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Antifreeze glycoprotein levels in Antarctic notothenioid fishes inhabiting different thermal environments and the effect of warm acclimation

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Abstract

A quantification method was developed to determine the concentrations of the major antifreeze glycoprotein (AFGP) isoforms in the blood of Antarctic notothenioid fishes. Serum samples were precipitated with 2.5% TCA and the supernatant containing AFGPs were chromatographed on an HPLC size exclusion column and the concentrations of the major AFGP size classes were determined from the areas of the corresponding peaks in the elution profile. Eight species of Antarctic notothenioid fishes were examined and their blood AFGP concentrations varied from 5 to 35 mg/mL. All of these fishes synthesized both the large and small AFGPs, but maintained higher levels of small AFGPs than the large ones in their blood. The species inhabiting more severe water environments (lower temperature and presence of ice) had higher serum AFGP levels than those in milder environments. The cryopelagic *Pagothenia borchgrevinki* decreased their blood AFGP concentrations in response to warm acclimation, but to a much lower extent in comparison to the antifreeze-bearing fishes in the Northern Hemisphere. After being warm acclimated at +4 °C for 16 weeks, the serum concentrations of the small and large AFGPs were decreased by about 60% and 20%, respectively.

Keywords: AFGP; Antarctic fish; Notothenioid; Antifreeze glycoprotein; Quantification; Warm acclimation; TCA precipitation; Thermal hysteresis

1. Introduction

Marine teleost fishes inhabiting both polar regions synthesize macromolecular antifreezes to avoid freezing in the ice-laden waters (DeVries, 1982; Fletcher et al., 2001). These biological "antifreezes" or hysteresis proteins are either peptides or glycopeptides and are found in unrelated northern and southern hemisphere fishes. So far, four types of proteins (AFP) and one type of glycoprotein (AFGP) have been isolated and characterized (Cheng, 1998). These hysteresis proteins depress the "freezing point" (temperature at which small seed ice crystals grow) of the body fluids in a non-colligative manner without significantly lowering the melting point (or equilibrium freezing point) (DeVries, 1971). The difference between the melting and freezing point is termed "thermal hysteresis " and the non-colligative freezing point as the hysteresis freezing point (DeVries, 1983; Cheng and DeVries, 1991; DeVries and Cheng, 1992; Ewart et al., 1999; Fletcher et al., 2001).

Northern fishes experience large seasonal variations in water temperature compared to the Southern Ocean fishes and show annual cycles in blood hysteresis protein levels, with the highest concentrations occurring during the winter season and the lowest during the summer. Other temperate water fishes such as the winter flounder *Pleuronectes americanus*, Atlantic tomcod *Microgadus tomcod* and Atlantic cod *Gadus morhua*, have high levels of AFP in the winter but have little or none circulating in their blood during summer (Petzel et al., 1980; Fletcher et al., 1978, 1982; Reisman et al., 1984). A few northern fishes such as the sculpin maintain blood AFPs year-round, but the concentrations are much lower during summer (Fletcher et al., 1984, 1985). Temperature acclimation and photoperiod studies show that the AFP cycle in the winter flounder is an endogenous one and that the onset of synthesis in the autumn can only be slightly modulated by water temperature and/or photoperiod, but the loss is accelerated by high acclimation temperatures

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during the spring season (Duman and DeVries, 1974a; Fletcher, 1981; DeVries, 1982; Fletcher et al., 1987).

In the high latitudes of the Arctic and Southern Ocean, water temperatures are near their freezing point/melting point (−1.9 °C) for most of the year (DeVries, 1983; Eastman, 1993), and hence, the hyposmotic fishes residing in these waters (body fluid melting points of approximately −1 °C) need to maintain high levels of AF(G)Ps throughout the year (DeVries and Lin, 1977; Fletcher et al., 1982). In McMurdo Sound, Antarctica, there is ice cover for 10–11 months of the year and the annual mean water temperature is about −1.9 °C (Littlepage, 1965). Below 100 m, it varies with season and depth by less than 0.1 °C. However, the temperature of the surface waters (<50 m) may rise to +1 °C for a few weeks during January (Hunt et al., 2003) depending upon the Ross Sea current patterns and ice cover during a particular year.

Based on hysteresis activity measurements, the notothenid fishes inhabiting the constantly cold environment of McMurdo Sound show no detectable variation in their AFGP concentrations throughout the year. Relatively low LD_{50}s (+6 °C) in several McMurdo Sound notothenids indicate they are extremely stenothermal (Somero and DeVries, 1967) and their upper lethal temperature changes very little after 1 month of warm acclimation at +4 °C (DeVries, unpublished). Thus, it is possible that these fishes have lost their capacity to acclimate to warmer temperatures. DeVries and Lin (1977) reported that the serum hysteresis freezing point of the Antarctic notothenid fish *Pagothenia borchgrevinki* rose only by 0.15 °C (from −2.75 °C to −2.60 °C) after the fish were warm acclimated at +4 °C for 60 days but the hysteresis freezing point of the dialyzed serum did not change. In another study, *P. borchgrevinki* acclimated at +4 °C for 7 weeks showed no significant change in the hysteresis freezing point (O'Grady et al., 1982). Although the hysteresis does not change in these studies, it is not possible to unequivocally state that the levels of AFGP do not change, because the concentrations of AFGP are in the plateau region of the hysteresis freezing point vs. concentration curve. In the plateau region of the curve, substantial increases or decreases in concentration have little effect on the hysteresis freezing point (DeVries, 1986). In addition, it has recently been shown in the McMurdo Sound fishes that when seed crystals of variable size are used, the resulting hystresis freezing points are highly variable and very small (5 μm) seed crystals give much lower hysteresis freezing points than large crystals (Jin and DeVries, 1997). Wöhrmann (1997) also reported that the hysteresis activity in serum from *P. antarcticum* was dependent on ice content in calorimetric freezing point determinations with small ice fractions correlated with low hysteresis freezing points. There were probably few or no large seed crystals remaining in the calorimeter cell sample after warming to reduce the ice fraction. It has also been shown that if frozen insect hemolymph is melted so that it makes up a very small fraction of the sample in a nanoliter sample well, then the hysteresis freezing points are much lower than when the ice fraction is large (Zachariassen and Husby, 1982). Although the McMurdo Sound notothenioid fishes show little if any change in the hysteresis with warm acclimation, closely related notothenioid fishes inhabiting the slightly warmer waters adjacent the Antarctic Peninsula clearly have higher blood hysteresis freezing points, correlated both with habitat depth and a milder thermal environment (DeVries and Lin, 1977). In some of these deeper water species levels of AFGP based on blood hysteresis measurements are insufficient to prevent freezing should they be exposed to ice in their environment (DeVries, 1988). Thus, the evolutionary response to a slightly less severe thermal environment has enabled these notothenioids to maintain lower levels of AFGP than their close relatives in the more extreme McMurdo Sound. Based on the correlation between the level of AFGP and the severity of the environment, it was suggested that the notothenioid fishes could respond to environmental temperature changes both in the long term and short term. In order to clarify the relationship between antifreeze level and the severity of the environment, we developed a method to quantify the levels of the major AFGP size isoforms in the blood of several notothenioid fishes and determine the effect of extended warm acclimation on one of the species.

One of the major difficulties in the isolation, separation and quantification of the AFGPs is the complex size heterogeneity. Originally, 8 major size isoforms of AFGP were described in the fish *P. borchgrevinki* and most other McMurdo Sound notothenioid fishes (DeVries et al., 1970; Komatsu et al., 1970). Using low-resolution acrylamide gel electrophoresis, the AFGPs were initially classified into a large size group (AFGPs 1–5) and small size group (AFGP 6–8) as indicated by their mobility on a non-denaturing gel. The largest isoform (least mobile) was numbered 1 and the smallest (most mobile) 8. The smallest AFGP (AFGP 8) contains only 4 tripeptide repeats and has a molecular mass of 2600 Da, while the largest one (AFGP 1) has about 56 tripeptide repeats and a molecular mass of about 34,000 Da. Using gradient gel electrophoresis and staining with fluorescamine, as many as 16 size isoforms have been identified in many of the notothenioid fishes (Cheng, 1996). The high-resolution gels also showed that AFGP 6 separated into 6 isoforms that differed from each other by one 608-Da glycotripeptide repeat. AFGP 5 was also resolved into two narrowly separated bands that also differed by 608 Da in molecular mass.

The small AFGPs are only about two thirds as effective as the large AFGPs in depressing the hysteresis freezing point on a mass basis (Raymond and DeVries, 1977; Schrag and DeVries, 1982; DeVries and Cheng, 1992). When antifreeze activities are used to estimate AFGP concentrations in different species, the error can be large if different species have proportions of the large and small AFGP isoforms that differ from those used to generate the standard curves.

The use of high-performance liquid chromatography (HPLC) to separate the AFGPs in trichloroacetic acid (TCA) supernatants of *P. borchgrevinki* serum has previously been reported (Burcham et al., 1984). This method was based on the fact that AFGPs are soluble in a TCA solution while the other serum proteins are precipitated. Although the method separated many