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Anomalous Depression of the Freezing Temperature in a Biological System

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Water relationships in biological systems present some of the most challenging problems in biology.[1,2] One of the most interesting is: How does a biological system survive against or succumb to freezing of water? Since all living things contain much intra- and extracellular water, the ability of the cell or of the organism to withstand the freezing of water is a major problem of adaptation.

There are at least two major ways that freezing of water can cause irreversible damage to cells. First, when a cell membrane has been rendered rigid by the cold environment, the formation of ice crystals from either intra- or extracellular water can cause mechanical damage. The irregular ice crystalline protrusions can puncture or bruise the membrane walls. There have been many efforts to ensure that these ice crystals, when formed, are small enough so as not to cause wall damage. For example, Franks and Skaer[3] recently examined cells in aqueous solutions containing poly(vinylpyrrolidone) (PVP). It was found that intracellular protection was provided even though the cytoplasm was not in direct contact with the medium containing the polymer.

A second major cause of cell damage due to freezing is cell dehydration. Even when intracellular water does not freeze, should the extracellular water freeze, the cells become hypoosmotic with respect to the external free water content. The resulting water outflow, in the direction to balance the chemical potential, leads to dehydration of the cell interior. This may lead to denaturation of proteins and changes of ionic strength. Directly or indirectly, these can do irreversible damage to the cell.

It is therefore obvious that, for organisms to survive at freezing temperatures, they must make special adaptations. Partial acclimatization can be achieved in animals without circulatory systems by increasing concentration levels of compounds such as NaCl, sugars, and glycerol, which lower the freezing point by normal colligative means. Animals with circulatory systems, and probably some others, eschew this approach, apparently because it would raise the osmotic pressure too high. Therefore, unless they could raise their internal temperatures by generating more heat, other noncolligative routes would need to be found.

Basic Types of Antifreezing Proteins Found in Fishes

Arctic and Antarctic fishes typically survive in seas in which the temperature is subzero. Some means must exist so that their aqueous interior can survive such a low temperature. In a series of articles from 1953 through 1962, investigators working with Scholander[4-6] reported that the blood serums of Arctic fish had lower freezing temperatures than did the blood serums of fish not adapted to the cold. They observed that there were no additional salts or possibly similar substances in the

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Table I
Freezing Temperature of Serums before and after Dialysis[a]

Serums	Freezing temperature, °C: Before dialysis	After dialysis
Human	−0.56	−0.014
Salmon (ocean)	−0.84	−0.011
Salmon (river)	−0.58	−0.008
Rainbow trout	−0.65	−0.010
T. borchgrevinki[b]	−2.07	−0.56
D. mawsoni[c]	−1.99	−0.49

[a] From ref 13. [b] Sample from pooled serums of 150–200 fish. [c] Average of individual samples from 4 fish.

serum which helped to lower the freezing temperature, and that on chemical fractionation of the serum these appeared in the fraction soluble in trichloroacetic acid. The original observation of Scholander that an antifreeze-like substance existed in Northern polar fish has been more recently confirmed by Scholander and Maggert[7] and Hargens[8] who found it in the Saffron cod, *Eleginus gracilis*. An antifreeze substance has also been reported in the winter flounder (*Pseudopleuronectes americanus*).[9,10] Our laboratory has recently found antifreeze glycoproteins in the polar cod (*Boreogadus saida*) from the Barents Sea north of Russia.[11]

DeVries and Wohlschlag[12] first reported on the antifreeze protein in Antarctic fish, and the first extensive chemical studies on the antifreeze proteins were with proteins obtained from Antarctic fish.[12–15] These were done under the auspices of, and with the facilities of, the National Science Foundation.[16]

Two Antarctic fish studied were *Trematomus borchgrevinki* and *Dissostichus mawsoni*, common inhabitants of the McMurdo Sound in Antarctica.[17] There the freezing point of the sea water is −1.9 °C and the freezing temperature of the fish blood is approximately −2.0 °C. As a point of comparison, the freezing temperature of human blood serum or of cold-fresh water trout serum is much higher, approximately −0.6 °C. The low blood serum freezing temperature of the *T. borchgrevinki* is due 70% to dialyzable salts found in temperate marine fishes and 30% to nondialyzable solutes, most of which are relatively high molecular weight glycoproteins (Table I) occurring in the serum at ~10 mg/mL concentrations.

The antifreeze glycoproteins (AFGP) from these two very different Antarctic fishes have very similar if not identical structures.[12–15,18,19] AFGP is a collective name

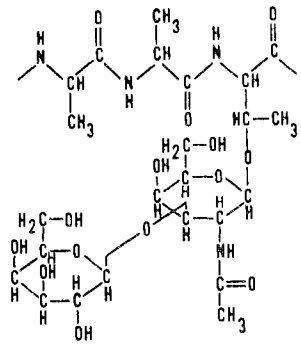

Figure 1. Polymer unit of the active antifreeze glycoprotein. From ref 20.

for a family of at least eight closely related glycoproteins. They were named AFGP 1–8, according to their relative migrations on gel electrophoresis. AFGP 1–5 (the larger ones) have similar antifreeze activity. AFGP 6–8 have little or no activity.

The active AFGP contain only two amino acids, alanine and threonine. The fundamental structure is that of a glycotripeptide composed of two alanines followed by a threonine with the sugars glycosidically linked to the threonines (Figure 1). The disaccharide is galactosyl-*N*-acetylgalactosamine with a β 1→3 internal linkage.[19,20] This fundamental subunit is repeated approximately 17–50 times in the active AFGP molecules. One or two alanines are added at the NH_2-terminal end of each polymer. The AFGP from the polar cod has been found to have nearly identical structure to the glycoprotein of the two Antarctic species.[21]

In contrast to these AFGP, the antifreeze proteins (AFP) from the Newfoundland winter flounder appear to exist as only one major protein having a molecular weight of 10 000,[22] but containing no carbohydrate.[10,22] Its structure is, however, similar to that of the glycoprotein in that alanine in both proteins accounts for approximately two-thirds of the total amino acids. In considering AFGP, we have postulated that the presence of a methyl group, rather than only hydrogen, on each amino acid residue on the backbone (i.e., alanine rather than glycine, and threonine, rather than serine) provides regularly spaced hydrophobic areas down the chain and that these methyl groups are critical for function.[19]

The flounder protein contains repeating residues of as many as six alanines. The carboxylic acids, aspartic and glutamic, constitute an appreciable fraction of the other amino acids.[22] Functionally, the flounder protein appears to have only approximately half the capability of lowering the freezing temperature as do the antifreeze glycoproteins.[23]

Fundamental Features of These Antifreeze Molecules

Pure water has been known to exhibit freezing temperatures of nearly −40 °C due to extensive su-

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Table II
Freezing and Melting of Water and Solution of Antifreeze Glycoproteins in Water[a]

Temperature adjustments		Observed changes	
Temp, °C	Direction of temperature change	In water containing ice crystals	In water solution of 1% antifreeze glycoproteins containing ice crystals[b]
0.0	Holding	Melt and freeze	Crystals melt
−0.1	Lowering	Frozen	Crystals do not melt, liquid does not freeze
−0.7	Lowering	Frozen	
−0.8	Holding	Frozen	Crystals grow, new crystals form until all solution frozen
−0.7	Raising	Frozen	All frozen, no melting
−0.1	Raising	Frozen	All frozen, no melting
0.0	Holding	Melt and freeze	Crystals melt

[a] From ref 25. [b] Water and antifreeze glycoprotein solution was initially frozen at −3 °C and then allowed to melt at +0.1 °C until 5-10% of solution remained as ice crystals. The temperature was then adjusted to 0.0 °C and periodically lowered and then raised as indicated. The times of each temperature intermediate between freezing and melting were 5-10 min. All observations were made microscopically.

percooling.[24] Such low freezing temperatures for pure water can be explained by considering the statistical probability that water molecules will cluster and exceed the critical dimension for crystal formation through favorable local enthalpic and entropic conditions. The large degree of supercooling reflects the fact that extensive structural rearrangement occurs in the process of freezing from the liquid phase. Seeding pure water with ice-structure promoters often leads to an appreciable decrease in the degree of supercooling. In effect, these substrates serve to simulate a more favorable ice-lattice bonding environment.

Conversely, there are several mechanisms for freezing point depression. Colligative depressants function simply by the fact that as they dissolve there is a free-energy decrease (free energy of mixing). Antifreezing molecules may function as inhibitors of ice nucleation or ice crystal growth by several means. These noncolligative mechanisms could include the poisoning of nucleation sites which were provided by seeding with heterogeneous nucleators, the randomization of the local tetrahedral hydrogen-bonding feature of liquid H_2O, the formation of a solution of new composition and structure, and finally, obstruction of ice surface growth sites.

In assessing the mechanism by which these antifreezing biopolymers function, we can exclude colligative phenomena on the basis of the low concentration of the polymer. Colligative effects can contribute only $^1/_{500}$th of the total lowering of the freezing temperature.[17] Nucleation site poisoning can probably be excluded because AFGP functions in the presence of ice crystals.[25] Consequently, only the three remaining possibilities need to be examined.

Four very specific features of the observed antifreeze behavior must be considered. These are shown in Figure 2 and Table II. (1) The active antifreeze glycoproteins depress the freezing temperature of water several hundred times more than do other comparably sized macromolecules. Lysozyme is the only protein shown in Figure 2, but tests on a series of other glycoproteins show that they act very similarly to lysozyme. (2) There is a saturation phenomenon at higher concentrations—the plateau. (3) There is an additive effect in mixtures of the glycoprotein with sodium chloride. (4) AFGP protein has little or no effect on the melting temperature (Table II).

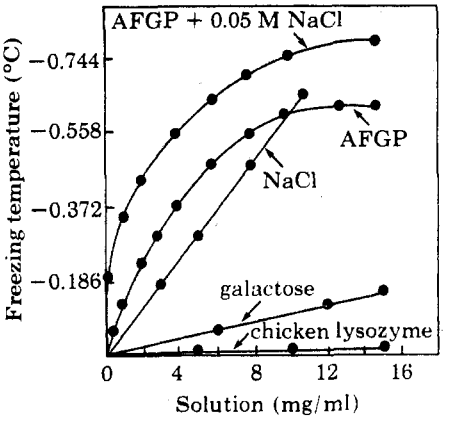

Figure 2. Freezing temperatures of solutions of sodium chloride, galactose, lysozyme, a mixture of antifreeze glycoproteins 3, 4, and 5 from *T. borchgrevinki*, and a mixture of glycoprotein and sodium chloride. AFGP = antifreeze glycoprotein. Reprinted with permission from ref 14. Copyright 1970, American Society of Biological Chemists.

Experimental Studies of the Possible Mechanisms of Antifreezing Function

Most of the discussion is related to the Antarctic AFGP, which has been the most thoroughly studied species.

Liquid-Phase Interaction. Certain hydrophilic groups of the protein or glycoprotein may be able to interact with H_2O molecules to disrupt the local tetrahedral coordination of liquid water. Should such disruption occur, the freezing process would be required to expend energy in reordering liquid H_2O and subsequently converting to ice structure. The excess energy needed for ordering could result in freezing point lowering.

That this is the correct mechanism for antifreeze action is, however, unlikely on several grounds. First, the number of disaccharide groups of the AFGP is very limited. Even allowing for a maximum of 10 H_2O per disaccharide group,[26] a 1% solution of AFGP could bind less than 0.2 M H_2O, far less than the total H_2O molecular content. Secondly, a series of studies measuring the hydrodynamic size of AFGP by a quasielastic light-scattering technique showed no dramatic con-

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Table III
Translational Diffusion Coefficients of Glycoproteins 4 and 8 Determined at Temperatures Close to Freezing in the Presence and Absence of Ice Crystals[a]

Antifreeze glycoprotein	Temp, °C	$D \times 10^7$, cm² s⁻¹	R_e, Å
4	22	6.11	37.2
	−0.2	3.82	29.7
	−0.2 + ice crystals	3.70	30.1
8	22	12.1	18.8
	0	8.4	13.1
	0 + ice crystals	9.2	12.3

[a] From ref 27.

formational change when the sample AFGP in H_2O was cooled from room temperature to 0 °C and then to −0.2 °C with ice seeds.[27] This result suggests that there is apparently no new mechanism of the AFGP operating to cause the creation of a much enhanced hydration sheath near the functioning temperature of −0.2 °C (Table III). Finally, Raman study[28] of the bulk liquid water with and without AFGP showed that the O–H stretching regions of these two samples are indistinguishable, again suggesting that no new H_2O–AFGP bonds are formed at the functioning temperature. Consequently, the possibility that liquid-phase interaction is the freezing point lowering mechanism is remote.

Ice-Phase Interaction. If AFGP were to enter into some form of solid solution with H_2O and thus completely alter the structure and properties of this system from that of pure H_2O, we should easily detect such an alteration by many methods. First, the new "solution" would exhibit a new melting point.[25] However, AFGP causes no changes in the melting point; $T_m = T_0$, the melting and freezing temperatures of pure water. Second, Raman spectra in the O–H stretching region of bulk ice frozen in the presence of AFGP indicate no difference from normal ice I_h structure.[28] Finally, x-ray examination showed no difference from normal ice.[29] Consequently, we do not believe that an ice–AFGP solid solution has been formed.

Interfacial Mechanism. Evidence for such a mechanism must necessarily be predicated upon the existence of an ice–liquid H_2O interface. We postulate that the existence of a precritical embryonic ice crystal is possible, and thus an ice–liquid interface can be conceived. Consistent with this postulate, there are several pieces of experimental evidence for the prevention of further growth of such a surface to postcritical size.

In the case of the AFGP, Raman spectroscopy of the COH region,[28] due primarily to the disaccharides, shows changes in spectral structure as the biopolymer goes from being in liquid water to being trapped in ice (Figure 3). Specifically, the 1403-cm⁻¹ band of AFGP liquid seems to vanish when AFGP is trapped in ice. Furthermore, such a band does not exist without H_2O. This spectral band was also not observed in the inactive AFGP. The enhancement of the spectra at 1403 cm⁻¹

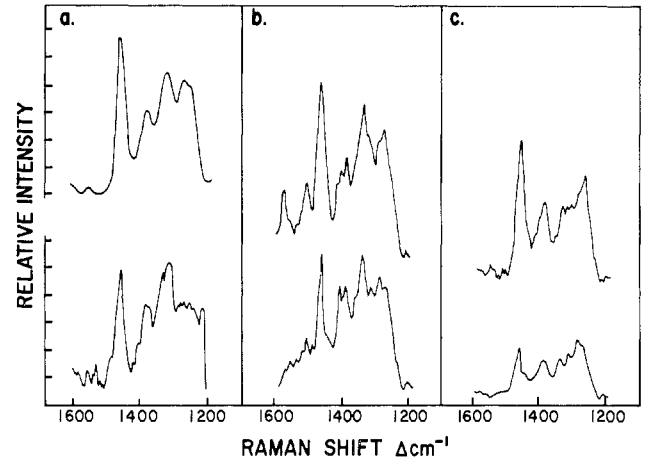

Figure 3. Raman spectra of AFGP samples 4 (lower trace) and 8 (upper trace). Perpendicular polarization experiments of (a) solid samples at T = 25 °C, (b) 5% solutions at supercooled temperatures of −4.3 °C for band 4 and −10.9 °C for band 8, and (c) frozen in samples at −10 °C. Note specifically the change in the spectra near 1400 cm⁻¹ due primarily to COH vibrations. Data from ref 28.

in the supercooled state is indicative of a new mode present. Conceivably this mode could be a vibration characteristic of the disaccharide–embryonic ice hydrogen bond.

There have also been studies on the growth pattern of ice in the presence of AFGP at low temperatures under crossed-polaroid examination. The *a*-axis growth is much impaired when the active forms of AFGP are present.[28] In contrast, the *a*-axis growth is normal in the presence of inactive AFGP (bands 6–8) or ovomucoid, a glycoprotein. This observation suggests that AFGP may be inhibiting ice formation by "shielding" certain growth sites. There are also observations that active AFGP becomes "entrapped"[28,30] as ice growth continues. Here entrapment means that the ice which is formed is in every way normal ice, but AFGP still is within the ice region. The way such a phenomenon can occur is if the grain boundaries of the ice crystals trap and hold the AFGP molecules within their boundaries. Such an AFGP entrapment picture again is consistent with an interfacial mechanism. One might even envision that the shorter sections of inactive AFGP (bands 6–8) are not as flexible and do not have enough ice disruptor sites at necessary spacings to shield the growth sites.

Further evidence that the role of the AFGP disaccharide is surface disruption is the fact that almost all chemical modifications on the disaccharide groups inactivate AFGP as an antifreeze agent.[31] Reversible binding of 2 mol of borate per disaccharide side chain caused complete loss of antifreeze activity. Both the binding and inactivation (Figure 4) were dependent on the pH, with an apparent pK near that for the ionization of borate, but the binding had little apparent effect on the rigidity of the molecule (Table IV).[32]

Circular dichroism spectra of AFGP showed highly random structures.[14] On the other hand, AFP, the nonglycoprotein from some Arctic species, contained

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Table IV
Effect of Borate on Sedimentation and Diffusion Coefficients of Antifreeze Glycoproteins[a,b]

Buffer	Ultracentrifugation $s_{20} \times 10^{13}$, s		Ultracentrifugation $D_{20} \times 10^{7}$, cm² s⁻¹		Quasielastic light scattering $D_{20} \times 10^{7}$, cm² s⁻¹	
	Antifreeze Glycoprotein 4	7	4	7	4	7
H_2O	1.48	0.645	5.64	11.60	5.04	9.30
0.1 M borate at pH 7.0	1.43	0.662	5.09	10.74	4.80	9.10
0.1 M borate at pH 8.6	1.44		4.97		4.68	

[a] From ref 32. [b] The molecular weights for antifreeze glycoproteins 4 and 7 are approximately 19 000 and 4400, respectively.

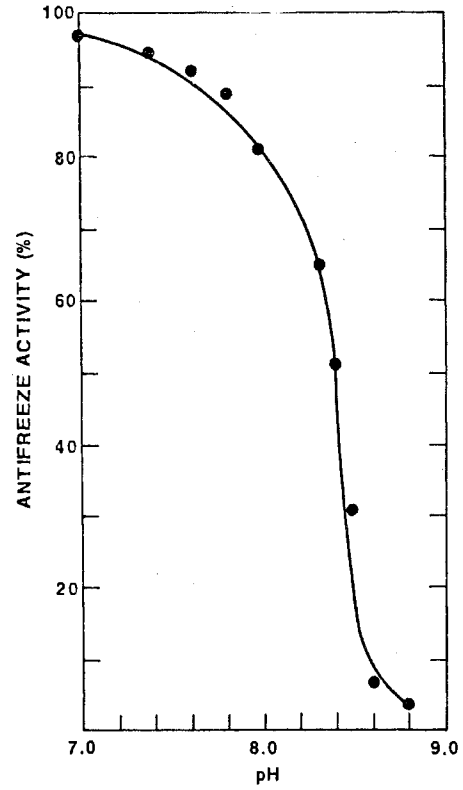

Figure 4. Effect of pH on antifreeze activity of antifreeze glycoprotein in the presence of borate. Antifreeze glycoproteins 1 to 4 (5 mg/mL) were measured in 0.1 M phosphate and 0.1 M borate and adjusted to the pH values indicated. The freezing temperatures of the solutions were measured by the sensing of the heat of fusion. The values for the freezing temperatures of control solutions were subtracted from the values for samples containing the antifreeze glycoprotein. Reprinted with permission from ref 32. Copyright 1976 American Society of Biological Chemists.

significant α-helical sections at low temperatures.[33] Since the AFP molecule does not have sugar side chains, it may require a more regular peptide spacing to compete effectively for the ordered OH groups of the ice surface. Conceivably certain carboxyl groups may be favorably aligned with the OH groups of the surface. To date, however, very few detailed interaction and physical studies of AFP have been undertaken.

A Model for Antifreezing Mechanism

Polymer adsorption on the surface of ice crystals was previously examined by Kuhn.[34] Assuming that a surface interaction energy density, σ, exists between any ice facet and polymer, Kuhn showed that anomalous freezing point depression can be far in excess of colligative effects. In particular,

$$\Delta T_{\text{lowering}} = \frac{RT_0^2}{\Delta \bar{H}_0}\left[\frac{4M\sigma}{aRT_0\rho_0}\right] \quad (1)$$

where T_0 is the normal freezing temperature for the pure solvent, M is the molecular weight of the polymer, ρ_0 is the density of ice, and ΔH_0 is the latent heat of fusion. The quantity a is the dimension of an edge of a microcrystal.

Raymond and DeVries extended this idea to include anomalous surface irregularities.[35] Since $(1/a) \propto dA/dV$, the surface to volume ratio, an irregularly shaped surface will lead to larger surface area for the same volume. Consequently, on the basis of ice surface coverage, "a" may be written as

$$a = [2r\alpha c]^{-1/2} \quad (2)$$

where r is related to polymer size, α is an entrapment coefficient of the polymer in ice, and c is the concentration of polymers in solution. Inserting eq 2 into eq 1, one finds that $\Delta T_{\text{lowering}} \propto c^{1/2}$. This result is attractive because it begins to exhibit qualitatively the functional saturation phenomenon.

A further refinement toward understanding the noncolligative antifreeze phenomenon is based on the initial stipulation that the polymer and the solvent H_2O together must form a solution. Consequently, we believe that some form of the Flory–Huggin's theory[36] must be incorporated. For such surface interaction as described previously, but now applied to a polymer solution, one has a result similar to one derived by Solms and Rijke:[37]

$$\Delta T_{\text{lowering}} \simeq \frac{RT_0^2}{\Delta \bar{H}_0}\left[\frac{4M\sigma}{R_0T_0\rho_0 a}\phi - (\chi - {}^1/_2)\phi^2\right] \quad (3)$$

Two additional features can be seen: First, there is a proportionality to ϕ, the mole fraction of solute, due to the fact that the total area of interaction is the sum of all interacting facets. Secondly, a term is now provided to account for the solution compatability of the polymer. χ is typically derived from an analysis of the enthalpy associated with lattice mismatch when a polymer solute is incorporated into the solvent, $\chi = \chi_H$. However, it has been pointed out by Flory[38] that χ must

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include rather dramatic entropic contributions. In fact, Flory has shown that polymers can exhibit $\chi < {}^1/_2$ or $\chi > {}^1/_2$, the difference mainly being in the entropic contribution. For example, hydrophobic surfaces of a polymer might lead to considerable ordering of the water in its immediate neighborhood, thus causing a decrease in residual entropy, ΔS^R. In view of the relationship $\chi = \chi_H + \chi_S$, and since $\chi_S = -\Delta S^R/(RT_0^2)$, a large negative ΔS^R will lead to a large positive value of χ_S and therefore of χ total. An examination of eq 3 indicates that $\chi > {}^1/_2$ would lead to a gradual decrease of the antifreezing effect, even though no a priori conditions of affinity such as those proposed by DeVries have been incorporated into the model.

It is of interest to consider an extension of the above idea. Zettlemoyer[39] has postulated that heterogeneous nucleation takes place with hydrophilic sites embedded in a hydrophobic matrix. In this respect we note that the AFGP polymer could behave bifunctionally. Immediately below T_0 the surface adsorption phenomenon is dominant at low concentrations (ϕ is small). However, as ϕ increases, the hydrophobic surface begins to confine the supercooled liquid phase, working against its own hydrophilic absorption sites. At what is called the functional saturation concentration, this hydrophobic matrix could actually assist in ice formation as a nucleator. However, since the physiological concentrations are lower than the concentration at which these matrices form, these molecules will behave as antifreeze agents.

This model explains much of the data concerning the antifreeze glycoproteins. The x-ray structure of this system[29] shows that the disaccharide repeat distance is either 7.7 or 8.7 Å. Raymond pointed out that 7.7 Å is within 5% of the ice *c*-axis repeat distance and 8.7 Å is within 4% of twice the ice *a*-axis repeat distance.

Next, it is significant that Duman and DeVries,[30] Raymond and DeVries,[40] and Tomimatsu et al.[28] have clearly shown by different techniques that the active AFGP is entrapped when freezing occurs, while the nonfunctioning low molecular weight species are entrapped much less. Ice *a*-axis growth inhibition has been consistently observed for these AFGP samples, as has inactivation by alteration of the disaccharide structure.

This model can also explain the observed fact that shorter sections of AFGP are of little or no functional value. Since these molecules are shorter, their ability to cover sufficient sites is correspondingly less. Consequently, these molecules do not function in an antifreeze capacity and are easily expelled into the liquid pool.

Finally, the qualitative behavior of eq 3 when $\chi > {}^1/_2$ is assumed can very clearly describe the functional behavior of AFGP, as shown by comparison of Figure 2 with Figure 5. This additional feature of our model, coupled with the assumptions underlying eq 2, provides sufficient generality to accommodate the slightly varying behavior of many of these species.[29]

As long as AFGP exists in solution in a fairly low concentration where salt–AFGP interaction is at a minimum compared with either salt–H_2O or AFGP–H_2O interactions, the independence of these two effects

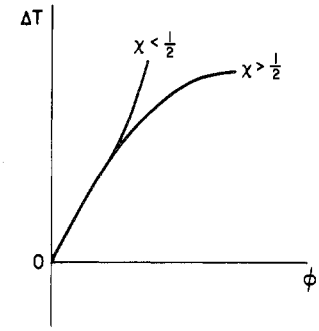

Figure 5. Freezing point lowering, ΔT, as a function of polymer mole fraction and according to eq 3. For a polymer with a large entropic contribution to χ, then $\chi > {}^1/_2$ becomes possible and after the large initial freezing point depression slope there is a tendency to saturate functionally. For $\chi < {}^1/_2$ this "saturation" phenomenon is not present except in the sense of eq 2.

on the freezing point lowering is reasonable. For an AFGP of molecular weight 10^4 daltons, at a concentration of 10 mg/mL, there are 6×10^{17} AFGP molecules per cubic cm of solution. The center-to-center spacing, S, is then $\approx 10^{-6}$ cm. Since an active sample of average size has $R_e \approx 30$ Å,[27] the average linear spacing between molecules is $S - 2R_e \simeq 40$ Å. At NaCl concentrations of 0.05 M (Figure 2) there can be only about four Na^+ or Cl^- in the volume of $(40\text{ Å})^3$. This means that interaction between AFGP and salt is indeed not significant.

In a more extreme situation, where almost 1.3 °C of the freezing point depression in the blood serum is due to smaller dialyzable compounds, corresponding to a total salt or small molecule concentration of 0.3 M, the $(40\text{ Å})^3$ volume must contain nearly 24 ions. For ionic diameters of 1.5 Å, there should still be enough space for a hydration sheath (~3 Å) to develop around each of these ions and not interfere greatly with the antifreeze molecules.

Even though empirically one can fit eq 3 to data concerning the functioning of Northern fish AFP, the available physical data present a problem for the model discussed above. Not only does the α-helical repeat distance of 5.38 Å seem incompatible with the *a*-axis spacing of 4.52 Å, but disaccharide side chains are absent. Understanding of the antifreeze activity of AFP requires that some different or additional ideas be incorporated.

Hew et al.[41] and Duman and DeVries[42] have suggested that Asp and Glu, both amino acids possessing ionizable COOH groups, may act in lieu of the disaccharide side chain. However, since the amino acid sequence is not completely known, it is difficult to evaluate this idea rigorously.

On the other hand, the most obvious similarity, the presence of about 60% of alanine residues in both systems, requires more detailed examination. The aliphatic side chain methyl groups are hydrophobic. A central question is: Does alanine have the same role in AFGP as in AFP? If so, are the alanine units the primary source of antifreezing activity? Or do these residues play a role as the hydrophobic matrix?[19] Much more information must be obtained about the structure of AFP before we can determine if it operates by an

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antifreeze mechanism different from that postulated for AFGP.

Conclusion

There is strong evidence that the basic mechanism for the functioning of antifreeze molecules found in polar fish serums is one of interfacial surface interaction.[19,35] Since the sugar contents of these proteins are quite different, but the amino acid units of both types of molecules are about 60% alanine, it is imperative that studies be continued in order to learn how these different structures function.

For the AFGP from Antarctic fish, a model combining the ideas developed for ice surface adsorption with solution hydrophobicity of the alanine units can, in principle, provide a good qualitative interpretation of the observed features of functional saturation, ice development impediment, additivity of effects in up to 0.3 M NaCl, and freezing temperature hysteresis. However, due to the preliminary nature of the data for the northern fish AFP, no comparable interpretation in terms of the model can be attempted without knowledge of what structural feature takes the place of the disaccharide side chains of AFGP.

For additional confirmation of the postulated model for the glycoproteins, studies are necessary on synthesis of the protein and the disaccharide side chain. The smaller inactive species contain some proline units in replacement of certain alanine units.[43,44] Specifically how this feature, together with the smaller size, combine to render the lower molecular weight AFGP inactive is yet unknown. What advantage might the organism gain by having inactive AFGP-like glycoproteins in its blood serum?

Since this model also places some emphasis on the rigidity of the basic AFGP molecule, experimental studies should be conducted to force artificially an even more rigid pattern of molecular alignment and to measure the associated antifreeze effects.

Our knowledge of these systems indicates that the following studies should be carried out:

(1) Additivity. What is the behavior of AFP in the presence of salts, other solutes, and detergents which might disrupt the α-helical structure?

What is the behavior of AFP in the presence of AFGP? Also, perhaps a convincing exhibition of AFGP's specific hydrogen bonding relationship to the H_2O system could be achieved if H_2O were substituted by some other solvent. Here systematic studies, including determination of the change of freezing temperature, the specific heats of the system, and the vapor pressure of the nucleated crystal, would be useful to determine if the AFGP–H_2O interaction is truly unique or if AFGP acts similarly to depress the freezing temperature of other solvents.

(2) Conformation of AFP in the Temperature Region Where the Molecule Functions. Quasielastic light scattering studies as well as Raman studies need to be carried out. The change in conformation of a highly organized α-helical structure to any other conformation is readily discernible. Certainly a complete primary amino acid sequence determination is necessary for theoretical interpretation of AFP activity.

(3) Direct Study of Order or Entropy in Solution in the Presence of These Molecules. A very recent study of macromolecular motion by Soret-type diffusion[45] suggests that one can not only measure diffusion of the macromolecule, but that with a clever arrangement of geometry and temperature gradient one can evaluate directly its order parameter. Such a measurement would enable direct entropic evaluation of the H_2O–AFGP or H_2O–AFP system.

(4) Synthesis of Antifreeze Proteins. The synthesis of a polypeptide with structural similarities to the AFP from the flounder has been recently reported.[46] The product is a random polymer consisting of alanine and aspartic acid in a molar ratio of 2:1. It is stated to have approximately one-third the antifreeze activity of the naturally occurring flounder AFP. Controlled syntheses of such synthetic polymers could be one of the most promising ways to investigate the structural requirements for antifreeze activity, and, in particular, the roles of the hydrophobic and hydrophilic groups as found in AFP and AFGP.

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