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Antifreeze glycopeptides and peptides in Antarctic fish species from the Weddell Sea and the Lazarev Sea

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ABSTRACT: Antifreeze glycopeptides and peptides have been isolated from 37 species of Antarctic fish representing the families Nototheniidae, Artedidraconidae, Bathydraconidae, Channichthyidae, Muraenolepididae, Liparididae, Zoarcidae and Myctophidae. Amino acid and carbohydrate analysis as well as antifreeze activity indicate that all investigated notothenioids contain antifreeze glycopeptides (AFGP). *Pleuragramma antarcticum*, *Lepidonotothen kempi*, *Bathydraco marri* and *Dolloidraco longedorsalis* synthesize additional antifreeze molecules. The non-notothenioid species possess antifreeze peptides (AFP), except *Muraenolepis marmoratus* and *Macrourus holotrachys*, which possess a glycosylated antifreeze peptide similar to the AFGP found in the notothenioid species. A novel glycopeptide comprised of the carbohydrate residue N-acetylglucosamine and the amino acids asparagine, glutamine, glycine, alanine, and traces of arginine, valine, leucine and threonine was isolated and characterized from *P. antarcticum*. The level of antifreeze concentration was dependent on the ambient water temperature, the depth of catch and life cycle of the species. Antifreeze activity of AFGP varies between 0.52 (*Neopagetopsis ionah*) and 1.20°C (*P. antarcticum*) at a concentration of 20 mg ml^{-1}. Antifreeze activity of AFP is lower than 0.50°C. A linear increase in activity of the AFGP could be demonstrated concomitant with decreasing ice content. The structural diversity of antifreeze molecules and their occurrence in a wide range of Arctic and Antarctic fish species suggest that they evolved from precursor proteins before the continental drift and recently during Cenozoic glaciation into the various antifreeze molecules.

KEY WORDS: Antifreeze · Notothenioidei · Antarctic fish · Evolution · *Pleuragramma*

INTRODUCTION

The sera of marine teleosts are hypoosmotic in relation to seawater, having approximately one-third its molarity of solutes. The colligative effect of the solutes in seawater (~0.45 M) depresses its freezing point to ca –1.9°C, whereas a typical teleost serum will freeze at ca –0.7°C (Holmes & Donaldson 1969). This discrepancy of ca 1°C in freezing points means that unprotected teleosts in polar and northern temperate waters would be at risk of freezing to death when their temperature fell below –0.7°C. Although there is evidence that some fish can survive at these temperatures in deep water in a supercooled state (DeVries 1980), this is not possible in shallow water or extremely cold, ice-laden deep water where contact with ice negates supercooling.

So far, 2 types of antifreeze have been isolated from polar and northern temperate fishes. They are either glycopeptides or peptides (AFGP or AFP, respectively). In all of the Antarctic fishes studied to date, with 2 exceptions, the antifreezes are glycopeptides (DeVries & Lin 1977, Schneppenheim & Theede 1982, Ahlgren & DeVries 1984). They have been characterized in detail in the family Nototheniidae of the suborder Notothenioidei. The glycopeptides are made up of a tripeptide repeat $(alanine\text{-}alanine\text{-}threonine)_n$ with a disaccharide moiety attached to the threonyl residues (Komatsu et al. 1970, Shier et al. 1975, Feeney & Yeh 1978). There are at least 8 different sizes (AFGP 1–8) and the range of molecular weights is between 2600

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and 34000 Da (DeVries et al. 1970, Duman & DeVries 1976, DeVries 1988). The 3 smaller glycopeptides (AFGP 6–8) differ from the larger ones in that proline replaces the first alanine in the glycotripeptide repeating sequence (Lin et al. 1972, Morris et al. 1978). The same 8 glycopeptides have also been isolated from the gadid *Gadus ogac* in the Arctic Labrador Sea (Van Voorhies et al. 1978). As more fish have been surveyed for antifreeze activity, 3 distinct AFP types have been characterized in addition to the AFGP (Davies et al. 1988). These are the alanine-rich, α-helical AFP of righteye flounders (Pleuronectidae) and sculpins (*Myoxocephalus* sp.) (type I) (Hew et al. 1980, Yang et al. 1988, Davies & Hew 1990), the cystine-rich AFP of the sea raven (*Hemitripterus americanus*) (type II) (Slaughter et al. 1981), and an AFP (type III) found in Antarctic and Arctic eel pouts (Zoarcidae) (Hew et al. 1984, Schrag et al. 1987, Cheng & DeVries 1989) which lacks distinctive features in its composition and sequence.

The AFGP and AFP make up 3.4% of the blood of many Antarctic fishes. Along with sodium chloride, they lower the fishes' freezing points below that of seawater. On a mass basis, both the glycopeptides and peptides are nearly as effective as sodium chloride in depressing the freezing point of water. On a molar basis, however, they depress the freezing point by 200 to 300 times more than can be expected on the basis of colligative relations alone (DeVries 1971a, b). The glycopeptides and peptides appear to lower the freezing point only in a non-colligative manner, but show the expected colligative effect on the melting point of the solid phase. The non-colligative lowering of the freezing point has been referred to as an antifreeze effect (also termed thermal hysteresis) and these molecules are referred to as 'antifreezes' (DeVries 1988).

Antarctic fishes inhabiting high-latitude coastal regions of Antarctica are potentially in year-round contact with ice. Seasonality of light, food resources and environmental factors such as water-mass distribution and current patterns also affect the lives of fish in the Antarctic. The Weddell Sea is the largest of several deep embayments of the Antarctic continent. The distribution of the principal water masses in the east wind drift (EWD) and coastal current leads to differentiated temperature conditions on the shelf. Water temperature in the eastern shelf water (ESW) is constantly below −1.8°C (Rohardt et al. 1990). At a depth of > 500 m, oceanic warm deep water (WDW) approaches the shelf and fills the deep trenches and innershelf depressions of the eastern shelf, providing temperatures between 0.0° and +0.8°C in near-bottom layers. The WDW is not present on the southern shelf or in the Filchner Depression, where cold Antarctic bottom water (ABW) is formed (Seabrooke et al. 1971, Carmack & Foster 1975, Fahrbach et al. 1987). During much of the year the surface waters are covered by sea ice, shallow bottoms are covered by anchor ice, and the upper 30 m of the water column contain small ice crystals.

Both the extremely cold ABW containing platelet ice and the ice-laden shallow water are inhabited by notothenioid and non-notothenioid fish. However, antifreezes are necessary for the evolutionary adaptation of Antarctic fish to the full spectrum of ice-laden habitats. Surprisingly, a few Antarctic notothenioids and non-notothenioids, e.g. *Pleuragramma antarcticum, Lepidonotothen kempi, Pogonophryne scotti, Pagetopsis macropterus* and liparidids or gadiforms, have been reported to lack antifreezes (DeVries & Lin 1977, Haschemeyer & Jannasch 1983, Eastman 1993). What is the reason that antifreezes have been reported as missing in these species living in the Weddell Sea and the Lazarev Sea?

This study provides new information on the distribution, chemical composition and function of AFP and AFGP purified from several members of Notothenioidei and members of the Antarctic fish families Muraenolepididae, Liparididae, Zoarcidae and Myctophidae in relation to ecological habitats, mode of life and evolution. The investigated fishes occupy diverse ecological habitats in the Weddell Sea and the Lazarev Sea, from the WDW up to the cold ice-laden surface water.

MATERIAL AND METHODS

Fish were collected in 1989 and 1991 during the expeditions ANT VII/4 and ANT IX/3 of the German RV 'Polarstern' in the Weddell Sea and the Lazarev Sea between 69° and 76° S latitude. The fish specimens were caught at 12 stations in water depths between 120 and 1400 m with a commercial 140 ft bottom trawl and an Agassiz trawl. Detailed information about the cruise and the scientific programmes is given by Hureau et al. (1990), Rankin et al. (1990) and Wöhrmann & Zimmermann (1992). Species, standard length (SL) and fresh weight (fresh wt) were determined aboard the ship. Species were identified according to the FAO identification sheets (Hureau & Fischer 1985), nomenclature follows Gon & Heemstra (1990). Samples were deep frozen at −80°C.

AFP were isolated as described previously (Wöhrmann 1993, 1995). Samples were passed over Bio-Gel TSK DEAE 5PW (diethylaminoethyl cellulose; Bio-Rad) ion exchange resin (column 75 × 7.5 mm i.d.), equilibrated with 20 mM Tris-HCl, pH 9.5, and a salt-concentration gradient (0.8 M NaCl in 20 mM Tris-HCl, pH 9.5). Collected fractions were further purified by high-performance liquid chromatography (HPLC) on a

Vydac C4 reverse-phase column (5 µm, 250 × 4.6 mm). A linear acetonitrile gradient was used to elute the peptides and glycopeptides which were detected by absorbance at 215 and 280 nm (Wöhrmann & Haselbeck 1992).

Fractions from the ion exchange chromatography were analysed by polyacrylamide gel electrophoresis according to the procedure described by Laemmli (1970), blotted on nitrocellulose according to Burnette (1981) and detected with the lectins peanut agglutinin PNA and wheat germ agglutinin WGA (Haselbeck & Hösel 1990).

Amino acid analysis was accomplished following acid hydrolysis of the antifreeze peptide in 5 µl phenol with 6 N HCl at 110°C for 24 h under a nitrogen atmosphere. After hydrolysis the samples were dried *in vacuo* and analysed on an Applied Biosystems (Foster City, CA, USA) model 420A derivatizer-analyser system. Norleucine was added to each sample as an internal standard.

The carbohydrate residues of antifreeze glycopeptides were analysed using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), consisting of a Dionex Bio-LC Series 5000 instrument (Dionex Corp., Sunnyvale, CA), a Dionex Bio LC gradient pump, a CarboPac™ PA-1 (4 × 250 mm) high-resolution strong anion exchange column, and a Model PAD-2 detector

Circular-dichroism (CD) spectra were obtained on a Jasco J-600 spectropolarimeter. The sample cell had a path length of 0.1 mm and was water jacketed for temperature control. Spectra were taken at 20°C. The peptide was dissolved in 50 mM KH_2PO_4, pH 7.4, at a concentration of 1 mg ml^{-1}.

Samples of reverse-phase HPLC were used for plasma desorption mass spectrometry (PD-MS) analysis. Measurements were performed on a 252Cf plasma desorption time-of-flight mass spectrometer (Applied Biosystems) with a flight tube length of 15 cm. Acceleration voltages were 15 kV in the positive- and negative-ion modes. Spectra measured in the positive and negative ionization modes were accumulated for 10 million fission events each.

The molecular weight was also obtained at 25°C by coupling on-line high-performance size exclusion chromatography (HPSEC) and a multi-angle laser light scattering photometer [MALLS detector, a DAWN-F fitted with a K5 flow cell and a He-Ne laser (λ = 632.8 nm) from Wyatt Technology Corporation, Santa Barbara, CA]. Proteins were chromatographed on a TSK-gel G3000SW column with an elution buffer 50 mM KH_2PO_4, 150 mM NaCl, pH 7.0, and a flow rate of 1.0 mg ml^{-1}. Weight average MW and root-mean-square radii were established with ASTRETTE and EASY software (v. 3.04) (Wyatt 1994).

In this study the differential scanning calorimetry (Perkin-Elmer DSC-7) used to determine antifreeze activity employed direct observation of the sample under slow cooling (1.0° and 0.1°C) in the presence of seed ice crystals; these conditions eliminate any supercooling of the sample which may give rise to non-physiological estimates of the freezing point depression. DSC allows for the observation of various thermal events including phase transitions. Analysis of antifreeze peptides or glycopeptides by DSC allowed for rapid assessment of activity which took into account the amount of ice initially present in the sample. With the DSC the thermal hysteresis is defined as the difference between the annealing temperature within the melt zone and the point of ice growth: starting temperature – onset point = antifreeze activity (Hansen et al. 1991).

Samples of AFGP (5, 10 and 20 mg ml^{-1}) and AFP (40 and 50 mg ml^{-1}) from Antarctic fish were dissolved in distilled water and analyzed for antifreeze activity. Samples of approximately 5 µl were placed in 10 µl aluminum pans. The samples were frozen (–40°C, 5 min) and warmed up to various partial-melt temperatures so that upon subsequent re-cooling (1°C min^{-1}) the samples would be inoculated with different amounts of ice. The samples were monitored as they were cooled in the presence of ice. The crystallization temperature and melting-point onset and area for each sample were noted by scanning 1°C min^{-1} between +10° and –40°C. The amount of ice present in the partial-melt runs was calculated by comparing the freeze area to the complete-melt area. For a detailed reference of all procedures see Wöhrmann (1993, 1995).

RESULTS

As shown in Table 1, all investigated notothenioids possess AFGP but in different concentrations. The highest AFGP content was found in *Trematomus pennellii*. The lowest content was determined in the benthopelagic channichthyid *Neopagetopsis ionah*. The maximal thermal hysteresis (antifreeze activity) of AFGP, measured at concentrations of 20 mg ml^{-1}, differ from 0.52°C in *N. ionah* to 1.20°C in *Pleuragramma antarcticum* (Fig. 1). *P. antarcticum*, *Lepidonotothen kempi*, *Bathydraco marri* and *Dolloidraco longedorsalis* possess further AFP of the same concentration as the AFGP. However, the antifreeze activity is lower (0.41 to 0.84°C at peptide concentration of 40 mg ml^{-1}). So far, these peptides could not be further characterized.

Antifreeze substances were also isolated from non-notothenioid species. *Muraenolepis marmoratus*, *Macrourus holotrachys*, *Paraliparis somovi* and *Lycenchelys hureaui* synthesize AFP at very low concentrations

Table 1. Antifreeze peptides (AFP), and glycopeptides (AFGP), region of catch, depth of distribution (m) (after Gon & Heemstra 1990), and depth of catch (m) of investigated notothenioids and non-notothenioids of the Weddell Sea and the Lazarev Sea. AF: antifreeze substance; % FRG: antifreeze substance % fresh weight; TH: thermal hysteresis (°C) of AFGP (20 mg ml^{-1}) and other antifreeze substances (indicated by *, 50 mg ml^{-1})

Species	AF	% FRG	TH (°C)	Region of catch	Distribution (m)	Catch (m)
Nototheniidae						
Gobionotothen gibberifrons	AFGP	0.0270	0.67	Elephant Island	5–750	200
Lepidonotothen kempi	AFGP	0.0636	0.52	Lazarev Sea	100–900	560
	AF II	0.0598	0.73*			
Aethotaxis mitopteryx	AFGP	0.0356	0.89	Lazarev Sea	100–850	702
Pleuragramma antarcticum	AFGP	0.0267	1.20	Weddell Sea	0–900	450–630
	AF II	0.0032	0.45*			
	PAGP	0.0143	3.21*			
Dissostichus mawsoni	AFGP	0.1053	1.10	Lazarev Sea	80–1600	626
Trematomus bernacchii	AFGP	0.1021	1.01	Weddell Sea	100–700	626
Trematomus eulepidotus	AFGP	0.1989	1.02	Weddell Sea	70–550	467
Trematomus lepidorhinus	AFGP	0.1351	0.97	Weddell Sea	200–900	617
Trematomus loennbergii	AFGP	0.1204	0.95	Weddell Sea	60–830	574
Trematomus pennellii	AFGP	0.3337	1.06	Lazarev Sea	0–730	405
Artedidraconidae						
Artedidraco loennbergi	AFGP	0.0977	0.85	Weddell Sea	230–600	343
Dolloidraco longedorsalis	AFGP	0.0879	0.81	Weddell Sea	200–2250	626
	AF II	0.1322	0.72*			
Pogonophryne marmorata	AFGP	0.1595	0.87	Weddell Sea	140–1400	626
Pogonophryne scotti	AFGP	0.1627	0.89	Weddell Sea	110–1200	830
Pogonophryne barsukovi	AFGP	0.1544	0.86	Lazarev Sea	220–1120	800
Pogonophryne permitini	AFGP	0.1509	0.87	Lazarev Sea	430–1120	830
Pogonophryne macropogon	AFGP	0.1487	0.85	Lazarev Sea	570–840	830
Bathydraconidae						
Bathydraco marri	AFGP	0.0279	0.85	Weddell Sea	300–1250	623
	AF II	0.0651	0.84*			
Bathydraco macrolepis	AFGP	0.0219	0.84	Lazarev Sea	450–2100	1400
Bathydraco antarcticus	AFGP	0.0231	0.85	Lazarev Sea	320–2250	1400
Gymnodraco acuticeps	AFGP	0.1973	0.90	Weddell Sea	0–550	197
Cygnodraco mawsoni	AFGP	0.1794	0.89	Weddell Sea	110–300	197
Gerlachea australis	AFGP	0.1643	0.84	Weddell Sea	200–670	407
Racovitzia glacialis	AFGP	0.1147	0.84	Weddell Sea	220–610	574
Channichthyidae						
Chaenodraco wilsoni	AFGP	0.2835	0.57	Weddell Sea	200–800	509
Chionodraco hamatus	AFGP	0.2576	0.80	Weddell Sea	0–600	407
Chionodraco myersi	AFGP	0.1544	0.89	Weddell Sea	200–800	623
Cryodraco antarcticus	AFGP	0.0920	0.65	Weddell Sea	200–800	623
Dacodraco hunteri	AFGP	0.0809	1.00	Lazarev Sea	300–800	623
Neopagetopsis ionah	AFGP	0.0621	0.52	Weddell Sea	20–900	799
Pagetopsis maculatus	AFGP	0.1796	0.94	Weddell Sea	200–800	453
Pagetopsis macropterus	AFGP	0.2498	0.97	Weddell Sea	0–650	506
Muraenolepididae						
Muraenolepis marmoratus	(AFGP)	0.0076	0.56	Lazarev Sea	20–1600	830
Macrouridae						
Macrourus holotrachys	(AFGP)	0.0031	0.13*	Lazarev Sea	150–1100	742
Liparididae						
Paraliparis somovi	AFP	0.0103	0.54*	Lazarev Sea	400–850	623
Zoarcidae						
Lycenchelys hureaui	AFP	0.0041	0.18*	Lazarev Sea	560–940	830
Myctophidae						
Gymnoscopelus opisthopterus	AFP	0.0070	~0.1*	Lazarev Sea	≥500	742

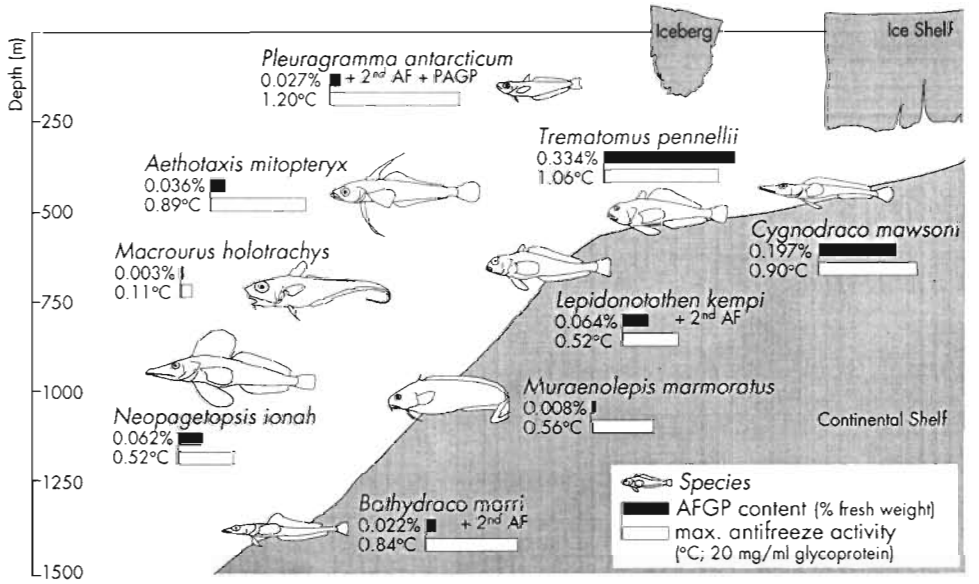

Fig. 1. Antifreeze glycopeptides (AFGP) of some investigated Antarctic fish species in relation to water depth and mode of life. Species shown here are representative of most of the ecological habitats of the Weddell Sea and the Lazarev Sea. The concentration of AFGP is given in % fresh weight, the antifreeze activity (°C) was measured at a protein concentration of 20 mg ml^{-1} with the differential scanning calorimetry

(Table 1); there was no thermal hysteretic effect observed in the blood serum. At peptide concentrations of 40 mg ml^{-1}, antifreeze activity could be measured (0.13°C in *M. holotrachys* up to 0.56°C in *M. marmoratus*). The antifreeze peptides of *M. marmoratus* and *M. holotrachys* were glycosylated in a fashion similar to that of the AFGP of the notothenioid species (Table 2).

AFGP were also isolated from *Pleuragramma antarcticum* caught in the southeastern Weddell Sea. The concentration of AFGP is about 80% less in comparison to other notothenioids from the same region. The results of the amino acid analysis (Table 2) showed that the larger glycopeptides were composed solely of the amino acid residues alanine and threonine in a 2:1 ratio. Analysis revealed only alanine and threonine and some proline in the lower molecular weight glycopeptide fractions. Carbohydrate analysis by HPAEC-PAD also revealed the presence of N-acetylgalactosamine (GalNAc) and galactose (Gal) after acid hydrolysis (trifluoroacetic acid) and of Galβ1→3GalNAc after β-elimination ($NaOH/NaBH_4$) and treatment with endo-α-N-acetylgalactos-aminidase. The same composition is present in the AFGP of *Aethotaxis mitopteryx*, *Pogonophryne scotti* and *Pagetopsis macropterus*. There is only a minor variation in the proportion of the amino acids alanine to threonine to proline. It seems apparent that although the molecular weights for the AFGP may vary in the range 35000 to 2600 Da from species to species, they still conserve the same structural subunits. The molecular weight of AFGP 7 and 8 were exactly determined by plasma desorption-mass spectrometry at 3277 and 2669 Da.

An additional glycopeptide, occurring in concentrations similar to those of the AFGP in *Pleuragramma antarcticum*, was isolated (Table 2). Amino acid analysis of this novel glycopeptide, called PAGP (*Pleuragramma*-antifreeze glycopeptide), shows glycine (23.9 mol%), alanine (20.9 mol%), aspartic acid (17.7 mol%), glutamic acid (15.8 mol%) and traces of valine, leucine, arginine and threonine as the amino acids. Sugar analysis by HPAEC-PAD indicate N-acetylglucosamine as carbohydrate residue. The molecular weight of PAGP is approximately 150 kDa, the root mean square radius 57.3 nm, as determined by laser-light scattering. The analysis of circular dichroism for PAGP obtained at temperatures of 20°C shows β-sheet (56%), α-helical (19%) and random chain (25%) characteristics. Both AFGP and PAGP are expanded in secondary structure. The antifreeze compounds total 2.46 mg ml^{-1} blood serum in adult specimens (SL 22 cm) of *P. antarcticum*.

There is a variation in the amount of both AFGP and PAGP present in relation to the age of *Pleuragramma antarcticum* (Fig. 2). Early post-larvae and maturing adults, abundant near the ice shelf in the southeastern Weddell Sea, possess higher antifreeze concentrations (AFGP: 0.283 to 0.295% fresh wt; PAGP: 0.188 to 0.219% fresh wt) than juvenile fish feeding on krill in the East Wind Drift (AFGP: 0.279% fresh wt; PAGP: 0.139% fresh wt). Moreover, the adult specimens possess the highest concentrations of high molecular weight AFGP (0.194% fresh wt).

The antifreeze activity was measured by DSC (Fig. 3). The sample was frozen at −40°C, then taken to various annealing temperatures (−0.2 to −1.0°C for 5 min) and cooled again. At −0.2°C no ice was present, and the sample supercooled and crystallized around −17°C (Fig. 3a). While water, non-antifreeze peptides and glycopeptides freeze immediately upon cooling when ice is present, whereas antifreeze glycopeptides

Table 2. Characteristics of antifreeze glycopeptides of *Pleuragramma antarcticum* (*Pa*), *Aethotaxis mitopteryx* (*Am*), *Pogonophryne scotti* (*Ps*) and *Pagetopsis macropterus* (*Pm*) and the antifreeze glycopeptide of *Muraenolepis marmoratus* (*Mm*). Amino acid and carbohydrate compositions were determined on the HPLC purified antifreeze components. Molecular weight was determined by SDS polyacrylamide gel electrophoresis, plasma desorption-mass spectrometry and laser light scattering. Thermal hysteresis (TH) was measured by differential scanning calorimetry (DSC) at a scan rate of 1°C min^{-1}, the secondary structure by circular dichroism

Species:	*Pa*	*Pa*	*Pa*	*Am*	*Ps*	*Pm*	*Mm*
Antifreeze:	PAGP	AFGP1–5	AFGP6–8	AFGP	AFGP	AFGP	(AFGP)
Amino acid (%)							
Aspartic acid	17.7						11.0
Glutamic acid	15.8						15.7
Serine							13.7
Glycine	23.9						
Arginine	5.1						6.4
Threonine	3.8	38.8	34.1	33.5	33.4	34.1	18.0
Alanine	20.9	61.2	62.4	63.6	63.5	62.6	18.4
Proline			3.5	2.9	3.1	3.3	10.2
Valine	6.4						
Leucine	6.2						
Carbohydrate (%)							
Galactose		50	50	50	50	50	50
GalNAc		50	50	50	50	50	50
GlcNAc	100						
Molecular weight (Da)	~150000[a]	~34000[a,c]	2667[b,d]				
Secondary structure	expanded in all cases						
TH (20 mg ml^{-1}) (°C)	0.16	1.20	0.92	0.89	0.89	0.97	0.56

[a]Determined by laser light scattering
[b]Determined by plasma desorption-mass spectrometry
[c]Molecular weight value is for AFGP1 only
[d]Molecular weight value is for AFGP8 only

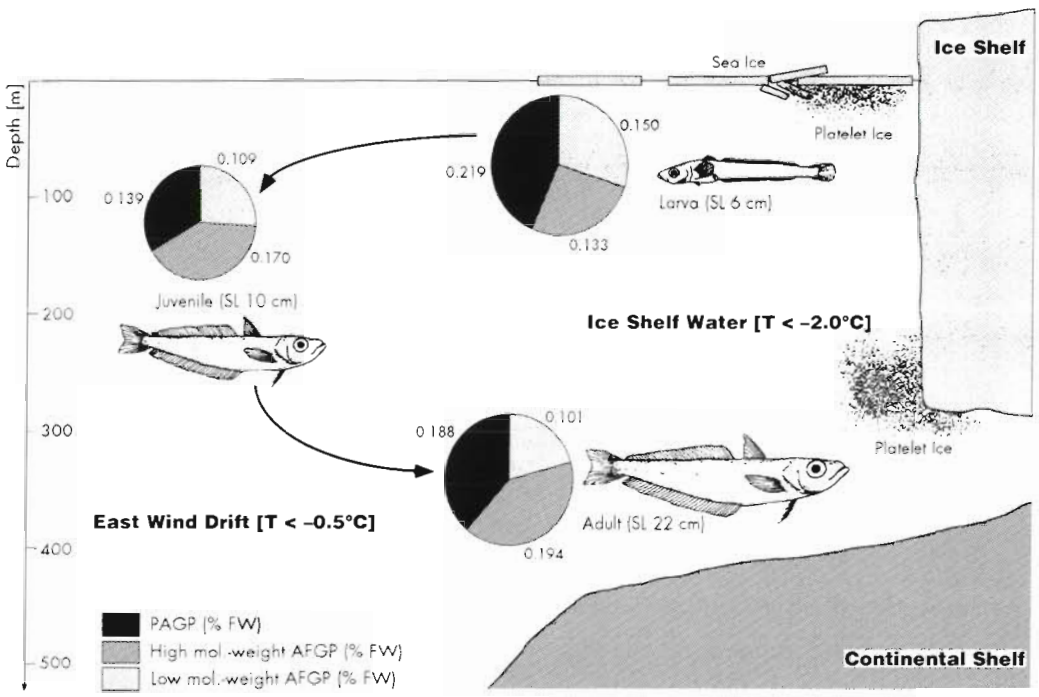

Fig. 2. Antifreeze glycopeptides in *Pleuragramma antarcticum* in relation to age of fish. AFGP 1–5: high molecular weight fraction; AFGP 6–8: low molecular weight fraction; PAGP: *Pleuragramma* antifreeze glycopeptide. Age of fish was calculated indirectly using standard length after Hubold & Tomo (1989)

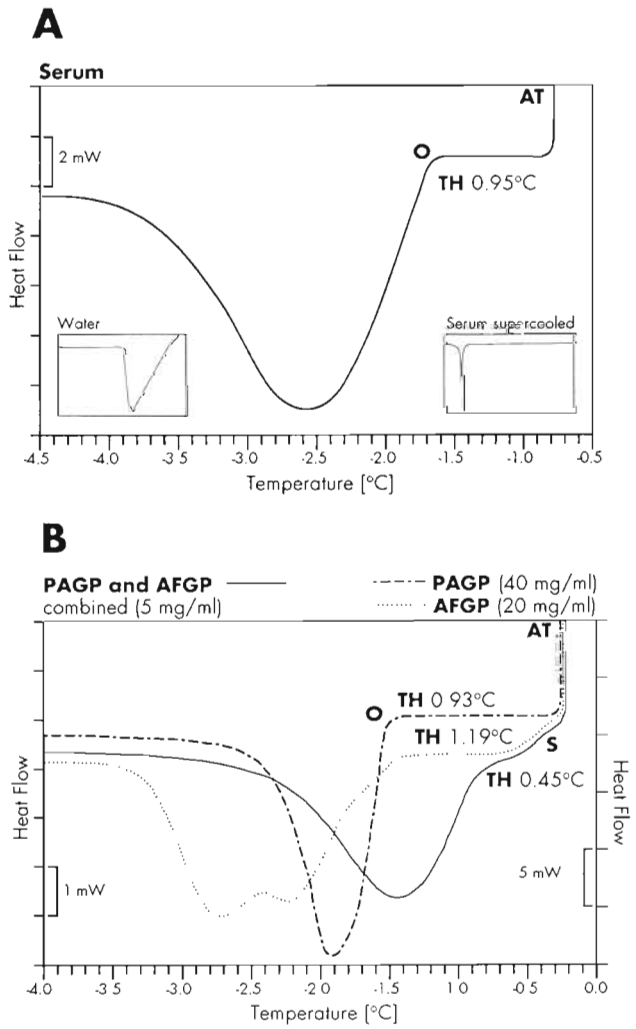

Fig. 3. Differential scanning calorimetry (DSC) of (A) serum and (B) AFGP and PAGP glycopeptides of *Pleuragramma antarcticum*. Concentrations of 20 and 40 mg ml^{-1} (5 µl) were tested for antifreeze activity. The typical thermogram revealed a delay in the exotherm onset upon cooling at 1°C min^{-1}. Samples that were cooled in the absence of ice supercooled and crystallized at temperatures below −17°C. Scale bars next to the thermograms represent heat flow in milliwatts

revealed an initial shoulder (*S*) upon cooling, followed by a delay before the onset (*O*) of the freezing exotherm (Fig. 3b). However, a shoulder is not observed when freezing blood serum (Fig. 3a) or the novel PAGP (Fig. 3b); instead, after cooling, there is a long delay before the onset of the freezing exotherm.

Thermal hysteresis seems to be ice-content dependent (Fig. 4). Below 75% ice content, AFGP demonstrated a linear increase in activity with decreasing ice content. The PAGP has no antifreeze activity at ice concentrations exceeding 60% and a high activity at low ice content (<20%). The serum of *Pleuragramma antarcticum* shows a very similar thermal hysteresis at

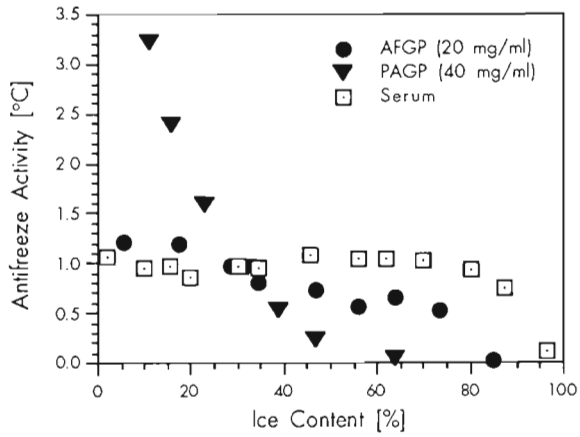

Fig. 4. Graph of antifreeze activity of AFGP, PAGP and blood serum of *Pleuragramma antarcticum* in relation to the ice content (%; different annealing temperatures). All samples shown were run at a scan rate of 1.0°C min^{-1}

different ice contents up to 80%. The freezing point of the serum is −1.9°C in adult specimens, and hence the same as the freezing point of sea water

As an additional measure of AFGP and PAGP similarity, the amount of thermal hysteresis as a function of protein concentration was measured. As can be seen in Fig. 5, the purified AFGP of *Pleuragramma antarcticum* show a good agreement with results obtained from the high molecular weight glycopeptides of *Dissostichus mawsoni*. This again would suggest that the molecular structure of the AFGP is conserved, and that only the number of structural repeating units varies. However, the thermal hysteresis of PAGP increases exponentially with increasing protein concentration.

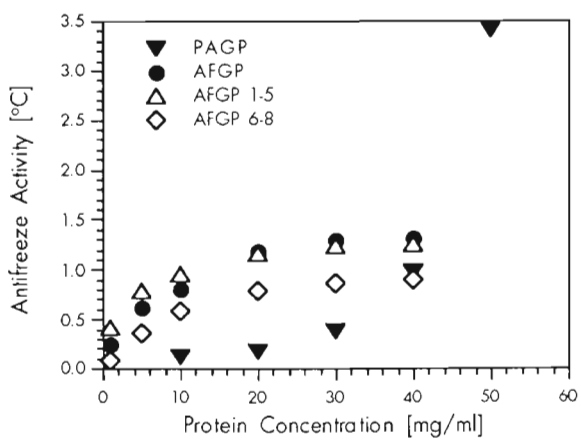

Fig. 5. Comparison of freezing-point depressing activity of the purified glycopeptides AFGP and PAGP of *Pleuragramma antarcticum* and high (AFGP 1–5) and low (AFGP 6–8) molecular weight fractions of antifreezes from *Dissostichus mawsoni* in relation to the protein concentration (mg ml^{-1})

DISCUSSION

The Antarctic Ocean hosts little more than 200 coastal fish species (Andriashev 1987, Kock 1992). About 50% of these species are found exclusively in Antarctic waters and belong to the suborder Notothenioidei (DeWitt 1970). The origin of this suborder dates back to the Lower Tertiary (50 million yr ago, Andersen 1984). Low temperatures have pervaded the Southern Ocean for some 40 million yr (Kennett 1977). Recent data suggest more or less constantly low temperatures (+3°C to −2°C; Hellmer & Bersch 1985) since about 13 million yr ago (Eastman & Grande 1989), which have led to the high degree of stenothermy and endemism of the fish fauna (DeVries & Eastman 1981).

Most adaptations of Antarctic notothenioids to the cold environment are based on biochemical adjustments (Eastman & Grande 1989) in metabolic pathways or important cell structures. Some notable examples are increased protein synthesis, low activation energies and enhanced low temperature activities in various enzymes, poorly developed glycolytic pathways combined with an increased role of the pentose-phosphate cycle, lipid storage in muscles instead of glycogen deposits, increased membrane fluidity, high conduction velocities and fully compensated synaptic events in the nervous system, and finally, cold-stable microtubules (for review see Macdonald et al. 1987, Eastman 1993). Antarctic notothenioids fill ecological roles normally occupied by taxonomically diverse fish in temperature waters (Eastman 1991).

The presence of AFP and AFGP in fish is an important adaptation which permits survival in freezing seawater. Because of this adaptation Antarctic fishes are found occupying most ecological niches of the Antarctic Ocean, including the surface and midwaters which are often rich in food but are ice-laden year round (for review see DeVries 1988, Cheng & DeVries 1991, Eastman 1993). Over the past 20 years DeVries and colleagues have isolated antifreezes in 15 species of notothenioids (Eastman 1990) and in 2 non-notothenioid Antarctic species (Cheng & DeVries 1989, Schrag et al. 1987). The results of this study of additional members of the notothenioids (32 species, Table 1), including the deeper living bathydraconids, channichthyids and non-notothenioid species, reveal that AFGP are common in all Antarctic notothenioids and probably present in the gadiform species *Muraenolepis marmoratus* and *Macrourus holotrachys*. It is striking that the amino acid and sugar composition of the isolated AFGP is the same in all those species. The other non-notothenioid species investigated (5 species of 5 families) also possess, without exception, antifreezes, though at very low concentrations.

Thermal hysteresis of antifreezes and blood serum measured by DSC was observed to depend on the amount of ice in the sample. Activity of AFGP of *Pleuragramma antarcticum* and other notothenioid species remained high at very high ice content, an important factor in the ice-laden water of the Antarctic. Below an ice content of 75%, activity of AFGP increased linearly with decreasing amounts of ice, exhibiting a maximal hysteresis of 1.19°C (8.8% ice). This behaviour is different from insect AFP (Zachariassen & Husby 1982, Hansen & Baust 1988) and PAGP, in which antifreeze activity increased exponentially with decreasing ice content. When the colligative freezing point depression effects of plasma solutes (0.8°C; Gordon et al. 1962) are added to the maximal thermal hysteretic effect of blood serum (1.06°C), the resulting protection from freezing (ca −1.9°C) is not as high as the lowest water temperature experienced by adult *P. antarcticum* (< −2.0°C). Adults of this species probably live in a supercooled state, and would freeze upon contact with ice in the surface water at <−1.9°C. The data also revealed that the low molecular weight AFGP 6–8 are less active than the high molecular weight AFGP 1–5. Also, the thermograms of AFGP revealed an initial shoulder in the exotherm direction upon cooling which correlates with observed *c*-axis ice growth (Raymond et al. 1989, Hansen et al. 1991); and, the loss of the shoulder during annealing experiments represents the end of the ice growth.

The thermogram of PAGP revealed no shoulder upon cooling, the ice growth is totally blocked. The subsequent exotherm would then be the explosive growth of ice normally observed with other antifreeze assessment techniques (DeVries et al. 1970, Hansen & Baust 1988). Antifreeze proteins, more appropriately called thermal hysteresis proteins, have also been described in insects (Block & Duman 1989, Duman et al. 1982). Since overwintering terrestrial insects usually face lower temperatures than fish, their thermal hysteresis proteins can cause a greater lowering of the freezing point than the 1°C that appears to be the maximum value for fish AFP and AFGP. However, the highly glycosylated PAGP at high concentrations shows an antifreeze activity of several degrees similar to that which has been reported for crude insect hemolymph. In the hemolymph there are different proteins and other substances depressing the freezing point to such a low level. In PAGP the high content of carbohydrates (N-acetylglucosamine, Table 2) probably causes the thermal hysteresis at about 3.4°C.

In the presence of ice, Antarctic fishes will freeze within a few tenths of a degree of the temperature at which ice will propagate in their blood. It has been shown in earlier investigations that the amount of AFGP present depends upon the fishes' habitats

(review DeVries 1988). Species like the nototheniid *Trematomus pennellii* as well as the channichthyid *Pagetopsis macropterus* or the bathydraconid *Gymnodraco acuticeps* possess large amounts of AFGP. There is a correlation between the blood freezing point, the concentration and composition of antifreezes, the temperature at which the fish will freeze in the presence of ice, and the habitat of the fish (e.g. water depth). Fishes living in shallow waters which may come in contact with anchor ice have high concentrations of both high and low molecular weight AFGP. They freeze at lower temperatures than those living in ice-free deep water such as the non-notothenioid species (e.g. *Macrourus holotrachys* or *Lycenchelys hureaui*), which will freeze in surface water at –1.9°C. In their natural deep water habitat, these species are in little danger of freezing because of the higher temperature (e.g. +0.5°C on the continental slope in the Lazarev Sea) and because of the effect of hydrostatic pressure which at 500 m lowers their freezing point to –2.3°C, a temperature well below that of the freezing point of seawater. However, in all species investigated, antifreeze molecules could be found.

The snailfish *Paraliparis somovi* was caught at a depth of 620 m in the Lazarev Sea, the temperature was about 0.5°C, no ice was formed in water, and no thermal hysteretic effect could be measured in the blood serum. However, this species possesses non-glycosylated antifreezes in low concentrations. Most Antarctic liparidids are benthic or epibenthic at depths of 300 m up to 3000 m (Andriashev 1986). They feed on a variety of invertebrates captured on or near the substrate. In McMurdo Sound, *Paraliparis devriesi* lives near the bottom at 500 to 650 m (Andriashev 1986). This species does not possess antifreezes but lives in a supercooled state (Eastman 1993). *Paraliparis* spp. are in no danger of freezing because of the absence of ice at that water depth, hence the body cannot be seeded by ice crystals (DeVries & Lin 1977). On the other hand, the presence of antifreezes in *P. somovi* suggests that this species also has the genetic potential to synthesize these peptides.

Near Balleny Island, 1200 km north of McMurdo Sound, *Lepidonotothen kempi* inhabits a +1°C layer of water. Slightly shallower and warmer water may have permitted western Antarctic species to become established at Balleny Island. DeVries & Lin (1977) reported that *L. kempi* does not possess antifreezes. However, *L. kempi* from the Lazarev Sea does synthesize AFGP, but in lower concentrations than other notothenioids from the same region (e.g. *Trematomus lepidorhinus, Gymnodraco acuticeps, Dacodraco hunteri*). Although the Balleny Island are less than 300 km off the Victoria Land coast in eastern Antarctica, the fauna lacks eastern Antarctica endemics and includes *L. larseni*, a species found primarily in western Antarctica and South Georgia (Andriashev 1965, DeWitt 1971). Like the other species distributed at Balleny Island, *L. larseni* possesses AFGP. Presumably the concentration of AFGP in *L. kempi* from Balleny Island was too low to be detected by the methods available in the 1970s. It is likely that all species of the genus *Lepidonotothen* and the *L. squamifrons* group (Schneppenheim et al. 1994) from the Antarctic Peninsula and the eastern and western Antarctic synthesize antifreezes.

The AFGP content in *Bathydraco marri* and *Dolloidraco longedorsalis* is relatively low compared with that of other notothenioids. However, they possess further antifreeze compounds in non-glycosylated peptides. Bathydraconids and artedidraconids are usually referred to as the 'typical high-Antarctic' species (DeWitt 1970) and species of the genus *Bathydraco* clearly prefer deep and cold areas (Ekau et al. 1986, Schwarzbach 1988, Ekau 1990). Bathydraconids are characteristic of the area of the Filchner depression, which is a deep trench running from the Filchner ice shelf to the continental slope. The prevailing water body is the Ice Shelf Water (ISW) with temperatures as low as –2.2°C and salinities of 34.6 to 34.7‰ (Hellmer & Bersch 1985). The relatively high abundance of bathydraconids in Gould Bay, which is adjacent to the Filchner Depression, could be due to the extremely low temperatures of –2.0 to –2.2°C, as proposed by Ekau (1990), or to the high pressure, or a combination of both and the possession of antifreeze compounds in addition to the AFGP.

Aethotaxis mitopteryx and *Pleuragramma antarcticum* belong to one taxonomic tribe (Pleuragrammiini, along with *Cryothenia peninsulae* and *Gvozdarus svetovidovi*; DeWitt et al. 1990), which is the most advanced amongst Nototheniidae and which may have developed fairly recently, possibly less than 10 million yr ago (Andersen 1984). All 4 species are more or less confined to a pelagic/benthopelagic mode of life. Some special adaptations (e.g. neutral buoyancy, antifreeze, blood characteristics) may be of relatively recent origin (Andersen 1984) and could be assigned to recent changes in lifestyle. Apparently, this unique mode of life for fishes, i.e. pelagic and sluggish, seems to be an energy-saving adaptation, providing advantages for fish life in the pelagial or at least not having any obvious disadvantages (Kunzmann 1991, Kunzmann & Zimmermann 1992). In contrast to *A. mitopteryx*, *P. antarcticum* has the PAGP and an additional antifreeze peptide in lower amounts. The content of both AFGP and PAGP seems to depend on the fish's ontogenetic migration and is related to the ambient water temperature (Fig. 2). This is particularly interesting because of the seasonal migration of *P. antarcticum* (Hubold 1985, Gerasimchook & Lanin 1988). Different water masses

are crossed during migration and functionally different antifreeze compounds could be helpful when environmental temperature varies.

Most notothenioids are bottom fish confined to water less than 1000 m deep, although the depth range of individual species may be considerable (Kock 1992). They lack swim bladders, are usually denser than seawater (Eastman & DeVries 1982, Eastman 1991), and commonly feed and reproduce on the substrate. In an ecological sense, the Southern Ocean is probably underutilized by fishes and could theoretically support more species. Assuming that all Antarctic fish species have the genetic potential to synthesize antifreezes, the low biomass of fish in the water column is due to other environmental factors, such as food supply. The waters south of the Antarctic Convergence are productive during the summer only, and contain relatively few non-notothenioid fish.

Of the more than 75 fish species occuring in the Weddell Sea, planktonic stages of less than half are known. Most of these appear only in the surface layer for a short time in the summer (Hubold 1991). There is an established seasonal sequence of species in the summer plankton of the Weddell Sea (Hubold 1990), as well as in other Antarctic areas, e.g. the Bransfield Strait (Kellermann 1989). In winter, pelagic stages of at least 7 species have been found in the Weddell Sea. The pelagic stages and species utilize the relatively high water column production and thus achieve higher stock sizes than the strictly benthic species. They are probably sensitive to environmental changes and represent a relatively young community which evolved in the interglacial periods (DeWitt 1970, Hubold & Ekau 1987). Planktonic stages concentrate in the surface mixed layer, where feeding conditions seem to be near the optimum, but where there is a risk of contact with ice crystals.

Eastman & Grande (1989) discussed factors that might have contributed to the paucity of non-notothenioid species in the modern Antarctic ichthyofauna. They concluded that low water temperature was not paramount, and that factors in the realm of ecological constraints were probably at least as important in restricting diversity. Given that antifreezes may have evolved independently several times in the course of evolution, as suggested by the presence of antifreeze peptides in 10 families of 5 suborders, it seems unlikely that low temperature as such has caused the paucity of non-notothenioid species in the Antarctic fish fauna (Clarke 1987, 1990). Rather, the limited shallow water habitat on the continental shelf and seasonal oscillation in the food supply are plausible ecological factors.

The 5 major antifreeze types characterized to date are distributed over 10 families, spanning at least 5 suborders (Fig. 6). Some closely related fish species inhabiting the same environment produce very different antifreezes, while others belonging to different orders and which are geographically isolated produce essentially identical antifreezes. Scott et al. (1986) suggested that the first antifreeze proteins of marine teleosts were established during the dramatic Cenozoic cooling events initiated approximately 36 million yr ago at the Eocene/Oligocene boundary (Clarke & Crame 1992) and that the identical AFGP of Arctic gadiforms, such as *Boreogadus saida*, and Antarctic notothenioids imply a close relationship. However, Eastman (1993) states that AFGP evolved independently in gadiforms and notothenioids, and that notothenioid AFGP appeared during the past 10 to 15 million yr, possibly even later. At the conclusion of the Eocene/Oligocene cooling event at 36 million yr, waters were simply too warm (5 to 7°C) to require the presence of AFGP. And, if gadiforms evolved in the Southern Hemisphere and possessed AFGP early in their history, we would expect them to be more extensively represented in the modern Southern Ocean ichthyofauna. However, the present investigation indicates that Antarctic gadiforms also possess AFGP. If this is the case, then AFGP have evolved independently at least 3 times. By contrast, there are no known examples in which unrelated proteins have sufficiently similar (and sufficiently extensive) sequences to warrant the descriptor 'sequence convergence' (Doolittle 1994). If it turns out that the AFGP from Antarctic and Arctic fishes are truly unrelated, this case would have to rate as the nearest thing to sequence convergence yet reported. However, I suggest that, before the continental drift occurred, precursor proteins (e.g. blood proteins or lectins) to the present antifreeze proteins existed and evolved during the Cenozoic cooling events initiated approximately 36 million yr, 16 million yr and 3 million yr ago (Kennett 1977, Clarke 1990) into the various antifreeze peptides and glycopeptides of marine teleosts which are found today.

CONCLUSION

Freezing resistance is an important adaptation which all Antarctic fishes have successfully developed. All high-Antarctic notothenioids possess AFGP; the non-notothenioid species, except for the gadiforms *Muraenolepis marmoratus* and *Macrourus holotrachys* possess non-glycosylated antifreeze peptides. The ambient water temperature near the freezing point is no selective mechanism for the distribution of Antarctic fish. The discovery, to date, of 5 different antifreeze structures in teleosts, with little relation to their distribution within the currently accepted phylogenetic scheme, has led to the suggestion that more types of antifreezes might be discovered as more species are

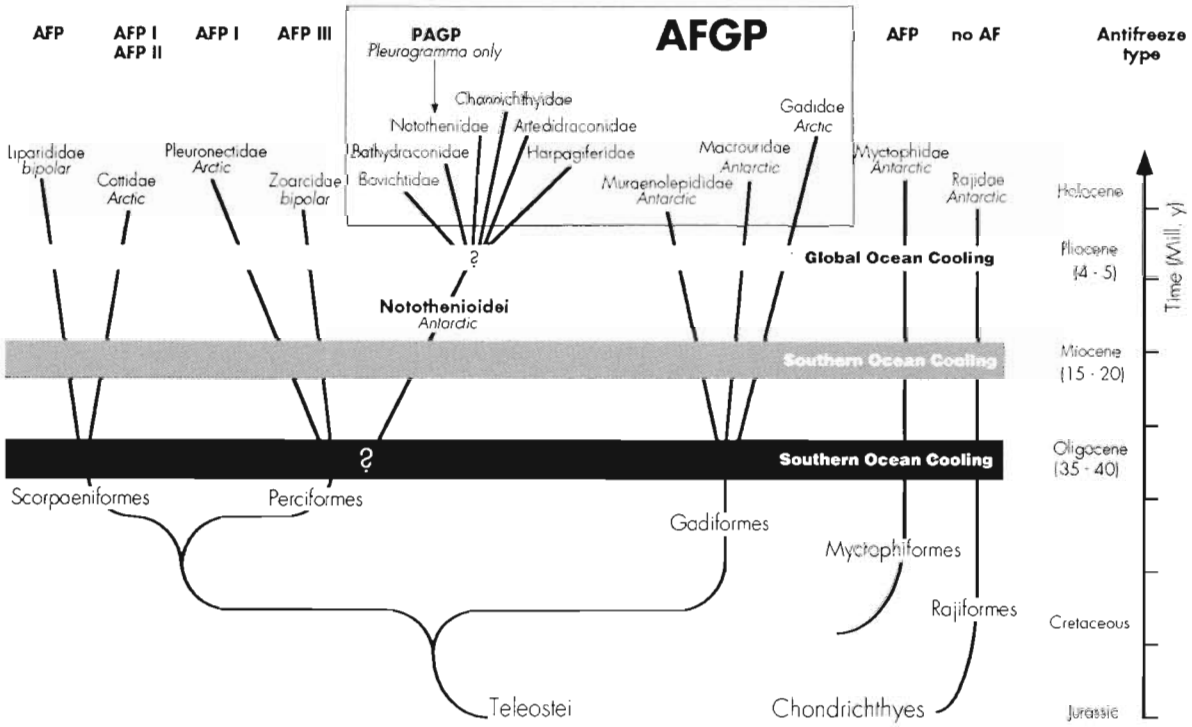

Fig. 6. Schematic outline showing the phylogenetic relationships of present-day fish (after Nelson 1984) that possess antifreeze peptides or glycopeptides. The broad bands at Oligocene, Miocene and Pliocene indicate the onsets of Antarctic and Arctic glaciation (Kennett 1977, Clarke & Crame 1992)

surveyed. At the present time, cysteine-rich antifreeze proteins in smelt and herring (Ewart & Fletcher 1990) and further proteins in deep-living notothenioids (Wöhrmann et al. unpubl.) are being characterized. Recent investigations into the genomic organization of antifreeze genes are beginning to offer additional insights into the dynamics of antifreeze evolution. On the one hand, the lineage of the more commonly studied proteins such as globins, crystallins, and cytochrome *c* can be projected back 108 to 109 million yr ago, i.e. at least some of the fish antifreezes developed as recently as 106 to 107 million yr ago. On the other hand, recent data about the mtDNA variation detected in notothenioid fishes shows it to be too low to agree with the age of notothenioid fishes as suggested by radiation (38 million yr ago) and might instead suggest a younger age (10 to 15 million yr ago) (Bargelloni et al. 1994).

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