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FISH SKIN: AN EFFECTIVE BARRIER TO ICE CRYSTAL PROPAGATION

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Summary

It has been well established that antifreeze peptides are responsible for the ability of many marine teleosts to survive in icy sea water at temperatures below the colligative freezing points of their blood. However, the *in vivo* site of antifreeze action has yet to be determined. One current hypothesis, the 'ice-exclusion hypothesis', suggests that antifreeze proteins act within epithelial membranes (skin, gills, gut) and block the entry of external ice crystals. This hypothesis was examined by measuring ice propagation temperatures across isolated skin samples from the winter flounder (*Pseudopleuronectes americanus*). The results obtained were consistent with the hypothesis, indicating that fish skin is an effective barrier to ice propagation and that the effectiveness of this barrier improves with the addition of antifreeze proteins. The results also demonstrated that the skin ice propagation temperatures were substantially lower than the lethal freezing temperatures of the fish. Therefore, some other epithelial tissue (possible gill) must be less effective than the skin in blocking ice crystal penetration into the fish.

Introduction

Despite the considerable body of literature on antifreeze proteins and their role in improving the freezing resistance of fish, it is not clear how these proteins protect fish from freezing (DeVries, 1984; Feeney *et al.* 1986; Fletcher *et al.* 1986, 1991; Davies *et al.* 1988; Davies and Hew, 1990). Two hypotheses have been proposed to explain the *in vivo* mechanism of antifreeze action (DeVries, 1986) and they can be termed (a) the 'ice-modification hypothesis' and (b) the 'ice-exclusion hypothesis'.

The ice-modification hypothesis is based on the mechanism proposed for antifreeze action *in vitro*. Detailed observations of ice crystal growth under the microscope reveal that antifreeze proteins not only lower the freezing point of an aqueous solution but they also modify the growth habit of the ice. The antifreezes

Key words: freezing resistance, antifreeze proteins, winter flounder, *Pseudopleuronectes americanus*, ocean pout, *Macrozoarces americanus*.

are believed to do this by preferentially binding to the prism faces of ice crystals, thereby inhibiting growth along the favoured direction (*a*-axis) by blocking access of water to the ice lattice (Scholander and Maggert, 1971; Knight *et al.* 1984; DeVries and Lin, 1977; Yang *et al.* 1988; Davies and Hew, 1990). The ice-modification hypothesis suggests that small, possibly embryonic, ice crystals can be present within the body fluids of fish, but are prevented from growing by the antifreezes that are bound to them (Franks *et al.* 1987).

The ice-exclusion hypothesis argues that, by inhibiting the growth of ice crystals, the antifreeze proteins prevent them from entering the fish by blocking their propagation across epithelial membranes (skin, gills, gut).

DeVries (1986) argues in favour of the ice-exclusion hypothesis and cites two lines of evidence in support of his argument. One piece of evidence stems from the fact that antifreeze glycopeptides are absent from the urine and ocular fluids of Antarctic nototheniids residing in the ice-laden sea water. Thus, antifreezes are unavailable to prevent ice crystal growth in these undercooled fluids (DeVries, 1982; Eastman *et al.* 1987). The second line of evidence comes from unpublished experiments cited by DeVries (1986) in which fish survived undercooling to −6°C. As DeVries (1986) pointed out, microscopic ice crystals could not have been present in the fish during the course of these experiments otherwise they would have propagated and killed the fish at −2.2°C, the lowest effective temperature of the plasma antifreeze proteins.

Although the arguments presented by DeVries (1986) support the ice-exclusion hypothesis, there is no direct evidence demonstrating that antifreeze proteins can prevent ice propagation across epithelial tissues. The present study examines the role of antifreeze proteins in blocking the movement of ice across fish skin.

Materials and methods

General procedures

Winter flounder (*Pseudopleuronectes americanus* Walbaum) and ocean pout (*Macrozoarces americanus* Schneider) were caught in Conception Bay, Newfoundland, by SCUBA divers. Most of the fish were maintained in 250 l aquaria at seasonally ambient seawater temperatures and photoperiod. This ensured a normal annual plasma antifreeze cycle (Fletcher, 1977).

Blood samples were collected from a caudal blood vessel using plastic syringes with 21 gauge needles and were transferred to Vacutainers containing sodium heparin (Becton Dickinson). Plasma was separated from red cells by low-speed centrifugation (3000 *g*) and stored at −20°C until analysis.

Mucus samples were obtained from intact fish by enclosing them in plastic bags at 2°C for a few minutes (Shephard, 1981). The mucus was removed from the bags and centrifuged to remove traces of cellular debris.

The fish were killed by a blow on the head. Skin samples from the main trunk of the fish were dissected free of muscle tissue and either frozen (in liquid N_2) for later analysis or, if they were to be used immediately, placed in a Petri dish at 4°C

with the dermal surface in contact with filter paper moistened with saline. Considerable care was taken not to damage the scales or the epithelial cell layer of the skin samples. Muscle tissue was removed from the anterior dorso-lateral region of the fish.

Extracellular fluid volumes of muscle and ocular-side (dark upper side) and blind-side (lower light side) skin were estimated from the chloride space (Manery, 1954). Note that the dark upper surface and the lower light surface of a flatfish are sometimes referred to as the dorsal and ventral surfaces, respectively.

$$Cl^- \text{ space} = \frac{Cl^-_t \times H_2O_p \times r_{Cl^-}}{Cl^-_p},$$

where t is tissue concentration in mmol kg^{-1} tissue water; H_2O_p is plasma water in g $100\,g^{-1}$; Cl^-_p is plasma Cl^-, in mmol l^{-1}; and r is the Gibbs–Donnan equilibrium ratio (0.977).

Extracellular space was also measured in blind-side skin by incubating samples in solutions containing [^{14}C]inulin (Lutz, 1972). In this method approximately 3 g of skin was placed in 25 ml of saline containing 2×10^6 disints min^{-1} [^{14}C]inulin and incubated at 4°C for 80 h. Sub-samples of the skin and incubation medium were removed at various times following the initiation of incubation. Equilibrium between the incubation medium and the skin extracellular space occurred between 60 and 80 h. Inulin space was computed at equilibrium:

$$\text{Inulin space (\% tissue water)} = \frac{\text{skin inulin (disints min}^{-1}\,\text{kg}^{-1}\,\text{water)}}{\text{saline inulin (disints min}^{-1}\,\text{l}^{-1}\text{)}} \times 100.$$

Tissue and plasma water were measured by drying samples to constant mass at 50°C. Skin samples was digested in nitric acid prior to chloride determinations. [^{14}C]inulin levels were measured in skin samples solubilized by Protosol. Radioactive determinations were carried out using a liquid scintillation counter (Minaxi β Tri-Carb 4000 series). Protosol and [^{14}C]inulin were purchased from New England Nuclear.

Chloride analyses were carried out on plasma and digested skin samples using a Radiometer CMT 10 chloride titrator. Freezing point depression was determined using a freezing point osmometer measuring small (50 μl) samples (Fiske OR, Fiske Associates). Antifreeze activity was measured using a nanolitre osmometer (Clifton Technical Physics, Hartford, NY). In this method, the melting and freezing behaviour of a small ice crystal is observed under a microscope. Thermal hysteresis, a measure of antifreeze activity, is the difference between the freezing and melting temperatures (Kao *et al.* 1986).

The fish saline (pH 7.8, freezing point depression approximately 0.63°C) used in the following experiments consisted of (mmol l^{-1}): NaCl, 175; KCl, 2.7; $MgCl_2 . 6H_2O$, 0.64; $CaCl_2 . 2H_2O$, 2.74; glucose, 2.22; Trizma, 3.0.

Antifreeze extraction from skin

All samples (2–4 g) were homogenized under liquid N_2 using a mortar and

pestle. 30 ml of distilled water was added to the resulting powder and the mixture incubated for 1 h at 2°C. Following incubation, the suspension was filtered under vacuum through Whatman no. 1 filter paper to remove suspended debris. The filtered debris was washed with 10 ml of distilled water and the original filtrates along with the washings were pooled and lyophilized. The resulting material was reconstituted to its original concentration by adding a volume of saline equivalent to the estimated extracellular fluid volume of the skin (Cl^- space). This method appeared to remove all of the antifreeze proteins from the skin since no antifreeze activity was found associated with the skin tissue debris.

Skin freezing points

Skin freezing points were determined by placing an amount of tissue (approximately 50 mg) in the Fiske osmometer tube sufficient to cover the thermistor probe. These samples were briefly rinsed in isotonic saline, blotted on filter paper and lightly coated in mineral oil to prevent water loss by evaporation.

Stability of undercooling in fish tissues

To preclude the possibility of spontaneous (heterogeneous) nucleation during ice propagation experiments, and to test the hypothesis that fish tissues are capable of significant undercooling in the absence of seed ice crystals, experiments were designed to test the ability of tissue homogenates to undercool without freezing.

Winter flounder tissues, consisting of whole blood (2 ml), muscle (5 g), gonad (2 g), liver (2.5 g), kidney (2 g), gill (2 g), dermis (2 g) and epidermis (0.2 g), as well as the tissue fluids, plasma (2 ml), bile (2 ml) and skin mucus (2 ml), were homogenized in saline (total volume 10 ml) and placed in 50 ml plastic centrifuge tubes. The test tubes containing the homogenates were then cooled to, and held at, −4°C for 30 min, when they were given a sharp tap to initiate freezing. The temperature of each homogenate was monitored using a digital thermometer (model 8502–25 Cole Palmer, Chicago, IL) coupled to a Varian A-25 strip chart recorder.

Ice propagation experiments

Ice propagation through dialysis membranes (Spectra/por, Spectrum Medical, LA, USA), Millipore filters and fish epithelial tissues was investigated using a modified Ussing chamber (Fig. 1) (Dobson and Kidder, 1968). The membrane of interest was placed between the two acrylic chambers, each of which was enclosed by a jacket containing circulating coolant. The temperature of the coolant was controlled by a Neslab cooling unit (Neslab Instruments Inc.). One of the chambers was filled with saline and the other with sea water (32 ‰), both of which had been filtered through a 0.45 μm cellulose acetate filter. Each chamber was continuously agitated using a modified stirring bar and the temperature was monitored using a digital thermometer as previously described.

In experiments using dialysis membranes or Millipore filters the chambers were

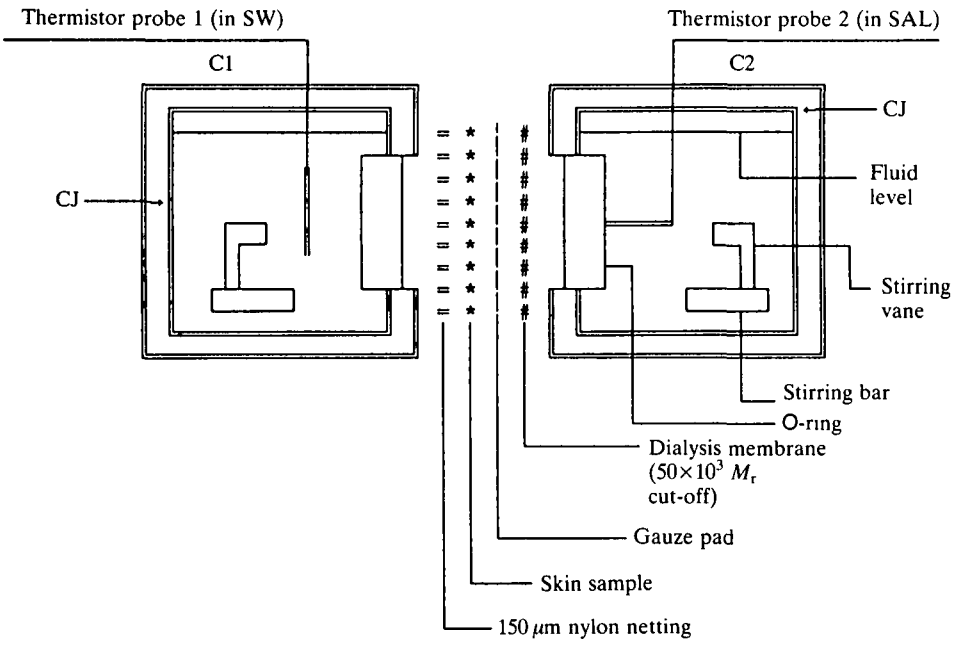

Fig. 1. Ice propagation apparatus. Drawing not to scale. Each chamber holds approximately 130 ml. C1, seawater (SW) chamber; C2, saline chamber (SAL); CJ, cooling jacket. The diameter of the membrane (skin sample, etc.) exposed to the two chambers is 2.6 cm. Therefore, the area of ice contact is 5.31 cm^2.

undercooled to −2°C and ice crystals were introduced into the seawater-filled side by touching the surface of the liquid with a wooden splint that had been pre-soaked in saline and frozen. The resulting suspension of ice crystals acted as a source of seed crystals for propagation across the membrane separating the two chambers. The initiation of freezing in the second chamber was recorded by the thermistor probe registering a rise in temperature (freezing exotherm).

In experiments using fish skin, the saline was added to the chamber on the dermal side of the skin, while sea water was added to the mucosal side. During each experimental run, the temperature control unit was programmed to reduce the temperature in the chambers linearly from 1.0 to −3.0°C over a period of 30 min. Reported ice crystallization rates are rapid (approximately 10 $mm\,s^{-1}$) (Blond, 1988). Therefore, the time lag between seeding the epidermal extracellular fluids of the skin and subsequent freezing of the undercooled saline compartment would be negligible. When the temperature of the chambers had reached the freezing point of the saline (dermal side), the frozen, water-soaked, wooden splint was placed in contact with the mucosal surface of the skin, where it remained until ice propagated across the skin. The temperature at which this occurred is termed the ice propagation temperature.

Initial experiments indicated that isolated skin samples were very susceptible to

mucosal surface damage, resulting in highly variable and unrepeatable ice propagation temperatures. This damage was prevented by placing a layer of nylon netting (150 μm pore diameter) over the mucosal surface of the skin.

Preliminary observations with fish skin established that the highly water-soluble antifreeze proteins rapidly diffused from the dermal surface of the skin into the saline-filled chamber. To retard the rate of loss of antifreeze proteins, a layer of surgical gauze soaked with blood plasma (0.4 ml) from the fish was placed on the dermal surface of the skin. The gauze was then covered by a layer of dialysis membrane with a relative molecular mass cut-off point that was freely permeable to ice crystal propagation (50×10^3 M_r) (see Results).

Two lines of evidence indicated that the dialysis membrane did not retard ice crystal propagation across the skin preparation. (1) The skin sample showed no evidence of being frozen until ice propagated from the sea water into the saline. That is, ice did not form in the skin or gauze solution prior to propagation. (2) Skin ice propagation temperatures were not altered when a small incision was made in the dialysis membrane when the skin was undercooled.

At the completion of each experimental run, the tissue and gauze were removed for analyses, and the chambers were cleaned and rinsed with ethanol, to ensure the removal of ice crystals, and then dried.

Results

Stable undercooling of fish tissues and sea water

All tissue homogenates and tissue fluids from winter flounder could be undercooled to −4°C for at least 30 min without spontaneous freezing occurring. In all cases, freezing was initiated immediately by a sharp tap on the side of the test tube containing the undercooled sample.

Ice propagation across dialysis membranes and filters

Dialysis membranes with a relative molecular mass cut-off of 25×10^3 M_r or less effectively blocked the propagation of ice from one chamber to the other, even when exposed to ice (−2°C) for periods of up to 2 h (Table 1). Ice propagation occurred rapidly across membranes of 50×10^3 and 100×10^3 M_r and across Millipore filters with a pore diameter of 0.45 μm. The addition of mineral oil to the Millipore filters blocked ice propagation.

Ice propagation across fish skin

Experiments were performed on winter flounder at two times of the year: spring, when blood plasma antifreeze levels would be expected to vary widely between fish, and summer, when antifreeze proteins are absent.

In most cases, ice propagation temperatures for ocular- and blind-side skin samples from each flounder were essentially identical, differing by less than 0.2°C (r=0.93, N=11, spring fish).

Table 1. *Ice propagation across dialysis membranes and Millipore filters*

Barrier (size)	Extent of undercooling (°C)	Duration of undercooling in presence of ice	Ice propagation (time)
Dialysis membranes			
1.0	−2.0	0.1–2 h	No
3.5	−2.0	0.2–0.3 h	No
12–14	−2.0	0.4–1.0 h	No
25	−2.0	0.5–2.0 h	No
50	−2.0	<1 min	Yes (<1 min)
100	−2.0	<1 min	Yes (<1 min)
Millipore filter			
0.45	−2.0	<1 min	Yes (<1 min)
0.45 (+mineral oil)	−2.0	0.2–1 h	No

Ice propagation time is the time between the introduction of ice crystals to the seawater side and the observation of a freezing exotherm on the saline side of the membrane.

Size is relative molecular mass cut-off ($\times 10^{-3}$) for dialysis membranes or pore size (in μm) for Millipore filters. One Millipore filter was presoaked in mineral oil.

Four to five separate experiments were carried out using each membrane except the 25×10^3 and 50×10^3 M_r sizes, where 10 experiments were conducted.

Experiments carried out during spring revealed that ice propagation temperatures were correlated with skin freezing points (Fig. 2) which were, in turn, correlated with blood plasma freezing points (Fig. 3). Ice propagation temperatures were also correlated with plasma freezing points (Fig. 3). Thus, it would appear that some of the variance observed in skin freezing and ice propagation temperatures can be accounted for by the variance in plasma freezing temperatures.

Antifreeze protein levels (thermal hysteresis) in the plasma samples ranged from 0 to 0.67°C and were highly correlated with plasma freezing points (r=0.93, N=11), suggesting that a component of the skin freezing point and ice propagation temperature was attributable to the antifreeze proteins (Fig. 3). There was a significant correlation between skin freezing points and plasma antifreeze thermal hysteresis (r=0.66, N=11). However, the correlation between ice propagation temperatures and thermal hysteresis was not significant (r=0.46, N=11). This lack of correlation seems to be attributable to the high variation in propagation temperatures when antifreeze proteins were absent or present at very low levels in the blood. Propagation temperatures were consistently low when plasma thermal hysteresis values exceeded 0.15°C (Fig. 3). The mean propagation temperature (−2.74±0.15°C, ±S.E., N=4) for the skin samples with the higher plasma thermal hysteresis values (>0.15°C) was significantly lower than that of the samples with little or no plasma antifreeze (−1.81±0.21°C, ±S.E., N=7). Propagation temperatures for the three samples with no detectable plasma antifreeze averaged −1.72±0.3°C (±S.E.).

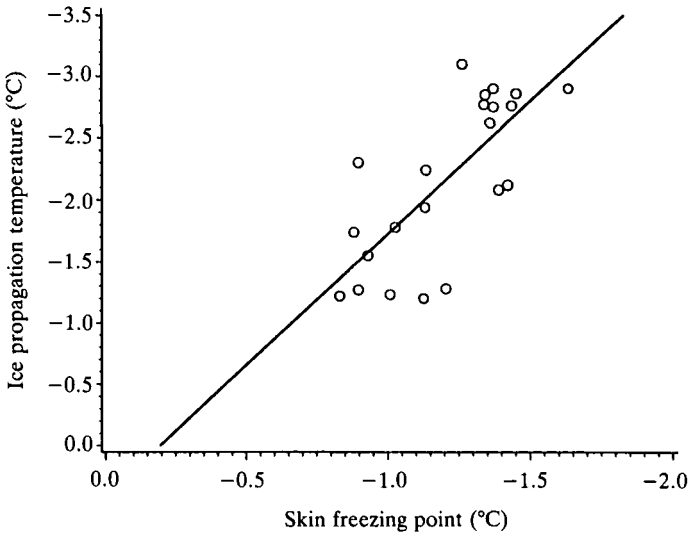

Fig. 2. Relationship between the ice propagation temperature (t_p) and the freezing point (FP) of skin. Each point represents a single measurement using an ocular-side or blind-side skin sample. Regression line: $y=2.15x+0.417$ ($r=0.739$, $N=22$, $P<0.01$). The intercept does not differ significantly from 0 ($P<0.05$). y=skin t_p, x=skin FP.

Experiments carried out during the summer months revealed that antifreeze proteins can decrease ice propagation temperatures. In these experiments the addition of blood plasma containing antifreeze proteins or an antifreeze protein solution to the skin preparation resulted in significant reductions in the propagation temperatures (Table 2).

Ice propagation temperatures were always lower than the respective skin freezing points, with the mean difference between the two being 0.96±0.11°C (±S.E., $N=22$). The differences between ice propagation temperatures and skin freezing points are illustrated in Fig. 3, where they are plotted against the plasma freezing points. Both regressions intercept the y-axis at approximately the same point (about 0.4°C). However, this value only differs significantly from zero in the case of the skin freezing temperature. It is apparent that, although ice propagation temperatures are lower than skin freezing points, the slopes of the regressions are such that the difference between the two increases as the plasma freezing points decline. The regression relating plasma freezing points to the differences between propagation temperatures and skin freezing points was significant at the $P<0.05$ level (Fig. 4).

Skin freezing temperatures were slightly lower than plasma freezing temperatures; the mean difference between the two being 0.156±0.038°C (±S.E., $N=11$). The differences between skin and plasma freezing points were not correlated with plasma freezing points. This indicates that the difference between these two measurements is essentially the same over the range of plasma freezing points observed.

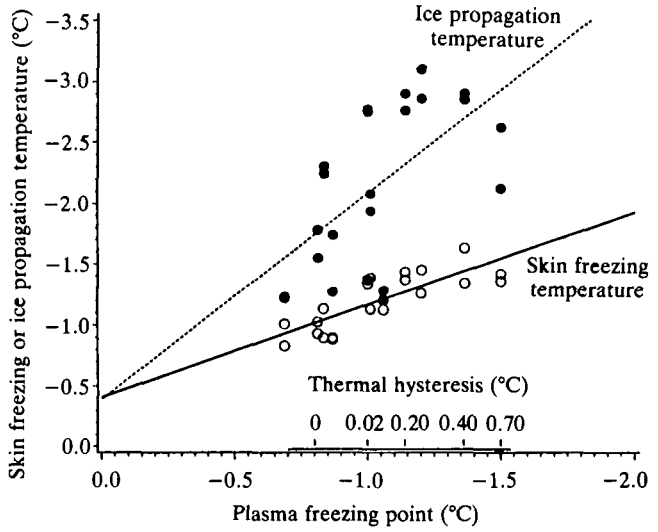

Fig. 3. Relationship between skin freezing point (FP), ice propagation temperature (t_p) and plasma freezing point. Closed circles, ice propagation temperature; open circles, skin freezing temperature. Each point represents a single measurement using an ocular-side or blind-side skin sample. Plasma thermal hysteresis (°C) indicates the amount of antifreeze activity observed in the plasma. Regression lines: y=skin t_p or FP, x=plasma FP. Dashed line (t_p) $y=1.70x-0.39$ ($r=0.612$, $N=22$, $P<0.01$). Solid line (FP): $y=0.761x-0.407$ ($r=0.797$, $N=22$, $P<0.01$). The intercept for the solid line (FP) differed significantly from zero ($P<0.05$).

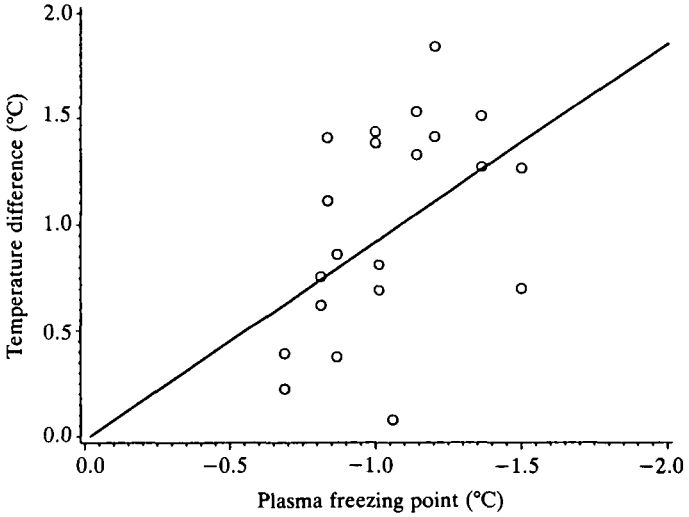

Fig. 4. Differences between the skin ice propagation temperatures (t_p) and the skin freezing points (FP). Each point represents the difference between the two temperatures for a single skin sample (ocular side or blind side). Regression line: $y=t_p$ or FP, x=plasma FP. $y=0.935x-0.017$ ($r=0.432$, $N=22$, $P<0.05$).

Table 2. *Effects of antifreeze proteins on ice propagation temperatures (°C)*

Fish number	Solution added to skin		
	AFP-free plasma	AFP plasma (difference)	AFP saline (difference)
1	−1.4	–	−2.02 (0.62)
2	−1.53	–	−1.81 (0.28)
3	−1.38	–	−1.80 (0.42)
4	−1.19	−1.38 (0.19)	–
5	−1.56	−1.99 (0.43)	–
Mean propagation temperature (°C)	−1.41±0.66	−1.69±0.19 (0.25±0.05)	−1.88±0.053 (0.44±0.1)

Experiments were carried out on winter flounder skin samples during July and August, when antifreeze proteins were absent from the blood.

AFP-free plasma (plasma containing no antifreeze proteins, AFP) was pooled from a number of fish during July (plasma freezing point=−0.61°C). AFP plasma was obtained from flounder during winter (freezing point =−1.5°C, thermal hysteresis=0.9°C). AFP saline was a 5 mg ml^{-1} winter flounder antifreeze solution dissolved in saline (freezing point=−1.12°C, thermal hysteresis=0.5°C).

Differences (in parentheses) are the differences (±S.E.) between propagation temperatures using AFP-free plasma and propagation temperatures using the AFP test saline.

Ice propagation temperatures were always lower than the respective blood plasma freezing temperatures (Fig. 2). The mean difference between average (ocular-side and blind-side) ice propagation temperatures and plasma freezing points was 1.11±0.16°C (±S.E., N=11) during the spring. There was no correlation between this difference and antifreeze protein levels in the blood. The low ice propagation temperatures relative to plasma freezing points were also evident from experiments carried out during the summer, when antifreeze proteins had completely cleared from the blood [mean difference=0.80±0.066°C (±S.E., N=5) (Table 2)].

Experiments carried out on ocean pout epithelia indicated that they were equally as effective as winter flounder skin in blocking ice crystal propagation (Table 3). All three epithelial membranes, skin, urinary bladder and gall bladder, had similar ice propagation temperatures, ranging from −2.6 to −2.9°C. Ocean pout skin differs from that of the winter flounder in that the scales are small and completely buried, leaving the mucosal surface smooth and slippery. Despite their anatomical differences, there were no differences between the skins in terms of ice propagation. As in the case of winter flounder skin, ice propagation temperatures across ocean pout skin were considerably lower than blood plasma freezing points, the difference between the two being approximately 1.1°C (Table 3).

Freezing behaviour of skin mucus

The freezing points of the gel and sol phases of winter flounder skin mucus did

Table 3. *Ice propagation temperatures across ocean pout tissue barriers*

Barrier	Gauze solution	Solution freezing point (°C)	Ice propagation temperature (°C)
Skin	Plasma	−1.52	−2.66±0.26
Urinary bladder	Urine	−1.47	−2.55
Gall bladder	Bile	−1.1	−2.88

Four experiments were carried out on ocean pout skin and one on each of the bladders.

In experiments using skin, sea water was added to the chamber on the mucosal side while saline was added to the dermal side. Ocean pout plasma was added to the gauze on the dermal side. In experiments using urinary and gall bladder epithelia, saline was added to both chambers and urine and bile collected from the respective bladders was added to the gauze on the serosal side of the membranes.

Values for skin ice propagation temperatures are presented as mean±S.E.

not differ significantly (sol=−1.64±0.057°C, ±S.E., N=5; gel=−1.65±0.064°C, ±S.E., N=5) and both were slightly higher than the freezing point of sea water (−1.75°C). There was no seasonal cycle in the mucus freezing points.

No evidence of antifreeze activity was found in most of the samples examined. In the occasional sample that did exhibit antifreeze activity, the values were very low (<0.05°C) and were associated with cellular debris indicative of epithelial damage.

Antifreeze proteins in skin

Preliminary analyses revealed that winter flounder skin contained substantial amounts of antifreeze proteins. This prompted us to measure the skin extracellular space in order to estimate skin antifreeze protein concentrations. Ocular-side and blind-side skin chloride spaces were essentially identical (approximately 69 %) and substantially greater than muscle chloride space (approximately 8.8 %) (Table 4). Inulin space, determined by the incubation method, gave a value that was

Table 4. *Extracellular fluid volumes in winter flounder skin and muscle*

	Ocular-side skin	Blind-side skin	Muscle	Plasma
Water (g 100 g^{-1})	70.6±0.68	74.7±0.47	81.2±0.35	97.3±0.28
Cl^{-1} (mmol kg^{-1} tissue H_2O)	132±3.5	131±4.1	16.9±1.6	182±1.3
Chloride space (% tissue water)	69.1±1.9	68.5±2.1	8.82±0.81	–
N	4	4	4	4
Inulin space (% tissue water)	–	78.2±1.25	–	–
N	–	2	–	–

Plasma water was measured as g 100 ml^{-1}. Values are presented as mean±S.E.

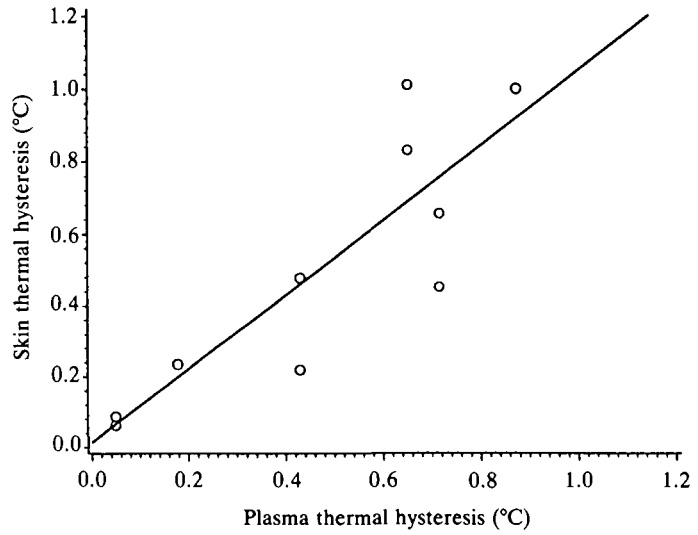

Fig. 5. Relationship between skin antifreeze activity and plasma antifreeze activity. Thermal hysteresis (TH) is a measure of antifreeze activity. Each point represents a single skin sample. Regression: y=skin TH; x=plasma TH. $y=0.0135+1.037x$ ($r=0.882$, $N=11$, $P<0.01$).

approximately 10 % greater than the chloride space (Table 4). The chloride space was used to calculate skin antifreeze protein concentrations.

Antifreeze proteins were extracted from ocular-side ($N=4$) and blind-side ($N=7$) skin of selected flounders sampled during winter (February, March, April) and during July. Skin antifreeze activity was highly correlated with plasma antifreeze activity (Fig. 5).

Antifreeze activity in the winter skin samples did not differ significantly from antifreeze activity in the blood plasma (plasma thermal hysteresis=0.569±0.12°C, ±S.E., $N=5$; ocular skin=0.568±0.18°C, ±S.E., $N=3$; blind-side skin= 0.681±0.17°C, ±S.E., $N=5$).

There was no evidence of antifreeze activity in July plasma samples; however, traces of antifreeze activity could be detected in the skin extracts (thermal hysteresis=0.03±0.008°C, S.E., $N=5$). The presence of low levels of antifreeze proteins in these summer skins was confirmed by the fact that ice crystal growth, observed under the microscope, was in the form of long spicules rather than hexagonal crystals, as would be the case with salt solutions (DeVries, 1982).

Discussion

The results of this study demonstrate conclusively that fish skin is an effective barrier to ice propagation and that this barrier is enhanced by the addition of antifreeze proteins. This conclusion is consistent with the ice-exclusion hypothesis

in that antifreeze proteins can act within epithelial tissues and assist in blocking ice movement from the external environment into the fish.

Additional evidence that the skin may be an important *in vivo* site of antifreeze action comes from studies on the European shorthorn sculpin (*Myoxocephalus scorpius*) and the cunner (*Tautogolabrus adspersus*). Schneppenheim and Theede (1982) found no evidence for antifreeze peptides in the blood plasma of shorthorn sculpin exposed to freezing conditions. However, they were able to isolate and purify these proteins from aqueous extracts of skin. Similar observations have been made on cunner, during the winter, where antifreeze activity was found in skin but not in blood (Valerio *et al.* 1990).

The present study establishes that antifreeze proteins are also present in the skin of winter flounder during winter. However, in contrast to the above observations on sculpin and cunner, the concentration of antifreeze proteins in flounder skin extracellular space appears to mirror blood plasma concentrations. This is in accordance with our current hypothesis that the liver is the source of antifreeze proteins in winter flounder (Davies *et al.* 1988; Fletcher *et al.* 1989). However, the presence of antifreeze peptides in the skin of sculpin and cunner lacking measurable levels of plasma antifreeze suggests the possibility that these proteins may also be synthesized in the skin (Schneppenheim and Theede, 1982; Valerio *et al.* 1990).

One striking feature of the present study was the observation that skin ice propagation temperatures were, on average, considerably lower than skin freezing points (spring mean was approximately 0.96°C). This suggests that some component of skin, in addition to antifreeze proteins and colligatively active solutes, participates in blocking ice propagation. A possible candidate for such a function is the structure of the skin itself.

Spatial restriction of ice crystal growth in small-diameter pores has been established in a number of non-biological (Solms and Rijke, 1971; Viaud, 1972) and biological (Bloch *et al.* 1963; Yingst, 1978) systems. In the present study, the spatial restriction of ice growth across dialysis membranes with a relative molecular mass cut-off of 25×10^3 M_r illustrates that this phenomenon can operate at pore diameters of 5–7 nm (based on Spectra/por product information – see Materials and methods). Reported values for cell junction intercellular spacing in vertebrate epithelia (desmosomes, 10–20 nm; gap junctions 2–4 nm, Novikoff and Holtzmann, 1976) are within this range; thus, ice propagation across fish skin may well be restricted by intercellular spacing.

It is evident from the literature that biological membranes can be very effective at blocking ice propagation. Turner *et al.* (1985) demonstrated that both the cornea and the corneal epithelium effectively block ice crystal propagation to the undercooled ocular fluids of Antarctic nototheniids. Similarly, Davenport *et al.* (1979) found that the chorion of caplin (*Mallotus villosus*) eggs allowed the eggs to be undercooled (−12°C) even when they were in close contact with ice. Harvey and Ashwood-Smith (1982) also observed that rainbow trout eggs could remain undercooled in the presence of ice for a period at −19°C.

Teleost fish skin is a complex membrane consisting of an external mucus coat, the cuticle, covering an avascular layer of epithelial cells resting on a thin gel-like layer of connective tissue, the basement membrane. The basement membrane overlies a relatively thick dermal layer of largely non-cellular connective tissue. The upper portion of the dermis contains scales, pigment cells and blood vessels (Bullock and Roberts, 1974; Burton, 1978; Burton and Fletcher, 1983). The large extracellular space observed for the winter flounder skin (approximately 70–80 %) (Table 4) clearly indicates that the bulk of this tissue is noncellular.

The complex nature of the skin makes it difficult to point to any single structural feature as being responsible for blocking ice crystal propagation. The scales do not seem to be important in this regard because they are well-separated from one another (μm) (Burton, 1978). Moreover, urinary bladder and gall bladder epithelial membranes were equally as effective as skin in retarding ice propagation. The spacing between vertebrate connective tissue collagen fibres varies considerably (8–95 nm) (Fujii, 1968) and does not appear to be small enough to exert a structural freezing point depression. One likely location for an ice barrier in fish skin is within the epithelial cell layer. This argument is supported by the results of a recent study by Eastman and Hikida (1991), who found that the epithelial cell membranes of an Antarctic notothenioid exhibited more extensive membrane specializations in terms of tight junctions, interdigitations and desmosomes than do the cell membranes of other species of teleost fishes.

The hypothesis that the epithelial cell layer may constitute a barrier to ice propagation is in accordance with the observations from the present study indicating that skin freezing points were closer to those of the blood plasma than they were to the ice propagation temperatures. In other words, the major freezing exotherm observed during measurements of skin freezing points was attributable to the large extracellular space of the dermal connective tissue compartment, rather than to the small cellular compartment.

The lower freezing temperatures observed for the skin relative to the plasma (approximately 0.16°C) suggest that the solute concentration in the skin extracellular space may be higher than that in the plasma by approximately 80 mosmol kg^{-1}. However, since the external surface of the skin was not washed extensively, it is possible that the samples were contaminated to a small degree by sea water. 80 mosmol kg^{-1} represents less than 8 % of the dissolved solutes in sea water. This argument is supported by the observation that the regression relating skin freezing temperatures to plasma freezing points intercepted the y-axis at a value greater than zero (approximately 0.4°C) (Fig. 3).

If the differences between ice propagation temperatures and skin freezing points are attributable to a structural component of the skin, it is evident from the results that the magnitude of this structural freezing point depression is directly affected by the dissolved solute concentration in the skin, both of antifreeze proteins and of electrolytes. The slope of the regression (Fig. 2) relating ice propagation temperatures to skin freezing points suggests that for every increase in skin solute concentration there is a doubling of the structural freezing point depression. We

are unaware of an explanation that would account for this interesting phenomenon.

One argument counters the hypothesis that the low ice propagation temperatures observed were attributable to a structural component of the skin. The *y*-intercept of the regression relating propagation temperatures to skin freezing temperatures did not differ from zero, suggesting that, in the absence of dissolved solutes, there would be no structural freezing point depression. A *y*-intercept value greater than zero would have been more consistent with the existence of such a phenomenon.

If there is no simple structural barrier to ice propagation across the skin, what accounts for the observed differences between propagation temperatures and skin freezing points? Perhaps the dissolved solute concentration in a minor compartment of the skin exceeds, but stays in equilibrium with, the bulk of the extracellular space. One possible candidate for such a compartment would be the interstitial space of the epithelial cell layer. Evidence for hypertonic cellular interspaces has been reported for fluid-transporting epithelia (Gupta and Hall, 1979). However, nothing is known about the interstitial space in fish skin. There is no evidence that antifreeze proteins can concentrate in interstitial spaces. However, a recent study by Rubinsky *et al.* (1990), indicating that antifreeze proteins can interact with cell membranes, opens up the possibility that this could occur. Clearly, the presence or absence of structural or concentrated solute barriers to ice propagation across fish skin will have to await further experimentation.

In previous experiments it was observed that the lethal freezing temperatures of winter flounder and other fish species in the presence of ice were consistently lower (0.05–0.2°C) than their blood plasma freezing points. This suggested that the epithelium was acting as a barrier to ice crystal propagation (Fletcher *et al.* 1986, 1988). The discovery in the present study that ice propagation across the skin could only occur when the water temperature was 0.8–1.11°C below the plasma freezing points indicates that the skin could not have been the route of ice crystal propagation into the intact fish.

Two other potential sites of ice propagation into the fish are the gut and gill epithelia, both of which have only a single layer of epithelial cells lying between the environment and the blood. Of the two, the most likely one would be the gills because of the relatively large volumes of water pumped across them during ventilation.

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