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Stabilization of Supercooled Fluids by Thermal Hysteresis Proteins

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ABSTRACT It has been reported that thermal hysteresis proteins found in many cold-hardy, freeze-avoiding arthropods stabilize their supercooled body fluids. We give evidence that fish antifreeze proteins, which also produce thermal hysteresis, bind to and reduce the efficiency of heterogenous nucleation sites, rather than binding to embryonic ice nuclei. We discuss both possible mechanisms for stabilization of supercooled body fluids and also describe a new method for measuring and defining the supercooling point of small volumes of liquid.

INTRODUCTION

Cold-hardy terrestrial arthropods may be exposed to minimum winter temperatures from just below 0 to as low as −60°C. Species that survive such temperatures by avoiding freezing must prevent inoculative freezing and often must promote the supercooling of their hemolymph, because depression of the equilibrium freezing point to below the temperatures encountered may be difficult to achieve. Many of these arthropods are known to synthesize thermal hysteresis proteins (THPs), which depress the non-equilibrium freezing point of their body fluids by up to 5°C without significantly affecting the melting point (Zachariassen, 1985; Duman et al., 1993b).

The supercooling point (SCP) has previously been defined as the temperature at which body fluids spontaneously freeze (see, e.g., Johnston and Lee, 1990). This definition is necessarily somewhat vague, because the nucleation of supercooled fluids is a stochastic process and many factors affect both the onset and the measurement of the nucleation temperature. If freeze-avoiding animals have body fluids that are supercooled and so by definition are lacking in ice crystals, what is the purpose of the THPs? Previous investigators have assigned the THPs the task of "stabilizing the supercooled state", without describing how such stabilization may come about (Block, 1991; Duman et al., 1993b). Zachariassen and Husby (1982) have suggested that the THPs might bind to microscopic ice nuclei and completely inhibit the ice growth, thus keeping the solution free of any macroscopic ice crystals, i.e., in a supercooled state. They argued that because the hysteretic activity increased with decreasing ice crystal size as measured on a nanoliter osmometer, for the microscopic sizes of embryonic crystals at typical supercoolings the hysteresis was sufficient to depress the freezing point completely and inhibit crystallization. They also suggested that THPs appeared unable to mask the effects of biological ice nucleation agents (INAs), and so any effect at very low temperatures was restricted to animals whose body fluids lacked such INAs.

It is worth noting that although some insect THPs may produce up to 5°C of thermal hysteresis, 1 or 2°C is the norm for those found in polar fish and most cold-hardy insects (Duman et al., 1993a). In contrast, INAs can raise the crystallization temperature by more than 10°C. This may indicate that the process of inducing crystallization of the metastable supercooled state is less difficult to facilitate than the process of inhibiting ice growth at temperatures below the equilibrium freezing point. It may also be that the levels of thermal hysteresis generated in nature by THPs and antifreeze proteins (AFPs) are all that are required by the animals involved in their respective cold-hardy strategies. This is certainly true for some Antarctic fishes that depress the non-equilibrium freezing point of their body fluids to just a few tenths of a degree below the freezing point of the seawater, because the temperature can never drop below this level (Duman and DeVries, 1972).

Freeze-avoiding animals can extend supercooling abilities in winter by three mechanisms: 1) accumulation of colligative solutes to depress the equilibrium freezing point, 2) removal of ice nucleators, and 3) masking or inactivation of either ice nucleators or embryonic ice crystals.

In this paper we describe a method for measuring the SCPs of solutions using a single sample rather than multiple samples, as has historically been the case (Zachariassen et al., 1982). We have measured the SCPs of a simple saline solution and of insect hemolymph containing ice nucleation proteins (INPs), before and after the addition of purified Antarctic fish AFP molecules. Fish AFP was used in place of purified insect THP because of difficulty in obtaining a sufficient amount of the latter material. Although our conclusions are based on the observed action of AFPs we suggest that the method of action of insect THPs in supercooled solutions is similar.

We discuss three possible models for the stabilization of supercooled body fluids by THPs. The first outlines the hindering of ice nucleation without any binding, but simply by the physical presence of protein molecules in solution. The

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second model involves the binding of THP molecules to all embryonic ice nuclei, thus disallowing further water molecules joining the ice and so decreasing the probability for complete nucleation. We argue that neither of the first two models can account for the observed stabilization of supercooled body fluids. The third model describes the masking by THPs of heterogenous nucleation sites, either passive sites such as dirt, membranes and container walls or active biological sites such as INPs.

A more detailed description of THPs, AFPs, and INPs is given in the Discussion section, where we also relate our results to the present theory of heterogenous nucleation.

MATERIALS AND METHODS

The basis for the experimental design was simply the repetitive supercooling and measuring of lag times of single fluid samples. A device was built based heavily on the design of an automatic lag-time apparatus (ALTA), which has been designed and used by Prof. A. D. J. Haymet at the University of Sydney (Haymet and Barlow, 1994). The idea of ALTA is to measure the lag times and the statistical distribution of lag times between supercooling a solution below its equilibrium freezing point and the first appearance of a crystal. The ALTA designed by Professor Haymet provides statistically meaningful data about the nature of the heterogenous nucleation event per se and is an updated version of an apparatus described by Baldwin and Vonnegut (1982). In both of those series of experiments a small liquid sample was repeatedly lowered to the same (supercooled) temperature perhaps 500 times and the lag times recorded. Thorough analysis of such data gives insight into the goodness of fit to a function where the probability of nucleation is proportional to the time interval (at a given level of supercooling). A plot of number of samples unfrozen after a given time versus time often shows a single exponential form, indicating a one-step nucleation process (A. D. J. Haymet and P. W. Wilson, unpublished results).

In contrast, the experiments described here assume heterogenous nucleation, which follows classical theory, and do not attempt any statistical analysis of data. We report only the SCPs as measured from the various samples run in ALTA and describe a method for defining the nucleation temperature. This is because we alter the temperature of the sample between runs, since we are interested in generating a spread of lag times caused by temperature and not by the stochastic nature of freezing of supercooled solutions. Statistical analysis to determine the nature of the heterogenous nucleation event is only valid if temperature is not a variable.

The design of the device used in the SCP determinations described here is shown in Fig. 1 (we call our device ALTA also). It incorporates a double-walled glass cell with vacuum between the walls and temperature-controlled fluid flowing around a sample capillary. This design eliminates condensation on the outside glass, which would block the optical detection of the freezing event in the capillary. The cooling and heating fluids were both ethanol, either one being pumped through the cell, the flow controlled by computer-switched, electronically operated solenoids.

Each sample of 3 μl was examined in a 5-μl capillary tube sealed into ALTA. The solidification event was determined optically, and temperature measurement was by a small thermocouple (32 gauge) mounted in the glass chamber of ALTA, placed on the outside of the capillary to avoid introducing nucleation sites and electric fields to the sample itself. Initial testing of temperature control and measurement showed that the difference between the temperature measured beside the capillary and that inside it after 30 s equilibration time was less than 0.3°C. Similarly, the difference between the set-point on the temperature controller for the cooling fluid and the temperature of the thermocouple was less than 0.2°C after 30 s. The data have not been corrected for this offset, because in each case it represents a simple linear shift of all points to slightly warmer temperatures and does not affect the conclusions.

Once the sample was frozen and the lag time recorded the cooling fluid was exchanged for heating fluid, at 25°C. The heating cycle used to melt

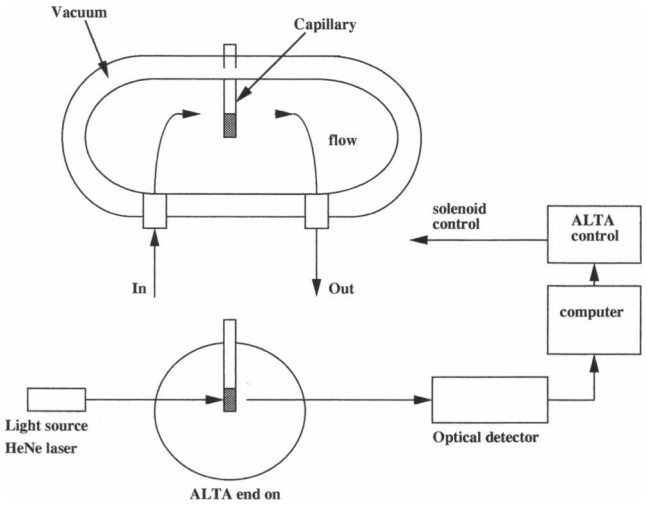

FIGURE 1 Schematic of ALTA showing the 50-ml double-walled glass cell and capillary. Computer-controlled solenoids directed either hot or cold ethanol through the chamber and around the 5-μl sample capillary. Optical detection of freezing meant non-invasive measurements. Temperature recording was performed by placing the thermocouple beside the capillary followed by analog-to-digital conversion.

the ice in the capillary was kept flowing for 3 min to ensure complete melting of all ice before the cooling cycle was restarted. The heating cycle reached its set-point temperature 30 s after the solenoids were switched on. Each hemolymph or saline sample was run through the cooling/heating cycle between 35 and 40 times, and the cold-flow temperature varied slightly each time to give a spread to lag times and to observe the effects of temperature on lag time. The temperature was lowered by 0.1 or 0.2°C increments in most cases.

Sample freezing events occurring within 30 s or less of the cooling fluid being turned on have all been categorized into the same group, i.e., having effectively no supercooling ability, because of the presence of either many or very efficient nucleation sites. Conversely, freezing events taking longer than ~300 min were classified as having high supercooling ability, with few or inefficient nucleation sites. Each sample was initially stepped from warmer to colder temperatures, and the spread of lag times recorded in each case. Once the short-time data points were gathered at colder temperatures the temperature was raised in increments until nucleation was much less probable and times all fell into the >300-min interval. No hysteresis or memory effects that depended on the direction of the temperature increments were found.

In each case samples were loaded into clean capillaries, which were flame-sealed at the lower end and sealed at the top with a silicon (not in contact with the fluid). In some cases capillaries were used straight from the dispensing container (Drummond microcaps, Broomall, PA) whereas in other cases capillaries were soaked in chromic acid, rinsed thoroughly in distilled water, and oven dried. We have previously done tests with multiple samples of the same fluid in different capillaries to determine whether imperfections or differences in the glass had any effect on the SCP. We found that transferring hemolymph from an alpine grasshopper, which lacks INAs, to five capillaries gave the same SCP in each case to within 0.3°C (P. W. Wilson, unpublished results), suggesting that the capillary glass did not significantly affect the nucleation process. There also appears to be no systematic dependency on capillary cleanliness, i.e., "new" or cleaned in-house. We have also sought to determine whether sample volume affects the nucleation temperature. We found that capillary size had little effect on the outcome of SCP determinations when INAs were present. For example, hemolymph from the New Zealand alpine weta *Hemideina maori* gave the same SCPs whether measured as 3-μl volumes in a 5-μl capillary or 20-μl volumes in a 50-μl capillary. However, with saline solutions or other fluids lacking efficient INAs, volume is significant; this is discussed further below.

Hemideina maori Hutton (Orthoptera; Stenopelmatidae) is a large alpine grasshopper (known locally as an alpine weta) from New Zealand, which is freeze-tolerant (Ramlov et al., 1992). It is exposed to numerous freeze-thaw cycles in its habitat and has been shown to tolerate mild freezing exposures ($T > -10°C$) for several hours in the laboratory (Ramlov and Westh, 1994). Specimens of *H. maori* were collected during winter from the top of the Rock and Pillar Ranges, New Zealand, at an altitude of 1500 m. It is known that in winter these insects have higher levels of INAs present in their hemolymph, and there is substantial evidence that these agents are INPs, synthesized specifically so that the insects avoid deep supercooling and so rapid extracellular ice formation. As part of another study we have measured the SCP of native weta hemolymph and found that it was −17.5°C after ultrafiltration through a 5000 molecular weight cutoff filter (MC 5000, Millipore Corporation, Boston, MA), arguing that the INAs responsible for the high SCP of −7.5°C before filtration had molecular weights >5000 and so were presumably proteins (P. Wilson, J. Bedford, and J. Leader, submitted for publication). We also noted that crude weta hemolymph is bright blue in color and that the filtered hemolymph was completely transparent, the blue-colored constituent remaining in the filter. Gel electrophoresis has previously shown that the blue color was associated with a protein of ~100,000 Da (H. Ramlov, personal communication). Further evidence for the proteinaceous nature of the INAs comes from the fact that heat-treated hemolymph had SCPs of between −24°C and −30°C depending on sample size (Ramlov, 1993).

The wetas were kept in plastic boxes at 4°C on a diet of apples and plants until use. Hemolymph was collected from the insects by puncturing the cuticle at the base of a leg. The hemolymph was drawn into 75-μl capillary tubes and transferred into Eppendorf tubes on ice, then stored at liquid nitrogen temperatures.

Purified antifreeze glycopeptides (AFGP) from the Antarctic cod *Dissostichus mawsoni* were donated by Prof. Art DeVries, University of Illinois (purified as in Cheng and DeVries, 1991). Mixtures of the five heaviest-size molecules (labeled AFGP 1–5, m.w. 10,500–34,000 Da) were used for these experiments in aqueous solutions at concentrations as described, where the equilibrium melting point of solutions was ~−0.2°C, but the non-equilibrium freezing point was ~−1.2°C.

A saline solution that matched the ionic makeup of the hemolymph of larvae of the mealworm *Tenebrio molitor* (Easton and Horwath, 1994) was used, made up as follows: osmolality 355 mOsm, 78 mM NaCl, 4.2 mM KCl, 11.2 mM $MgCl_2{*}H_2O$, 19 mM $MgSO_4$, 3.6 mM $CaCl_2$, and 32 mM sucrose, equilibrium freezing point of ~−0.7°C.

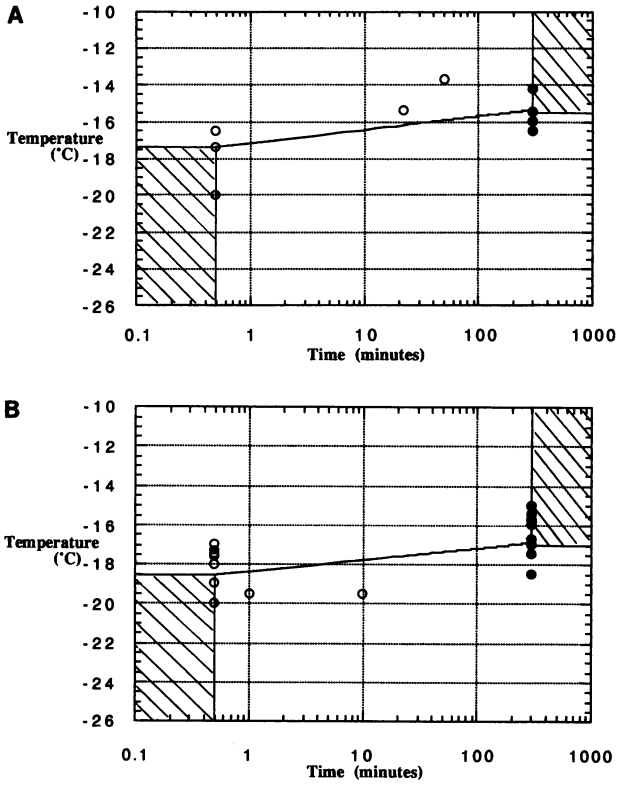

FIGURE 2 (*A*) Plot of measured lag times of a single sample of *Tenebrio* saline solution cooled repeatedly to the temperatures indicated. (●) Events where the sample was still unfrozen after the time indicated. Hatched areas indicate that 15 runs froze within 30 s at temperatures below −17.4°C, and 15 runs were unfrozen after 300 min at temperatures greater than −15.5°C. The solid line represents a best-fit line for the data points shown, used to calculate the SCP by being taken as the intercept at a time of 10 min. In this case the SCP was −16.5°C, and the solid line has the equation $T = -17.1 \mp 0.73 \log(t)$, where T is temperature and t is time. (*B*) Plot of lag times after AFGP molecules have been added to saline solution to give a final concentration of 10 mg/ml. The SCP has shifted to −17.7°C, the equation now being $T = -18.3 \mp 0.60 \log(t)$. Hatched areas have the same meaning as in *A*.

RESULTS

In Figs. 2 and 3 the filled circles indicate that the sample was unfrozen when the lag time was recorded, at 300 min for the saline solutions, and >70 min in the case of the weta hemolymph.

Fig. 2 *A* shows a plot of lag times of a single sample of saline solution cooled repeatedly to the temperatures indicated. The hatched area at short time intervals indicates an area where 15 runs froze within 30 s, all at temperatures <17.4°C. Most of the actual data points are not shown but are within the hatched area, although all data points that represent freezing times >30 s and <300 min are shown. Thirty seconds was chosen as the minimum time for recording nucleation, because accuracy in temperature control was limited at shorter times. The hatched area at times greater than 300 min indicates that 15 runs were unfrozen at temperatures warmer than −15.5°C. Again, this time was chosen simply for experimental reasons, because 15 runs at more than 300 min each makes the data-collection time excessive. The solid, best-fit line (for the data points shown) indicates the transition between high and low nucleation rates, and it spans the range of −15.4°C to −17.4°C. Note that the data points falling between 30 s and 300 min span a 6.2°C range. We quote only the span of data points between 30 s and 300 min in what follows, because the span of all the data points is very large because lag times less than 30 s can correspond to any temperature less than the top of the left-hand hatched area (Fig. 2*A*). Similarly, any temperature below the equilibrium melting point and the bottom of the right-hand hatched area is a valid "long" time measurement. The SCP has been defined here as the temperature of the intercept of the best-fit line at time $t = 10$ min. Although this is an arbitrary choice, it does signify a temperature where the lag time varies significantly with small changes in temperature, and represents an ideal experimental time scale. In the case of Fig. 2 *A* the SCP is then −16.5°C.

Fig. 2 *B* shows the results of adding AFGP molecules to the saline, to give a final concentration of 10 mg/ml. The symbols have the same meanings, and it can be seen that the SCP has shifted from −16.5°C to −17.7°C for the best-fit line.

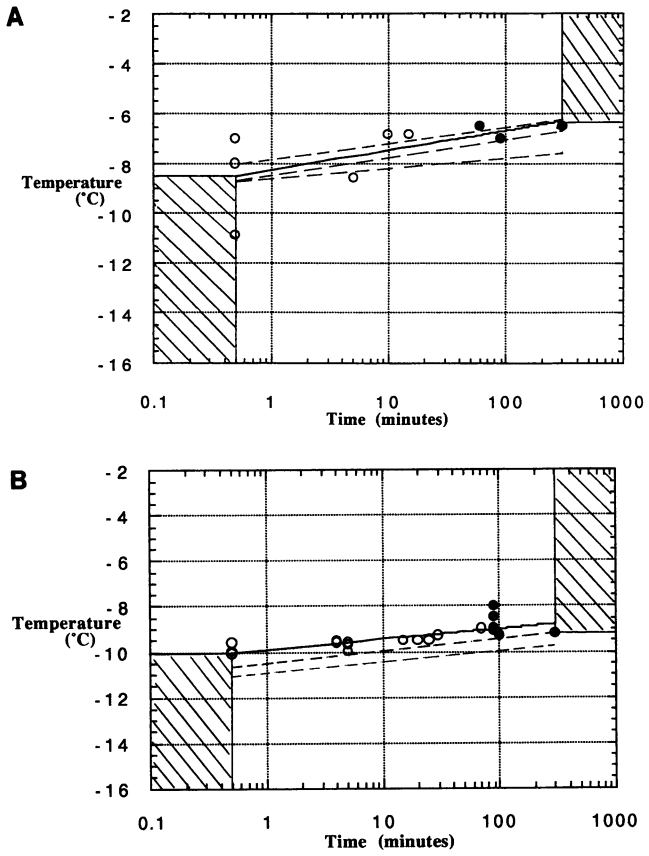

FIGURE 3 (*A*) Plot showing data points of lag times of a single sample of native weta hemolymph. Hatched areas have the same meaning as in Fig. 2. (———) Best-fit line for the data points shown; its SCP is −7.3°C, and the equation is $T = -8.2 \mp 0.79 \log(t)$. (- - - - -) Three other samples; although the data points are not shown in these cases their spread is similar to the single set shown. (*B*) Plot of the lag times for three samples of weta hemolymph to which aqueous AFGP 1–5 has been added such that the final AFGP concentration was 10 mg/ml. Data points are shown for one sample only, and the best-fit lines are shown for all three. The equation for the solid line, corresponding to the data points shown, is $T = -9.888 \mp 0.44 \log(t)$, giving a 10-min SCP of −9.4°C.

The span of data points shown is 5.2°C, and the slope of the best-fit line is similar to that in Fig. 2 *A*. This observed lowering of the SCP cannot have been due to the lowering of the equilibrium freezing point by the addition of the AFGP molecules, because at 10 mg/ml the equilibrium, or colligative freezing point, is depressed by only 0.2°C (Cheng and DeVries, 1991). However, the non-equilibrium freezing point, or temperature of crystal growth, is depressed by ~1.2°C; this may be responsible for the observed lowering of the SCP, or the supercooled state may have been stabilized somewhat by the addition of AFGPs. We discuss these possibilities later in this paper.

Fig. 3 *A* shows a plot of lag times of a single, representative sample of native weta hemolymph. The hatched areas have the same meaning as in Fig. 2, *A* and *B*. The best-fit line (*solid*) shows that the measured SCP is −7.3°C, while the data points falling between 30 s and 300 min span 4.4°C. Note that the relatively high value of the SCP is due to the presence of INPs in the hemolymph. Samples of hemolymph from three other insects collected at the same time of the year had SCPs of between −6.9 and −8.2°C and had a similar scatter of data points. The data points of those samples are not included in Fig. 3 *A* but the dashed lines are best-fit lines of those three data sets.

Fig. 3 *B* shows the lag times for three samples of weta hemolymph to which aqueous AFGP 1–5 has been added such that the final AFGP concentration was 10 mg/ml. The data points from only one sample are shown, and corresponding best-fit line is indicated by the solid line, having an SCP of −9.4°C, while the corresponding data points span 2°C. The best-fit lines for the other two samples are shown as broken lines, having SCPs of −10.0°C and −10.5°C, both having data sets spanning ~2°C.

The average SCP has been lowered from −7.5°C (with a 4.4°C spread for data points) for native weta hemolymph to −10.0°C (with a 2.0°C spread for data points) by the addition of AFGP molecules. This lowering by 2.5°C is greater than the spread of data points in each case and indicates that some INPs have been masked or at least had their efficiency reduced by the addition of AFGP molecules. In contrast the addition of AFGP to saline solution lowered the SCP from −16.5 (6.2°C spread for data points) to −17.7°C (5.2°C spread for data points).

Although multiple freeze-thaw cycles are known to cause protein denaturation in some cases, we have not found any such denaturation or loss of efficiency of the INPs found in the weta hemolymph. Each sample of winter hemolymph underwent typically 40 freeze-thaw cycles, and we found no change in the measured SCP that could be described as due to this cycling. Similarly fish AFGP molecules are known to exhibit their hysteresis activity regardless of how many times it is measured, and they undergo a freeze-thaw cycle in a Ramsay Brown chamber (Duman and DeVries, 1972; P. W. Wilson, unpublished results).

DISCUSSION

Cold-hardy arthropods

THPs are found in many cold-hardy insects, spiders, and mites (reviewed in Duman et al., 1993a) and have also been found in many freeze-avoiding hibernating insects (Zachariassen, 1985). These THPs generally fall within the 14–20-kDa molecular weight range, and all lack the carbohydrate residues found in some fish AFPs (Duman et al., 1991, 1993a). Insect THPs are not able to affect the hysteretic freezing point below about −10°C, but are found in body fluids that supercool to −30°C or −40°C. The THP hysteretic activity has been shown to plateau at ~30–40 mg/ml in aqueous solutions. This is similar to the physiological level of AFPs found in polar fishes (Cheng and DeVries, 1991) and is thought to represent the levels occurring in insect hemolymph (Duman et al., 1993b).

The SCPs reported in both insect whole-body and hemolymph studies have always been defined to within a small

temperature range (see, e.g., Johnston and Lee, 1990). A typical example of recorded SCPs is that of Gehrken (1989), in which the adult bark beetle *Ips acuminatus* was shown to have a whole-body SCP of typically −18.0°C ∓ 0.3°C. In another example it was reported that adults of the Antarctic mite *Alaskozetes antarcticus* supercooled to approximately −30°C on average for most of the Austral winter, with little variation in individual SCPs (Cannon and Block, 1988). It has been suggested that such well-defined SCPs may be caused by the segregation of water into smaller volumes (Franks et al., 1984), given that volume plays an important role in determining the supercooling ability of aqueous solutions. Examples of such volume dependency can be found in Duman and Patterson (1978), who reported that 10-μl samples of distilled water supercooled to −17.4°C ∓ 2.4°C, while Neven et al. (1986) found that 1-μl samples supercooled to −20.0°C ∓ 1.6°C. Similarly, the volume of the animal in whole-body SCP measurements has been reported to be significant (Johnston and Lee, 1990).

The presence of solutes can also affect the supercooling ability of solutions (MacKenzie, 1977). In many cases solutes such as glycerol have been reported in animals that supercool to low temperatures, although many species are able to supercool to between −24 and −30°C in the absence of glycerol (Ring, 1981). Extremely high concentrations of glycerol do occur in some overwintering species, causing the hemolymph melting points to be depressed and the SCPs to be as low as −50°C (Zachariassen, 1985). Somme (1964) and Block and Young (1979) have reported that glycerol has depressed the SCP by more than three times the equivalent melting point depression, although Zachariassen (1985) has suggested that this nonlinear depression factor is closer to two.

INPs

Biological INAs or INPs are believed to act by mimicking the structure of an ice crystal surface, thus imposing an ice-like arrangement on the water molecules in contact with the nucleating surface and so lowering the free energy necessary for ice nucleation and growth of a macroscopic crystal (Duman et al., 1991). INPs associated with certain strains of bacteria range in molecular weight from ~150 kDa for those active at −12°C to ~9 MD for the largest, or most efficient nucleation sites, active at −3°C (Burke and Lindow, 1990). It is thought that these large sites are aggregations of smaller subunits. Of the several species of insects and arthropods that possess INPs, the most thoroughly studied is one found in the larvae of the crane fly *Tipula trivittata* (Duman et al., 1985), where the INP is a lipoprotein of some 800 kDa.

Often whole-body SCPs are warmer than hemolymph SCPs because of the presence of ice nucleation sites in the gut. Where INPs are present in the hemolymph the SCP depression is equal to the equilibrium melting point depression. Some insects that have low polyol levels, and thus high subzero hemolymph melting points, can still supercool to temperatures below −20°C, probably by the removal or inactivation of INAs. Many such insects promote evacuation of the gut contents, thus decreasing the probability of freezing by removing ice nucleating material (Somme and Block, 1982). We confine our discussion to hemolymph supercooling and will not speculate on the ice nucleation properties of gut contents such as dirt and bacteria, which may be efficient nucleators and require masking or eradication before supercooling can take place.

Polar fish AFPs

In the case of Antarctic fishes, ice crystals suspended in the water column are ingested into the hypo-osmotic intestinal tract and are inhibited from growing by the presence of the AFPs. The method of action of AFPs has been the subject of many studies, although it is still not completely understood (reviewed in Cheng and DeVries, 1991; see also Duman and DeVries 1972, Raymond et al., 1989, and Wilson et al., 1992). What is clear is that the AFPs must depress the rate of nucleation in undercooled fluids where ice is not normally present and must poison all possible crystal growth sites in fluids where ice may be present. It is known that AFPs adsorb to specific faces of ice crystals, and the spacing of oxygen molecules at the ice surface has been suggested as an important factor in facilitating such adsorption (see, e.g., Raymond and DeVries, 1977; Knight et al., 1991).

The blood of these fishes has an equilibrium freezing point warmer than the seawater temperature and so is supercooled, if ice is never present. The chances of inoculation of ice into the bloodstream are not known. If inoculation occurs at the gills many ice crystals would be circulated in the blood, each of which must presumably be inhibited from growing. Ice has never been found in the blood of *D. mawsoni*, e.g., but this species and many other Antarctic fish tested have been found to have ice present in the spleen (A. L. DeVries and P. W. Wilson, unpublished results). On the other hand, if inoculation by ice does not occur then large volumes of blood are supercooled throughout the life of the fish, more than 30 years in some cases. It is possible that nucleation does not occur because AFPs circulating in the blood are able to stabilize it in the supercooled state. It may be significant that there is only 1°C of supercooling involved in this case, compared with that of insects that may experience perhaps 20 or 30°C of supercooling, given that such a driving force directly affects the probability of a nucleation event occurring.

In much of this discussion we use the term THP, but this could equally be replaced by AFP or AFGP, because the size, amino acid makeup, and hence, degree of hydrophilicity of insect THPs are known to be similar to those of fish antifreeze molecules (Duman et al., 1993a). Although there are several distinct classes of fish AFPs (Cheng and DeVries, 1991), they are thought to lower the non-equilibrium freezing point of macroscopic ice crystals by a similar mechanism, and there is no evidence that insect THPs cause thermal hysteresis by any different mechanism (Wilson, 1993).

Heterogenous nucleation

If nucleation of ice occurs on foreign particles the ice is said to form by heterogenous nucleation. If the ice phase is initiated by water molecules alone combining together to form an ice embryo that can grow spontaneously, homogenous nucleation has taken place. Zachariassen (1985) has suggested the term semihomogenous nucleation to describe situations where the SCP is as low as −30°C, resulting from nucleation sites of low efficiency, rather than homogenous nucleation.

Homogenous nucleation of pure water can be made to occur in small volumes (10–100 μm in diameter) at about −40°C; however, with heterogenous nucleation the onset of crystallization is highly unpredictable and depends strongly on the specific sample and its treatment. As mentioned volume has a large influence, because large samples may contain more nuclei to initiate crystallization. Nucleation frequently occurs on the container surface, foreign particles, or other heterogeneities that catalyze ice formation. The time at which spontaneous nucleation occurs is also proportional to temperature and solute concentration. The actual relation between temperature of the sample, the amount of supercooling, and the probability of crystallization is not well known.

Reports of "nucleation temperature" are difficult to analyze because there is no unique temperature at which nucleation occurs. However, because the nucleation rate $J(T)$ is an inverse fifth-order function of T (Franks et al., 1987), the process is extremely sensitive to temperature changes, and the "nucleation temperature" is often the temperature at which nucleation becomes sufficiently rapid for its experimental measurement.

The present theory for heterogenous nucleation, such as described in Hobbs (1974), has not changed significantly in the past two decades and is based on the classical theory of homogenous nucleation, developed largely by Fletcher (1958). The classical model describes nucleation as a process whereby a cluster of water molecules grows by stepwise addition of molecules until it reaches a critical volume from which it can grow spontaneously into a macroscopic crystal. The critical radius for an ice embryo is given as

$$r^* = \frac{2\sigma_{12}}{\Delta G_v} \tag{1}$$

where ΔG_v is the free energy difference between water as liquid and solid and the surface tension at the ice/water interface is σ_{12} where the subscripts 1 and 2 denote the ice and water, respectively. Then r^* determines the number of water molecules gathered together in an ice-like cluster that are necessary before the free energy can decrease and solidification of the sample proceed. The critical free energy is given as

$$\Delta G^* = \frac{4\pi\sigma_{12}^3 f(\theta)}{\Delta G_v^2} \tag{2}$$

where $f(\theta)$ is a geometrical factor depending on the contact angle, volume, and surface area of the ice embryo. This factor $f(\theta)$ has been discussed recently in Wilson (1994a), and although Fletcher (1958) considers embryonic nuclei to be polyhedral there is no experimental evidence for this; the theory applies equally to spherical embryos. Following the notation of Franks et al. (1984) the nucleation rate is given by

$$J(T) = L(\sigma T)^{1/2}\phi^2 \exp\left[-\Delta G_v/RT\right]\exp\left[-Q\sigma^3/(\Delta T)^2T^3\right] \tag{3}$$

where L is a function of the densities and molar volumes of the two phases, and

$$Q = (bV_{ice}/k)T^4(\Delta H_c)^2]. \tag{4}$$

We denote ϕ as the volume fraction of water, ΔT the degree of supercooling, b a shape factor depending on the geometry of the nucleus ($b = 16\pi/3$ for spherical nuclei), V_{ice} the partial molar volume of ice, and ΔH_c the latent heat of crystallization. The temperature dependence of ΔH_c is discussed in Franks et al. (1984). As in Franks et al. (1987) we simplify Eq. 3 to become

$$J(\tau) = A\, exp\,(B\tau) \tag{5}$$

where

$$\tau = [(\Delta\theta)^2\theta^3]^{-1} \tag{6}$$

θ is the reduced temperature (T/T_f) and $\Delta\theta$ is the reduced degree of supercooling [$(T_f - T)/T_f$], T_f being the equilibrium freezing temperature. This nucleation rate dependence on temperature to the fifth power is discussed further below.

Historically a freezing event within 1 s of cooling has constituted a high probability of nucleation, and a lack of nucleation in 1 or 2 h has been evidence that the SCP has not been reached. Fletcher (1958) used $J(T) = 1\ s^{-1}$ as an appreciable, or "high" nucleation rate. We have used one nucleation event in 30 s, $J(T) = 0.03\ s^{-1}$ or less to be a "high" nucleation rate and lack of freezing in 300 min or more to be a "low" rate, $J(T) = 5.5 \times 10^{-5}\ s^{-1}$. These values have been set purely for experimental reasons.

As mentioned earlier the presence of solutes, especially those with high molecular weight and low diffusion constant, can markedly affect the nucleation of ice. Generally $J(T)$ is reduced by the addition of solutes, one reason being that they reduce the volume fraction of water available for formation of the ice embryo. However, it should be stressed that the volume fraction is not significantly reduced by protein molecules such as THPs at concentrations of only 3.5% w/v, although in such solutions $J(T)$ is still reduced because ΔG is proportional to the slowest diffusing species in the mixture (Franks et al., 1984). Before macroscopic growth an ice cluster becomes surrounded by a region impoverished in water, and $J(T)$ is further reduced in this way. Michelmore and Franks (1982) reported that in aqueous solutions of polyethylene glycol (PEG) (m.w. = 44000) $J(T)$ was reduced, an effect that they attributed to the effects of the PEG molecules on the diffusional motions of the water molecules. They also found that the addition of 1% AFGP to a variety of solutions reduced $J(T)$ by amounts identical to those where PEG was added. They concluded that the hysteretic effects of AFGP required the presence of macroscopic ice crystals, i.e., $r > r^*$.

When calculating or determining $J(T)$ one critical quantity is σ, the interfacial free energy, because $J(T)$ is proportional to σ^3. This quantity is not well known for macroscopic ice

or embryonic-sized ice crystals and is discussed in Wilson (1994b). Values of 25 or 30 erg/cm^2 are commonly used for general ice/water interfaces but when considering polygonal ice, anisotropy must also be taken into account (Wilson, 1994b). The values of σ for embryonic-sized ice are not known, and since the ice/water interface is diffuse with a typical transition length of 10 or 20 Å (Beaglehole and Wilson, 1993), a spherical embryo with a radius of, e.g., 50 Å will consist largely of an interfacial region with unknown and perhaps very unstable σ. If σ changes value with r the present theory would require significant alteration.

In Fig. 4 we have replotted the lag times of saline and weta hemolymph as a function of freezing temperature. We have also plotted points corresponding to the function $J(T) = A \log(BT^5)$ for 4, 5, and 6°C supercoolings and 16, 17, and 18°C supercoolings, where A and B are numerical constants. The measured dependence of $J(T)$ on temperature fits the above theory well for low levels of supercooling but at colder temperatures $J(T)$ increases more rapidly with every further degree of supercooling. These results do, however, help to explain why animals that supercool have such well-defined nucleation temperatures.

Previous studies on heterogenous nucleation have produced results that varied markedly because small differences in experimental method can drastically affect the measured nucleation temperatures. Most previous studies have utilized finely dispersed droplets of water of μm dimensions in an inert carrier fluid to determine nucleation rate $J(T)$ (see, e.g., Franks et al., 1987). In such studies crystal growth rates have been rapid compared with nucleation time (Harrison et al., 1987), and the same is true for the results presented here.

The first possible mechanism we consider for the stabilization of supercooled solutions is that the THP molecules hinder the formation of critical ice nuclei by a volume exclusion effect, i.e., they physically hinder the ice embryo and do not allow it to grow large enough to escape the free energy barrier and grow to macroscopic size. Secondly, we examine the possibility that the THP molecules diffuse to, bind to, and inhibit every cluster of water molecules that becomes "large" and approaches or exceeds the critical size r^*. Finally, we consider the possibility that THP molecules bind to heterogenous nucleation sites, be they physical or biological, and inactivate or mask them from the bulk water, thus reducing $J(T)$.

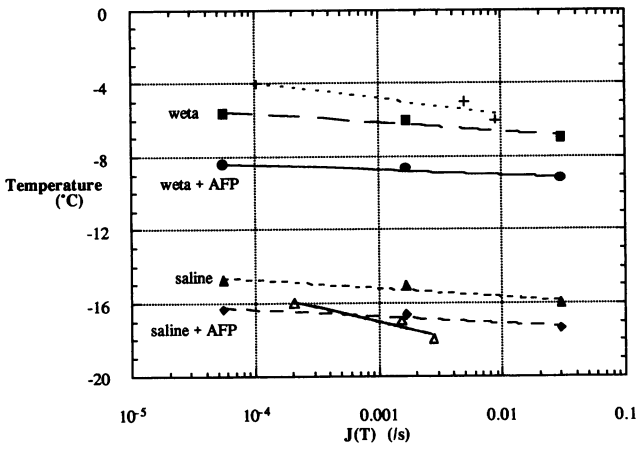

FIGURE 4 Plot of saline solution and weta hemolymph lag times before and after the addition of AFGP. In each case the SCP has been lowered. Shown also are calculated points corresponding to the function $J(T) = A \log(BT^5)$ for 4, 5, and 6°C supercoolings and 16, 17, and 18°C supercoolings, where A and B are numerical constants. The measured dependence of $J(T)$ on temperature fits theory well for small levels of supercooling, but at colder temperatures $J(T)$ increases more rapidly with every further degree of supercooling.

Physical hindrance of ice embryos

We consider here the possibility that protein molecules in supercooled solutions inhibit the formation of embryonic ice nuclei simply by virtue of taking up space in the solution. The argument that follows could equally apply to any hydrophilic protein and is not specific to those expressing thermal hysteretic properties. The hydrophilicity of THP molecules will ensure that they have a significant hydration shell. They will be, on average, X Å apart in solution at a given concentration, ignoring any sedimentation and assuming translational diffusion due to Brownian motion only. We consider here several specific molecular weights of AFP molecule and calculate X at the concentration of 35 mg/ml in each case.

The AFP from the winter flounder *Pseudopleuronectes americanus,* labeled AFP1, and AFGP 8 from the Antarctic cod *Dissostichus mawsoni*, both have molecular weights of ~3000 Da and are thought to be extended helices, which can be simplified to cylinders ~10 Å in diameter and 50 Å long. This volume corresponds to a sphere of diameter 20 Å. Spherical geometry has been used in what follows for ease of calculation only, but cylinders give similar results.

Fig. 5 shows a schematic of two AFP molecules separated by a typical, averaged intermolecular distance. Simple space-filling calculations show that at a concentration of 3.5% w/v AFP1 or AFGP 8 are on average 60 Å apart from center to center. If we assume that ice embryos will not include the space taken up by the protein, then the space available for an ice embryo at any instant in time is on average 40 Å in diameter. This is in a simple two-dimensional picture, and in real solutions the three dimensions make this ~65 Å.

Estimates of the size of theoretical ice nuclei at given temperatures (Wilson, 1994a) give $r^* = \sim 50$ Å at −12°C, with the contact angle constant $m = 0.97$ and at −20°C $r^* = \sim 30$ Å. These dimensions are similar to the $r = 33$ Å available if the protein molecules are at their most spaced positions and not diffusing. In the case of the larger antifreeze molecules, AFGP 1–5 (avg. m.w. = 20,000 Da) $X = 150$ Å, leaving ample room for an ice nucleus of critical size to form. These calculations suggest that the THP molecules do not inhibit the formation of ice embryos simply by being in solution. THP molecules of, e.g., 14 kDa would be separated by spaces of about 100 Å, large enough for embryos at moderate supercoolings.

The clustering of water molecules during homogenous nucleation or even the diffusion of water molecules to an ice cluster during heterogenous nucleation is more rapid than the

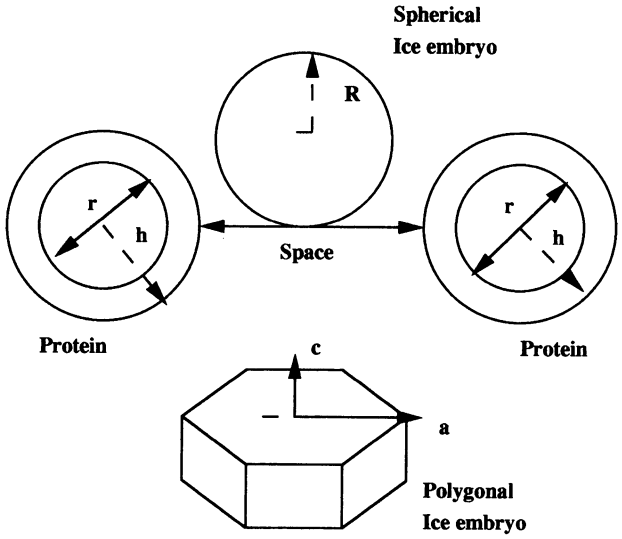

FIGURE 5 In this simple representation there are two protein molecules, idealized to be spherical, which take up space required by the ice embryo. The radius r of certain AFPs becomes radius h with a hydration shell added. R is therefore the allowable radius of an ice embryo if it is to fit in between the protein molecules. We show the situation in two dimensions, but the calculations described in the text have been carried out for three dimensions. The ice embryo may in fact be polygonal as shown but the calculations give similar results.

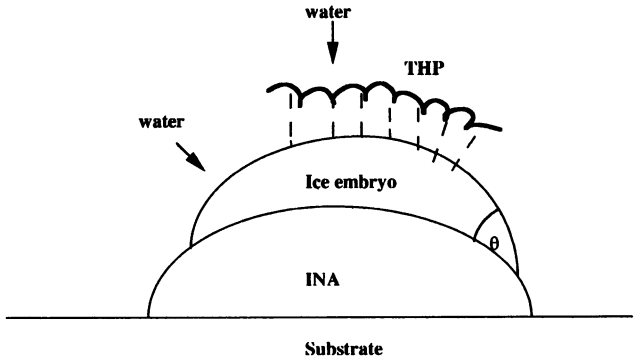

FIGURE 6 Cartoon of a THP molecule that has diffused to and bound to an embryonic ice nucleus formed on an INA. The INA could be a protein or some nonbiological nucleation site about which water molecules gather. The contact angle θ determines the efficiency of the nucleator. As well as reducing the surface area available for further water molecules to join the nucleus, the adsorbed THP may present a hydrophobic surface to the bulk water. Inhibition of growth of the ice may be due to the Kelvin effect, but this would require many THPs binding to the embryo (adapted from Wilson, 1994a).

diffusion of THP molecules in solution. This time-constant phenomenon is considered in more detail in the next section.

THPs binding to embryonic ice nuclei

We now consider the possibility that the THP molecules bind to each embryonic ice nucleus that forms and in doing so inhibit the subsequent growth of the ice to macroscopic size. This model involves considering that the THP molecules recognize and bind to ice rather than the INA itself. Fig. 6 shows the INA bound to a substrate but equally it could be freely diffusing in solution, or in fact the model could apply to homogenous or semihomogenous nucleation. The embryonic ice nucleus has one or more THP molecules adsorbed to it and is inhibited from growing by some mechanism, perhaps the Kelvin effect (Wilson, 1993). Although much less is known about insect THPs than about fish AFPs it is known that THPs typically have more hydrophilic residues, and it is thought that like AFPs they are amphiphilic, having a hydrophilic side attached to the ice and a hydrophobic side presented to the bulk water (Duman et al., 1993a). However, the following factors suggest that such inhibition of embryonic nuclei is unlikely.

1) The radius of an embryonic ice nucleus is in the 50 to 500 Å range at the levels of supercooling we are concerned with here (Wilson, 1994a). Small scales such as these mean that there may be insufficient room for a number of THPs to bind to the ice and so to cause the increase in surface area to volume ratio required by the Kelvin effect (Wilson, 1993), because each THP molecule will be on the order of 50–100 Å long.

2) The amount of thermal hysteresis found with even the most potent insect THP is only 5–8°C. Supercooled animals may depress the crystallization temperature by 20°C; the discrepancy is difficult to account for by the Kelvin effect model, contrary to the findings of Zachariassen and Husby (1982).

3) Franks et al. (1987) found that AFGP was not able to depress the homogenous nucleation temperature any more than polyvinyl-pyrrolidone (PVP), a synthetic polymer lacking hysteretic activity. This suggests that the AFGP molecules could not inhibit the embryonic ice clusters and only exhibited hysteretic activity when macroscopic ice was present.

Even if the THPs could stabilize the embryonic ice nuclei by some means other than the Kelvin effect the rate of diffusion of molecules in solution suggests that they may not have time to reach and inactivate every embryonic nucleus that forms. Once ice nuclei become critical in size and are over the free-energy hump, growth is very rapid. This rate of crystallization is dependent on the level of supercooling, and the rate of translational diffusion of the THPs is limited.

We consider here the measured diffusion coefficients D of some THPs in aqueous solution and show that diffusion becomes a severe limitation. The larger fish AFGPs 1–5 have a measured diffusion coefficient in aqueous solution, at 0.1 mg/ml, of $D = 2.5 * 10^{-7}$ cm^2 s^{-1}, and the smallest glycopeptides AFGP 8 (m.w. = 2600 Da) were found to have $D = 2.6 * 10^{-6}$ cm^2 s^{-1} (Wilson and DeVries, 1994). The insect THPs from *Tenebrio molitor* (m.w. = 14 kDa) had $D = 1 * 10^{-6}$ cm^2 s^{-1} (Wilson, P. and K. Horwath, unpublished results). A non-THP molecule such as ribonuclease, also of 14 kDa, has $D = 1.2 * 10^{-6}$ cm^2 s^{-1}. All of these were measured at 4°C, and it is known that D changes with temperature, depending on the hydrodynamic shape of the molecule. For example, in extended molecules such as AFGPs, D increases by ~20% when the temperature is reduced by 10–15°C (Wilson and DeVries, 1994).

The self-diffusion constant of water at 0°C is $5 * 10^{-6}$ cm^2 s^{-1} and at −15°C is $1 * 10^{-5}$ cm^2 s^{-1} (Angell, 1982). This suggests that in supercooled, aqueous solutions water molecules can diffuse to heterogenous binding sites at the INA perhaps 10 or 50 times faster than THPs. Also, there are significantly fewer THPs than water molecules in a physiological strength solution. This time constant discrepancy together with the three problems mentioned above lead us to the third model for stabilization of supercooled solutions.

Masking of nucleation sites by THPs

We now consider the possibility that THP molecules bind directly to heterogenous nucleation sites and so render such sites inactive. The sites may be biological (INP) or physical (INA), such as membranes within invertebrates or, in experimental situations, dirt or defects in the container walls. For such sites to act as INAs they must be able to cluster a number of water molecules together, presumably by hydrogen bonding, such that $r > r^*$. In the case of INPs the polar residues along the protein backbone should have a repeat spacing that matches one of the ones found on the surfaces of ice (Wilson, 1994a). If this is the case then perhaps THP (or AFP) molecules can bind to INAs, because the repeat spacings of THPs are thought to match those of an ice crystal (to produce the hysteresis measured in the presence of macroscopic ice) (Raymond et al., 1989; Duman et al., 1993a). Such binding would mean that water molecules in solution would then be presented with the hydrophobic side of these molecules, if THPs are as amphiphilic as AFPs, and significantly less water binding to the INA would take place.

Parody-Morreale et al. (1988) reported that AFGPs from *Dissostichus mawsoni* inhibited the ice nucleating activity of membrane vesicles from the bacterium *Erwina herbicola*. They used a drop-freezing assay to characterize the populations of ice nucleators and reported saturation at high AFGP concentrations, suggesting to them a simple binding mechanism between the AFGP and the INPs. They also found that the larger molecular weight AFGPs were more effective inhibitors of INPs, and their conclusion was that given that AFGPs bind to ice and INPs mimic ice, then the AFGPs bind to and inhibit the INPs. Similar experiments with insect THPs have shown that they have the ability to inhibit the ice-nucleating activity of certain INPs (Duman et al., 1991).

It is this scenario that we propose as a general mechanism for stabilization of supercooled solutions. Our results show that AFGPs reduce the $J(T)$ of weta hemolymph and are in accord with this general hypothesis. In Fig. 7 a generalized schematic is shown whereby a THP molecule has diffused through solution and bound to a possible nucleation site, exposing a hydrophobic side to the solution and inhibiting the clustering (binding) of water molecules to the INA, thus lowering the SCP, since water molecules must cluster elsewhere or the temperature must be reduced before $J(T)$ increases. We have drawn two smaller clusters of ice, neither of which has space available on the INA to allow $r > r^*$.

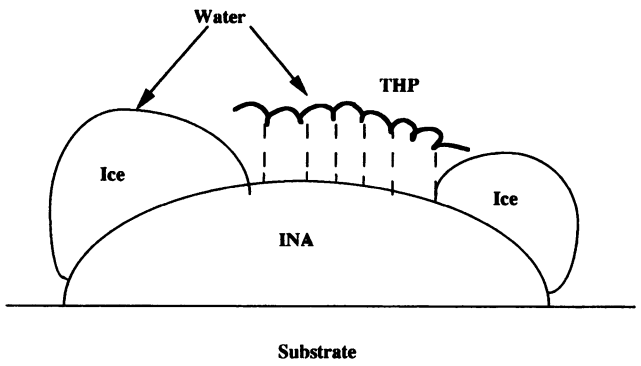

FIGURE 7 Cartoon showing a single ice nucleator (either an INP or a physical INA) bound to a substrate, with a single THP bound to it. The water molecules in solution must form smaller ice embryos, each of which may not be able to reach critical size.

CONCLUSION

We describe a new method for the measurement of and definition of the SCP of small volumes of fluid using the repeated freezing of a single sample rather than multiple samples. Defining the SCP requires choosing an experimental nucleation rate that is as independent of external parameters as possible. Measuring lag times at a variety of temperatures provides a simple way of determining a useful "nucleation temperature". The technique does however suffer from time constraints; to derive statistically meaningful data many more data points than we have given would be required (within the 30 s–300 min. interval), but since small changes in temperature drastically affect the lag time many of those data points gathered would actually fall within the "hatched areas" (see Figs. 2, A and B, and 3, A and B) and would be unused in any statistical analysis. Nevertheless the technique we have described is a relatively simple way to determine with reasonable accuracy the SCP of any given solution.

It has previously been shown that fish AFGPs partially inactivate INAs found on bacteria (Parady-Morreale et al., 1988), presumably by binding to them. Franks et al. (1987) found that AFGP was not able to depress the homogenous nucleation temperature any more than PVP, a synthetic polymer lacking hysteretic activity. This suggests that the AFGP molecules could not inhibit the embryonic ice clusters. We have shown that adding fish AFGP to a supercooled Ringer's solution does not stabilize the supercooling ability of that solution by as much as a solution containing INPs. We argue that the reduced stabilization of the Ringer's solution is due to a scarcity of efficient nucleation sites to which the AFGPs could bind compared with the readily available sites on the INPs. Obviously there must be some nucleation sites or the homogenous nucleation temperature would be able to be reached, but such sites are less active than those found when INPs are present.

From these observations we conclude that the AFGP molecules, and therefore probably THP molecules, bind to nucleation sites rather than diffusing to and binding to the actual heterogenous ice nuclei formed in a supercooled solution. Our results suggest a trend whereby AFPs can bind more

readily to those nucleation sites that are more efficient, i.e., more ice-like. We found ~2.5°C of supercooling reduction when the AFPs were added to solution containing INPs and only ~1.2°C of reduction when saline solution was used. The results of Franks et al. (1987) showed that when no nucleation sites were present the homogenous nucleation temperature was unchanged for AFGP compared with a non-antifreeze polymer. This "diminishing returns" principle also argues for the binding to sites that have an ice-like pattern.

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