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Antifreeze Proteins: Structures and Mechanisms of Function

Yin Yeh*,† and Robert E. Feeney‡

Departments of Applied Science and Food Science and Technology, University of California, Davis, California 95616

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Contents

I. Introduction

Polar and near polar fishes typically survive in seas where the temperature is subzero, frequently as low as −1.9 °C. In a series of articles from 1953 through 1972, Scholander's group (Scholander and co-workers[1,2] and Scholander[3]) reported an abnormally low freezing temperature of blood serum from Arctic fishes, and that these were not due to the presence of additional salts or other colligatively acting substances. Publications first by DeVries and Wohlschlag[4] from our laboratory and the Marine Science Institute of the University of Texas (work started at Stanford University), and then from our laboratory (DeVries et al.,[5] Komatsu et al.,[6] DeVries et al.[7]) described the existence of a glycoprotein in the sera of Antarctic fishes that lowered the freezing temperature without increasing the osmotic pressure, which would have been lethal to the fish.

Since these early studies, there have been many reports on these and similar acting proteins, some of which are not glycoproteins. Many reviews have appeared, some on the overall characteristics of these substances (Feeney,[8] Feeney and Yeh,[9] Eastman and DeVries,[10] Feeney,[11] Davies and Hew,[12] Cheng and DeVries,[13] Duman et al.[14]) and others devoted toward the mechanisms of functioning (Yeh and Feeney,[15] Feeney et al.,[16] Ananthanarayanan,[17] Hew and Yang[18]).

There are two main families of proteins that possess the capability of depressing the freezing temperature. They are the *antifreeze glycoproteins*, commonly given the acronym AFGP and the *antifreeze proteins* or AFPs which posess similar properties. The structures of both AFGP and AFP have been extensively studied for several decades. The principal amino acid found in both the AFGP and the most commonly found AFP (type I) is alanine (Ala), each family possessing about 67% of this amino acid. However, structurally, AFGP and AFP are completely different. In the AFGP molecule, the second most abundant amino acid is threonine (Thr) with Ala-Ala-Thr arranged in repeating tripeptide units. Some AFGP species have proline (Pro) replacing Ala after some threonines. All these proteins have disaccharide groups extending from the Thr residues. There is no α-helix content found in the AFGP molecules. On the other hand, the AFP (type I) is almost exclusively α-helical in secondary conformation, but there are no regular peptide repeats. It is without a doubt that their unique structures play significant roles in their specialized functions. These will be discussed in detail in sections III, IV, and V of this review.

† Department of Applied Science.
‡ Department of Food Science and Technology.

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Yin Yeh was born in Chungking, China, in 1938. He immigrated to the United States in 1949. In 1960, he received his B.Sc. degree in Physics from the Massachusetts Institute of Technology. His Ph.D. degree in Physics was obtained in 1965 from Columbia University in the area of laser scattering spectroscopy. After postdoctoral years at the Columbia Radiation Laboratory and the Lawrence Livemore National Laboratory, he became a staff member of LLNL in 1968. Since 1972, he has been on the faculty of the Department of Applied Science of the University of California at Davis. He was Guest Academician at the Solid State Laboratory of ETH, Zurich, Switzerland, from 1980 to 1981. In 1985, he was appointed Advisory Professor of the East China Normal University, Shanghai, China. He was one of the codevelopers of the techniques of quasielastic light scattering and laser doppler anemometry; these methods have been applied to physical chemistry and biophysics problems. Current research interests include the use of optical techniques to probe the structure and function of motor molecules and antifreeze proteins as they affect ice growth and crystalline morphology changes.

Robert E. Feeney was born in Oak Park, IL, in 1913. He received his B.S. in Chemistry in 1938 from Northwestern University and his Ph.D. in Biochemistry from the University of Wisconsin, Madison, in 1941. Following a postdoctoral at Harvard Medical School, he spent three years in the U.S. Army (United States and New Guinea) and seven years at the USDA Western Regional Laboratory, Albany, CA. He was then Professor of Chemistry and Chairman of the Department of Biochemistry and Nutrition at the University of Nebraska, Lincoln, NE, from 1953 to 1960. In 1960, he became Research Biochemist in the Agricultural Experimental Station and Professor in the Department of Food Science and Technology, University of California, Davis. He became Emeritus in 1984. His longtime area of research and teaching has been in the chemical modification of proteins with applications to proteins forming complexes, such as the iron-complexing transferrins and inhibitors of proteinases found in egg white and blood serum. These led to six trips to Antarctica and an equal number to northern areas and studies on the antifreeze proteins from polar fish.

The most well-documented observations of how both families of proteins function are as follows:

(1) Even though there is always evidence for freezing temperature lowering by these proteins, the melting temperature for ice that formed after having

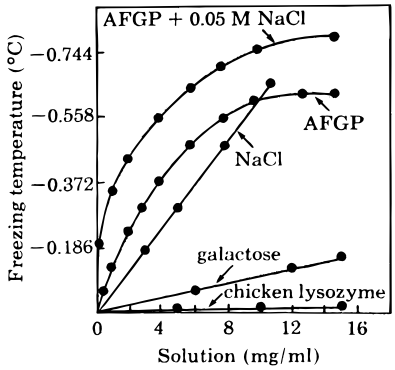

Figure 1. Freezing temperatures of solutions of NaCl, galactose, lysozyme, a mixture of AFGP species, and a mixture of AFGP and NaCl as a function of solute concentration (in mg/mL). (Reprinted with permission from ref 5. Copyright 1970 Journal of Biological Chemistry.)

exceeded the limit of the lowering capacity of the freezing temperature has always been nearly 0 °C. This disparity between the melting temperature and the freezing temperature is called *thermal hysteresis*. The presence of this hysteresis indicates that the mechanism is a noncolligative one.

(2) The level of this noncolligative activity is high. For some species, the observed freezing temperature lowering can be as high as 500× that of the colligative salts on a molal basis (Figure 1). In all species of antifreeze proteins, there seems to be an asymptotic concentration where the activity saturates. That is, solution concentration of the protein in excess of the saturation level will not increase the level of activity significantly.

(3) When the supercooling temperature of the solutions exceeds the level of freezing temperature suppression, growth morphology differs significantly from that of ice growth in pure water. Bipyramidal crystallites and columnal spicules form instead of sheets. Conversely, the existing crystal morphology of ice and the level of solution supercooling play important roles in whether a species of the AFP or AFGP will remain functional. These observations point toward the importance of a site coverage mechanism on the growth surface of the ice crystal.

(4) The effect of these proteins and glycoproteins on the rate of crystal growth is also significant. Rates of linear spicule growth can be much faster than dendritic growth into pure, supercooled water under free growth conditions. Furthermore, when the phenomenon of ice recrystallization is observed, the presence of very low solution concentrations ($\sim 10^{-8}$ M) seems to have pronounced effect in retarding the rate of recrystallization.

(5) Among the glycoprotein species, potentiation of function has been observed. When the ice morphology is unfavorable for the shorter AFGP molecules to be actively preventing growth of ice, the presence of the longer species of AFGP has been reported to uniquely facilitate the full function of the shorter species, seemingly via cooperation of the species in the presence of ice surface. Mixtures of AFGP and AFP do not have such cooperative effects.

As it is often the case in the growth and maturation of a field of study, experimental studies proliferate to define some of the parameters governing the

mechanism of action. In the antifreeze protein/glycoprotein field, the initial studies had tried to bring about a sense of the "right" molecular conformation in solution, and to see how these conformations might influence either the ice nucleation process or the growth of ice crystals. The present level of theoretical understanding of the phenomenon of freezing temperature depression has become quite sophisticated. In section II of this review, we will focus on those theoretical concepts that have been advanced to describe the mechanism of AFGP or AFP function in ice growth inhibition. Within each of the theories, we will discuss the experiments that have been conducted to provide some support to that theory. In order to provide a basis of comparison, we start with a review of the freezing *point* lowering produced in the presence of a colligative substance. The next aspect to be examined will be the pinning of growth by virtue of the Kelvin effect. This is a popular concept because it is easy to understand how pinned growth can lead to freezing *temperature* depression. More recently, it has been realized that the Kelvin effect itself may not be adequate; several improvements will be discussed. Reversible adsorption of the polymer on the ice surface has been postulated; other proposed concentration-dependent mechanisms will also be explored. Finally, the polymeric chain nature of the protein/glycoproteins needs to be considered carefully, particularly in regard to the manner in which they will adsorb onto a facet of the ice surface. As a distinctively different hypothesis, nucleation inhibition ideas will also be described. Each of these theories will be examined to see how it explains particular experimental observations.

II. General Postulates for the Mechanisms of Function of Antifreeze Proteins

A. Colligative Phenomena

For *ideal* solutions, colligative freezing point lowering is a manifestation of the entropy of mixing between the two constituents. For such a system consisting of species *a* (solvent) with *b* (solute), the freezing point lowering is (Eggers et al.[19])

$$\Delta T = T_o - T = \frac{kT_o^2}{\Delta H_o} x_b \qquad (1)$$

where T_o is the equilibrium phase transition temperature of pure solvent *a*, and ΔH_o is the enthalpy change (assumed to be independent of temperature) upon phase change at the temperature T. This is the commonly known colligative expression for the freezing *point* depression. ΔT is proportional to the concentration of the second species that has replaced a small amount of the solvent species. The important point of this relationship is that it is a thermodynamic specification. Given a particular x_b, we will have a well-defined lowering of the freezing point. Hence the phenomenon is colligative in that it is directly proportional to the concentration of the solute species regardless of its chemical nature.

If a solution is not *ideal* but *regular*, we introduce the feature that the enthalpy of solute replacement is not zero, but finite. The free energy of solution now must include enthalpic and entropic contributions. If the coordination number for the nearest neighbors is defined as z, and the energy of interaction between dissimilar species is given by w_{AB}, we then have (Eggers et al.[19])

$$\Delta T = \frac{kT_o^2}{\Delta H_o}[x_b - (\chi - {}^1/_2)x_b^2] \qquad (2)$$

where $\chi = zw_{AB}/kT$.

If the solute is a polymer, with m segments per chain, then the large solute molecule must occupy many spaces within the solvent volume. Approximating the system by the lattice model, Flory[20] showed that the dominant terms of the freezing temperature depression in this case will be given by

$$\Delta T = \frac{kT_o^2}{\Delta H_o}\left[\frac{x_b}{m} - (\chi - {}^1/_2)x_b^2\right] \qquad (3)$$

Consequently, each of the m segments of the polymer chain plays the role of a small molecule.

Colligative freezing point depression is a thermodynamic phenomenon, lowering the freezing temperature by an amount that is always proportional to the molal concentration of the solute molecule or molal concentration of the segments of a polymer in solution. As a thermodynamic phenomenon, this effect will also set the new temperature of melting of the solution. Hence there is no difference between the freezing point and the melting point in a the colligative phenomenon. A second point of note is that if we consider only the first term as the dominant term for the colligative effect, the freezing point lowering is entirely species independent. The only variable is the molal quantity of the solute species. From early studies[5] of the freezing point lowering by AFGP, it was determined that the physiological concentration of AFGP should be able to depress the freezing temperature of ice by only 1/500 of its observed, actual capability. A freezing temperature lowering to −0.8 °C requires about 0.43 M of the solute (or in the Flory−Higgins segmental polymer, solute segments). The observed freezing temperature lowerings by these antifreeze proteins or glycoproteins are manifested by 10^{-3} M concentration or lower. Obviously, the antifreeze action by these biological antifreeze molecules is definitely a noncolligative phenomenon.

B. Adsorption Inhibition

1. Gibbs–Thomson (Kelvin) Model

From a noncolligative point of view the first consideration should take into account the manner in which the ice crystal actually will grow. Solution growth of a crystal clearly involves a surface: the interface between the crystalline phase and the solution or melt phase. The total free energy of such a system must now include the contribution of the interfacial part as well as the volume part (Tiller[21]). In particular, the growth of the crystalline phase from a finite-sized seed crystal will include, as a portion of its driving force, the creation of new

interface area. The total free energy change, ΔG, for the creation of new crystalline mass from the solution will include the volume free energy change, ΔG_v, and the surface free energy change, ΔG_s:

$$\Delta G = \Delta G_v + \Delta G_s \quad (4)$$

Here, the relationship $G = N\mu$ has been used, where N is the number of molecules comprising the solid and μ is the chemical potential. For a spherical ice particle of radius ρ, and isotropic surface energy, the chemical potential of the solid phase is

$$\mu_{ice}(\rho) = \mu_{ice}(\infty) + \frac{2\Omega\gamma}{\rho} \quad (5)$$

where γ is the isotropic surface energy, Ω is the molar volume of ice, and $\mu_{ice}(\infty)$ is the chemical potential for the bulk ice with a surface of infinite radius ($\rho = \infty$). A stable ice crystal must have its chemical potential optimized, corresponding to one with a minimum radius, ρ_{min}. For the solution phase to be at equilibrium with such an ice surface, the chemical potential of the solution, μ_{soln}, can be decomposed into the bulk contribution and a term that suggests the contribution to the chemical potential is due to a freezing temperature that is different from the equilibrium melting temperature:

$$\mu_{soln} = \mu_{soln}(\infty) + \frac{\Delta H_o(T_o - T)}{T_o} \quad (6)$$

where ΔH_o is again the latent heat of fusion, and $T_o - T$ is the difference between the freezing temperature T and the equilibrium melting temperature.

For a system at equilibrium, the chemical potential for the standard state of the solution, $\mu_{soln}(\infty)$, is equal to the chemical potential for the standard state of the ice phase, $\mu_{ice}(\infty)$. By further equating the maximum chemical potential of the solid phase with that of the solution phase, hence $\mu_{ice}(\rho_{min}) = \mu_{soln}$, we have, from eqs 5 and 6,

$$\frac{\Delta H_o(T_o - T)}{T_o} = \frac{2\Omega\gamma}{\rho_{min}} \quad (7)$$

Thus the freezing temperature lowering due to the presence of the added spherical surface of ice with radius, ρ_{min}, is

$$\Delta T = T_o - T = \frac{2\Omega\gamma T_o}{\rho_{min}\Delta H_o} \quad (8)$$

This equation describes the freezing temperature *lowering*, due to the presence of finite-dimensioned crystals. It can be seen that once ice is formed the ρ_{min} quantity apporaches ∞, and ΔT becomes zero. Hence eq 8 describes a freezing hysteresis, showing that it is directly proportional to the interfacial energy and inversely proportional to the radius of the sphere in question.

This simple expression has served as a major theoretical hypothesis for explaining the function of AFP or AFGP molecules. Raymond and DeVries[22] were the first to point out that should an AFGP molecule lodge onto the crystalline surface and obstruct the growth at that point, further growth will have a radius of curvature that is smaller than that of the main part of the crystal. Hence, the growth front will experience a freezing temperature lower than the bulk system. Knight and DeVries have expanded this idea in several papers that show experimentally that certain facets of the growing ice crystal acquire significant AFGP or AFP molecules by adsorption; consequently these facets will not grow freely in solution. Implicit in their argument is the idea that these polymeric proteins are adsorbed essentially irreversibly onto certain favored facets. Hence it should be possible to predict the growth curvature from the amount of freezing temperature lowering on any particular growth facet.

The assumption that molecular adsorption onto a surface is essentially irreversible is based on estimating the number of possible hydrogen bonds that need to be broken before one AFGP or AFP molecule can be released. Knight et al.[23] have argued that upward of 24 hydrogen bonds can be made per tripeptide group of the shorter AFGP 7−8 molecules. They claim that despite the dynamic nature of the hydrogen bonding, the probability of all hydrogen bonds being released simultaneously must be very low.

Two fundamental questions associated with this adsorption−inhibition model need to be answered. First, are there experimental data that fit eq 8? It had been pointed out by Kuroda[24] that the actually observed radius of curvature of the Kelvin instabilities, ρ_{min}, is much too large to account for the measured freezing temperature lowering. Wilson[25] estimated that at the measured coverage of the ice surface by the antifreeze proteins and for the measured temperature lowering, the size of the surface inhomogeneities would require detection by very careful atomic force microscopy measurements; they cannot be discerned by the naked eye. A second ambiguity with regard to the apparent radius of curvature of the growing regions is the uncertainty in the magnitude of the interfacial energy. Both Kuroda[24] and Wilson[26] have independently raised this point. Wilson also extended his analysis to include the possibility of an anisotropic interface and suggested that such anisotropy could be large enough to account for the experimental observations.

2. Reversible Adsorption/Desorption Equilibrium

Kuroda[24] was the first to express doubt that adsorption is completely irreversible. He simplified the representation of the AFGP molecules by assuming that they are of rigid cylindrical shape; r being the radius and b is the length of the cylinder. Then Kuroda modified the Langmuir equation to show that the number of these rodlike molecules adsorbed onto a surface of ice, N_e, should be

$$N_e = \frac{c_a}{2r^2 b c_a + \exp(-E_a/kT)} \quad (9)$$

where c_a is the concentration of these AFGP molecules in solution and E_a is the binding energy for these molecules on the ice surface. The minimum distance that will allow for the curvature ρ_{min} of the ice crystal to develop will be the free spacing, L,

between these adsorbed molecules:

$$\rho_{\min} = \frac{L - (2b/\pi)}{2} \tag{10}$$

where L may be estimated by $\sqrt{N_e}$. In this manner, reversible adsorption equilibrium is introduced into the problem. The result of having introduced eqs 9 and 10 is that

$$\Delta T = \frac{2\Omega\gamma T_o}{\rho_{\min}\Delta H_o} = \frac{4\Omega\gamma T_o}{[\sqrt{N_e} - (2b/\pi)]\Delta H_o} \tag{11}$$

By setting the adsorption energy E_a to be the latent heat of melting, Kuroda found that the curvature of the freezing temperature lowering ΔT vs c_a is qualitatively correct but that the magnitude of the depression is too large, indicating that perhaps E_a has been overestimated. Kuroda further argued that as long as E_a is smaller than ΔH_o, the melting point elevation due to adsorption would be negligible. Essentially, long before the water molecules are pulled off the crystalline phase into the liquid phase, the weakly adsorbed AFGP molecules would have gone back into solution. Hence, melting temperature raising by a surface adsorption noncolligative mechanism will not be the inverse of the freezing temperature lowering effect. Knight and DeVries[27] have reported experimental evidence measuring a melting temperature raising, suggesting that the Langmuir adsorption explanation may be insufficient. However, this point will require further experimental verification. We shall point to several experimental studies that show a reversible adsorption/desorption equilibrium in later sections.

Hew and Yang[18] (1992) have taken note of the distinction in the static (Kelvin) model and the reversible binding model and suggested a possible compromise model. They propose that perhaps the binding of these molecules to the ice surface is reversible. However, while bound to the surface, the effect is to allow the Kelvin effect for freezing temperature lowering to manifest itself. This suggestion is consistent with Wen and Laursen,[28] who have further shown that AFP data indicate there may be a two-step mechanism: At low concentrations of the proteins, a reversible hydrogen-bonding mechanism exists. However, once the concentration of the proteins becomes high, the AFP molecules on the surface begin to pack and interact with each other. They further suggest that the greatly enhanced effective binding and the resultant antifreeze activity are the result of this cooperative intermolecular interaction. The concept of cooperativity or functional potentiation had previously been coined by Osuga et al.[29,30] when they showed that the activity of shorter AFGP molecule was able to be expressed more fully in the presence of the longer AFGP species.

3. The Polymeric Adsorption Model

In a series of two articles, Li and Luo[31,32] developed a Flory−Huggins model for the adsorption of polymeric proteins onto the surface of ice. The introduction of polymeric interaction with the surface constitutes a significant increase in sophistication in modeling the antifreeze function.

The first part of their analysis introduces a polymer of m units into a solution. This Flory−Huggins result has already been used by Feeney and Yeh.[9] The new result comes about from the introduction of an analogous mathematical apparatus to describe the interfacial adsorption of polymers in a manner similar to the theory of polymers in solution. There are now two distinct interactions: one is between the polymer and the solution and the other is the polymer−ice interaction. Assuming isotropic interaction, Li and Luo introduced a second interaction constant, K_2 for the polymer interaction with ice while keeping K_1 as the interaction constant between the polymer and the solution. Hence,

$$K_1 = [(z - 2)E_{wp}/kT_o] \tag{12a}$$

$$K_2 = [(z - 2)E_{pi}/kT_o] \tag{12b}$$

with z again being the nearest-neighbor coordination number, E_{wp} being the interaction energy between water and polymer and E_{pi} that between polymer and ice. They show that the freezing temperature lowering (ΔT) comes from the interaction of the polymeric species with the ice, diminished by the interaction of the same number of polymeric species with water. Combining these interactions, they arrived at the following expression:

$$\Delta T = \frac{2\Omega R T_o^2}{\Delta H_o}\left[(K_2 - K_1)\frac{m n_3 n_2}{n_3 + m n_2} - n_3 \ln\frac{n_3}{n_3 + m n_2} - n_2 \ln\frac{m n_2}{n_3 + m n_2}\right] \tag{13}$$

where n_2 and n_3 are molal concentrations of the protein and water, respectively.

All other symbols are as defined from previous sections. The first term on the right side of eq 13 is the difference in enthalpy contribution at the interface while the latter two terms are the entropic contributions from the ice and solution, respectively. Their fitting of experimental thermal hysteresis data from both the AFP (Figure 2a) and AFGP (Figure 2b) molecules shows significant improvement when compared with the Kelvin model. Indeed, the accounting of the polymeric nature of the adsorbate, even in this isotropic surface interaction model, does lead to a much better fit between experiment and theory.

C. Nucleation Inhibition

An often-asked question related to the function of these antifreeze proteins is, "Why do they wait until crystals have already been formed before adsorbing onto their surfaces and poisoning their growth sites? Why not simply prevent the nucleation of ice crystals completely?" Theoretically, this question can be answered by establishing the criterion to achieve the smallest critical nucleus for nucleation of a seed crystal. From classical considerations, the free energy for crystal nucleation depends on a volume free energy term and a surface term. By calculating the derivative of the total free energy with respect to the

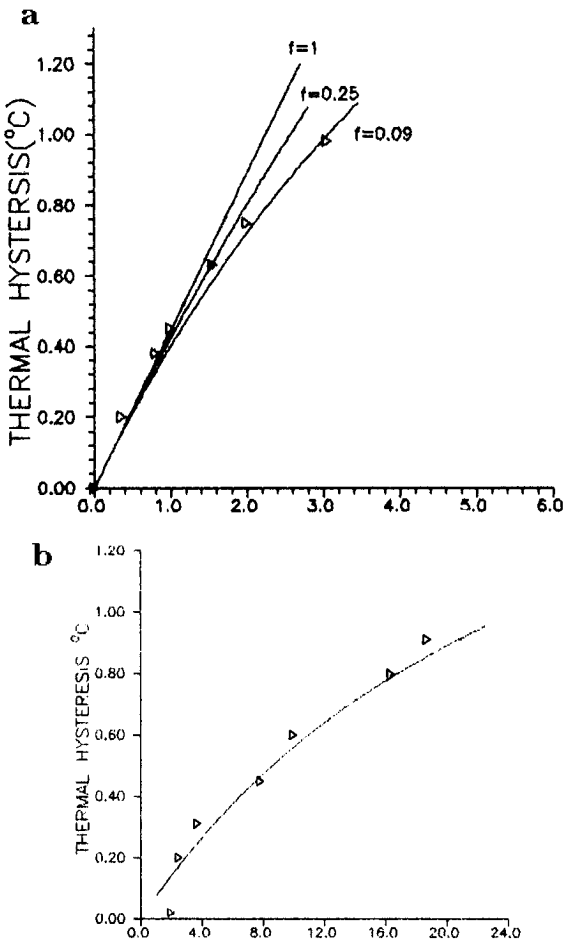

Figure 2. (a) Theoretical freezing point depression (thermal hysteresis) for AFP as a function of concentration (in mmol) given the parameter f = ratio of number of ice molecules to the number of ice + water molecules on the interface. $0 \leq f \leq 1$ (solid lines). Experimental data shown are from Davies and Hew.[12] (b) Theoretical freezing point depression for AFGP as a function of molecular weight (in kD) of the glycoprotein (solid line). Experimental data shown are from Schrag et al. (Schrag, J. D.; O'Grady, S. M.; DeVries, A. L. *Biochim. Biophys. Acta* **1982**, *717*, 322). (Reprinted with permission from ref 31. Copyright 1993 Elsevier. b: Reprinted with permission from 32. Copyright 1994 Elsevier.)

number of molecules involved and setting this to zero, we obtain a relationship for the critical radius for homogeneous nucleation. Seed sizes larger than this critical nucleus will spontaneously grow, while those smaller than the critical radius will dissipate into the melt or solution again (Hobbs[33]). This critical radius is given by

$$r^* = \frac{2\gamma}{n_s \Delta\mu} \tag{14}$$

where γ is the ice−water interfacial energy, n_s is the number density of the solid phase, and $\Delta\mu$ is the difference in chemical potential between the solid and the melt or solution phase. The nucleation rate at r^* is given by

$$J(r^*) = \frac{nkT}{h} \exp[-\Delta g/kT] \exp\left[-\frac{16\pi\gamma^3}{3n_s kT(\Delta\mu)^2}\right] \tag{15}$$

where Δg is the activation barrier for increasing the size of the seed crystal by one more water molecule, appropriately positioned, and the second exponential quantity provides the structural barrier due to lattice mismatch.

If the nucleation process is via a heterogeneous mechanism, then in place of a spontaneously developed seed crystal, "impurities" (defects, molecules, or structural incongruities) provide the matrix for nucleation. The rate of growth is now given by

$$J(r^*) = \frac{n_c kT}{h} 4\pi R_i^2 \exp[-\Delta g/kT] \exp\left[-\frac{\Delta G^*}{kT}\right] \tag{16}$$

where n_c is the number of water molecules in contact with the impurity site of radius R_i and ΔG^* is the free energy for attachment onto the "impurity" surface and has the general feature of

$$\Delta G^* = \frac{A\gamma^3}{\Delta G_v^2} \tag{17}$$

where ΔG_v is the volume free energy difference upon phase change, the factor A contains all aspects of the geometry of this "impurity" site, and γ is again the interfacial energy between ice and water.

We can immediately see how the antifreeze protein could possibly function to decrease the nucleation rates. In either the homogeneous or the inhomogeneous nucleation process, there will be an activation energy barrier. Diffusional processes near the interface between seed and solution are accounted for by this part. The other structural component is more specific. In the homogeneous nucleation case, growth becomes favorable when γ decreases. In heterogeneous nucleation, a larger fitting surface R_i and a minimized ΔG^* will lead to more rapid growth of the seed. If the antifreeze molecule acts so as to increase the interfacial barrier for water attachment, it will effectively serve as a nucleation retardant. Blocking interfacial sites by adsorption onto these sites is one way. Increasing the interfacial energies is another. Franks et al.[34] had shown that AFGP does not seem to alter the rate of homogeneous nucleation at very low temperatures. More recently, Wilson and Leader[35] have shown that interaction between the antifreeze protein with an ice nucleating agent (INA) does impede the development of these seed crystals.

In summary of this section, we can say that antifreeze proteins are postulated to function either by prevention of nucleation or inhibition of crystal growth. The argument for nucleation prevention is very persuasive from a functional point of view. Why chance the eventuality of unwanted growth of a crystal when it may be more efficient to bar the presence of any crystals from the start? We will show that experimentally, the evidence for nucleation prevention has yet to be fully convincing. On the other hand, a valid argument can be also advanced for favoring the ice growth prevention mechanism. Since the ecological niche for these polar fishes is the ice-laden sea, they swim and encounter ice. If the portion of the body that touches this ice is in a less saline state, then these crystals might serve as

nucleators to start ice growth since the ambient temperature is still below freezing at −1.9 °C. Under those conditions, it may be more efficient to prevent the growth of these small ice crystals rather than to seek out newly evolving supercritical ice nuclei.

III. Molecular Structures of Antifreeze Proteins

In the previous section, theoretical analyses have been based on mostly rather simplified representations of the proteins. In order to examine the detailed mechanisms of function of these antifreeze proteins, we first review the efforts that have led to the characterization of the solution structures of these molecules. The first of these systems that was examined in some detail is the glycoprotein found in the fishes from the Antarctic. The well-defined structural order of these glycoproteins were initially thought to be a unique requirement. More recently, even the ordered secondary structure of the so-called type I AFP has been shown to be unnecessary as a requirement for function with the discovery of type II and type III AFPs. We examine these solution structures first.

A. AFGP

1. Primary Structure

Initial isolation and fractionation studies from the blood serum of *Trematomus borchgrevinki* (later reclassified as *Pathogenia borchgrevinki*) and *Dissostichus mawsoni* yielded glycotripeptide repeats. The peptide backbone is composed of alanine-alanine-threonine (Ala-Ala-Thr) repeating tripeptide units. The Thr peptide has a disaccharide residue (3-*O*-(*β*-D-galactosyl)-D-*N*-acetylgalactosamine) where the two sugar groups are linked 1→3.[36] Eight distinct fractions of these proteins have been isolated, mostly the difference is in the number of the tripeptide repeats, which ranged from 50 to 4 for bands 1−8, respectively.[8] The corresponding molecular weights ranged from 32 kD to 2.7 kD.[7] The C-terminal sequence is Ala-Ala except for AFGP molecules from one species (*Eleginus gracilis*), which has a penultimate arginine.[16] As for bands 7 and 8, occasionally, Pro has substituted some of the intermediate Ala residues.[37] Recently, Hsiao et al.[38] have succeeded in isolating the gene structure of AFGP from an Antarctic nototheniid.

2. Secondary Structure

DeVries et al.[5] first determined that AFGP molecules do not have much α-helical content. Hence, this protein differs from many others. Attempts to characterize the secondary structure were made using solution Raman spectroscopy,[39] vacuum circular dichroism (CD),[40] and finally proton NMR (Bush et al.,[41] Bush and Feeney[42]). The best experimental evidence suggests that these glycoproteins exist in solution as left-handed extended 3-fold helices. The uniqueness of such a conformation is that given the three-residue repeat, this conformation allows all disaccharides to be positioned on the same side of the molecule while the relatively hydrophobic Ala groups occupy the primary position on the opposite side.

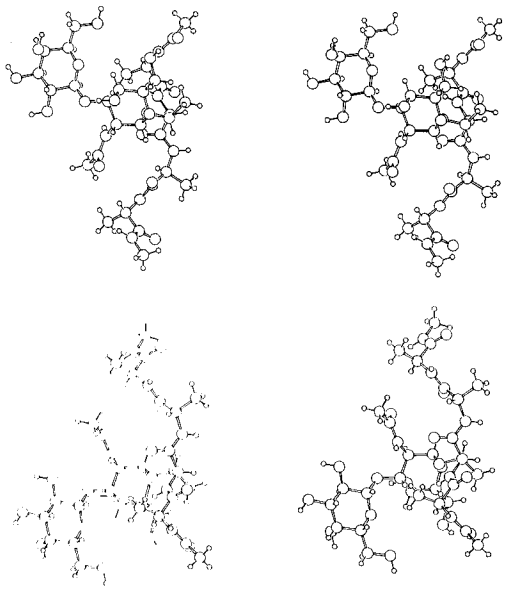

Figure 3. Stereopair representation of the glycotripeptide of AFGP molecule. Upper pair is the view from the hydrophilic side, and lower pair is the view from the hydrophobic side. (Reprinted with permission from ref 42. Copyright 1986 Munksgaard.)

Furthermore, Bush and Feeney argue on the basis of the NMR data that the disaccharide groups are tucked against the backbone of the polypeptide so as to present a stable, hydrophilic, hydrogen-bonding side, capable of interacting with the ice surface (Figure 3). A recent NMR study by Mimura et al.[43] of model glycopeptides indicates that GalNAc forms a hydrogen bond between the NH proton and the C═O oxygen of the Thr residue, thereby stabilizing the carbohydrate structure against the backbone. These authors suggest that such stabilization may be a model for disaccharide−peptide interaction in the AFGP molecule as well. Mimura et al. also suggested that the Pro found in the shorter AFGP species could act to stabilize the poly-L-proline type II of helical conformation. Dill et al.,[44] however, showed on the strength of their NMR proton exchange data that the intramolecular hydrogen bonding suggested by Mimura et al. is not a strong one, at least not for AFGP 7 or 8. Furthermore, Rao and Bush[45] could not find any significant difference in conformational energies between the larger AFGP 1−4 and the smaller AFGP 8. They suggest that instead it is the terminal sequence of Pro-Ala that is responsible for functional differences between AFGP 1−4 and AFGP 8. Recent CD and NMR data by Filira et al.[46] suggest that the helical content of the AFGP must be very small, at least at the temperature of their measurements (T = 25 °C). This is consistent with Rao and Bush's findings that evidence for a conformation that has exactly three residues per turn is not overwhelmingly strong. Recent Raman data obtained by Drewes and Rowlen[47] also question the existence of polyproline II structure, as suggested by Bush et al. On the strength of a very high amide I band at 1684 cm^{-1}, Drewes and Rowlen believe the backbone actually exists in a γ-turn motif. Even though such a conformation is structurally very stable, it calls for a different mechanism of action since there is no longer the convenient hydrophilic−

hydrophobic sides of the molecule in this model. They argue that perhaps cooperative hydrogen bonding over the length of the polymer may sufficiently alter the surface of the ice crystal so that water molecules cannot order efficiently at the surface to promote ice growth.

3. Tertiary Structure

The earliest characterization of the tertiary structure of AFGP was done by Ahmed et al.[48] The object was to see if these molecules change their size/shape upon cooling to their functioning temperature. Quasielastic light scattering (QELS) studies of AFGP 4, 5, 7, and 8 bands obtained at both 22 and −0.2 °C revealed that the structure is extended and rodlike. Pure species yielded translational diffusion coefficients from 5.6 to 12.1 × 10^{-7} cm^2/s for bands 4−8, respectively. From these, the hydrodynamic radius ranged from 3.7 to 1.8 nm, respectively, for band 4 and 8. Little difference in size was seen within the range of temperature change studied. Complexing with borate, which inactivates the species, did not change the hydrodynamic size.[49] Recent QELS data by Wilson and DeVries[50] led to translational diffusional coefficients of 2.5 × 10^{-7} cm^2/s for AFGP 1−5 and 26 x10^{-7} cm^2/s for AFGP 8. These numbers are consistent with the earlier values considering that Wilson and DeVries used a mixture of the larger species. Moreover, recent advances in QELS instrumentation has allowed for higher precision measurements of the smaller sized species.

B. AFP Type I

1. Primary Structure

The existence of these nonglycoproteins was first reported by Duman and DeVries[51] and Hew and Yip.[52] The most extensively studied species comes from the winter flounder (*Pseudopleuronectus americanus*). These AFPs are rich in Ala (~65 mol %), like the AFGPs. A recent review by Ananthanarayanan[17] summarized the many successful efforts to synthesize these species. Two major components have been isolated by HPLC methods. They are

HPLC-6
DTASDAAAAAALTAANAKAAAELTAANAAAAAAATAR

HPLC-8
DTASDAAAAAALTAANAKAAAKLTADNAAAAAAATAR

where the conventional single-letter symbol for amino acid representation has been used. As can be seen, the sequence is highly conserved, allowing for serious efforts to conduct site-specific mutagenesis studies. Wen and Laursen[53] have recently shown that systematic rearrangement of the Thr, Asn, and Asp residues in synthetic analogs of the HPLC-6 results in moderate to complete loss of antifreeze function. These authors further concluded that the ratio Asn/Asp is of major significance in defining function, probably as much so as the Thr residues.

2. Secondary Structure

Ananthanarayanan and Hew[54] and Raymond et al.[55] independently determined that the flounder AFP has predominantly α-helix for its secondary structure, the percentage increasing to over 85% at or near the functioning temperature of −1 °C (Figure 4). This highly ordered molecule was first crystallized by Yang et al.[56] X-ray diffraction data from these crystals showed that the residues implicated in ice binding are localized on one face, while the nonpolar Ala residues reside primarily on the other side. These investigators were not able to find the compatible match between the potential binding sites of the protein and either the a- (prismatic) or c- (basal) facets of ice.

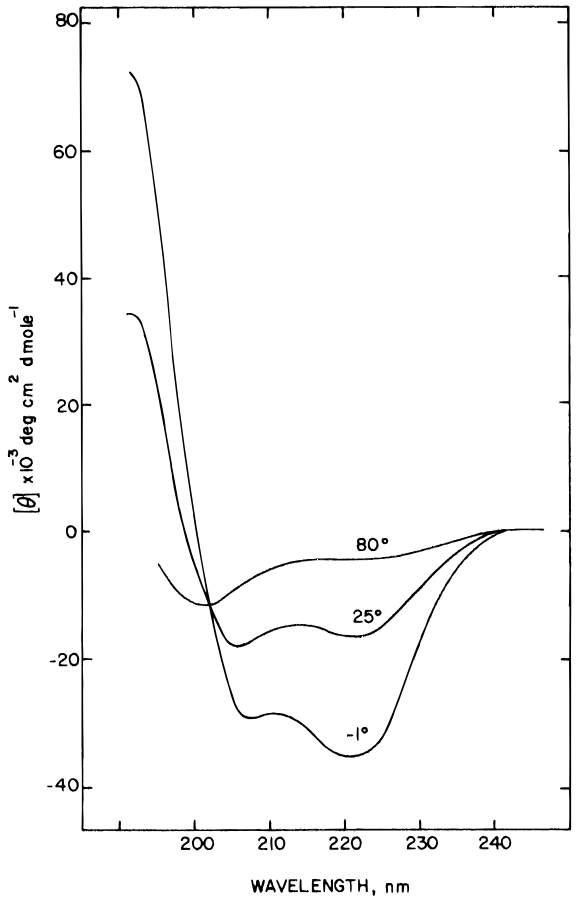

Figure 4. Circular dichroism spectra of the winter flounder AFP at three different temperatures (in °C). High α-helical content exists at both 25 and −1 °C. (Reprinted with permission from ref 54. Copyright 1977 Academic Press, Inc.)

3. Tertiary Structure

In a recent study by Sicheri and Yang,[57] X-ray crystalline structure of the HPLC-6 to 1.5 Å resolution revealed that there are four repeating ice-binding motifs, the side chains of which are inherently rigid or restrained by pair-wise side-chain interactions to form a flat binding surface. There is also an elaborate terminal cap structure at both the amino and carboxy ends.

4. Genetic Engineering of AFP

One of the most obvious applications of the antifreeze proteins is to introduce them into other species that originally did not have these protectives. The idea is to introduce these proteins through genetic manipulation. Over the intervening years, both

cDNA and genomic structure of the AFP types have been characterized by Davies et al.[58] Isolation of these genes means that they can be introduced into other fishes that inherently did not have such genes. Fletcher et al.[59] have successfully implanted this gene into the Atlantic salmon. Expression of the AFP gene in transgenic salmon over a period of 5 years showed that the Atlantic salmon can properly express the inserted winter flounder gene and secrete the proAFP into the blood. So far, the blood still lacks the enzyme systems necessary to process it to mature AFP molecules.

A series of peptide analogs to the AFP type I has also been generated by site-specific cleavage of engineered proteins synthesized in *Escherichia coli* by Warren et al.[60] Mueller et al.[61] had previously fused a gene to the 3′ end of a truncated staphylococcal protein A gene. When expressed in the budding yeast, the new protein again was shown to exhibit positive recrystallization-inhibition properties.

C. AFP Type II

1. Primary Structure

One of the most well-studied type II AFP comes from the sea raven *Hemitripterus americanus*.[62] Other type II AFPs have been found in insects by Patterson and Duman[63] and in the spruce budworm by Hew et al.[64] More recently, Ewart and Fletcher[65] isolated and characterized the primary structure of antifreeze proteins from smelt (*Osmerus mordax*) and Atlantic herring (*Clupea harentus harengus*). The molecular weights of these latter two antifreeze proteins are 24 and 14.6 kD, respectively, making them some of the largest AFP molecules. The principal fraction of the sea raven AFP also has a molecular weight of 14−16 kD. The most significant feature of these AFPs is that they are cysteine rich (8.3 mol % for the sea raven and up to 9.1 mol % for the Atlantic herring). Although Ala still is a dominant amino acid (as much as 14.4 mol % for the sea raven), there are several other major amino acid components including Asn, Gln and Thr. For the sea raven, activity at 5 mg/mL is about 0.5 °C freezing temperature lowering. AFP from both the Atlantic herring and the smelt have significantly lower activity per unit weight in depressing the freezing temperature although their molar depression activity is similar. Antifreeze action of all of these type II AFP is reduced in the presence of dithiothreitol, indicating the importance of disulfide bonds in maintaining the structural integrity necessary for function.

2. Higher Level Structure

Contrary to the type I AFP molecules, there is an absence of significant α-helical content in these proteins. Sönnichsen et al.[66] reported that for the sea raven AFP, there are 18% helices, 38% β-sheets, and 44% random coils. The 10 half-cystine residues are all probably involved in disulfide bonds. A detailed study of these bonds by Ng and Hew[67] confirmed three sets of pairing with a possibility of two additional pairs. A recent model proposed by Sönnichsen et al. indeed has specific assignments for all of the half-cystine residues. Their model further shows that the region of the primary chain that is exposed to the aqueous environment through the foldings dictated by the disulfide bonds is composed mainly of hydrophilic residues dominated by Thr, Asn, and Gln, each of which is capable of hydrogen bonding. Such a role for the disulfide bonds strongly enhances the idea that these bonds are necessary to maintain the structural motif both for stability and for function.

D. AFP Type III

1. Primary Structure

AFP type III found in the ocean pout (*Macrozoarces americanus*) is neither Ala rich nor contains any cysteine residues.[68] These proteins are not dominated by any particular amino acid among their 62−66 residues. Eel pout from both the Arctic and the Antarctic have also been found by Schrag et al.[69] to possess these proteins.

2. Higher Level Structure

A recent NMR study by Sönnichsen et al.[70] led to the finding that in solution, this 66-residue protein (from pout) has an unusual fold in which eight β-strands form two sheets of three antiparallel strands and one sheet of two antiparallel strands. Furthermore, the triple-stranded sheets are packed orthogonally into a β-sandwich. These proteins are basically globular; hence their ice-binding motif may be different from others discussed so far. These authors obtained a hydrophobicity factor of 5.0 for this protein, comparing it favorably with the AFP type I and distinctively different from most proteins, where this factor lies between −6.0 and 0.0. Even though no long, linear array of polar side chains is found, there are hydrophilic areas (4−8 Å apart) between β-strands which could bind to the ice surface. However, these hydrophilic surfaces are on opposite sides of the protein. Single crystals of the type III AFP has recently been obtained by Xue et al.[71] We look forward to important elucidations of the structure of this system by X-ray diffraction studies.

IV. Experiments Testing Nucleation Inhibition Hypotheses

Experiments designed to test the hypothesis that these proteins function via the process of inhibiting nucleation must first of all assure that no nuclei of ice exist. If the process to be studied is homogeneous nucleation, then there is the added problem of preventing heterogeneous nucleation from taking place, thus masking the homogeneous nucleation temperature prematurely. We discuss the experiment designed to test the effect of AFGP on homogeneous nucleation first.

A. Homogeneous Nucleation

In order to obtain supercooled water system free of nucleators for initiation of freezing, Franks et al.[34] emulsified their solution in silicon oil producing droplet sizes only about 2.5 μm in radius. A small volume that is not in contact with any solid walls has a minimum volume such that it does not have

appreciably large number of impurity nucleators. Their pure solvent (water) emulsions yielded a homogeneous nucleation temperature of 232.1 K, lower than the experimental 234 K that has been cited in Hobbs.[33] Upon the introduction of solutes such as AFGP or polyvinylpyrrolidone (PVP) at the level of 1% w/w neither was able to further depress the nucleation temperature by more than 0.2 K, a number that is probably within the limits of experimental error. This suggests that the AFGP proteins are no more unique than PVP in preventing the formation of a critical nucleus of water clusters. Wilson and Leader[35] suggest that since the size of the embryonic ice crystal at this temperature may be comparable to the size of these protein molecules, there may not be enough room on the surface of the embryo for binding and for the Kelvin effect to develop. We suggest an alternative mechanism that does not require stable embryos and fixed adsorbates. Perhaps the nonfunctionality noted here is a question of rates: dissipation rate of embryonic ice vs the surface adsorption rate of the proteins.

B. Heterogeneous Nucleation

An experimental study of heterogeneous nucleation requires that the "impurities" sites be controllably introduced and identified. Wilson and Leader[35] tested the possibility that AFGP molecules are actually poisoning heterogeneous nucleation sites in a system that has a controllable quantity of ice nucleating agents (INAs). In this study, approximately 5 μL of pure water or solution was captured and sealed in a capillary tubing. These authors had prepared the capillary so that without the INAs, the walls of the tubing had relatively few nucleation sites thus the solution can be held to below −20 °C of supercooling. In the presence of a solution of a native *weta hemolymph* from *Hemideina maori* (a large alpine grasshopper), the supercooling point (SCP) was determined to be −7.3 °C. When AFGP 1−5 mixture was introduced into such a solution at a concentration of 10 mg/mL, the SCP was depressed to −9.4 °C. This decrease of nearly 2 °C in the capacity of the INA was attributed to the poisoning of nucleation sites by the AFGP molecules. Hence, these authors postulate that the remaining nucleation sites on the INA cannot elicit clusters of ice large enough to become critical size for spontaneous growth.

A review of the ice nucleating proteins by Hew and Yang[18] provides an up-to-date summary of the current status of the INA field. We cite the findings that have given us an understanding of their mechanism of action: In all postulated models of the INA (Warren and Wolber,[72] Mizuno[73]) the contacting crystal planes of ice were found to be the basal plane [0001]. The assembly of fundamental repeating units seems to be primarily of hexagonal symmetry. Hence it is possible that the action of these groups of an INA molecule when they bind to the basal planes is to promote prismatic (fast) facet growth. Upon the interaction between AFGP molecules and these INAs, it is conceivable that the disaccharide groups now impede binding of the INA to the ice surface by blocking off certain sites of the INA such that the fast-growing prismatic facet is no longer favored to grow.

V. Experiments Testing Crystal Growth Inhibition Hypotheses

A myriad of experiments have shown crystal growth inhibition by these proteins, and several parameters that were thought to be important have turned out to be indeed significant in the description of the freezing temperature lowering phenomenon (or thermal hysteresis). These parameters are the size of the polymer, the lattice match between polymer and ice, and the interaction energy between polymer and water or ice. What remains to be resolved are the rates of adsorption and desorption so that studies such as those of Burcham et al.[74,75] can also be explained. In the 1986 Burcham et al. studies,[75] the nature of the reversible adsorption isotherm is found to be a function of the concentration and the type of AFGP present. Secondly, there is the phenomenon of altered crystal growth habits whenever the temperature for complete growth inhibition is exceeded. Finally, we will examine the recrystallization phenomenon and see if the AFP/AFGP effectiveness as a recrystallization inhibition agent can be explained completely in the context of the present analysis.

A. AFGP

1. Facet-Selective Ice Growth Inhibition

Early studies of the effect of AFGP molecule on the growth of existing ice crystals showed that over a certain limited range of temperature (approximately from 0 to −0.8 °C) nothing grew. Hence over this range of temperature, there is a hysteresis effect between ice growth and ice melting (at 0 °C). However, it was noted in the early works of Raymond and DeVries[76] that upon exceeding that growth prevention temperature, ice will again grow, but the gross morphology of that ice was entirely different from the ice growing in a pure ice−water system. Knight et al.[77] first showed that the microscopic images of the ice crystals growing in solutions of AFGP are narrow spicules developing from the existing crystals oriented in directions almost perpendicular to that of the basal facet. Kerr et al.[78] conducted a linear growth experiment showing that ice from the seed crystal will normally propagate along the length of the tube if the system is pure ice−water. However, in the presence of AFGP solution, the direction of movement of the spicular growth was perpendicular to the direction of the original pure ice−water growth. Hence in these narrow plastic tubes, directed growth essentially stopped when crystals turned almost completely towards the *c*-axis, confined by the narrow tube walls. Growth cessation was also observed in the study of Raymond et al.[79] whereby large single crystals with exposed basal facets developed hexagonal pits upon growth. The growth proceeds until the pits are large enough so that there are no flat surfaces left on the basal plane. Both of these experiments demonstrate that AFGP definitely affected growth direction, and the latter showed it is because of growth inhibition along certain facets

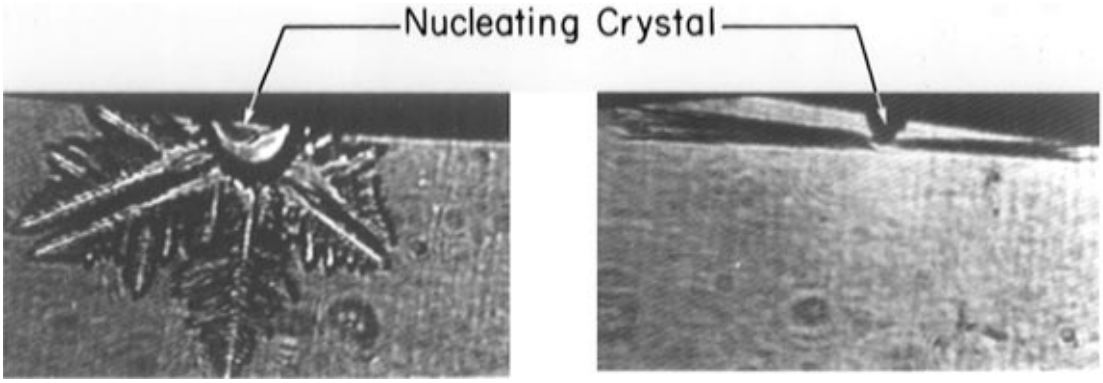

Figure 5. Ice crystal growth from seed crystal inserted into (a, left) pure water, with *c*-axis normal to the plane of paper, and (b, right) solution of AFGP 4 (5 mg/mL) with *c*-axis in the plane of the paper (oriented seed crystal is rotated 90° from the a case). Both supercooling temperatures are at −1.25 °C. (Reprinted with permission from ref 80. Copyright 1987 Nature.)

within the hexagonal pits. In an effort to prevent wall effects from playing a role, free growth conditions were used by Harrison et al.[80] and by Feeney et al.[81] In these experiments, preformed seed crystals of well-defined orientations were introduced into a solution supercooled to various temperatures. Figure 5 shows crystal growth of an oriented ice seed crystal in pure water (Figure 5a) and in a solution of AFGP (Figure 5b). Continued growth was monitored on a video camera and later analyzed with the aid of VCR playback capabilities. Not only were the growth orientations captured on film, the growth rates were measured for the various conditions. Strong correlation was established between growth rate, the degree of initial supercooling and the concentration of AFGP samples in solution. The onset of growth upon exceeding the freezing temperature is marked by rapid spicular growth along the *c*-axis. As the degree of supercooling is increased, or as the solution concentration is decreased, growth becomes more like that of the pure ice−water system. These different growth facets at the different sample conditions were attributed to facet-selective binding of AFGP to the ice surface.

Knight et al.[27,82,83] developed another way of examining the specific facet of AFP (or AFGP 7 and 8) adsorption onto the ice surface. In these studies, the authors grew a large ice crystal into a solution containing AFP (AFGP) at some defined concentration. The growth rate was held at approximately 0.5 cm/h. Upon the growth having taken place, they removed the crystal from the solution and scraped the remaining solution from the surface. The exposed crystal is then allowed to etch by evaporation in a cold room held at −10 to −15 °C. Different facets are then examined either as surfaces or after cross-sectional slices were made. Those surfaces with a motley appearance are considered to be affected by the proteins, whereas those facets with no protein adsorption show glassy, smooth appearance. Quantitative estimation of the amount of adsorbed protein is made using tritium-labeled proteins where the scintillation counts were registered as a function of the facets cut. These studies gave good qualitative account of the adsorption process: prismatic planes have strong adsorption, whereas the basal planes were essentially free of proteins. Scintillation data are more ambiguous because successive cuts along the same facet orientation yielded high and low counts. These authors have postulated that the strong adsorption planes are those with favorable inhibition capability, whereas the smooth facets (basal) will facilitate growth. They further pointed out that there is a very attractive lattice match between the 9.31 Å repeat spacing of the AFGP assuming a 3-fold helical form when compared with the 9.038 Å dimension, which is twice the *a*-axis repeat distance. In a recent extension of those studies, Knight and DeVries[83] have examined similar coverage patterns by AFGP 1−5. Unique features such as grooves running parallel to the *c*-axis are found. When the AFGP concentration is increased, fibrous texture replaces the groove/pit instability. Although these data support irreversible adsorption, other studies suggest that this model needs some reconsideration. First of all, the growth rates indicated are about 10-fold faster than those rates that were used in the studies of Brown et al.[84] and Vesenka et al.,[85] where attempts were made to obtain the reversible equilibrium constant for the adsorption. At these fast growth rates, it is well known that filamentary structures exist, suggesting trapping of the macromolecules between spicules, hence rendering an interpretation based on monolayer adsorption interpretation problematical. Secondly, the suggestion based on calculation that upward of 24−30 hydrogen bonds can form for each AFGP 7 or 8 respectively is very interesting; however, there needs to be some tangible spectroscopic evidence to support the proposed model. Early Raman data by Tomimatsu et al.[39] indicated additional spectral features besides that of ice and protein; however, there were no serious attempts to quantitate this signature.[15] Finally, these new results on the longer AFGP 1−5 suggest possibly dynamic interfacial stability, hence a reversible equilibrium on a microscopic scale.

2. Quantitative Coverage of the Growth Facet

Vesenka et al.[85] explored the use of quasielastic light scattering at the crystal−solution interface to see if there is a possibility of measuring the amount of AFGP on a particular growth facet. Vesenka and Yeh[86,87] had previously argued that since a dominant light scattering mechanism at the pure ice−water interface is from the extruded microbubbles, the presence of adsorbed proteins may affect the extru-

Table 1. Comparison of the Size Increase of Bubble Radii and Polydispersity During Steady-State Crystal Growth with the Percent Surface Tension Reduction (Modified from Vesenka et al.[85])

solute	quasi-elastic light scattering data: factor increased[a]	quasi-elastic light scattering data: polydispersity	surface tension % reduction[b]
water (*a*- and *c*-axis)	0	0.30 ± 0.11	0
lysozyme (*a*- and *c*-axis)	2	0.39 ± 0.07	–
ovomucoid (*a*- and *c*-axis)	10	0.42 ± 0.12	15
detergent (*a*- and *c*-axis)	2	0.28 ± 0.04	57
AFGP-8 (*a*- and *c*-axis)	0	0.44 ± 0.07	1
AFGP-4 (*a*-axis)	80	0.90 ± 0.20	5
AFGP-4 (*c*-axis)	0	0.42 ± 0.12	5

[a] 50 μg/mL in bulk solvent, error bars are ±10%. [b] 10 mg/mL in bulk solvent, error bars are ±10%.

sion process, hence the strength of the light scattering signal from these microbubbles. Furthermore, protein molecules pushed away from the slowly growing interface have higher concentration near that interface; hence its effects on the size of the microbubble would provide an indication of the amount of AFGP near that interface. These studies were very sensitive to low levels of solution AFGP concentrations (50 μg/mL), and they yielded quantitative results showing that the *a*-axis growing facet affect microbubble signal far more than the *c*-axis-growing crystal, suggesting that there is a strong tendency (nearly 80×) for AFGP 1−5 to congregate near the *a*-axis prismatic plane in comparison with the basal facet (Table 1). However, because there are two competing phenomena affecting the size of the microbubble, surface tension and gas infusion, estimates of the absolute amount of proteins on the interface is difficult to obtain. Wilson et al.[88] used ellipsometry to provide an estimate of actually adsorbed protein on specific facets. In this experiment, because the presence of even a monolayer of protein on the interface between ice and solution will alter the interfacial optical polarization properties, light reflected from the interface will suffer differential polarization change depending on the amount of material present. The saturation coverage suggested by their data of 0.16 for the basal plane and 0.3 for the prismatic plane, leads to an average distance between adsorbed molecules of 50 Å (AFGP 8) and 165 Å (AFGP 1) for the basal plane and 33 Å (AFGP 8) and 120 Å (AFGP 1) on the prismatic plane. Clearly basal coverage is not as complete as that of the prismatic facet, hence more growth inhibition is expected from the prismatic planes. These authors went on to estimate the interfacial distortion should the Kelvin effect be the dominant mechanism of freezing temperature depression. They showed that when temperature depression is 1 °C, the interfacial height should only be 17 Å. Such height characterization could require rather sophisticated force microscopies to detect. To date, there has not been any definitive results along these directions.

3. Kinetics of the Interfacial Adsorption Reaction

Two experiments done in our laboratories attempted to investigate the kinetic process of AFGP adsorption onto the surface of ice. In one, Kerr et al.[89] adapted the technique of Jones and Chadwick[90]

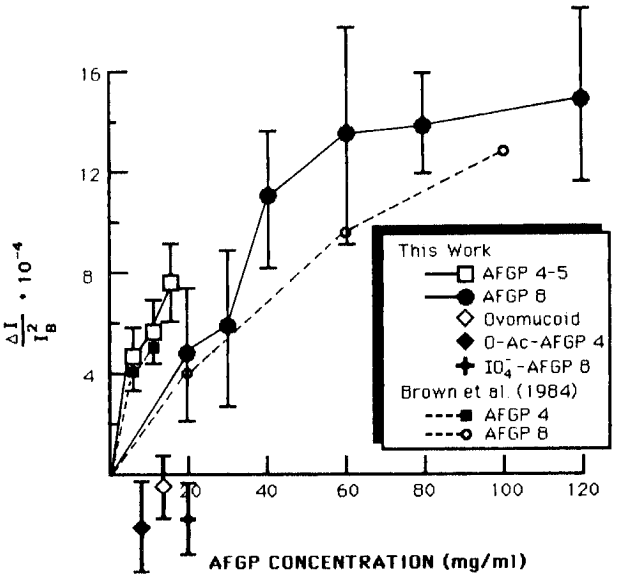

Figure 6. Surface second-harmonic generation intensity of light (at 532 nm) at the solution−ice interface vs AFGP concentration for various cases studied. Error bars are standard deviation (From ref 91.)

to examine quantitatively the change in the grain boundary curvature upon adsorption of AFGP. Grains of ice crystals were allowed to grow in a thermal gradient. When the fluid part was changed from pure water to AFGP solutions, grain boundary curvature changed. From the measured change in curvature, a change in the interfacial energy was calculated as a function of the AFGP concentration. Such a measurement yielded an excess concentration of adsorbates on the interface. A plot of this excess concentration of adsorbate vs solution concentration can in turn yield an equilibrium constant for the adsorption−desorption reaction. Both AFGP 1−5 and AFGP 7−8 were studied over a range of concentration. The results up to 10 mg/mL of AFGP 1−5 and 72 mg/mL of AFGP 7−8 did not show full saturation of coverage. From these results and the assumed validity of the Langmuir isotherm, the dissociation coefficient K_d for AFGP 1−5 was calculated by Feeney et al.[81] to be $(2.8 \pm 0.2) \times 10^{-4}$, while for AFGP 7−8, $K_d = (70 \pm 5.0) \times 10^{-4}$.

Another way to measure the amount of surface adsorbent is to have a surface-selective monitor of the adsorption process. Unlike the ellipsometry experiment whereby the interfacial signal from the pure ice−water system has to be subtracted from the ice−solution signal, the surface second-harmonic generation experiment (SSHG) provides a direct indication of the amount of adsorbed material onto the interface. Due to low optical polarizabilities of both ice and water, the pure ice−water interface gives a negligible SSHG signal. Hence the detected signal in this experiment is only the result of interfacial adsorbents. Figure 6 shows the SSHG intensity as a function of AFGP concentration. Such a concentration dependence study on the normally fast growing *a*-axis prismatic plane yields an equilibrium constant for the adsorption−desorption reaction on that facet. Brown et al.[84] obtained data that suggest no site saturation for either AFGP 4 or AFGP 8, at physiological concentrations. From these data

and those carried out by Kerr,[91] the equilibrium constant for adsorption−desorption on the prismatic facet was calculated to be $K_d = (6-14) \times 10^{-4}$ for AFGP 1−5. Low signal-to-noise ratio precluded the determination of similar coefficient for AFGP 8. The rather poor certainty in the SSHG data suggests that another approach is needed to ascertain these numbers quantitatively. Guyot-Sionnest et al.[92] have recently developed another surface-sensitive nonlinear optical technique, called sum frequency generation (SFG) to examine the resonant spectral features of adsorbed species on a surface. In this experiment, a visible light signal is coherently summed with a tunable IR source to yield a sum frequency in the optical regime. By tuning the IR source across the vibrational bands, the sum frequency detected is both surface selective and resonantly enhanced. We are currently exploring the use of this technique for more detailed quantitative adsorption studies.

Burcham et al.[75] explored another way to obtain equilibrium constants that are associated with the adsorption of AFGP molecules on ice surface. On the premise that inhibition of the ice growth process is derived solely from AFGP adsorption onto existing ice surface, they ascribed the function of freezing temperature depression to percentage coverage on the surface of ice. From such activity vs solution mole concentration studies at different degrees of supercooling, a Langmuir adsorption isotherm was obtained. From these, the dissociation constant, K_d, as well as the level of segmental cooperativity, n, were computed. These dissociation constants were nearly identical to those obtained from the interfacial tension studies: $K_d = (2.9 \pm 0.025) \times 10^{-4}$ for AFGP 1−5 and $K_d = (68 \pm 0.067) \times 10^{-4}$ for AFGP 7−8. The fact that these numbers were obtained using the freezing point osmometer means that no facet specification can be obtained.

Adsorption/desorption kinetics was also noted in a series of studies which were called "potentiation of function" and cooperativity. In these studies (Osuga et al.,[29] Mulvihill et al.[93]), the level of antifreeze activity was shown to vary for the shorter AFGP 8. At deeper supercooling conditions and/or in the presence of dendritic ice, AFGP 8 does not function as effectively as it does at less supercooling. However, the full activity can be reestablished if AFGP 8 were to operate in the presence of small quantity of the longer AFGP 1−5. These results suggest (1) molecular competiton for the sites on the growing ice surface is constantly taking place between water molecules and the AFGP molecules, and (2) surface-induced interaction between AFGP 1−5 and AFGP 8 can effectively keep the shorter AFGP 8 molecules competing more favorably for those surface binding sites.

4. Recrystallization

When small, polycrystalline samples of ice are held for a prolonged period of time at a sub-melting temperature, grains of ice will change size and shape. This is the process of recrystallization. Smaller grains with negative curvatures are gradually swallowed up by larger, positive-curvatured grains. The driving force is that which will minimize the overall grain boundary energy. In the presence of impurities, certain grains are inhibited from growth, either by decreased diffusion or by altering the interfacial energy. When a solution freezes via a splat-cooled method at −80 °C, the grains of ice crystallites should exclude most of the solute molecules. Thus the boundaries are highly concentrated in extruded solutes. Accordingly, very low solution concentrations of solutes such as AFGP can effect the grain boundary migration dramatically. Figure 7 shows typical recrystallization assays where the splat crystallites are examined after 5 h at a holding temperature of −5.4 °C, where the solute conditions will determine the grain sizes.

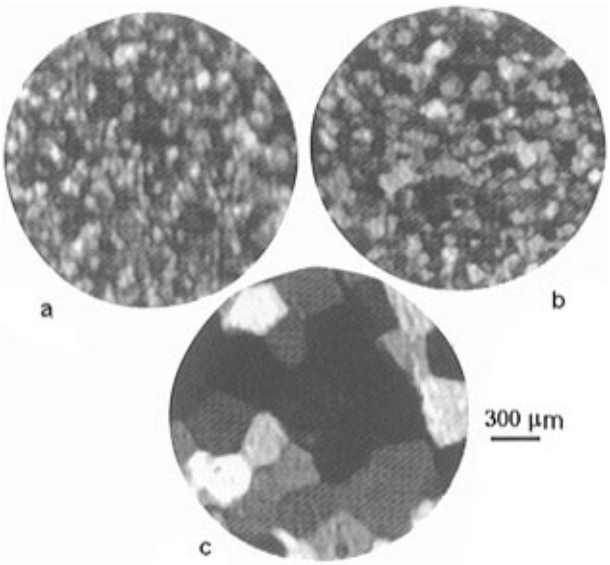

Figure 7. Video images of grain sizes of ice after 5 h of recrystallization under different solution conditions at $T = -5.4$ °C: (a) AFGP 4 solution at 0.1 μg/mL, (b) AFGP 8 at 0.5 μg/mL, (c) pure water. (Reprinted with permission from ref 96. Copyright 1994 John Wiley & Sons, Inc.)

Initial effort that showed recrystallization inhibition is significant for solutions of AFGP was made by Knight et al.[94] McKown and Warren[95] used this effect to identify genetically altered antifreeze proteins. Yeh et al.[96] have studied recrystallization to see if interfacial activation energy can be determined from an Arrhenius plot over a limited range of temperature. The values of the activation energy Q_g were calculated to be $(6.61 \pm 1.02) \times 10^5$ J/mol for AFGP-4 and $(5.71 \pm 2.39) \times 10^5$ J/mol for AFGP 8. These values show little distinction in the activation barrier for the two species of AFGP, however, they are a factor of 2 or 3 larger than the Q_g of pure ice−water system. It is reasonable to find comparable activation barriers among the different species since that quantity is basically governed by the diffusional limitation of water molecules to the site of grain boundary addition or subtraction. Knight et al.[97] have recently shown that peptides that do not have the antifreeze property can also inhibit ice recrystallization because they simply inhibit water mobility in the grain boundaries that have low free-water concentration. On the other hand, if there is a water layer in between the grains, these nonantifreeze active peptides cannot inhibit recrystallization, whereas the AFGP or AFP molecules continue to function as viable recrystallization inhibitors. They

argue that, given the liquid space, these surface adsorbents can migrate to the binding sites and effect site-specific binding and subsequently inhibit grain boundary movement in the true growth inhibition sense.

B. AFP Type I

The principal efforts to identify a mechanism of AFP action have been to find selective matching residues of the ordered helix with some direction of the ice crystal. The fact that Yang et al.[56] were not able to identify either the basal or the prismatic facets for good matching suggested either a more complicated mechanism of action (beyond the Kelvin effect) is needed or some other facet needs to be implicated. The observation that bipyramidal crystals grow upon exceeding the thermal hysteresis point opens the door for new experimentation and interpretations. Chakrabartty and Hew[98] showed that α-helicity is a key element for function of the AFP molecules; however, the relationship between structure and function may be rather complex. Modeling studies of the AFP in vacuum and solution by McDonald et al.[99] showed that in an aqueous medium, the α-helical conformation is stabilized by a combination of main-chain hydrogen bonds, salt−bridge interactions, and solute−solvent hydrogen bonds. Indeed, Chakrabartty et al.[100] suggested that the high hydrogen-bonding potential of the helical face of AFP may be able to form hydrogen bonds with the water molecules of the ice surface.

1. Dipole–Dipole Interaction

In unpublished notes, Sicheri and Yang[101] show that in order for the dipole of the α-helix to have a significant effect on the ice surface, it must interact with that surface via dipole−dipole interaction. Their calculations show that the energy required to induce a dipole on the surface of ice is too large compared with the actual dipole−dipole attractive energy.

2. α-Helix Match with the Ice Dimensions

Chou[102] showed the importance of Thr repeats in AFP type I at every 11 amino acid residues interval (2, 13, 24, 35). In a vacuum−ice interface simulation, he showed that energy-minimized hydrogen-bonding interaction of Thr (interval of 16.1 Å) with oxygen is achieved along the [01$\bar{1}$2] direction of the ice facet at intervals of 16.6 Å. Chou suggests that this model can describe the data of Knight et al.[82] where the etching data showed AFP deposits on the 12 equivalent (20$\bar{2}$1) planes. It was pointed out by Knight et al. that the adsorption planes could have been chosen to intersect along five of the 11 equivalent [01$\bar{1}$2] directions. In the simulation studies of Jorgensen et al.,[103] care was taken to have the molecule exist in a solution−ice interface, not vacuum−ice interface. It is found that a stable structure indeed still exists for the Thr of HPLC-6 to interact with the ice lattice. These authors tested other variants with either a more varying Thr position or a spatially protruding amino acid residue. In both cases, the stabilized adsorption of the protein to the ice surface is eliminated.

3. Dual Mechanism

Wen and Laursen[28,104,105] studied various site-substituted synthetic AFPs, all of which are essentially 100% α-helical in secondary conformation. However, their levels of antifreeze activity are quite different, suggesting very site-specific residue dependency. In particular, they show that the ratio of Asn/Asp plays an important role. In their model, on the plane (20$\bar{2}$1) there is only one favorable orientation for the AFP to reside. This is along the [$\bar{1}$102] direction, with the amino terminus oriented toward the apex of the hexagonal bipyramid. Rotation of AFP by 180° would place Asn and one of the Thr in positions not so favorable energetically along the [01$\bar{1}$2] direction. Hence their model shows adsorption to only the [$\bar{1}$102] direction on the (20$\bar{2}$1) plane (Figure 8). In fact, when Wen and Laursen[106] synthesized the D-enantiomer, activity was the same as the L-form of the native AFP. However, they postulated the adsorption to be along the [01$\bar{1}$2] direction on the same (20$\bar{2}$1) plane. Because of this ordered, parallel binding onto a plane along a unique direction, Wen and Laursen suggested that initial adsorption could be randomly situated on that surface, but with higher concentration of adsorbates; there is a side-by-side intermolecular hydrophobic interaction, aligning the AFP molecules so as to saturate the binding sites. The slow bipyramidal growth then stops, and the crystal will no longer grow, leading to total growth inhibition. This mechanism is similar to the interaction that AFGP 1−5 and AFGP 8 exhibited in eliciting full activity. In related work, these authors show that if certain specific residues are altered (made bulky), steric hindrance will prevent effective association of AFP molecules, rendering function minimal.[104] Lal et al.[107] further affirmed this adsorption mechanism, showing in their modeling study that along this crystallographic direction ice has several "ridges and valleys" which produce nearly the perfect match for the AFP molecule. These authors pointed out that there is a similarity between this case and enzymatic functioning requiring a "lock and key" match.

4. Flat Ice-Binding Motifs

The recent X-ray crystallography data of Sicheri and Yang[57] led these authors to suggest that the models of Knight et al.[82] and that of Wen and Laursen[28] may no longer be adequate. They point to the fact that (1) a sufficiently close spatial match between the AFP and ice is not apparent along the directions proposed by these other groups; (2) the ice-binding groups do not really protrude as much as suggested to clear hindering groups; (3) the trigonal planar (SP2) coordination of Asn and Asp hydrogen-bonding groups differ from the tetrahedral (SP3) coordination of water molecules in ice. Instead, these authors suggest that AFP binding to ice is defined by a less stringent hydrogen-bonding criterion. Since the hydrogen-bonding groups extend only minimally from the AFP's flat ice-binding surface, it is the water molecules on the [01$\bar{1}$2] ridges of the (20$\bar{2}$1) ice plane that are the most probable binding sites, and it is the rigid ice-binding motifs of the HPLC-6 that allows this type of binding to take place (Figure 9).

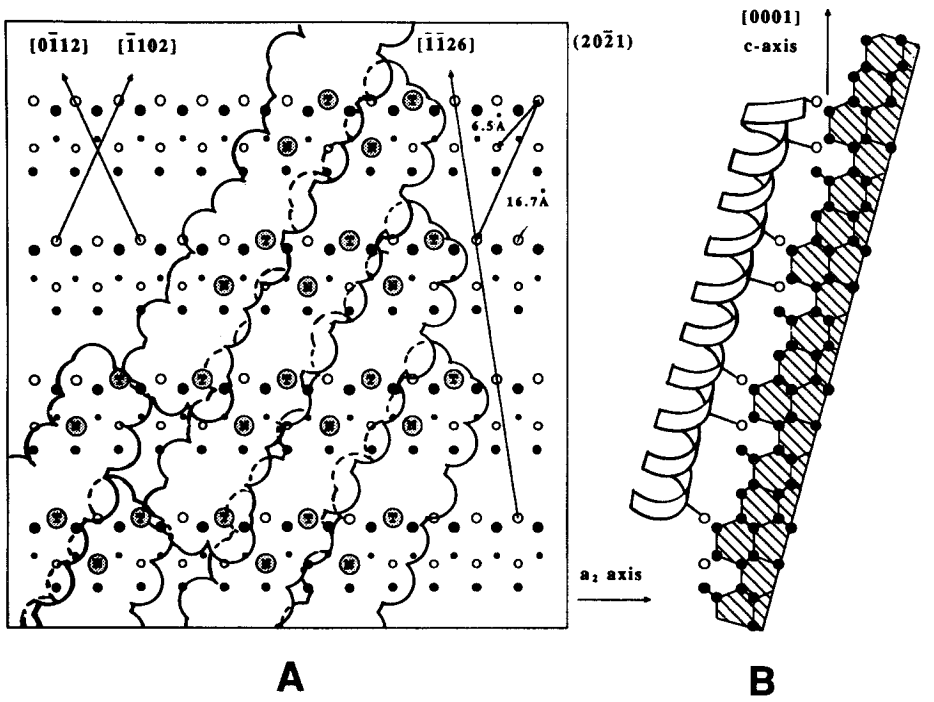

Figure 8. Proposed Wen and Laursen model for AFP binding on ice. (A) Schematic view of ice normal to the hexagonal bipyramidal plane (20$\bar{2}$1). Note the 16.7 Å Thr−Asn distance on AFP. They are postulated to lie on the [$\bar{1}$102] or equivalently [01$\bar{1}$2] direction. (B) Shows the profile of the (2021) surface viewed from the a_2-axis. (Reprinted with permission from ref 28. Copyright 1992 Biophysical Society.)

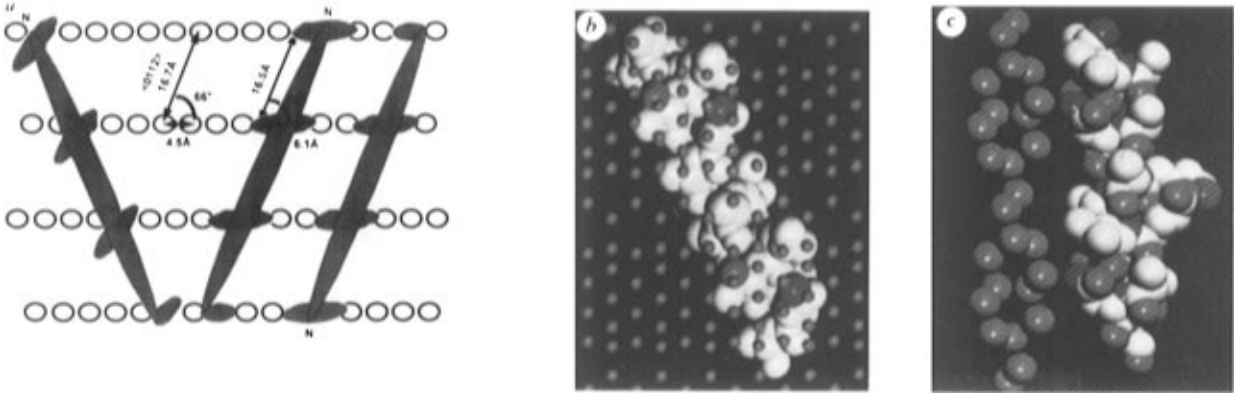

Figure 9. Proposed model for AFP binding on ice by Sicheri and Yang. Panel a illustrates the match between AFP structures and the topology of (20$\bar{2}$1) ice plane. The AFP molecules are arranged along the [$\bar{1}$102] binding axis. Because of the flatness of the ice-binding motif of AFP, binding is favorable along either direction of the [01$\bar{1}$2] axis. Panel b is the top view of the AFP molecule (large-sphere chain) docking from behind onto a surface layer of water molecules arranged in the ice lattice (small spheres). The darker large spheres of the AFP are oxygen and nitrogen atoms making bonds to the water molecules. Panel c shows the side view docking along the [01$\bar{1}$2] axis of the (20$\bar{2}$1) plane where the most prominent features are the nitrogen atoms (darkest in shade) contacting the water molecules of the ice surface on the left. (Reprinted with permission from ref 57. Copyright 1995 Nature.)

5. Kinetic Studies

Two recrystallization experiments have been done using AFP. In the study of Carpenter and Hanson,[108] cryomicroscopy showed that AFP inhibits recrystallization in the extracellular space during the process when frozen cells are warmed up. Even at low concentration of 5 μg/mL, AFP enhances the survival of red blood cells cryopreserved in hydroxylethyl starch solution. These investigators find that increased concentration (1.54 mg/mL) leads to ice growth around the cell itself, culminating in a high percentage of cells suffering hemolysis. If water migration can still take place in the recrystallization-inhibited sample, then this water might flow toward the proteins of the RBC membrane, crystallizing around the cell. Perhaps, the water layer between the ice grains, postulated by Knight et al.[97] (1995) to be necessary for the AFP migration within the water layer to proper binding sites on ice, might also be the source for the unfavorable environment as far as the cells that needed cryoprotection are concerned.

C. Relationship to Biomineralization

It is of interest to note that preventing or modifying ice growth is related to the broader subject of biomineralization. There are many biological molecules that adsorb onto crystalline matrices in ways that the growth of the crystal after adsorption is altered by specific adsorption−inhibition mechanisms. Some of these systems are more stable than the ice system,

therefore more easily studied. Ng and Hew[67] had noted the similarity in the sequence of disulfide bonding pattern in the pancreatic stone protein which binds to $CaCO_3$ to prevent growth of those crystals. They suggest a possible common fold between these proteins and the AFPs, even though the type of crystal and the facets where growth has been inhibited are totally different. Mann[109] has examined biomolecular processes that result in the construction of higher-order mineral architectures and predicts the development of new strategies for the controlled synthesis of organized inorganic and composite materials. Examples of these concepts are the consolidated biominerals such as bone, enamal, shell, invertebrate teeth, and siliceous plant materials, all of which are based on the nucleation of calcium phosphate in nanospaces organized within the supramolecular assembly of collagen fibrils. Sikes et al.[110] have also shown that polyamino acids are effective antiscalants, corrosion inhibitors, and dispersants of mineral particles such as calcium carbonate, calcium phosphate, kaolin, and iron oxide. These authors have examined the result of the interaction between polyamino acids and the crystalline surfaces using atomic force microscopy. Using synchrotron X-ray diffraction, Berman et al.[111] have further shown that the manipulation of calcite crystal texture in different organisms is under biological control and that crystal textures in some tissues are adapted to specific function of that organism. In a broad sense, both the antifreeze protein and the polypeptides that control mineralization processes are manifestations of species survival and adaptation.

VI. Summary

The deep interest that so many investigators have had with the function of these biological antifreeze molecules, coupled with much fertile imagination of how these molecules might be applied in cryoprotection, has led to significant enhancement in the knowledge of the mechanism(s) of action of these molecules. Thermal hysteresis can now be explained by the adsorption−inhibition mechanism of these polymers onto available adsorption sites of the ice crystal. Apparently, the competition with water molecules on certain growth facets remains favorable for substantial levels of supercooling. This leads to growth inhibition as well as to altered growth facets, as reported by many studies. The remaining theoretical task is to relate crystal growth anisotropy to adsorption anisotropy, making it possible to establish a relationship between dynamic site occupancy and inhibition of crystal growth. Such a task will require much more experimental underpinning. Use of various designed structures of the flounder protein by chemical synthesis or molecular biology should continue to give information on possible fittings on surface sites. X-ray crystallography of protein crystals and modeling studies will continue to reveal accessible sites on the ice surface. Gross identification of adsorbing facets have already been accomplished. What is needed are experimental studies that will provide information on bonding angles and the rates of adsorption/desorption as a function of facet and molecular details. New tools, such as the spectroscopically sensitive sum frequency generation (SFG) technique, could be useful in assessing bonding angles and rates of adsorption. Versions of the scanning probe microscopy (SPM) might be developed to identify spatial localization at the molecular level. Finally, the use of recrystallization inhibition (RI) as a monitor of grain boundary dynamics might provide more accurate activation energy measurements.

Another area of interest is the application of AFGP or AFP molecules to cell survival. The studies of Rubinsky and DeVries[112] on red blood cell survival with AFP was extended by Ishiguro and Rubinsky[113] into directional freezing conditions. It was found that AFP actually causes cell damage under certain ice growth conditions. The effect of different types of AFP or AFGP on the integrity of thylakoid membrane of spinach was studied by Hincha et al.[114] Arav et al.[115] have shown that AFP is effective in protecting oocytes from freeze−thaw damage. Rubinsky et al.[116] have also reported that freezing mammalian livers with glycerol and AFP affords an additional level of cryoprotection. These mixed results suggest that possibly AFP and AFGP interact with membranes in a more complicated fashion than simply acting as a cryoprotectant. Hays et al.[117] have found that AFGP will inhibit leakage from liposomes during thermotropic phase transition. It is clear that much remains to be done in defining new role for these proteins.

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