Antifreeze activity of Antarctic fish glycoprotein and a synthetic polymer

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Antifreeze glycoproteins (AFGPs) and proteins isolated from the sera of some polar fish species and overwintering insects are able to depress the freezing temperature of the aqueous body fluids (and of water) by means of a non-colligative mechanism[1,2]. All previous measurements of the antifreeze effect have been performed on bulk samples under conditions where ice nucleation would be catalysed by particulate impurities, giving limited and indeterminate degrees of undercooling. We report the first measurements of homogeneous (spontaneous) ice nucleation rates in deeply undercooled (<233 K) solutions of AFGP and polyvinyl pyrrolidone (PVP), a well-characterized polymer which finds use as a cryoprotectant. Antifreeze activity is said[3] to derive from the sorption of AFGP molecules on the active growth sites of ice crystals, preventing normal growth and inducing unusual crystal habits. We have performed experiments on the inhibition of ice crystal growth in solutions containing AFGP and PVP under standardized conditions, and find that the homogeneous nucleation and crystallization rates are sensitive to low concentrations of both substances, but AFGP is remarkably effective at inhibiting ice crystal growth.

Homogeneous nucleation rates, J (nuclei $s^{-1} m^{-3}$), were measured by differential scanning calorimetry (DSC) on <1 mg sample solutions emulsified in silicone oil as previously described[4]. The dispersed aqueous droplets had mean radii of 2.5 μm and controls were run on solutions that did not contain antifreeze polymers. All nucleation measurements were performed on a Perkin-Elmer DSC-2 instrument, fitted with a subambient accessory, at a cooling rate of 1.25 K min^{-1}, which is below the threshold where J becomes dependent on the cooling rate.

According to the classical theory of nucleation of a crystalline phase in the melt[5], $(J)T$ is expressed as

$$J(T) = L(\sigma T)^{1/2}\phi^2 \exp[-\Delta G^{\ddagger}/RT] \exp[-Q\sigma^3/(\Delta T)^2 T^3] \quad (1)$$

where L is a function only of the densities and molar volumes of the two phases, and $Q = (bV_{solid}/k)[T^4(\Delta H_c)^2]$. In these equations, ϕ is the volume fraction of solvent (water), σ is the interfacial tension between the coexisting solid and liquid phases, $\Delta G^{\ddagger}$ is the free energy of activation of self-diffusion, ΔT is the degree of undercooling, b is a shape factor which depends on the geometry of the nucleus and ΔH_c is the latent heat of crystallization, which is itself a function of the temperature. The validity and limitations of the theory, as applied to the nucleation of ice in undercooled aqueous media, have been discussed[6], but in the present work a modified form of (1) was used in the analysis of the freezing exotherms, because it had previously been found to account reasonably well for the nucleation of ice in water and aqueous solutions of polyethylene glycol[4]. Equation (1) can be written in a simplified form: $J(\tau_\theta) = A \exp(B\tau_\theta)$, with $\tau_\theta = [(\Delta\theta)^2\theta^3]^{-1}$, where θ is the reduced temperature (T/T_f) and $\Delta\theta$ the reduced degree of undercooling $[(T_f - T)/T_f]$, T_f being the equilibrium freezing temperature. Reduced, rather than actual, temperatures were used to permit direct comparisons of solutions with different equilibrium freezing points. The AFGP was isolated from dialysed blood serum of *Notothenia neglecta* and purified as previously described[7]. The PVP was fractionated by gel permeation chromatography, and the fraction with a number-average molecular weight 44,000 was used.

Nucleation experiments were performed on water and various aqueous solutions of identical osmolalities, to determine whether interactions could be detected between the polymers and the low molecular-weight solutes, whose effects on $J(\tau_\theta)$ are known[8].

The steady-state dimensions of ice crystals were determined by following their growth from solutions held at 1.5 K below their melting points. The AFGP and PVP were added to 20% (w/w) sucrose solutions, to provide enough unfrozen solution for ease of analysis. The apparatus consisted of a Mettler FP52 microscope temperature stage and controller equipped with a liquid-nitrogen cooling source and a video microscope from which images could be copied at regular intervals. The ice crystals were formed between a glass microscope slide and cover slip (using 2 μm glass beads as spacers) by quenching the solution to 245 K. They were then allowed to mature at 1.5 K below the melting point of the ice. It was found that the final crystal dimensions were achieved after 3 h at the maturation temperature. With the aid of an automated image analysis system a video copy of the crystals was analysed and a mean equivalent circular diameter estimated.

Table 1 shows a summary of the nucleation results. A and B in (1) were calculated from linear regressions. All values cited in Table 1 are averages of at least three independent determinations; estimated uncertainties of B are shown in parentheses.

Homogeneous nucleation measurements are difficult to perform and calculations of $J(\tau_\theta)$ are subject to some uncertainty[8], but the results are more likely to provide fundamental information about the mechanisms that govern the formation of critical-size embryos in the undercooled liquid than are previous measurements of freezing in the bulk, which is always catalysed by particulate impurities of unknown origin. There appears to be a definite trend in the results shown in Table 1. The addition of 1% (w/w) AFGP or PVP to any solution affects $J(\tau_\theta)$ identically, in the sense that B becomes more negative, that is, nucleation becomes more sensitive to temperature. Although the influence of the polymers on B may seem to be minor, it must be emphasized that according to (1), τ_θ is an inverse fifth-order function of temperature, so that the effects on $J(\tau_\theta)$ are quite pronounced. Reference to (1) shows that B contains several thermodynamic quantities. Of these, σ, the interfacial free energy between the growing embryo and the undercooled liquid, is most likely to be affected by low concentrations of antifreeze polymers. Considerable reductions in σ, measured between ice and water (at the equilibrium melting temperature) in the presence of AFGP have recently been reported[9]. Although the conditions are not strictly comparable to those in the deeply

Table 1 Homogeneous nucleation data for undercooled solutions

Sample	T^* (K)	ln A ($m^{-3}s^{-1}$)	$-B$
Water	232.1	39	0.82 (0.02)
Water + 1% AFGP	232.0	43	0.94 (0.02)
20% Sucrose	228.5	36	0.78 (0.01)
20% Sucrose + 1% AFGP	228.3	40	0.82 (0.02)
20% Sucrose + 1% PVP	228.7	42	0.98 (0.03)
2.45% NaCl	230.0	34	0.69 (0.02)
2.45% NaCl + 1% AFGP	229.8	44	1.05 (0.02)
2.45% NaCl + 1% PVP	230.2	45	1.03 (0.02)
12% Fructose	230.0	38	0.80 (0.03)
12% Fructose + 1% PVP	229.6	42	0.98 (0.03)
5% Urea	230.4	—	—
5% Urea + 1% PVP	230.4	—	—

T^* is the temperature corresponding to the maximum nucleation rate. The mean droplet volume is taken as 2×10^{-17} m^3. Concentrations are w/w for AFGP and PVP, and w/w for sucrose, NaCl, fructose and urea.

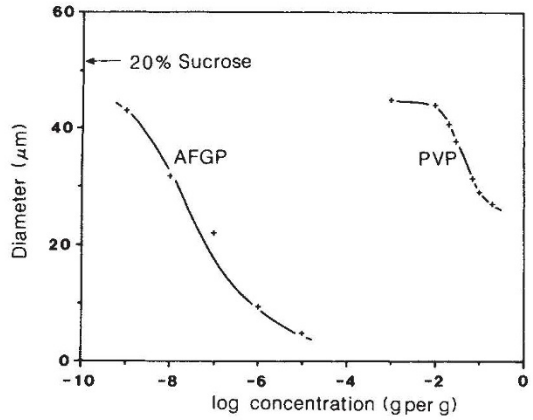

Fig. 1 Mean ice crystal diameter plotted against concentration of AFGP and PVP in 20% (w/w) sucrose solutions measured after 3 h growth at 1.5 K below the melting point of ice in the solution. The solutions had previously been quench-cooled to 233 K and rewarmed.

undercooled liquid, the measurements provide an indication of the trends in σ resulting from polymer sorption. Since the expression for B contains σ^3, even a small change in σ can produce measurable effects in $J(\tau_\theta)$ (ref. 6).

In view of the almost identical influence of AFGP and PVP on the nucleation of ice in undercooled aqueous media, it is surprising that they differ markedly in their ability to suppress ice crystal growth in quenched solutions, as Fig. 1 shows. Trace quantities of AFGP inhibit the growth of ice crystals in a highly nucleated system under conditions where maturation would normally take place quite rapidly. There are approximately seven orders of magnitude in concentration between equivalent crystal diameters of ice grown in the presence of either PVP or AFGP. The dramatic difference between PVP and AFGP suggests that entirely different modes of growth inhibition are involved. The effect of PVP on maturation could possibly be attributed to the increase in viscosity of the unfrozen phase under conditions of freeze concentration. AFGP is believed to function by sorption to the active sites of the growing ice crystals[3], and direct evidence for such sorption has recently been reported[10].

Various attempts[7,11] have been made to relate the hypothetical structure of the antifreeze molecules in solution to the spacings of the water oxygens in ice, in efforts to account for the poisoning of normal growth sites and the appearance of abnormal morphological forms. The preferred solution conformation of AFGP is still a subject of debate[12], but model-peptide studies[13] show that at a water/apolar solid interface some peptides can adopt specific conformations, depending on the peptide sequence.

Any sorption and growth-inhibition mechanism for *in vivo* antifreeze activity presupposes that, at subzero temperatures, submicroscopic ice crystals can exist in the body fluids of animals (fish, insects) that rely on the peptides for their cold survival. On a practical level, the proposed mechanism of sorption on ice crystals calls into question earlier measurements of the 'freezing temperature depression', as performed by osmometry[2] because AFGP is now thought to inhibit only the growth of crystals, not their initial formation. It follows then that ice crystals do form in the serum, but only to certain steady-state critical sizes, possibly not large enough for the evolution of a measurable latent heat. Small-angle light-scattering measurements should be able to resolve this question.

In bulk aqueous media, the temperature range of the antifreeze activity of AFGP, measured by crystal growth, only extends to 271 K, below which the natural antifreeze glycopeptide loses its ability to prevent freezing. On the other hand, our studies have shown that the homogeneous nucleation in the temperature range 228–233 K is much more sensitive to low concentrations of AFGP and PVP than would be predicted from normal effects of solute concentration[8]. The question remains whether physiological antifreeze activity is primarily due to a depression of $J(T)$ or the poisoning of growth sites on existing ice crystals in the body fluids.

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The spring in the arch of the human foot

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Large mammals, including humans, save much of the energy needed for running by means of elastic structures in their legs and feet[1,2]. Kinetic and potential energy removed from the body in the first half of the stance phase is stored briefly as elastic strain energy and then returned in the second half by elastic recoil. Thus the animal runs in an analogous fashion to a rubber ball bouncing along. Among the elastic structures involved, the tendons of distal leg muscles have been shown to be important[2,3]. Here we show that the elastic properties of the arch of the human foot are also important.

Figure 1*a* shows the principal forces that act on the foot in the middle part of the stance phase. The heel is rising from the ground and the centre of pressure for the ground force is on the ball of the foot[4]. The forces exert bending moments on the foot, tending to flatten the arch.

The same pattern of forces was simulated on an amputated foot (Fig. 1*b*). Note that the tension in the Achilles tendon in Fig. 1*a* is represented by pressure of a steel block on the calcaneus in Fig. 1*b*. The skin and adipose tissue of the heel had been removed so that this force was applied directly to the bone. The actuator of the testing machine was made to move up and down sinusoidally, and the forces required were recorded (Fig. 2*a*). Some stress relaxation occurred in the first few cycles after an increase in amplitude, but successive cycles after the first ten gave almost identical records.

Figure 2*a* shows that the foot is capable of storing strain energy and returning it in an elastic recoil. Notice that the hysteresis loop is fairly narrow: the energy dissipations[5] shown by this and 26 similar records, from six feet, were $22 \pm 1\%$ (mean ± s.e.m.). X-radiographs of the feet under static loads in the testing machine showed that the deformations observed in the experiments involved flattening of the longitudinal arch.

We have shown that the arch of the foot has spring-like qualities. We now consider whether it can store enough strain energy to make useful energy savings in running. Figure 2*b* shows how the strain energy stored in our experiments depended on the load applied. A load of 6.4 kN would be needed to imitate the pattern of forces applied in running at 4.5 m s^{-1} (Fig. 1*a*). The loads applied in our experiments were limited by crushing