Special Issue: Crystal Growth Controlled by Macromolecules

Journal of the Japanese Association for Crystal Growth
Vol. 35, No. 3 (2008)

Directly Probing the Antifreeze Protein Kinetics at the Ice/Solution Interface

Salvador Zepeda, Yukihiro Uda, and Yoshinori Furukawa

Antifreeze proteins (AFP) and glycoproteins (AFGP) help fish, plants, insects and bacteria survive sub-freezing environments. It is well known that these proteins function via some surface interaction, but the exact mechanism has eluded scientists. Aside from mutagenesis experiments directed towards examining the functional importance of specific residues, conclusions about the mechanism have been drawn from indirect studies or more precisely from studies that describe the proteins effects on the ice interface. Here, we will review recent work aimed at directly studying the protein kinetics at the ice interface. fluorescent microscopy is used to determine interaction planes, surface concentrations as well as adsorption characteristics, while fourier transform infra-red attenuated total reflectance (FTIR-ATR) is used to determine the protein structure vs. temperature in the liquid and solid states as well as the ice interface characteristics. Although the functions to some degree are the same, it becomes somewhat evident that the AFGP, AFP III, and spruce budworm AFP (sbwAFP) kinetics at the ice/solution interface, as well as the mechanism, can be rather different.

1. Introduction

Instead of avoiding sub-freezing environments, living things find ways to survive. Humans and other warm-blooded creatures simply bundle up or find some other means to stay warm. Some frogs[1] and turtle hatchling[2] are known to completely freeze during the winter months and avoid freeze damage by dehydrating essential body parts. Some microorganisms, through a set of complex cellular component adaptations, can thrive in sub-freezing conditions[3]. Fish produce antifreeze proteins or glycoproteins that adsorb to existing nuclei, effectively lowering their freezing temperature[4]. Currently, AF(G)Ps are used in some frozen foods[5] and are known to improve the storage of rat islets and human blood platelets[6, 7]. It is important from both a scientific standpoint as well as a technological one to understand the fundamental mechanism of AFPs in general and has been the focus of much research to date. The proteins act by adsorbing onto the interface and preventing ice crystal growth either by physically blocking some growth sites modifying the surface free energy or disrupting the transition region between the solid and the liquid. Aside from mutagenesis experiments, it can be argued that most experimental studies have described the AFP effect on the crystal morphology and not protein action itself. Many observations describing antifreeze action (freezing point depression) were made during growth or outside the temperature range of the main function and are used to infer which ice facet the protein interacts with, to some extent the binding characteristics and the mechanism. In order to more appropriately address the question of the mechanism, Pertaya *et al.*[8, 9] and Zepeda *et al.*[10, 11] have labeled antifreeze proteins with fluorescent labels to follow the AFPs during growth and inhibition. In addition Uda *et al.* have carried out FTIR-ATR measurements aimed at exploring the conformation of AFGPs while in the liquid, super-cooled and solid states of the water[12]. Although from previous studies differing mechanistic scenarios have emerged for the AF(G)Ps, the general consensus is that there is some general mode of action for all the proteins at the ice/solution interfaces. But one emerging feature of these direct studies is that the different AF(G)Ps while having nearly the same functions can have some strikingly differing protein kinetics, and hence different mechanisms, and each AF(G)P must be studied individually.

There exists an extensive body of literature with several reviews already written describing the general characteristics of the AF(G)Ps from evolutionary to a mechanistic aspects[4, 13-17]. Here we will only briefly summarize some main points as they pertain to the following discussion and then concentrate on recent developments made towards addressing the mechanism question by directly exploring the kinetics of AFPs to determine the protein incorporation, segregation, surface coverage, binding characteristics

Institute of Low Temperature Science, Hokkaido University, Kita-19, Nishi-8, Sapporo, Japan 060-0819
Correspondence should be addressed to : sz, E-mail : zepeda@lowtem.hokudai.ac.jp

and protein structure at the ice interface by fluorescent microscopy and FTIR-ATR spectroscopy.

2. General description of structure, function and mechanisms

2.1 Structures

Since the AFGPs were first identified in *trematomas borchgrevinki, bernachii* and *hansoni*[18], four other classes of non-glycosylated AFPs in fish[4, 19], plants[20], and bacteria[21, 22] as well as several distinct insect AFPs[23, 24] have been identified. The composition of the various AFPs although quite diverse generally all have a high content of hydrophobic residues[4]. Likewise the structures are quite diverse ranging from tight to extended flexible helices and globular forms. AFGPs are a repeating alanine-alanine-threonine tri-peptide unit with a disaccharide joined to the hydroxyl group of the threonine, ranging in weight from 2.6 kDa to 33 kDa (AFGP-8 to AFGP-1)[25] and can best be described as a highly flexible extended helix[26]. SbwAFP form a β-helix with a triangular cross-section and a repetitive array of threonines implicated in ice-binding[27]. The AFP III are a globular protein with a "flat" surface that has been identified to complement the prism plane of ice[28]. Despite the large differences between them to some extent they all accomplish the same functions.

2.2 Functions

There are three particular functions that characterize AF(G)Ps that make then unique. 1. They lower the freezing temperature of water by inhibiting the growth of existing nuclei. Seawater in the polar and near polar regions can drop to roughly 2 ℃ and while the fish can carry enough salt to lower their freezing temperature about 1 ℃, they rely on AFPs or AFGPs to keep them from freezing. Hence, their originally given name "antifreeze glycoproteins" and "antifreeze proteins." Interestingly, the melting temperature of AF(G)Ps solutions remains unchanged since the molar freezing point depression is negligible, their second given name "hysteresis proteins". 2. In addition, these proteins are known to highly modify the overall shape of the ice crystal, "ice structuring proteins" (ISPs), this name was also given to disassociate AF(G)Ps with the "antifreeze" ethylene glycol. Ice grows in a rough mode from the melt near the freezing temperature with the c/a axis growth ratio less than 1 giving the overall shape of a flat disc or flat dendritic crystal. In AF(G)P solutions, the ice crystal shape depends greatly on the concentration of AF(G)P and the supercooling, but growth usually switches to a layer-by-layer mode becoming highly faceted and at higher concentrations the c/a ratio can be greater than 1. Within the hysteresis region, the crystal will grow to take on a bipyramidal shape before completely stopping growth, these crystals are usually a few tens of microns at most. 3. Finally, the recrystallization inhibition (RI), or Ostwald ripening, function has emerged as perhaps the most important function due to its applicability in the frozen foods industry. At concentrations as low as a few μg/ml quantities, AF(G)Ps significantly reduce the recrystallization process of poly-crystalline samples. The grain coarsening process can damage flavor molecules and can significantly alter the taste and texture and is usually the factor that determines the shelf life of just about any frozen food. AFPs are currently used in some frozen foods in the US and the EU.

2.3 Mechanism

From early on it was know that only small amounts of protein are required to achieve the full activity (freezing point depression); the solution effect is small and it became clear that the proteins did not lower the freezing temperature in the way salt does, for example. Instead, it has become well accepted that the antifreeze effect is an anisotropic interfacial phenomenon. With DLS experiments Vesenka *et al.* found the AFGP effect to be more prominent in the prism planes when compared to the basal plane[29, 30]. The exact details have yet to be worked out and much work in answering the question of exactly how the proteins interact with the ice interface is ongoing. A better understanding of this could then lead to the design of more efficient antifreezes tailored to a specific function, instead of strictly relying on empirical evidence. For example, there may be certain compositions or configurations that can lead to a better RI effect with little freezing temperature lowering, ΔT_f, or vice versa tayloring proteins for specific functions can become an important asset to industry. Essentially, two distinct interpretations of how the protein interacts at the interface or near the ice interface have emerged.

2.3.1 Adsorption-inhibition

DeVries *et al.* first proposed an adsorption-inhibition mechanism whereby the protein molecules bind to the interface and growth is terminated at a curved interface between the pinned sites[18]. The driving force for lowering the freezing temperature comes from changes in the surface free energy brought about by this curved overgrowth, the Gibbs-Thomson (G-T) or Kelvin effect. Since then, workers have developed schemes where hydrogen-bonding capable planes on the proteins surface are identified and matched up with specific complementary ice planes[31, 32], although recently the focus has turned to other motifs where hydrophobic groups are thought to play the larger role in keeping the proteins bound to the ice[33-35]. These planes are

identified by either growing the ice crystal in AF(G)P solution within the hysteresis region until a faceted bipyramid forms or by locating macroscopically roughened surfaces on sublimation etched ice hemispheres decorated with AF(G)P[36]. The Type I AF(G)Ps, perhaps the simplest and the most studied, are an alanine rich and α-helical AFP with a 16.5 Å repetitive moiety that is matched up to the $\{20\bar{2}1\}$ planes along the $<01\bar{1}2>$ directions of the ice crystal[32]. It is generally understood that other AFPs follow a similar mechanism, but with different complimentary planes and binding moieties. Here, the proteins are required to bind permanently, leading some to adopt "ice-binding proteins" as another alias for AFPs. An equilibrium on-off type of scenario would allow water to creep under the proteins and attach to the ice interface resulting in a finite growth rate and propagation of the ice interface. If growth resumes by lowering the supercooling beyond ΔT_f, due to the strong binding the AFPs become incorporated into the bulk ice crystal. Ice hemispheres grown under high temperature gradients have been examined to confirm the inclusion of AFPs into the ice with values ranging from 10 % to over 100 % of the solution concentrations![37, 38] However, we must note that these ice hemispheres were grown below ΔT_f well outside of the functional temperature range in a rough growth mode and not a smooth one as known to occur within the hysteresis region. Additionally, in some of these studies the ice samples were polycrystalline[37].

2.3.2 Hydrophobic interaction within the transition region

However, this scenario has received much scrutiny over the last decade or so due much in part to the ambiguity of dealing with the ice/solution interface. While there is substantial experimental evidence of an ample surface melting or quasi-liquid layer (QLL) at the ice/vapor surface, ice/substrate interface as well as in the grain boundaries[39-43], directly probing the ice/water interface has proven to be a harder challenge. Molecular dynamics simulations predict at least a narrow transition region of about 10-15 Å whose properties gradually change from the solid to the liquid phase[44]. Experimental evidence suggests that the transition region must be on the same order as the calculation and highly anisotropic with respect to the basal and prism planes[45]. Recently, Wierzbicki *et al.* saw that the AFP I tends not to bind to the solid bulk at all, instead they found the protein within the transition region and closer to the liquid phase than the solid finding a balance with it' s highly amphiphilic content[46]. Additionally, it was seen that the hydrophobic surface, not the hydrogen bonding surface of the protein faces the ice. In contrast, Nada *et al.* found that a hydrophobic mutant, using a six-point water model[47, 48], has a much closer interaction with the bulk ice with the hydrophobic residues facing the ice and hydrogen bonding taking place with side serines[49]. Mutagenesis experiments aimed at determining the importance of the hydrogen bonding convincingly demonstrate the importance of the hydrophobic interaction. The Threonine (hydrogen bonding capable residues) were replaced with alanine (hydrophobic residues) and retained nearly all the native function, while replacements with serine (hydrogen bonding capable residues) lost all function[35, 50]. These experiments disproved the necessity of hydrogen bonding by way of the repetitive Threonine array that matched rather well with the ice surface and placed more emphasis on the electrostatic interactions. What has become clear is the importance of the hydrophobic contribution and the effect of the somewhat narrow transition region at the ice interface, leading many to question the "irreversible requirement" for AFP action.

3. Protein Incorporation and Segregation

The fundamental nature of the AF(G)P interaction should determine to what extent proteins are included in the solid and more so into the crystal matrix itself. The partitioning of proteins into the growing solid will depend on the binding strength as well as the protein kinetics relative to the crystal growth rates for example. This has been addressed to some extent, but not at the relevant growth rates and within the hysteresis region, as we will discuss below. One-directional growth experiments have been used quite extensively to examine the interfacial properties of the growing crystal with its melt and interacting solutes for several materials including ice. For example, Furukawa *et al.* have explored instabilities at the ice interface and determined the solute concentration profiles in the solvent, by using conventional bright field microscopy and interferometry[51]. These techniques are adequate to determine the gross morphology of the growing interface, but the solute concentrations and the concentrations derived from the kinetics must be large enough in order to detect any changes in the refractive index. On the other hand, fluorescent microscopy lends itself to exploring solution concentrations that are several orders of magnitude smaller and can map out the 3-dimensional distribution of AFPs within the solid.

Zepeda *et al.* have labeled AFGP-8, the smaller and less active of the AFGP (AFGP 1-8, MW: 33 kDa-2.6 kDa), with fluorescein isothiocyanate (FITC) to conduct fluorescent microscopy studies with a one-directional growth apparatus[10]. A growth cell made of two cover slips with a thin spacer is placed within a temperature gradient and is pulled towards the cold side to observe growth. At the slower

growth velocities, more relevant to the supercoolings that the fish may experience, no instabilities were found during the time frame studied, the interface remained flat, and the FITC-AFGP8 was expelled from the ice crystal. Additionally, protein accumulated at the interface, increasing the near interface effective solution concentration that is sampled to nearly 2 orders of magnitude greater than the original solution concentration of 0.1 mg/mL as seen Figure 1. This large concentration gradient creates a significant driving force for protein diffusion away from the interface into the solution and thus we can easily determine the diffusion coefficient. Our value of 1.0×10^{-10} m^2/s is in good agreement with previous NMR measurements, validating our technique[52].

At the faster growth rates, large instabilities develop at the interface and form sub-crystalline structures with the largest amounts of protein incorporated at the boundaries, measuring up to 3 orders of magnitude the initial solution concentration as seen Figure 2. Large pockets of protein are also seen within the solid ice regions. These features are present in both the fluorescent and the phase contrast images and thus correspond to protein that becomes trapped between the ice crystal and the glass cover-slide. Regardless of the growth velocity no protein is incorporated into the ice crystal. This is in contrast to the work by Knight *et al.* that measured a partitioning of between 0.1-0.15 for AFGP7-8[38]. However, trapped species can explain the results by Marshall *et al.* who found a partitioning coefficient of roughly 1 for the AFP II, AFP III and two insect AFPs[37]. As mentioned earlier, in the latter study the ice sample was polycrystalline and an effect similar to that seen in Figure 2 should have influenced the results making it impossible to distinguish between grain boundary inclusions and crystalline incorporation that are fundamentally different. One drawback from both the 1-directional growth and ice hemisphere growth experiments is the large temperature gradients.

On the other hand, free solution growth experiments under isothermal conditions and batch growth experiments on a smaller scale can more carefully examine these phenomena under a relevant supercooling. Pertaya *et al.* have labeled AFP III and sbwAFP with green fluorescent protein (GFP) to observe similar phenomenon in a batch growth apparatus[8, 9]. AFP solution was sandwiched between two cover slips and placed on a cold stage. Initially multiple crystals are nucleated and the entire solution is solidified at temperatures well below the freezing. The ice is then melted back to reduce the number of grains and then grown within the hysteresis region to form the typical hexagonal bypyramidal crystals shown in Figure 3a. Ice grown in

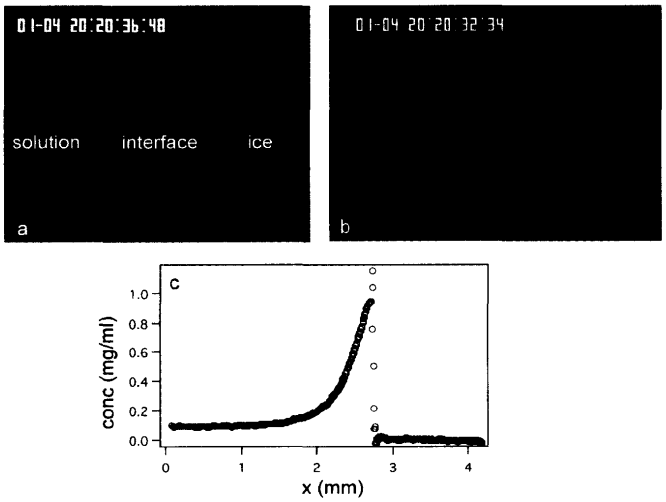

Fig.1 a) Phase contrast image of the solution ice interface of a 0.1 mg/ml sample grown at 0.5 μm/s in a 1-directional growth experiment with a temperature gradient G = 1.5 K/mm. b) Corresponding fluorescent image taken approximately 4 seconds after the phase contrast image. c) Concentration profile of the blue box in b). Each point represents the average intensity along the width of the box and x represents the position along the length of the box. Both images are 4.2 mm across. Reproduced with permission from Royal Society of Chemistry © 2007.

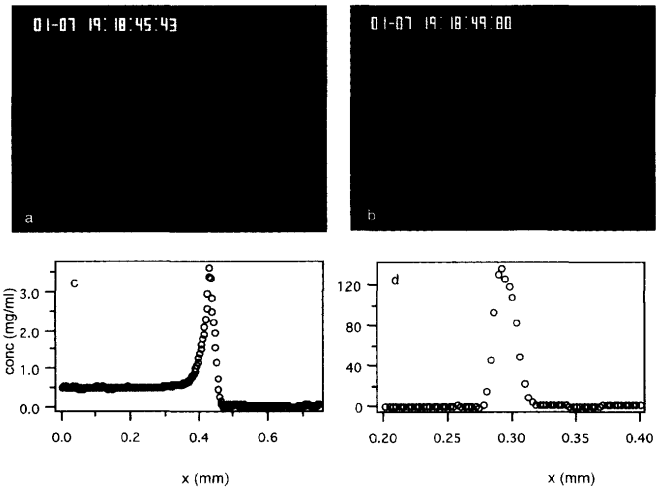

Fig.2 a) Phase contrast image of the solution ice interface of a 0.1 mg/ml sample grown at 5 μm/s. b) Corresponding fluorescent image taken approximately 4 seconds after the phase contrast image. c) and d) Protein distribution along the length of the blue and red rectangles in b), respectively. Each point in c) and d) represent the average intensity of the width of the rectangles in b. X indicates the position along the length of the box from left to right and from top to bottom of the blue and red rectangle, respectively. Reproduced with permission from Royal Society of Chemistry © 2007.

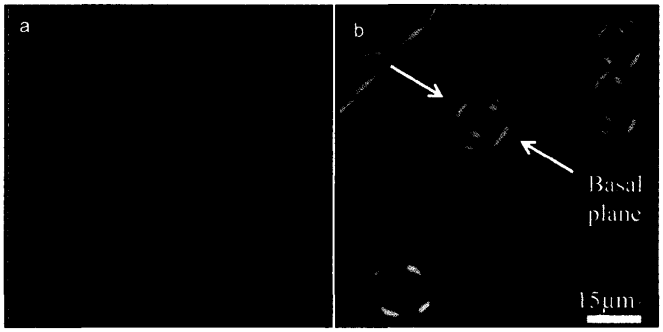

Fig.3 Hexagonal bipyramidal crystal from batch growth in GFP-AFPIII solution. The bright center of the crystal was grown from deeply supercooled solution, whereas the tips of the bipyramid were grown within the hysteresis region. In b, a crystal grown in GFP-sbwAFP with similar orientation. Note the high concentration of protein at the prism corners (bright spots midway between the basal planes) and basal planes (white arrows). The additional adsorption on the basal planes significantly increases antifreeze freezing point depression. Reproduced with permission from Figure 7 *Biophys. J*, (2007), *92, 3663-3673* © 2007 Biophysical Society and Figure 2 of *Biophys. J.* (2008), 95, 333-341 © 2008 Biophysical Society.

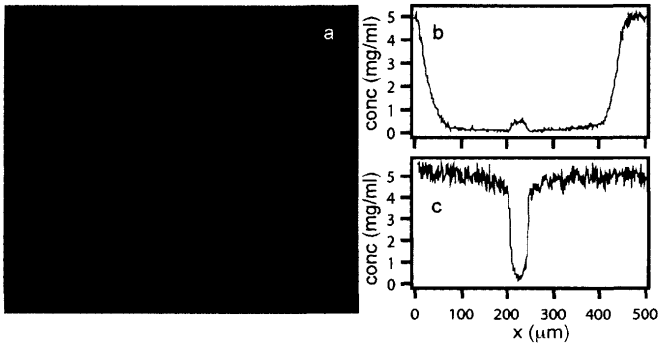

Fig.5 Fluorescent microscopy measurements of an ice crystal growing from a capillary into an isothermal solution of AFGP 2-5. The crystal was rotated 90° with respect to the images in Figure 6 to obtain a clear confocal section of the solid. The graphs in b) and c) represent the average concentrations along the red and blue rectangles without the background subtraction. Some edge effects are seen in b) but in the center the intensity drops to the background levels, 0.25 ± 0.08 μg/ml, as measured in c) along the glass capillary, 0.16 ± 0.07 μg/ml. Reproduced with permission from *Cryst. Growth Des.*, (2008), ASAP Article © 2008 Am Chem. Soc.

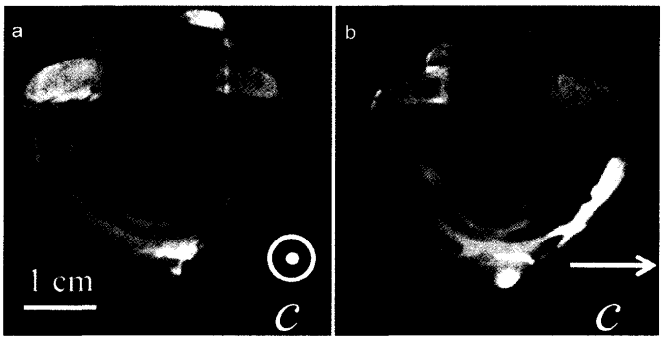

Fig.4 Ice hemisphere grown in GFP-AFPIII solution from a cold finger. The cold finger is visible in the center of the crystal. The c axis was oriented normal to the cold finger and is normal to the figure plane in a and parallel in b. The hemisphere was photographed under UV illumination. In a, the center of the crystal is transparent, while the sides (prism and pyramidal) directions are illuminated by the fluorescence of the adsorbed GFP-AFPIII. Reproduced with permission from Figure 4 of *Biophys. J.* (2008), 95,333-341 © 2008 Biophysical Society.

GFP-AFP III solution at large supercoolings formed many thin icicles and trapped much protein in between them before solidifying, seen as the bright center in the solid of Figure 3a. However, the subsequent growth of the grains within the hysteresis region did not show any detectable amounts of protein within the crystalline ice, but protein did accumulate at the interface. Furthermore, ice hemispheres, as described above, were grown with GFP-AFP III and when viewed from the basal plane appeared transparent or void of fluorescence figure 4a and only present when viewed perpendicular to the c-axis as seen figure 4b. The GFP-sbwAFP on the other hand, did not exhibit spicular formations and did not trap protein when grown below the freezing temperature, but did exhibit similar protein rejection when grown within the hysteresis region. Upon further growth below the freezing temperature, protein that accumulated at the interface was completely frozen into the ice crystal, but solute protein was not "efficiently" incorporated as the ice region appeared dark with protein being pushed away from the ice into the solution (see Figure 5 of Pertaya *et al.*[9]).

Zepeda *et al.* grew a single ice crystal from a capillary into a solution of FITC labeled AFGP 4-6 of concentration 5 μg/ml[11]. In these experiments the temperature was held at less than 0.05 ℃ and imaging was carried out during ice crystal growth and inhibition simultaneously, i.e. just around the freezing temperature, presumably due to small temperature variations formed by heat released during growth. The capillary was easily rotated and the fluorescent signal inside the ice was directly measured with confocal imaging showing no protein inclusion in the ice crystal as seen in Figure 5.

The proteins studied here represent a wide range of the different types of AF(G)Ps discovered thus far and none incorporate into the ice crystal when grown within the hysteresis region, but do so to some extent below the freezing temperature. However, incorporation into the solid

does occur in veins as trapped species, but this cannot necessarily be a measure of the binding strength of the protein, since they need not necessarily be adsorbed to an ice interface when trapped in the solid in this manner.

4. Protein adsorption and interface concentration

The interface concentration has never been verified by direct measurement as done here and this is essential since the freezing temperature lowering depends inversely on the density of the adsorbed species and is given by

$$\Delta T = \frac{2\,\Omega\gamma\,T_O}{\rho\,\Delta H_O},$$

where Ω is the molar volume of ice, γ is the surface free energy, T_O is the equilibrium freezing temperature, ΔH_O is the latent heat of fusion, and $2\rho = d$ is the spacing between the adsorbed molecules. Previously, Grandum *et al.* measured grooves on the ice surface using STM and attributed these to the adsorption sites of AFP I[53]. The spacing between grooves measured was d = 4-20 nm for AFP I solutions of concentration 2 mg/ml. By directly measuring the adsorbed protein density of the ice interface with fluorescently labeled FITC-AFGP 4-6 as seen in Figure 6, Zepeda *et al.* measured an interface density that corresponds to d = 21 nm ± 4 nm for FITC-AFGP 4-6 solutions of concentration 5 μg/ml[11]. And in batch growth experiments Pertaya *et al.* measured a spacing of d = 20 nm ± 5 for Type III AFP in solutions with concentrations ranging from 0.3-3 mg/ml[8]. For the GFP-sbwAFP studied, large amounts of protein were adsorbed onto the a-axis corners and onto the basal planes truncating the bipyramidal structure as seen in Figure 4b. The larger freezing point depression seen for the sbAFP has been attributed to basal plane adsorption not seen with other AFPs[54]. No molecular spacing was reported. For a spacing of 20 nm, the G-T model predicts a freezing temperature lowering of 5 ℃. Despite the large amount of adsorbed protein, the largest freezing point depression observed is roughly 1 ℃, contrary to the 5 ℃ predicted by the G-T model. It has been postulated that only the first proteins to adsorb are responsible for growth inhibition and subsequent over-adsorption does not play a role in the inhibition process[55]. This was verified by Zepeda *et al.* for FITC-AFGP 2-5, Growth modification occurred with no significant amount of protein adsorbed at the interface and full inhibition was seen to occur at similar interface coverage that followed several seconds later by the over adsorption to the final coverage stated above. The GFP-AFP III and GFP-sbwAFP also show a similar build up after growth has been stopped[8,9]. Interestingly, the adsorbed molecular spacing is

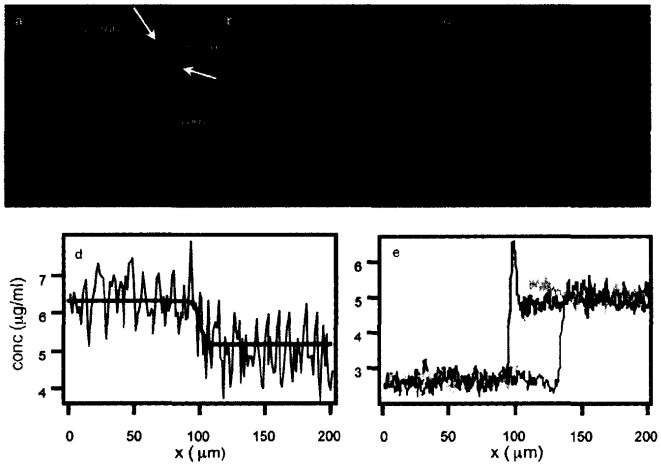

Fig.6 The concentration along the interface, blue dashed line in a), is shown graphically as the solid blue line in d). The black line is the fit to the data as a guide. At the arrows in a) new surface along the halted interface is formed. Growth continues in the [01-10] direction, when the interface reaches the apex, growth is halted and proceeds in the [1-100] direction, b). Protein adsorbs on the (10-10) face while the protein on the (1-100) face desorbs during growth. e) Average intensities along the length of the rectangles in a) - c), color-coded accordingly. The plots show adsorption at the interface (black), desorption and protein diffusing into the solution (gold), and protein distribution during growth (red) with the interface concentration at solution level. Additionally, in c) where the peak was in a), the concentration drops to levels as the rest of the solid showing removal of the adsorbed protein. Reproduced with permission from *Cryst. Growth Des.*, (2008), in press © 2008 Am Chem. Soc.

the nearly the same for the FITC-AFGP 2-5, AFP I and GFP-AFP III despite nearly 3 orders of magnitude of difference in the solution concentrations. The freezing temperature shows a strong non-linear dependence on the solution concentration and one would expect the surface coverage to show a similar dependence.

5. Reversible vs. irreversible binding for the adsorbed protein

Another point of debate has been whether the adsorbed protein responsible for inhibition is permanently or reversibly attached. Pertaya *et al.* explored this by photo-bleaching GFP-AFP III adsorbed on the stopped interfaces and monitoring for fluorescence recovery after photo bleaching (FRAP)[8]. Exchange between solute molecules would eventually lead to an increase in the fluorescent signal, but none was observed over the time frame studied, and hence they concluded the GFP-AFP III to exhibit a pseudo-permanent interaction with the ice. Hence, all the proteins, the proteins that initially inhibit the growth and those that adsorb

afterwards, bind with a similar affinity. While the over-adsorbed proteins are not necessary for inhibition, their interaction should be equivalent to proteins that initially halt the growth. The adsorption is not necessarily as depicted by Sander *et al.*[55], where the over-adsorbed proteins bind onto the curved surface between pinned sites. During batch growth experiments the adsorbed GFP-sbwAFP became "entombed" into the crystal during re-growth below the freezing temperature, although the solute was not incorporated. In the latter, the rejection of the solute is attributed to the protein kinetics required to find a fit onto the substrate as being slow compared to the growth rate of the ice crystal. On the other hand, Zepeda *et al.*, observed the FITC-AFGP 4-6 that was adsorbed onto the halted interfaces completely removed upon re-growth as shown in Figure 6. Additionally, in the following section we will see that AFGP 4-6 undergo a slight structural change before adsorbing onto the interface.

6. AFGP conformation at the interface

It is well known that the AFGPs are the most flexible and the only glycosilated class of AFPs and the overall structure can be characterized as an extended helix. While several studies exist of the protein conformation in solution and even in the frozen state, none have examined the changes near the freezing temperature and more in particular during warming from the solid to the liquid state. Nor has a correlation been made between the protein kinetics and the dynamics of the QLL experimentally. Uda *et al.* have carried out Fourier Transform Infra- Red Attenuated Total Reflection (FTIR-ATR), to examine exactly this[12]. A thin layer of protein solution was prepared on a ZnSe substrate. The sample was then cycled from 15 ℃ to −25 ℃ to observe the protein structure in the solution, supercoooled, and frozen states during cooling. Interestingly, there were no significant changes in the peak position or the relative contribution to the deconvoluted signal for the assigned structures when cooling from 15 ℃ down to −13 ℃ to the supercooled state. However, upon freezing the unordered and α-helix peaks decreased and increased in the relative peak area, respectively as shown Figure 7. That is the secondary structure of the AFGP 4-6 becomes slightly more ordered when adsorbed at the ice interface. Previous NMR measurements depicted many energetically similar solution conformations[26]. This flexibility can be advantageous in locking in the most efficient interaction.

In contrast to the abrupt change upon freezing, while warming the sample from the solid to the liquid state produced a gradual decrease in the α-helical content that approached the solution state conformation at the melting

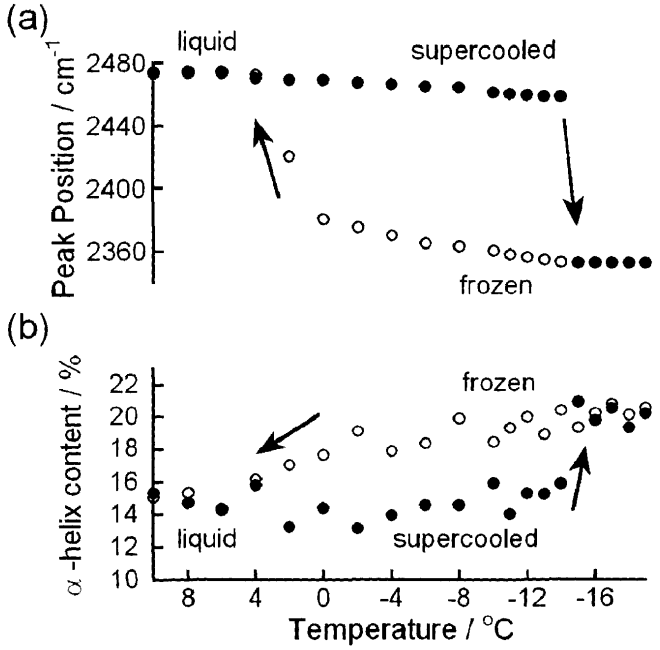

Fig.7 FTIR-ATR measurements of the protein structure of AFGP 4-6 at the ice/substrate interface. Plotted is the temperature dependence of the peak maximum position of the O-D stretching band of D_2O in (a) and the a-helical content of the AFGP in the AFGP/D_2O solution films in (b) during the cooling (solid circles) and heating (open circles) processes. Arrows indicate the gradual change during melting and the abrupt change during solidification. Reproduced with permission from *J. Phys. Chem. B* (2007), *111*, 14355-14361

point. This trend correlates very well with findings from previous studies that the QLL thickness increases gradually as one approaches the melting point from the frozen state and does so quite rapidly within a few degrees from the melting point[56]. To explain this QLL/protein structure correlation, the authors noted the possible contributions from the signal. The ice samples in this study are polycrystalline and the grain size were much larger than the penetration depth of the evanescent wave and thus most of the signal came from the ice/QLL/prism interface. At the lower temperature range studied the QLL thickness measured only a few nm constraining the protein to a rather narrow region. Above $\Delta T = 5$ ℃ the thickness of the QLL increases to several times the thickness of the protein diameter. The thickening QLL eliminates the physical constraints placed on the AFGPs and this is especially apparent at less than $\Delta T = 1$ ℃, where the QLL is nearly 15 nm and the protein conformation becomes nearly identical to the conformation in solution. The proteins gradually relax their conformation to the solution like conformation as more liquid becomes available.

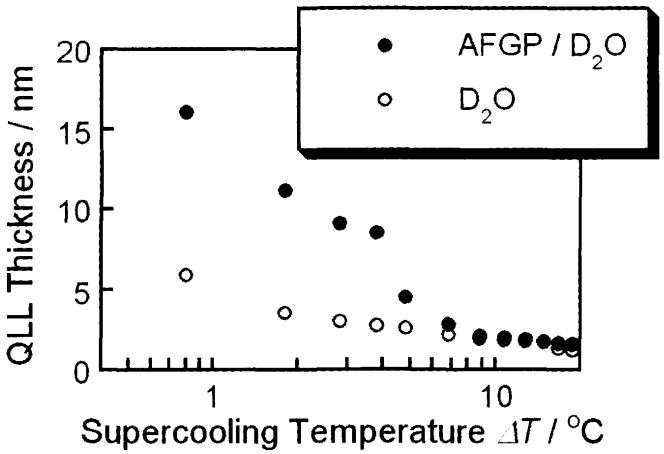

Fig.8 The QLL thickness between the ZnSe and ice in the pure phase and in AFGP 4-6 solution during the heating process. The spectra where no liquid signature was present was used as the baseline measurement. At higher temperatures this ice signature was subtracted and the remaining spectra was attributed to the QLL. Reproduced with permission from *J. Phys. Chem. B* (2007), *111*, 14355-14361

The QLL thickness was also seen to increase up to 3-fold in the presence of the AFGPs as seen in Figure 8. Through a simple thermodynamic argument, the authors note that this increase is consistent with a lowering of the surface free energy in solution when compared to the pure case. The wetting parameter is defined as $\Delta\gamma_{\infty} = \gamma_{is} - (\gamma_{il} + \gamma_{ls})$, where γ_{is}, γ_{il}, and γ_{ls} are the interfacial free energies for ice/substrate, ice/liquid, and liquid/substrate, respectively, at the ice/substrate interface. Since AFP solutions are known to have a lower surface free energy than that of pure water, we can expect the term in parenthesis to decrease. This enhances wetting at the interface, i.e. an increase in the QLL. A reduction in the surface free energy however, would result in surface roughening during growth, when the opposite is observed with all of the AFPs. Furthermore, the G-T model should produce a rough interface between the adsorbed species and we should expect a similar gross morphology not a faceted crystal. The results from this study indicate the strong correlation that exists between the proteins structural dynamics and the QLL dynamics as well as the tendency for the protein to be within the QLL. Although, the thermodynamics play a considerable role in the interaction at the interface, the mechanism responsible for antifreeze action must come from purely kinetic means.

7. Adsorption at a rough interface not smooth facets

We also note that the hemispheres grown in GFP-AFP III AFP solution by Pertaya *et al.*[9] necessarily have rough interfaces (Figure 4) and do not exhibit any well-defined planes similar to those formed during growth in the hysteresis region that are generally defined as the "ice-binding planes" of the AFPs. Nevertheless, significant adsorption does take place in the general directions perpendicular to the c-axis where one would expect a large prism direction step density while adsorption vanishes in the c-axis direction where no prism steps are presents. The protein/ice interaction could be described by anisotropic effect on the growth rates, and hence the kinetic coefficient, in the a- and c-axis. Similarly, describing the different shapes seen with varying concentration and supercooling: in this case a hemisphere, long ice needles, and pyramidal forms.

8. Summary

One drawback when comparing the protein kinetics here are the differences in concentrations and the supercoolings studied. It is likely that the proteins dynamics are comparable to that of the propagating ice interface and a crossover can occur within the temperature ranges studied. Additionally, the differences in the nature of the ice surface, i.e. QLL thickness, can vary dramatically near the melting point. Nevertheless, there are apparent similarities and differences between the proteins behavior at the surface that can be discussed. The FITC-AFGP are loosely bound where as the GFP-AFP III and GFP-sbwAFP lock on to the surface more strongly. The most notable difference is in the apparent binding strength or lifetime, when growth resumed below the freezing temperature the AFGPs were removed from the surface whereas GFP-sbwAFP became entombed into the crystal and the GFP-AFP III are "pseudo-permanently" bound. Despite the strong attachment by some of the proteins as thought to be required for the G-T model, there are several similarities between the proteins that are clearly inconsistent with the G-T model. 1. The surface concentrations are nearly identical for the FITC-AFGP, the AFP I, and GFP-AFP despite very different solution concentrations whereas the actual antifreeze freezing point depression is concentration dependent and different in these studies. 2. The $\Delta T_f \sim 5$ ℃ predicted by the G-T model from the observed protein surface coverage is much lower than observed experimentally. 3. Ice crystal growth in AFP solutions is smooth whereas the G-T model by nature would necessarily produce a rough morphology.

Previously, it has been understood that although the AF(G)P types can have drastically different composition and structure but rather similar functions, the mechanism, whether it be a permanent binding G-T scenario or one

where the protein disrupts the QLL, should be the same. However, this may not necessarily be the case and we look forward to further exploring the AFP kinetics by similar direct studies.

References

1) Costanzo, J.P., Bayuk, J.M. and Lee, R.E., Rana sylvatica, J. Exp. Zool., **284** (1999) 7-14
2) Costanzo, J.P. and Clausen, D.L., J. Exp. Zool., **254** (1990) 228-232
3) Margesin, R., Neuner, G. and Storey, K.B., Naturwissenschaften, **94** (2007) 77-99
4) Yeh, Y. and Feeney, R.E., Chem. Rev., **96** (1996) 601-617
5) Wang, J.H., Cryobiology, **41** (2000) 1-9
6) Matsumoto, S., Matsusita, M., Morita, T., Kamachi, H., Tsukiyama, S., Furukawa, Y., Koshida, S., Tachibana, Y., Nishimura, S. and Todo, S., Cryobiology, **52** (2006) 90-98
7) Inglis, S.R., Turner, J.J. and Harding, M.M., Applications of type I antifreeze proteins: Studies with model membranes & cryoprotectant properties, Curr. Protein Pept. Sci., **7** (2006) 509-522
8) Pertaya, N., Marshall, C.B., DiPrinzio, C.L., Wilen, L., Thomson, E.S., Wettlaufer, J.S., Davies, P.L. and Braslavsky, I., Biophys. J., **92** (2007) 3663-3673
9) Pertaya, N., Marshall, C.B., Celik, Y., Davies, P.L. and Braslavsky, I., Biophys. J., **95** (2008) 333-341
10) Zepeda, S., Uda, Y., Yokoyama, E. and Furukawa, Y., In Physics and Chemistry of Ice, Kuhs, W., Royal Society of Chemistry: Cambridge, (2007)
11) Zepeda, S., Yokoyama, E., Uda, Y., Katagiri, C. and Furukawa, Y., Crys. Growth Des., (2008), ASAP Article
12) Uda, Y., Zepeda, S., Kaneko, F., Matsuura, Y. and Furukawa, Y., J. Phys. Chem. B, **111** (2007) 14355-14361
13) Venketesh, S. and Dayananda, C., Crit. Rev. Biotechnol., **28** (2008) 57-82
14) Cheng, C.H.C. and Detrich, H.W., Philos. T.R. Soc. B, **362** (2007) 2215-2232
15) Moffatt, B., Ewart, V. and Eastman, A., Physiol. Plantarum, **126** (2006) 5-16
16) Harding, M.M., Ward, L.G. and Haymet, A.D.J., Euro. J. Biochem., **264** (1999) 653-665
17) Ewart, K.V., Lin, Q. and Hew, C.L., Cell. Mol. Life Sci., **55** (1999) 271-283
18) DeVries, A.L. and Wohlschlag, D.E., Science, **163** (1969) 1073
19) Deng, G.J., Andrews, D.W. and Laursen, R.A., Febs Lett., **402** (1997) 17-20
20) Atici, O. and Nalbantoglu, B., Phytochemistry, **64** (2003) 1187-1196
21) Gilbert, J.A., Hill, P.J., Dodd, C.E.R. and Laybourn-Parry, J., Microbiololgy, **150** (2004) 171-180
22) Kawahara, H., Iwanaka, Y., Higa, S., Muryoi, N., Sato, M., Honda, M., Omura, H. and Obata, H., Cryoletters, **28** (2007) 39-49
23) Graham, L.A., Liou, Y.C., Walker, V.K. and Davies, P.L., Nature, **388** (1997) 727-728
24) Graether, S.P. and Sykes, B.D., Euro. J. Biochem., **271** (2004) 3285-3296
25) DeVries, A.L., Komatsu, S.K. and Feeney, R.E., J. Biol. Chem., **245** (1970) 2901-2908
26) Lane, A.N., Hays, L.M., Tsvetkova, N., Feeney, R.E., Crowe, L.M. and Crowe, J.H., Biophys. J., **78** (2000) 3195-3207
27) Graether, S.P., Ye, Q.L., Davies, P.L. and Jia, Z.C., J. Struct. Biol., **126** (1999) 72-75
28) Chao, H., Sonnichsen, F.D., Deluca, C.I., Sykes, B.D. and Davies, P.L., Protein Science, **3** (1994) 1760-1769
29) Feeney, R.E., Fink, W.H., Hallett, J., Harrison, K., Osuga, D.T., Vesenka, J.P. and Yeh, Y., J. Cryst. Growth, **113** (1991) 417-429
30) Vesenka, J.P., Feeney, R.E. and Yeh, Y., J. Cryst. Growth, **130** (1993) 67-74
31) Wen, D.Y. and Laursen, R.A., J. Biol. Chem., **268** (1993) 16396-16400
32) Sicheri, F. and Yang, D.S.C., Nature, **375** (1995) 427-431
33) Wierzbicki, A., Dalal, P., Cheatham, T.E., Knickelbein, J.E., Haymet, A.D.J. and Madura, J.D., Biophys. J., **93** (2007) 1442-1451
34) Dalal, P., Knickelbein, J., Haymet, A.D.J., Sonnichsen, F.D. and Madura, J.D., Phys. Chem. Comm., **4** (2001) 32-36
35) Haymet, A.D.J., Ward, L.G. and Harding, M.M., J. Amer. Chem. Soc., **121** (1999) 941-948
36) Knight, C. A., Cheng, C. C. and DeVries, A. L., Biophys. J., **59** (1991) 409-18
37) Marshall, C.B., Tomczak, M.M., Gauthier, S.Y., Kuiper, M.J., Lankin, C., Walker, V.K. and Davies, P.L., Biochemistry, **43** (2004) 148-154
38) Knight, C.A., Driggers, E. and DeVries, A.L., Biophys. J., **64** (1993) 252-259
39) Wei, X., Miranda, P.B., Zhang, C. and Shen, Y.R., Phys. Rev. B, **66** (2002) 085401
40) Petrenko, V.F., J. Phys. Chem. B, **101** (1997) 6276-6281
41) Bluhm, H., Ogletree, D.F., Fadley, C.S., Hussain, Z. and Salmeron, N., J. Phys.: Cond. Matt., **14** (2002) L227-L233
42) Bluhm, H. and Salmeron, M., J. Chem. Phys., **111** (1999) 6947-6954
43) Furukawa, Y., Yamamoto, M. and Kuroda, T., J. Cryst. Growth, **82** (1987) 665-677
44) Hayward, J.A. and Haymet, A.D.J., J. Chem. Phys., **114** (2001) 3713-3726
45) Beaglehole, D. and Wilson, P., **97** (1993) 11053-11055
46) Wierzbicki, A., Dalal, P., Cheatham III, T.E., Knickelnein, J.E., Haymet, A.D.J. and Madura, J.D., Biophys. J., **93** (2007) 1442-1451
47) Nada, H. and Furukawa, Y., Journal of Crystal Growth, **283** (2005) 242-256
48) Nada, H., van der Eerden, J.P. and Furukawa, Y., Journal of Crystal Growth, **266** (2004) 297
49) Nada, H. and Furukawa, Y., Journal of Physical Chemistry B, **112** (2008) 7111-7119
50) Loewen, M.C., Chao, H.M., Houston, M.E., Baardsnes, J., Hodges, R.S., Kay, C.M., Sykes, B.D., Sonnichsen, F.D. and Davies, P.L., Biochemistry, **38** (1999) 4743-4749
51) Furukawa, Y. and Nagashima, K., J. Phys. Chem. B, **101** (1997) 6174-6176
52) Krishnan, V.V., Fink, W.H., Feeney, R.E. and Yeh, Y., Biophys. Chem., **110** (2004) 223-230
53) Grandum, S., Yabe, A., Nakagomi, K., Tanaka, M., Take-

mura, F., Kobayashi, Y. and Frivik, P.E., J. Cryst. Growth, **205** (1999) 382-390
54) Scotter, a.J., Marshall, C.B., Graham, L.A., Gilbert, J.A., Garnham, C.P. and Davies, P.L., Cryobiology, **53** (2006) 229-239
55) Sander, L.M. and Tkachenko, A.V., Phys. Rev Lett., **93** (2004) 128102
56) Sadtchenko, V. and Ewing, G.E., J. Chem. Phys., **116** (2002) 4686-4697

(Received August 21, 2008)