they have certainly prompted us to reexamine our thinking and views about the physiological functioning of AFPs in fish.

The topic of AFPs in fish was discussed in the *Annual Review of Physiology* 1983 (5), and there are a number of excellent recent reviews on AFPs to which the reader should refer (4, 6–10). Rather than try to present all aspects of AFPs in fish, the present review focuses on topics that are of current interest: (*a*) the enduring puzzle of how AFPs bind to ice; (*b*) the largely unexplored area of AFP gene expression, including tissue specificity, by which protection from freezing is afforded to the whole organism in a metabolically cost-effective manner; and (*c*) the remarkable examples AFPs provide of recent evolution in response to environmental change.

ANTIFREEZE PROTEINS AND ICE

It has been more than forty years since Scholander and colleagues traveled to the coast of Labrador to determine why marine teleosts do not freeze during the winter when the water temperature (−1.9°C) declined a full degree below the freezing point of their body fluids (−0.7°C) (11). They never found out why the fish did not freeze. However, they did discover that although some fish could survive in an undercooled state, if they were brought into contact with ice they immediately

froze and died. This established the fundamentals of the problem for teleost fish: the combination of undercooling and ice contact is lethal.

Ten years later DeVries & Wohlschlag (1) found that the answer to Scholander's question resided with a family of plasma proteins that could lower the freezing temperature of the blood several hundred times better than any other known dissolved solute. This non-colligative lowering of the freezing point or thermal hysteresis (12) is entirely attributable to the structure of these unique proteins that enables them to bind to and prevent embryonic ice crystals from growing (13). These plasma proteins, collectively termed AFPs, are primarily synthesized in the liver and secreted into the blood stream to be distributed throughout the extracellular and interstitial space.

Antifreeze Activity Assays: Thermal Hysteresis

Thermal hysteresis is readily measured in vitro with the use of a Clifton nanoliter osmometer. Sub-microliter volumes of AFP solution are introduced into an oil droplet held by surface tension in a cylindrical well drilled into a metal plate, which is placed on a cooling stage and viewed under a microscope (32x magnification). Sample temperature is controlled by a Peltier device with a read-out in mOsmols, where 1000 mOsmols corresponds to 1.86°C. Observation through a microscope provides opportunities for still and video photography to record ice crystal morphology and monitor the absence of growth during the thermal hysteresis measurement.

One variable that can influence the thermal hysteresis reading is the rate of cooling. We lower the stage temperature by 10 mOsmol increments at 15 s intervals. When these parameters are followed, the readings are consistent and reproducible when compared both in-house and on nanoliter osmometers at other locations, and thermal hysteresis is not significantly increased by slower cooling. However, more rapid cooling can substantially decrease the non-equilibrium freezing point and hence the thermal hysteresis value. This is particularly noticeable when insect AFPs are being assayed. The latter may have thermal hysteresis values of 5–6°C (14), such that each measurement would take well over an hour to complete at this rate of cooling.

Another potential variable that is hard to control or even quantify is the size of the ice crystal present during the thermal hysteresis measurement. We note that larger ice crystals tend to burst at higher temperatures than do small crystals. This can be rationalized by considering that the surface area under containment by AFPs is a function of crystal size and that freezing can occur with the breakdown of that containment at any point of the crystal surface (Figure 1*B*). A complicating factor counterbalancing this effect is that starting crystal size is a reflection of AFP activity. The greater the AFP activity, the smaller the crystal formed in the melt.

Do AFPs Bind Irreversibly to Ice?

It has been argued by Knight & DeVries (15) that AFP binding to ice must be irreversible. Dissociation of an AFP from the ice at temperatures within the thermal

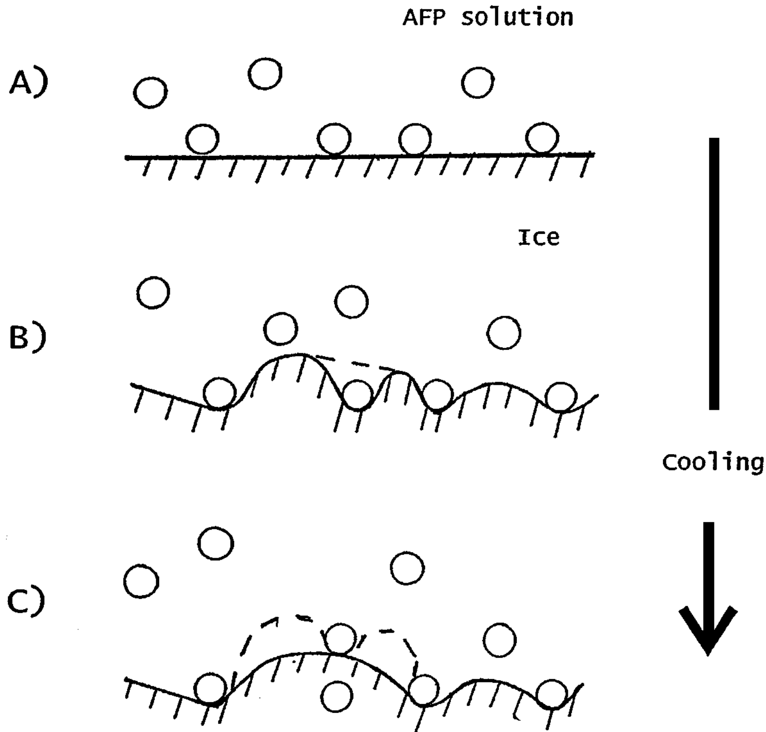

Figure 1 Inhibition of ice crystal growth following AFP adsorption. (*A*) AFP (open circles) in solution and in contact with the ice front (hatched line) at 0°C. (*B*) The ice-water interface at an undercooling approaching the non-equilibrium freezing point, where the curvature between bound AFPs leads to ice growth inhibition by the Kelvin effect. The dotted line represents the overgrowth of a bound AFP. (*C*) Binding of an AFP from solution to the point of overgrowth in (*B*). Here the dotted line represents subsequent stabilization of the ice-water interface around the newly bound AFP. This figure is based on the two-dimensional representations of ice-growth inhibitions displayed in Knight & DeVries (15) and Knight (17).

hysteresis range would provide an opportunity for water to join the crystal lattice, particularly since water is present at 55M and AFPs at mM concentrations. Even if the AFPs rapidly rebound to the crystal, there would be some addition of water to the ice. Given that the ice crystals observed during thermal hysteresis measurements ($\sim$50 μm in length) might be bound by millions of AFPs, the net effect would be slow growth of the crystal. This would be lethal to fish unless they had a mechanism for removing or melting the ice. In practice, we have observed an ice crystal during thermal hysteresis readings for as long as five days without seeing any signs of growth.

The idea of irreversible binding between a ligand (ice) and receptor (AFP) without covalent bond formation is difficult to accept. Even the most avid binding interactions have a finite dissociation constant. However, unlike most ligand-receptor interactions, the contact site is potentially extensive and may involve multiple interactions—all of which must be broken simultaneously for the AFP to escape. We argue that this event has zero probability of occurring with wild-type AFPs. However, when the ice-binding site is modified by mutations, particularly those that spoil surface-surface complementarity, the altered AFP typically allows slow growth of the ice crystal (16). With mild mutants, growth is so slow that it is only detectable by video microscopy, in which case an end-point to the assay is observed as the ice crystal bursts at the non-equilibrium freezing point. Typically, however, this thermal hysteresis value is lower than that of the wild-type at all concentrations tested, and the activity of the mutant can be expressed as a percentage of the wild-type's thermal hysteresis value (Figure 2*A*). With more severe mutations, growth is obvious during the 15 s delay between cooling increments and, by definition, the non-equilibrium freezing point has been exceeded (e.g. A21L in Figure 2*A*,*B*). Indeed, growth can be so rapid that an obvious burst point is not reached before the ice crystal fills the aqueous compartment in the well. With the most severe mutations, affinity for ice is completely lost, as evidenced by the absence of ice crystal shaping, and the protein is no longer recognizable as an AFP (e.g. A17L in Figure 2*B*).

We can think of AFP binding to ice as having a threshold value above which it is irreversible, below which there is a gradation of affinities for ice that is reflected in how rapidly the ice crystal grows. Through natural selection, AFPs have evolved to bind irreversibly to ice, i.e. to be above the threshold value. But this leads to another quandary. If binding is irreversible, an ice crystal surface could be readily saturated in the presence of excess AFPs. Why then is thermal hysteresis dependent on AFP concentration (15, 17)? To some extent the local concentration of AFP may influence the surface density of AFPs because of diffusion effects. Note that various studies support the model of ice growth inhibition with incomplete coverage of the ice surface by AFPs (18). A further explanation might lie in events that occur immediately prior to the end-point of thermal hysteresis, i.e. at the new non-equilibrium freezing temperature. This end-point is likely to be triggered by the overgrowth of an AFP and the coalescence of the surrounding ice fronts (Figure 1B). This stochastic event could presumably occur at any point on the crystal as determined by the random spacing between millions of bound AFPs. There would be two possible outcomes. One would be uncontrolled addition of water to the ice. The other would be the re-establishment of control by binding of additional AFPs to the coalesced ice front (Figure 1*C*). In essence, there would be competition between water and AFP for binding to ice. The higher the local AFP concentration the more likely an antifreeze would bind. Because there would not be sufficient time for diffusion to bring AFPs to the site of growth, the lowering of the non-equilibrium freezing point would be related to local AFP concentration.

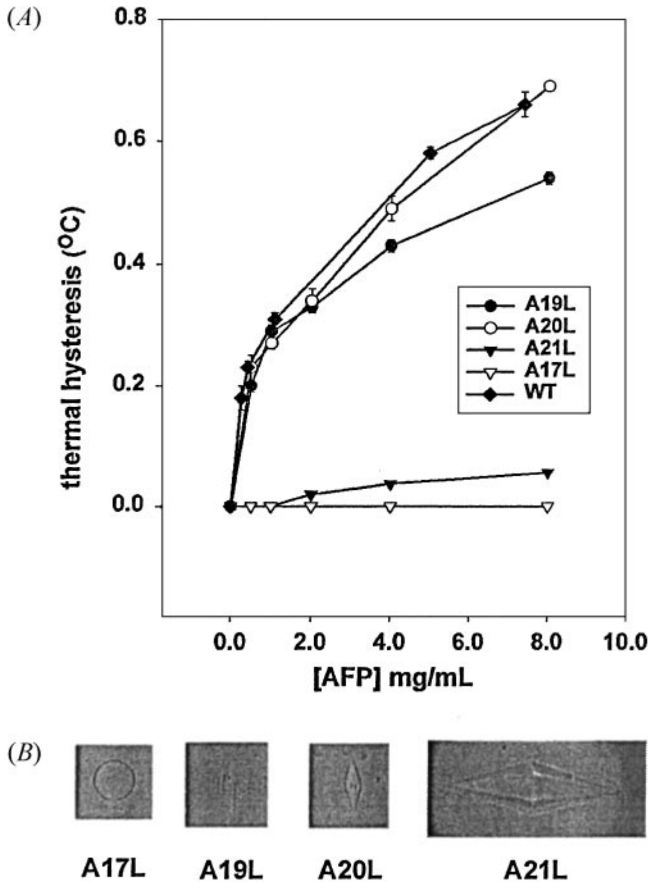

Figure 2 Effect of steric mutations on thermal hysteresis activity and ice crystal morphology produced by type I AFP. (*A*) Plot of thermal hysteresis (°C) as a function of AFP concentration (mg/mL) for the synthetic HPLC-6 isoform of type I AFP (WT) and variants where single Ala residues were replaced with Leu. WT (♦), A17L (∇), A19L (•), A20L (○), A21L (▾). (*B*) Ice crystal morphology obtained by the four different variants. Taken from Baardsnes et al (48) with permission.

AFP Diversity

Following discovery of antifreeze glycoproteins (AFGPs) in the blood plasma of Antarctic Nototheniids in the late 1960s and early 1970s, some very different AFPs were found in fishes of the northern hemisphere as more and more marine teleosts were surveyed for the presence of thermal hysteresis activity (Figure 3). Type I AFP was reported in winter flounder (19), type II in sea raven (20), type III in ocean pout (21), and most recently type IV in longhorn sculpin (22). A

comparison of their properties can be found in several recent reviews (4, 6–10). Briefly, AFGPs are made up of 4 to more than 50 tandem repeats of Ala-Ala-Thr with a disaccharide (galactosyl-*N*-acetylgalactosamine) attached to each Thr OH. It is thought that AFGPs fold as an amphipathic polyproline type II helix. Type I AFPs are alanine-rich, amphipathic α-helices. Type II AFPs are globular proteins with mixed secondary structure. Type III AFP is made up of short β-strands and one helix turn that gives it a unique flat-faced globular fold. Based on homology to serum lipoproteins and partial proteolysis studies, type IV AFP is thought to be a helix-bundle protein (23). The classification scheme of Davies & Hew (24) has proved useful and durable. With the exception of type IV AFP, newly discovered AFPs from unrelated fishes have fitted into a pre-existing type (Figure 3). For example, AFGPs in the northern cods (25) are remarkably similar to the Nototheniid AFGPs in sequence and distribution of size classes based on the glycotripeptide repeat. The sculpin AFPs are alanine-rich, single α-helical peptides that clearly resemble the flounder type I AFPs. Type I AFPs have also been recently detected in liparids and the cunner (RP Evans, MH Kao & GL Fletcher, unpublished data). Also, lectin-like Type II AFPs have subsequently been found in smelt (26) and herring (27).

One explanation for AFP diversity is that ice can present many different surfaces for binding. Although ice is made up solely of oxygen and hydrogen, with the O atoms in a tetrahedral arrangement linked by hydrogen bonds, the spacing between these atoms and the resulting surface contours will typically be different on different planes. Binding to any nonbasal plane is sufficient to inhibit ice growth and shape its crystal into a hexagonal bipyramid. Different AFP types do indeed bind to different ice planes, as was established by Knight et al (28), who developed the technique of ice etching to determine the plane (and in some cases the direction) of binding of AFPs to ice. In this method, an oriented hemisphere of ice is allowed to continue growing in the presence of a dilute solution of the AFP. The AFP concentration is insufficient to halt growth of the hemisphere. Instead, the proteins bind to their preferred planes and are continuously overgrown. On removal of the hemisphere to a walk-in freezer, the binding surfaces are revealed during sublimation by the residue of AFP.

This technique has worked particularly well for the winter flounder (type I) AFP, which binds to the pyramidal plane {20–21}. This binding should produce hexagonal bipyramidal crystals with a *c*- to *a*-axis ratio of 3.3:1, which is close to that observed during thermal hysteresis measurements using the nanoliter osmometer (Figure 2). Other fish AFPs like the AFGPs and type III AFP have been shown to bind on or close to a prism plane (29). They also form hexagonal bipyramidal crystals, but with *c*- to *a*-axis ratios that are lower than expected and that vary slightly with AFP concentration. One explanation for this ice crystal morphology is that the pyramidal surfaces might be formed by step-growth inhibition on the molecular scale, where the AFP binds to a prism plane (riser) but covers part of the basal plane (step) (16, 30).

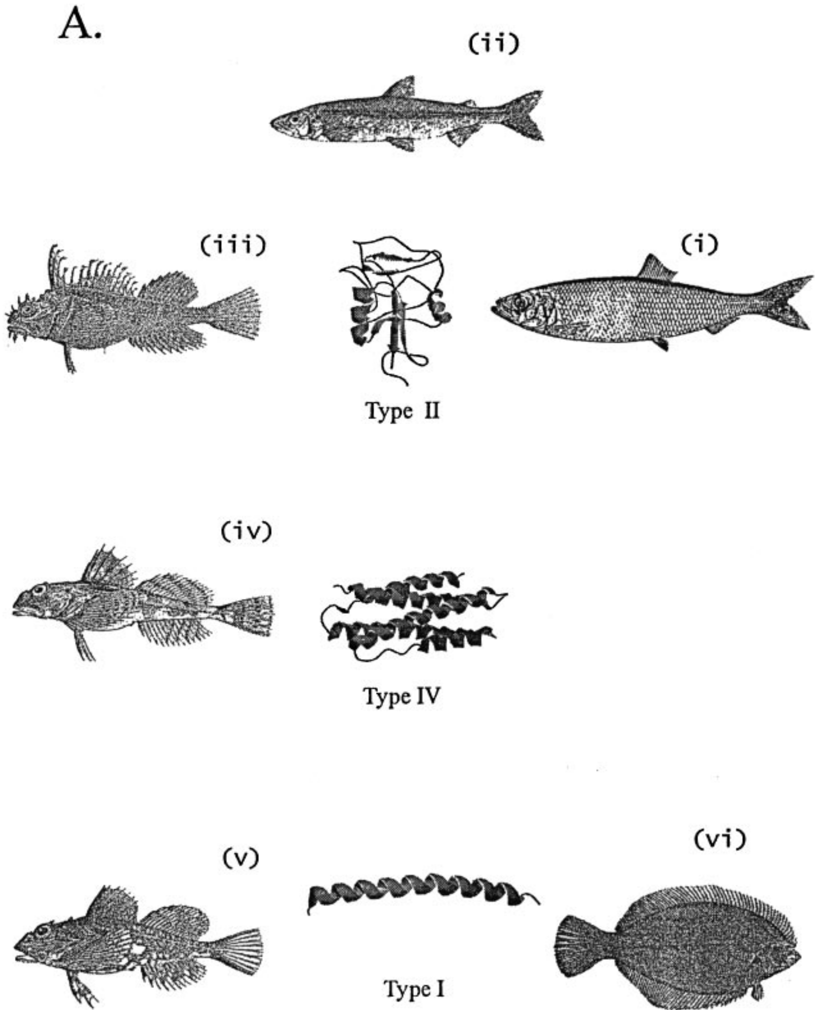

Figure 3 AFP structures and their distribution in fishes. Ribbon presentations of AFP structures from Davies & Sykes (7) are shown alongside fishes in which they occur. The AFGPs of cods and Nototheniids are represented by their glycotripeptide repeat. (*A*) (*i*) Atlantic herring (*Clupea harengus harengus*), (*ii*) American smelt (*Osmerus mordax*), (*iii*) sea raven (*Hemitripterus americanus*), (*iv*) longhorn sculpin (*Myoxocephalus octodecimspinosus*), (*v*) shorthorn sculpin (*Myoxocephalus scorpius*), (*vi*) winter flounder (*Pleuronectes americanus*). (*B*) (*vii*) *Dissostichus mawsoni*, (*viii*) Arctic cod (*Boreogadus saida*), (*ix*) ocean pout (*Macrozoarces americanus*), (*x*) *Lycodichthys dearborni*. Fish pictures were reproduced from Leim & Scott (142) with the exception of *vii* and *x*, which came from Eastman (143). The figure is based on an earlier representation (9).

B.

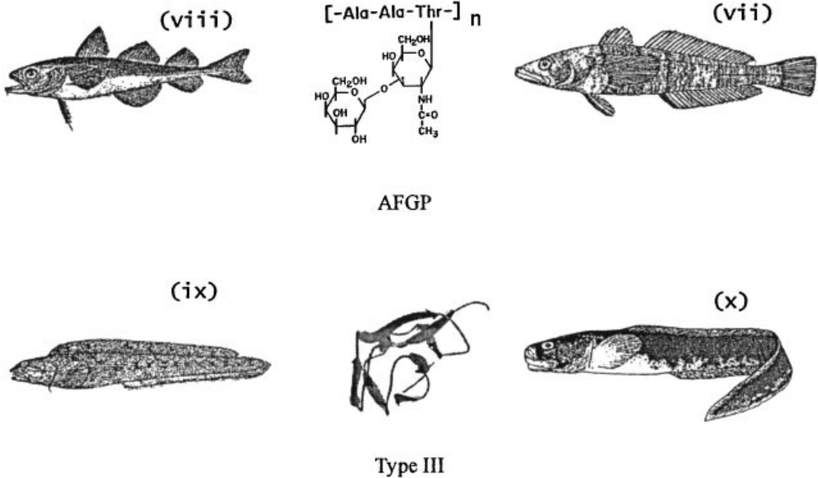

Figure 3 (*Continued*)

Structure-Function Relationships in the AFPs

Although the properties and amino acid sequences of the fish AFP support their classification into five nonhomologous types, this does not rule out the possibility that the AFPs might share a common three-dimensional ice-binding motif or principle. The drive to solve their three-dimensional structures has been largely fueled by the need to understand their structure-function relationships that are key to binding ice.

Type I AFP

Type I AFP from the winter flounder was the first AFP to have its three-dimensional structure determined. As anticipated by CD measurements and secondary structure predictions (31), it does indeed exist as a single, long, amphipathic, α-helix (32). The higher resolution X-ray structure revealed elaborate N- and C-terminal helix capping structures (33), which together with the internal salt bridge and high Ala content are the main reasons why such a long helix has stability, at least close to 0°C (31). The high-resolution structure also revealed the orientation of the putative ice-binding amino acid side chains, which was an issue for fitting the AFP to ice. When the first type I AFP amino acid sequence was determined by DeVries & Lin (34), these authors suggested a mechanism for AFP adsorption to ice whereby

the regularly spaced hydrophilic Thr and Asx residues might hydrogen bond to oxygen atoms on the primary prism plane of ice. Subsequently, Knight et al (28) demonstrated that the adsorption surface was the {20–21} pyramidal plane of ice rather than a prism plane and that the helix was oriented in the ⟨01–12⟩ direction on this plane. In effect, the same mechanism was invoked, only with a different periodicity where the threonines, spaced 16.5 Å apart at the start of each 11-amino acid repeat on the same side of the helix, matched specific O atoms on the binding surface that repeated at 16.7 Å intervals. The first of many models was produced where the $i,i{+}3$ Asx also hydrogen bonded to ice with the same spacing, and the $i,i{-}1$ Leu were thought to provide some van der Waals interactions with the ice surface (35). This trinity of residues was dubbed an ice-binding motif, even though there was little if any experimental evidence that these residues contacted ice (33).

In the interim, concerns about the paucity of hydrogen bonds that could be involved in binding the AFP to ice were addressed by two imaginative proposals. Wen & Laursen suggested that type I AFPs might bind to ice as a patch of helices similarly oriented and stabilized by side-by-side protein-protein interactions (35). Only with cooperative binding of the helices to ice would the number of hydrogen bonds be sufficient for irreversible adsorption. An elegant test of this hypothesis was set up with the synthesis of an all D-amino acid type I enantiomer (36). This bound to the same {20–21} plane, but in the mirror image direction to the all L-amino acid enantiomer. The mixture of these two enantiomers was predicted to provide some interference to helix patch formation. This was not observed, as a 50:50 mixture of the two enantiomers was just as active as the all-D or all-L forms. A similar result was obtained with mixtures of three AFP types (I, II, and III) which, coming from different fishes, were intrinsically unlikely to form protein-protein interactions, but also bound to different planes of ice (37). Although the type I AFP results were rationalized in terms of helix sorting into patches of the same handedness (36), our interpretation was that each AFP bound singly and was independently active. This was proven for type III AFP by fusing this 7-kDa protein to proteins that were large enough to block side-by-side associations of the AFP (38).

The other proposal for bringing more hydrogen bonds to bear on AFP binding was the suggestion that functional groups such as the Thr hydroxyls might occupy an O atom site in the ice surface (39). In this instance, the O atom of the amino acid side chain would be hydrogen bonded to three ice oxygen atoms (and covalently linked to the AFP—thus having a tetrahedral arrangement). In a sense, the AFP would be frozen to the ice surface through its Thr side chains. However, the orientation of the Thr hydroxyl revealed by X-ray crystallography was toward the helix backbone, which is not suitable for penetration of the ice lattice (33). NMR studies showed that while the Thr side chain did occupy other rotameric conformations in solution, its most preferred conformation matched that seen in the crystal structure (40).

Although these data from the three-dimensional structures do not rule out a Thr side chain rearrangement on binding to ice, a more serious challenge to the

hydrogen bonding hypothesis has come from amino acid replacement studies. When the central two of four Thr were replaced by Ser, there was a major decrease in thermal hysteresis activity (41). The Ser mutant had no activity below 2 mg/mL and had greatly reduced activity above this concentration. Similar results were obtained by Zhang & Laursen (42) and Haymet et al (43), who replaced all four Thr with Ser. This was surprising because the absence of the methyl group in the Ser side chain should have made it easier for the OH group to occupy an ice lattice O atom. Also surprising was the observation that Val was a relatively good substitute for the central two Thr, with only a 20% loss of thermal hysteresis activity (41). This result was confirmed and extended by Haymet who showed that a type I variant with all four Thr replaced by Val was functional, although less active than the wild-type (43). To keep this variant in solution it was necessary to add two more salt bridges (44). These results suggested that the Thr methyl group might have an important role in AFP binding which, together with the realization that fish AFPs are rather hydrophobic proteins (45, 46), has called into question the primacy of hydrogen bonds in fish AFP adsorption to ice (41).

Subsequent amino acid replacement studies indicated that Asx and Leu might be important for peptide solubility but failed to find a role for these two amino acids in ice binding (47). Indeed, a comparison of type I AFP isoforms from right-eye flounders shows that Asx and Leu are not well conserved. The region of the type I AFP that is perfectly well conserved is the Ala-rich face and the adjoining Thr residues. By substituting the bulky amino acid Leu for Ala at intervals around the helix at the mid-point of the molecule, we were able to establish the importance for ice binding of Ala 17 and Ala 21, the Ala residues immediately next to the Thr methyl group (48). Thus, although the nature of the binding forces are not established, it does appear that the hydrophobic face of the type I AFP helix is the ice-binding surface rather than the traditional ice-binding motifs defined by Sicheri & Yang (33).

Type III AFP

The type II AFP structure was determined by NMR methods and is of medium precision (49). Also, because of the difficulties of making and producing mutants of this AFP, its ice-binding site has not been pinpointed (50). The only other fish AFP for which there is a detailed structure and a finely mapped ice-binding site, is type III AFP. A high-resolution X-ray structure (to 1.25 Å) and a high precision NMR structure have been obtained for a recombinant QAE isoform (30, 46) and an X-ray structure for a natural SP isoform (51). The ice-binding site was identified by site-directed mutagenesis after targeting conserved, hydrophilic, solvent- exposed residues that were exposed on one face of the protein (52). In retrospect, the whole ice-binding surface is relatively hydrophobic, and the potential for the hydrophilic side chains to hydrogen bond to ice is limited. Tight packing of the side chains on this surface would not allow them to project into the ice surface or even to change orientations to adopt a more favorable conformation. Thus, there are notable

similarities between AFP types I and III in the overall hydrophobicity of the ice-binding surface and the difficulty of modeling a large number of hydrogen bonds to ice. In marked contrast to these two fish AFPs, one (and possibly both) of the recently solved insect AFP structures has solvent-exposed Thr, which are oriented in such a position that their hydroxyls can occupy O atom positions in the ice lattice without steric hindrance (53, 54). It is not known if this structural distinction has any bearing on the higher thermal hysteresis activity shown by insect AFPs compared with fish AFPs.

In light of the known and predicted fish and insect AFP structures, it is possible to reclassify them as repetitive and nonrepetitive. The repetitive AFPs include the AFGPs, type I AFP with its underlying 11-amino-acid repeat, and the two insect AFPs, where each β-helix turn brings a Thr-Xaa-Thr (where Xaa is any amino acid) ice-binding motif into register on one side of the coil. These repetitive structures typically suggest a lattice match to ice. AFP types II and III show no signs of repeating elements in their amino acid sequences or three-dimensional structures, although there is evidence for a secondary structure repeat in type III AFP (51). Type IV is difficult to classify at this time. However, it has been pointed out that some of its homologs have an obvious 22-amino-acid repeat (8). Irrespective of how fish AFPs are classified, these diverse protein structures binding to different planes of ice have remarkably similar thermal hysteresis activities, approaching 1°C at mM AFP concentrations.

PHYSIOLOGICAL FUNCTION OF ANTIFREEZE PROTEINS

As a general rule, cells are well protected from intracellular freezing at high freezing temperatures owing to the nature of the cell membrane and the spatial requirements for ice growth (55). Therefore freezing of intact tissues, organs, and whole organisms at high-freezing temperatures is, in the first instance, confined to the extracellular fluids. Thus it is evident that the addition of AFPs to the extracellular fluids of fish will not only protect these fluids from freezing, but also protect the entire animal. There is abundant evidence to demonstrate that the freezing temperature of blood plasma is a reasonable approximation of the freezing point of intact fish (56–58). In addition, intraperitoneal injections of purified AFP into rainbow trout improved their freezing resistance in direct proportion to the levels of AFP found in the blood (59).

In order for an undercooled fish to freeze, ice must propagate into it from the external environment. Several studies have shown that biological membranes can be effective at preventing ice propagation to undercooled fluids (55, 60, 61). Valerio et al (61) used a modified Ussing chamber to determine the temperature at which ice would propagate across isolated winter flounder and ocean pout skin, and ocean pout urinary and gall bladder epithelia. This study demonstrated that

not only did these epithelial tissues act as effective barriers to ice propagation, but that the ice propagation temperatures were lower than the freezing point of seawater (4). In acute experiments, where fish that have had no prior exposure to ice are incrementally cooled below their freezing point prior to ice exposure, it is evident that the nucleating ice must enter the fish at a location other than the skin. Of the two possible routes, gills and gut, the most likely would be the gills where the epithelium consists of a single-cell layer (62).

Two indirect lines of evidence indicate that ice crystals do gain entry to fish inhabiting ice-laden seawater without the fish suffering lethal consequences. DeVries (63) reported on a number of experiments whereby fish that had been residing in or exposed to ice-laden seawater would freeze when exposed to ice-free water at temperatures well below the freezing point of seawater (−2.7°C). He concluded from these observations that fish inhabiting icy waters contain ice crystals. Another line of evidence comes from a study by Verdier et al (64) who used ice nucleation as an assay to detect the presence of anti-ice Igs in the blood sera of two marine fish species (ocean pout and Atlantic herring) that produce AFP and inhabit icy waters during winter. Anti-ice Igs were not found in rainbow trout, tilapia, and bighead carp, freshwater species that had no prior exposure to ice at temperatures below the colligative freezing points of their body fluids. The presence of circulating anti-ice Igs suggests that ice crystals do gain entry into the marine fish species and remain for a time sufficient to elicit an immune response.

The evidence that ice can enter and reside within fish that produce AFPs is consistent with our current understanding of the mode of antifreeze action. As ice crystals enter the extracellular fluids of fish, they are prevented from further growth by being bound up by AFPs. Only when the fish encounter temperatures that are too low for the AFPs to inhibit ice crystal growth will they suffer the lethal consequences of freezing.

Although the hypothesis that plasma AFPs function to prevent the growth of ice crystals that gain entry into the fish seems robust, it raises questions as to the mechanisms of freeze resistance availed by other fish that produce little or no plasma AFPs yet reside in the same icy environment. One such example is the cunner (*Tautogolabrus adspersus*), which inhabits Newfoundland waters. This marine species overwinters in relatively shallow areas where it can be exposed to ice at temperatures as low as −1.6 to −1.8°C each winter. Plasma freezing points approximate −0.77°C, and we have rarely seen any evidence for the presence of plasma AFPs. On the occasions that we have obtained some evidence, the levels have been low as judged by thermal hysteresis measurements (<0.1°C) (65; GL Fletcher & MH Kao, unpublished data). This species clearly overwinters in an undercooled state, and we speculate that it likely survives by entering a near-torpid state, finding refuge in rock crevices and relying on an epidermis fortified by antifreeze to provide a barrier to ice propagation that is only penetrated under extreme conditions.

REGULATION OF PLASMA ANTIFREEZE PROTEIN PRODUCTION

All the antifreeze-producing teleosts that inhabit the waters off the Northeastern coast of North America show a distinct seasonal cycle in the level of AFPs observed in the blood plasma. There is, however, considerable species variation in the timing of this cycle. Within the coastal waters of Newfoundland, where the water temperatures range from 16°C during summer to as low as −1.8°C during winter, shorthorn sculpin (*Myoxocephalus scorpius*) and winter flounder (*Pleuronectes americanus*) produce AFPs well before environmental freezing conditions occur (66, 67). Juvenile cod (*Gadus morhua*) also produce AFGP prior to the onset of winter, whereas adults synthesize them only in response to freezing conditions (66, 68). Ocean pout (*Macrozoarces americanus*) possess high concentrations of AFP year round, although winter levels are several-fold greater than those observed during summer (69).

There are also significant population differences in the levels of AFPs produced by ocean pout and juvenile cod (69–71), and in winter flounder, there are clear differences in the time of the onset of antifreeze production between geographically distant populations (72). At the molecular level there appears to be a strong positive correlation between antifreeze gene dosage, antifreeze protein levels, and the environmental freezing conditions to which the fish are exposed. Newfoundland winter flounder residing in shallow coastal waters produce high concentrations of plasma AFP (10–15 mg/ml) during the winter and have approximately 30–40 copies of the liver-specific AFP genes (73). The yellowtail flounder (*Pleuronectes ferrugenia*), a close relative of the winter flounder, can also be exposed to subzero temperatures. However by virtue of its deeper water habitat it faces little or no danger of freezing by ice contact. This species has only one third of the AFP gene copy number observed in winter flounder and produces considerably less plasma AFP (2–4 mg/ml) (74).

A more striking example of the relationship between gene dosage and plasma AFP levels comes from the ocean pout. Plasma AFP levels in Newfoundland populations of ocean pout are five to ten times higher than those found in ocean pout from a more southerly New Brunswick population, where subzero water temperatures and ice occur considerably less frequently than in Newfoundland (69). Genomic Southern blots reveal that the basis for these differences likely lies with AFP gene dosage. Newfoundland populations have approximately 150 AFP gene copies, whereas ocean pout from New Brunswick have 30–40 (75).

The distinct seasonal cycles of plasma AFP concentrations in these cold-temperate fish provides a unique opportunity to study environmental, physiological and molecular factors involved in AFP gene regulation. Most of our research into the regulation of AFP production has been carried out on the winter flounder. This is largely because of its year-round accessibility in Newfoundland coastal waters and because it was the first nonglycosylated AFP to be described.

The annual cycle of AFP production and secretion into the blood has been extensively studied in the winter flounder. All the flounder within a particular geographical location are highly synchronous in their annual cycle, with little year-to-year variation in timing (72, 76). In Newfoundland waters the annual cycle of plasma AFP levels correlates closely with the annual cycle of seawater temperatures. AFP appears in the plasma during November as the water temperature declines below 8°C, reaches peak levels of 10–15 mg/ml during winter, and clears from the plasma as the temperature rises above 0°C. Peak levels of AFP during winter reduce the plasma freezing temperature to approximately −1.7°C. Because plasma freezing temperatures are a good indicator of a fish's freezing temperature, it is evident that the AFP improves the flounder's freeze resistance to the freezing point of seawater (59).

The liver was found to be the source of the plasma AFP, and cDNA cloning demonstrated that the AFP was encoded in liver mRNAs as preproproteins. The pre-sequence is removed cotranslationally, and the proAFP is secreted into the blood where the pro-sequence is removed within 24 h to yield a 37-amino-acid mature AFP (77–81). With the use of these cDNA probes it was determined that AFP mRNA appears in the liver in October, approximately one month prior to the appearance of AFP in the plasma, and declines during March and April, when water temperatures are still less than 0°C (82, 83).

The environmental factors regulating this annual cycle have been reviewed in detail by Davies et al (84) and Chan et al (85). Water temperature does not appear to play a major role in initiating or preventing the initiation of AFP mRNA or AFP synthesis in the fall, nor does it appear to be involved in terminating AFP production in the spring. However, it is important that the temperature be sufficiently low (<8°C) for AFP mRNA to accumulate and direct the synthesis of winter levels of plasma AFP (86–88). Similar effects of temperature on the turnover of type I AFP and its mRNA have been observed in transgenic tobacco and *Drosophila*, where the mRNA is destabilized at room temperature (89, 90). Photoperiod appears to act as the zeitgaber for the onset of AFP production by the liver in the fall. Long day lengths (>14 h) result in delays of several months in the appearance of liver AFP mRNA and plasma AFP levels, and in some instances complete suppression. Short day lengths (4–8 h) did not have any effect on the onset of AFP appearance in the plasma, suggesting the possibility that it is the loss of long-day lengths in the fall that allows AFP synthesis to proceed on schedule (83, 86, 91).

This effect of photoperiod on the annual cycle of plasma antifreeze production likely acts through the central nervous system to control the release of GH from the pituitary. Hypophysectomy during the summer results in the induction of liver AFP synthesis and subsequent elevation of plasma AFP levels. Pituitary transplants or the administration of pituitary extracts or purified growth hormone (GH) represses AFP gene transcription, thereby preventing the synthesis of AFP and its subsequent accumulation in the plasma (91–95).

Precisely how GH controls the annual cycle of antifreeze synthesis by the liver is unknown. It is assumed that there is an annual cycle of GH release from the pituitary gland that regulates the synthesis of insulin-like growth factor (IGF-1) by the liver, as has been observed for other teleosts (96–98). Recent experiments have demonstrated that IGF-1 can inhibit liver-type AFP gene enhancer activity in transient expression assays in cell culture. This inhibition was reversed by the use of wortomanin, a P13-kinase inhibitor believed to be one of the downstream factors for IGF-1 signaling. These results suggest that the GH/IGF-1 signaling pathways are important for liver AFP gene regulation (M Miao, SL Chan, GL Fletcher & CL Hew, unpublished data).

The hypothesis that growth hormone is responsible for regulating the annual cycle of plasma AFP levels by repressing AFP gene transcription during the summer is consistent with what is known about the annual life cycle of the winter flounder. Newfoundland winter flounder do not feed or grow during the winter months, a time when liver AFP mRNA and plasma AFP levels are at their peak. They resume feeding in April, when liver AFP mRNA has declined to background levels and cease feeding during October when AFP mRNA first appears in the liver (91, 99).

SKIN ANTIFREEZE PROTEINS

Until the 1990s it was generally accepted that the synthesis of AFPs was confined to the liver from where they were secreted into the blood. The significance of a report by Schneppenheim & Theede (100) on the isolation of AFP from the skin of European shorthorn sculpin, where none was evident in the plasma, went unrecognized. Subsequently, Valerio et al found evidence for antifreeze activity in the skin of cunner, another species that appeared to have no AFP in the plasma (65). However, the importance of these findings was not fully realized until Gong et al (101) demonstrated the presence of AFP mRNA transcripts in a variety of tissues in winter flounder and ocean pout, indicating that AFP synthesis was widespread in these fish. Gong et al (3) solidified this concept when, upon a detailed examination of a skin cDNA library from the winter flounder, they discovered that there were two distinct antifreeze gene families: the already well-known liver-type (wflAFP) whose expression is highly liver specific, and a skin-type (wfsAFP) that is expressed in many tissues, but most abundantly in the external epithelia (Table 1). Another observation made in this seminal paper was that the skin-type AFPs differed from the liver-type AFPs in that they lacked the pre- and pro-sequences. This lack of a secretory signal sequence (pre-sequence) suggests that these skin-type AFPs might remain and function intracellularly. Recently, skin-type AFPs have been discovered in shorthorn and longhorn sculpins (102; WK Low, Q Lin, C Stathakis, M Miao, GL Fletcher & CL Hew, unpublished data), suggesting that the production of AFP in external epithelia is a widespread phenomenon.

The functional significance of these skin-type AFPs remains an interesting question. The abundance of skin-type AFP mRNA found in gills and skin suggests that

TABLE 1 General features of liver-type (wflAFPs) and skin-type (wfsAFPs) AFPs

	Liver-type (wflAFP)	Skin-type (wfsAFP)
Tissue and cellular localization	Liver-specific; synthesized as preproAFP extracellular secretory proteins	Widely distributed in peripheral tissues; synthesized as mature AFP intracellular proteins?
Gene structure and organization	40 copies, primarily as tandem repeats; 2A-7b, a representative gene contains two exons and one intron	40 copies, primarily linked but irregularly spaced; F2, 11-3 as examples contain two exons and a larger intron
Seasonal and hormonal regulation	500–700-fold seasonal variations; inhibited by GH	6–10 fold seasonal variation; not affected by GH
Transcriptional control	Intron as liver-specific enhancer, contains DNA motifs for C/EBPα and AEP	Intron as ubiquitous enhancer, contains AEP and other DNA binding motifs; C/EBPα site destroyed by TA insertion

substantial amounts of AFP are produced in these tissues. Because external epithelia would come into intimate contact with ice crystals in an ice-laden environment, it may be that these tissues have a requirement for additional freeze protection over and above that conferred by AFP produced and secreted by the liver into the circulatory system. Although the absence of a pre-sequence suggests that the skin-type AFP might be retained to function within the cell, recent studies indicate that this may not be the case in all tissues. Through the use of immunohistochemistry, HM Murray, K Kao, CL Hew & GL Fletcher (manuscript in preparation) found in winter flounder that the skin-type AFP was, as expected, restricted to the cytoplasm of the gill epithelial cells. However, in the skin these AFPs could be located only outside of the cells in the interstitial space. Therefore, despite the lack of a secretory signal sequence, the skin-type AFPs appear to have been exported from these cells. Alternative pathways for protein export that bypass the Golgi apparatus have been described by Mignatti et al (103).

The localization of the skin-type AFP within the extracellular space of the skin is consistent with the idea that the AFP would assist in blocking ice propagation into and through the skin. In this case, localized concentrations of AFP could serve two functions: The first and possibly most important role would be to prevent the damaging effects of freezing within the epidermis itself, and the second would be the deterrence of ice propagation through the epidermis and into the blood.

A primary function of the gills is to facilitate the rapid diffusion of respiratory gasses between the fish and its aqueous environment. Therefore, the diffusion distance between water and blood is kept to a minimum by limiting the epithelium directly involved in gas exchange to a single-cell layer a few microns in thickness

(62). The consequence of this is that gill epithelia may be more susceptible to ice propagation and damage than any other external tissue. This risk would be exacerbated by the fish actively pumping ice water across the gills in order to meet its respiratory requirements. The presence of AFP within the gill epithelial cells implies that there is a need to lower the freezing temperature of the cytoplasm in order to prevent it from freezing. This would suggest that there is a danger of ice forming within or being propagated into these cells when ice-laden sea water is pumped across the gills. Given the role that this epithelium plays in ion regulation and gas exchange, it seems unlikely that the cell membrane on the apical face of the epithelial cell would be any more permeable to ice propagation than any other cell. However, the relatively delicate structure of the gill lamellae does render it susceptible to structural damage.

The inability to detect AFP in the intercellular space between the gill epithelial cells may be related to the tightness of the junctions that exist within this cell layer. The intercellular space between the skin epithelial cells is considerably greater than it is between the gill epithelial cells. Therefore, intercellular AFP may be detected more readily in the skin than it would be in the gill tissue. Consequently, it is possible that AFPs find their way out of gill cells in the same manner as the skin epithelial cells and remain undetected by the immunohistochemical methods used in this study (HM Murray, K Kao, CL Hew & GL Fletcher, manuscript in preparation). Quite apart from protecting the cytoplasm from ice damage, any AFP that is present in the interstitial space would serve to inhibit ice propagation through this cell layer.

In contrast to the liver-type AFP genes, the skin-type genes appear to be expressed constitutively. Although the levels of AFP mRNA observed in the non-liver tissues peak during winter, they are only several-fold greater than those observed during summer. This contrasts to the several-hundred-fold seasonal change that occurs with the liver-type AFP mRNA. In addition, the skin-type AFP mRNA levels do not appear to be influenced by hypophysectomy. Taken together these results indicate that the two sets of genes are not regulated by the same mechanisms (104).

REGULATION OF ANTIFREEZE PROTEIN GENE EXPRESSION

The recent discovery of two different gene families encoding liver-type and skin-type AFPs makes the winter flounder an interesting model in which to study environmental, hormonal, and tissue-specific AFP gene regulation. Extensive studies have been conducted on the DNA elements controlling winter flounder liver-type AFP gene transcription. The gene investigated (clone 2A-7b) codes one of the most abundant AFP isoforms found in the plasma. It is less than 1 kb in length and consists of two exons and a single intron of 0.5 kb.

Promoter analysis of the 2 kb 5′ upstream sequences of clone 2A-7b did not reveal any presumptive *cis*-acting sequences important for the liver-type AFP gene

transcription (105). Instead, the enhancer was located within the intron and, with the use of transient expression assays, was found to be liver specific (106, 107). The liver specificity of this gene was confirmed in transgenic salmon in which the 2A-7b gene was integrated into their genome (108). Biochemical studies, including deletion, mutagenesis, foot-printing, and mobility shift assays, defined the core enhancer as a 19 bp fragment designated Element B (106). An examination of Element B indicated that it contained presumptive DNA-binding motifs for the C/EBPα transcription factor and a novel AP-1 binding complex termed antifreeze enhancer-binding protein (AEP).

The use of C/EBP oligonucleotides and specific antibodies for the C/EBP family of proteins established evidence for C/EBPα binding to the intron enhancer. C/EBPα is a transcription factor with limited tissue specificity (109, 110), which raises the possibility that interactions between C/EBPα and the enhancer may be part of the basis for the tissue specificity of the liver-type AFP genes. In addition it has been demonstrated that treatment of adipocytes with IGF-1 causes dephosphorylation of C/EBPα, an effect that was correlated with the down-regulation of the glucose transporter gene GLUT4 (111). The demonstration that IGF-1 can significantly reduce the enhancer activity of liver-type AFP in expression assays suggests the possibility that IGF-1 may prevent the expression of the liver-type AFP genes in winter flounder during summer (M Miao, SL Chan, GL Fletcher & CL Hew, unpublished data).

The presence of a presumptive AEP and evidence for its interaction with Element B of the liver-type AFP gene were demonstrated using mammalian and flounder hepatocyte nuclear extracts (106, 107). The identity of the AEP analog was revealed by screening a rat liver cDNA expression library using Element B as a probe (M Miao, SL Chan, GL Fletcher & CL Hew, submitted) and found to belong to an AEP family of proteins that appear to be expressed in a wide variety of tissues (112–115).

In contrast to the liver-type AFP gene intron, the skin-type intron was found to function as an enhancer in a variety of cell types. An examination of the C/EBPα-binding site revealed that the skin-type AFP enhancer is disrupted by the central insertion of a TA dinucleotide. This lack of a functional C/EBPα-binding site may, in part, be responsible for the more ubiquitous tissue expression of the skin-type AFP genes. It may also help explain why the skin-type AFP genes are largely unaffected by season or hormones.

A working model of our current hypotheses regarding the various environmental, physiological, and molecular factors regulating the annual cycle of AFP production by the liver is illustrated in Figure 4. During the summer months, the release of pituitary GH stimulates the production of IGF-1, which in turn results in dephosphorylation and deactivation of C/EBPα and/or alters the level of AEP expression, resulting in transcriptional inhibition of the liver-type AFP genes. With the loss of long-day length in the fall, the production of GH is inhibited by the CNS. C/EBPα and AEP are left active to enable AFP gene expression.

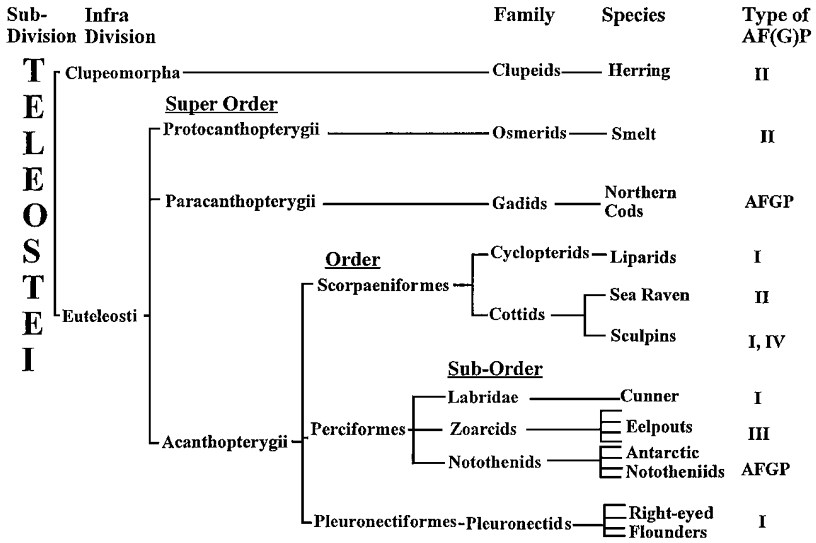

Figure 4 Seasonal regulation of AFP gene expression in winter flounder. Abbreviations: CNS, central nervous system; GHRH, growth hormone-releasing hormone; GH growth hormone; IGF-1, insulin-like growth factor-1; C/EBPα, CCAAT/enhancer-binding protein a; AEP, antifreeze enhancer-binding protein.

RECENT EVOLUTION OF AFPs

A perplexing feature of the distribution of AFP types in the teleosts is that it does not match taxonomic relationships. Rather than having one type confined to one branch of the teleost radiation, AFP types appear to be almost randomly distributed (Figure 5). Especially puzzling is the occurrence of different types in closely related species, a good example of which is the shorthorn sculpin producing type I AFP and the sea raven producing type II AFP (Figure 3). Both species are Northern hemisphere cottids belonging to different genera of the same family. Even more striking is the occurrence of type IV AFP in the longhorn sculpin, a species in the same genus as shorthorn sculpin.

The remarkable diversity in AFP structure in closely related cottids suggested that the need for an antifreeze occurred very recently in geological time, in some cases after the present speciation had been established. A search of the literature on climate change revealed that the first geological evidence for ice in seawater dates back ~10–30 million years ago in the southern hemisphere (116–118), but only 1–2 million years ago in the northern hemisphere (119). Both recent cooling events were the consequences of continental drift, the isolation of Antarctica by a circumpolar current, and the pinching off of the Bering Sea, respectively. Thus all but the very latest stages of the teleost radiation that began 175 million years ago took place in the absence of the threat of freezing. The sea-level glaciation

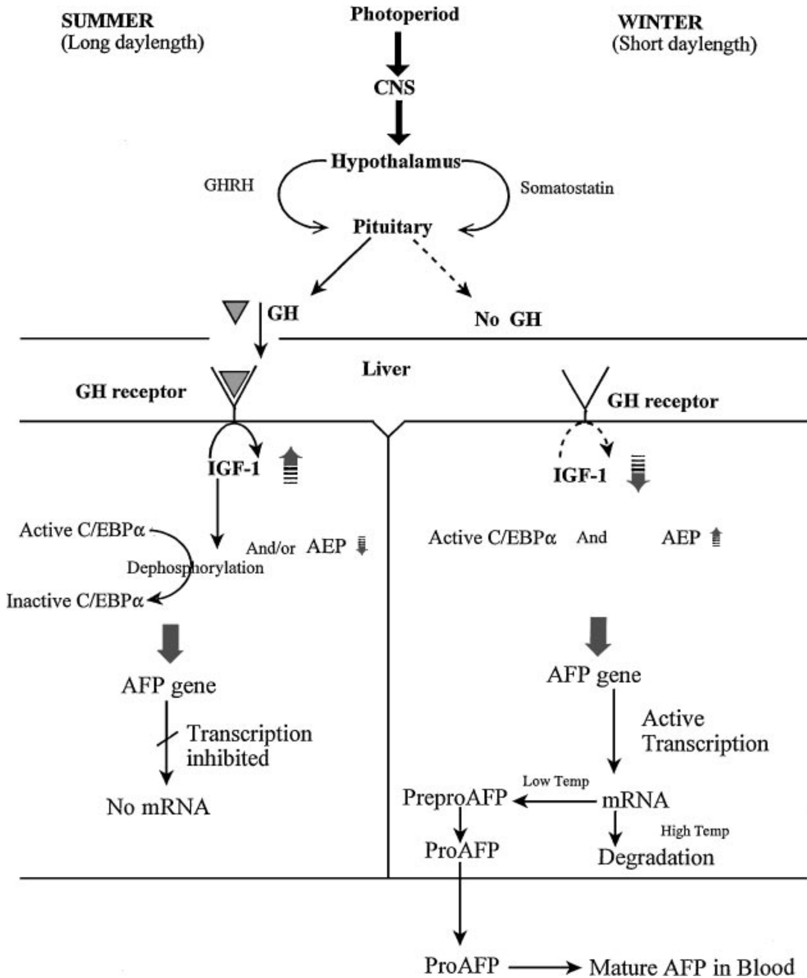

Figure 5 Phylogenetic tree of AFP evolution in fishes. This simplified scheme has been adapted from that shown in Davies et al (1993) and the original version of Scott et al (1986). The relationship of AFP-producing fishes within the teleost radiation was based on Greenwood et al (144) and Nelson (145).

hypothesis, first put forward by Scott et al (120) and reiterated in subsequent review articles (84, 121), states that AFPs were evolved (or elaborated from pre-existing proteins) as a direct response to the recent appearance of ice in seawater. The lateness of this selective pressure meant that some present day species developed radically different AFPs to bind and control the growth of ice. It appears that the same conclusions were arrived at autonomously by Cheng (8). Her evolutionary tree of AFP origins has many similarities to the original design and especially its

later refinement (120, 121). The plausibility of the sea-level glaciation hypothesis can only be enhanced by this convergence of opinion from two independent studies.

As noted above, a second contributing factor to the evolution of novel AFP types is the fact that ice can present a variety of binding surfaces. This is a very unusual situation where the ligand (ice) can present many different faces for recognition by different receptors (AFPs). Consequently, any protein with an affinity for an ice surface (other than the basal plane alone) has the potential to be an antifreeze. If binding were strong enough to produce a thermal hysteresis activity, the host species should be able to survive or invade this newly formed niche. Natural selection has presumably improved the ice-binding capabilities of AFP through mutation and ensured through gene amplification that there is an adequate quantity of the AFPs.

Convergent Evolution

One potentially confounding observation to the hypothesis of recent AFP evolution is the occurrence of similar AFPs in unrelated fishes. In some cases these fishes inhabit different hemispheres. For example, the AFGPs in the Nototheniids of the Antarctic Ocean bear a remarkable resemblance to AFGPs in the cods of the northern hemisphere. Did these AFPs evolve much earlier on in the teleost radiation, before the division into superorders (Figure 4)? If so, are they present in a wide range of fishes but without being expressed? Or are they the product of recent convergent evolution?

Evidence from the Cheng laboratory supports the latter scenario. An AFGP gene was first isolated and characterized from *Notothenia coriiceps neglecta* and shown to encode the AFGP peptide backbone as a polyprotein that contained over 40 units of the smaller AFGPs separated by tripeptide spacers (122). Processing would require excision of the flanking spacers by a presumed chymotryptic-like proteinase, as well as glycosylation on the threonines. A gene homolog was subsequently isolated from another Nototheniid, *Dissostichus mawsoni*, but this was embedded in a trypsinogen gene with most of the trypsinogen-coding region exons removed (123, 124). It appears that the fundamental tripeptide repeat Ala-Ala-Thr might have arisen from a 9 bp sequence at the first intron-exon boundary that underwent repeated duplication and unequal crossing over events to produce the large polyprotein sequence. When an AFGP gene was cloned from a cod (*Boreogadus saida*) it was also found to encode a polyprotein version of the antifreeze (125). Despite this remarkable coincidence (polyproteins being rare in eukaryotes) and the fact that the cod AFGP gene was isolated using the Nototheniid AFGP-coding region as a hybridization probe, the two genes appeared to be unrelated (126). 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. In place of the hydrophobic tripeptide spacers (e.g. Leu-Ile-Phe and Phe-Asn-Phe) in the Nototheniid polyprotein, the cod sequence is punctuated by arginine. Cleavage of the cod polyprotein by a

trypsin-like proteinase would account for the presence of this basic amino acid at the C terminus of the cod AFGPs. Although the precursor of the cod AFGP gene has not been identified, it does not appear to be a trypsinogen gene (125).

Convergence in Parallel?

A variation of convergent evolution of AFPs may account for the presence of the type II AFPs found in sea raven, herring, and smelt (Figure 3). All three AFPs show sequence and structural homology to the carbohydrate recognition domain (CRD) of C-type lectins, proteins that bind to sugars in a Ca^{2+}-dependent manner (45). It seems very unlikely that the three type II AFPs are related by direct descent, that is by the conversion of a C-type lectin to an AFP at a time before all three fishes diverged from their common ancestor. If this were so, type II AFP would pre-exist in a large section of the teleost radiation (Figure 4), a section including species that have subsequently produced totally different AFP types. Why would they have evolved other AFPs when they already had one that would do the job? A more parsimonious explanation is that type II AFPs have evolved from C-type lectins on separate occasions (convergence in parallel). The C-type lectins are present in all fishes as a protein superfamily related by descent. Only in isolated cases would there have been the chance conversion of a lectin family member into an AFP that has given the host fish a selective advantage in resisting freezing.

Like the C-type lectin CRDs, from which they have evolved, the type II AFPs from herring and smelt are dependent on Ca^{2+} for their activity. The AFPs show no thermal hysteresis activity if Ca^{2+} is absent or chelated by EDTA (127). In the lectins, X-ray crystallography has shown that Ca^{2+} is directly involved in binding to the sugars (128), and there is evidence this metal is required at the ice-binding site of the AFPs. For example, although the herring and smelt AFPs are only 30% identical to some of the well-characterized CRDs (from mammalian lectins), they have retained the key amino acids that coordinate Ca^{2+}. Replacement of Ca^{2+} by other divalent metal ions has noticeable effects on both thermal hysteresis activity and ice crystal morphology (127). Also, two amino acid replacements at the putative ice-binding site of the herring AFP eliminated ice binding (129). These replacements were equivalent to those that changed the sugar specificity of a CRD from galactose-binding to mannose-binding (130, 131).

The herring and smelt AFPs share a surprising degree of identity (80%). This fact alone led Ewart et al (10) to question the classification scheme that places herring and smelt into different orders of fish, the Clupeiformes and Osmeriformes. Nevertheless, it is conceivable that the two AFPs have been derived independently from the same CRD, one that has been well conserved during the long separation of these orders. A third member of the group, type II AFP from sea raven, is only 40% identical to the herring and smelt AFPs. It lacks the requisite amino acids for Ca^{2+} chelation and does not require Ca^{2+} for activity, yet its NMR structure clearly shows that it has the same fold as the C-type lectins (49). Site-directed mutagenesis studies indicate the sea raven AFP's ice-binding site is quite distinct from that of

the Ca^{2+}-dependent type II AFPs (50). The sea raven AFP has presumably been derived from a different member of the C-type lectin protein superfamily. It will be interesting to survey the new databases emerging from the genome sequencing projects for clues to the identity of the type II AFP ancestors.

The appearance of type I AFPs in right-eye flounders and some sculpin species again raises the same question of similarity by descent or by convergent evolution from a common non-antifreeze precursor. Convergence from two unrelated sources has all but been ruled out by the following tenuous connections. The skin and serum AFPs in winter flounder appear to be extreme isoforms encoded by their own multigene families (3). They share appreciable amino acid and DNA sequence identity, and their relationship by descent is established by the isolation of a genomic clone showing physical linkage of the skin and serum isoform genes (132, 133). Although the serum AFPs in the shorthorn sculpin have not yet been viewed at the DNA level, they bear a striking resemblance to the type I AFP isoforms found in winter flounder skin, even beginning with the same N-terminal sequence Met-Asp-Ala-Pro-Ala (3, 32). The recent discovery (GL Fletcher, RP Evans & MH Kao, unpublished data) of type I AFPs in a liparid (from the same order as sculpins) and in the cunner (a perciforme species) makes it even more likely that type I AFPs have been derived from a common lineage on more than one occasion. There are other small alanine-rich sequences in the databases, such as the *kin*-encoded proteins in plants (134, 135). However, they have no antifreeze activity, and there is no indication of homology other than their high content of alanine.

Type III AFPs are unique (so far) to zoarcid species and their relatives and are presumably all related. There is one interesting evolutionary issue that has to do with the zoogeography of this suborder. These are the only AFP-containing fishes found in both polar oceans, and it is not at all clear how species containing the same AFPs have ended up literally poles apart. The similarity of some type III isoforms shared by northern and southern hemisphere zoarcids is greater than the most diverse isoforms within one species (24). Therefore, we presume that type III AFPs predate the present dispersion of zoarcid species. If type III AFP evolved in response to sea level glaciation, it would be logical for it to have emerged in the south with subsequent dissemination to the northern hemisphere. There is no indication yet of a progenitor from which type III AFP might have been derived.

AFP Gene Amplification

While AFP diversity provides one of the best examples of natural selection in action, one of the hallmarks of this rapid response to environmental cooling is gene amplification (136). In those fishes (and insects) that show significant thermal hysteresis activity, and for which an AFP gene probe is available, the AFPs are invariably encoded by large gene families. Estimates range from ~10 AFP genes in yellowtail flounder (74), 30–50 copies in winter flounder (73, 137), 80 copies in the wolffish (138), to 150 copies in a population of ocean pout from Newfoundland waters (75). Where extensive sequencing has been done at the protein, cDNA,

and/or genomic levels in fish or insects, there is evidence for multiple isoforms, many of which differ by only a few conservative amino acid replacements. It is unlikely that these very similar isoforms have specialized functions because they are typically not well conserved from species to species (compare, for example, serum AFPs in the yellowtail flounder and winter flounder, or the wolffish and ocean pout). These small differences might be the result of genetic drift. There are, however, some isoforms that display greater divergence (50% identity) and even tissue specificity, such as the flounder skin AFP isoforms (mentioned above), which is an indicator of functional divergence. The skin isoforms in flounder are themselves encoded as a multigene family that is as extensive as the liver-specific serum isoforms (3). Another indicator of the rapidity of this evolutionary event is the tandem amplification of certain genes that accounts for the dominance of a specific isoform or sets of isoforms such as the HPLC-6 and -8 isoforms of type I AFP in flounder serum (73). These abundant isoforms are encoded by 1 kb genes in 7–8 kb tandem-direct repeats that may be kept relatively homogeneous by gene conversion and/or unequal crossing over (139). Gene amplification has been seen in many artificial systems where there is intense selective pressure to overcome an environmental insult, such as the treatment of mosquitoes with insecticide (140) and the poisoning of cells with methotrexate (141).

ACKNOWLEDGMENTS

This work was supported by grants from the Canadian Institutes for Health Research (formerly Medical Research Council) to PLD and CLH, and from the Natural Sciences Engineering Research Council to GLF. We are extremely grateful to Dr. Margaret Shears and Sherry Gauthier for help in the preparation of this manuscript, and to the many research assistants, postdoctoral fellows and graduate students who have made valuable contributions to the research on AFPs in our laboratories over the past 25 years.

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