Antifreeze Glycoproteins Bind Irreversibly to Ice

Konrad Meister[1], Arthur L. DeVries[2], Huib J. Bakker[1] and Ran Drori*[3]

[1]NWO Institute AMOLF, Science Park 104, 1098 XG, Amsterdam, The Netherlands, [2]Department of Animal Biology, University of Illinois, Urbana, Illinois 61801, United States, [3]Department of Chemistry and Biochemistry, Yeshiva University, New York, New York 10016, United States

Supporting Information Placeholder

ABSTRACT: The irreversible binding of antifreeze glycoproteins (AFGPs) to ice has been questioned and remains poorly understood. Here we used microfluidics and fluorescence microscopy to investigate the nature of the binding of small and large AFGP isoforms. We found that both AFGP isoforms bind irreversibly to ice, as evidenced by microfluidic solution exchange experiments. We measured the adsorption rate of the large AFGP isoform and found it to be 50% faster than that of AFP type III. We also found that the AFGP adsorption rate decreased by 65% in the presence of borate, a well-known inhibitor of AFGP activity. Our results demonstrate that the adsorption rate of AFGPs to ice is crucial for their ice growth inhibition capability.

Antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs) collectively abbreviated as AF(G)Ps are a unique group of proteins that enable the survival of a wide range of organisms in freezing and subfreezing habitats[1-2]. Their common characteristic is the depression of the freezing temperatures of an ice crystal without significantly affecting its equilibrium melting point. This phenomenon leads to a difference between the freezing and melting temperature, which is termed thermal hysteresis (TH) and is commonly used to detect and quantify antifreeze activity. AF(G)Ps also affect the morphology of ice crystals and inhibit ice recrystallization, two properties that make them highly interesting for food and biomedical applications[3-4].

All AF(G)Ps are assumed to function by an adsorption-inhibition mechanism, in which the proteins irreversibly bind to embryonic ice crystals and thereby prevent macroscopic ice growth[5]. A central premise in the adsorption-inhibition mechanism is the irreversible binding of AF(G)Ps to ice, which means that AF(G)Ps will remain bound to the ice surface until melting occurs. The irreversible nature of AF(G)P binding has been questioned as it is difficult to reconcile with the experimentally observed concentration dependence of thermal hysteresis[6]. Compelling evidence for irreversible binding of non-glycosylated AFPs to ice was provided recently by using fluorescently-labeled AFPs and microfluidic devices[7-9]. For AFGPs, however, both experiments and molecular dynamics simulations reported reversible binding[10-11]. Using confocal fluorescence microscopy, Zepeda *et al.* showed that the binding of a mixture of $AFGP_{4-6}$ to ice is reversible and that the proteins are not incorporated into ice crystals grown at low supercooling (<0.05 °C)[11]. Mochizuki and Molinaro reported reversible binding of AFGP to ice based on a calculated weak binding free energy of $AFGP_8$ and further suggested that binding occurs via the hydrophobic groups of the protein, rather than the hydroxyl groups of the disaccharide[10].

AFGPs have a special role among all identified AF(G)Ps. They were the first to be discovered, occur in several natural isoforms and have a fully flexible solution structure. AFGPs further show a capacity to inhibit ice recrystallization that is orders of magnitude greater than other AFPs[12], which makes them the primary target for the development of synthetic analogues[13-14]. A typical AFGP consists of the repeating tripeptide unit as shown in the inset of Figure 1. AFGP isoforms are typically grouped into size classes with $AFGP_1$ being the largest and $AFGP_8$ the smallest. A simplified version groups them into large ($AFGP_{1-5}$ and small $AFGP_{7-8}$ isoforms with the latter having 60% of the activity of the large on a weight basis. The reason for the much higher activity of $AFGP_{1-5}$ has not been resolved to date. Here we study the ice binding interactions of large ($AFGP_{1–5}$) and small ($AFGP_{7–8}$) isoforms using fluorescence microscopy and microfluidic devices.

Antifreeze glycoproteins were purified from the Antarctic toothfish *Dissostichus mawsoni* and fluorescently labeled using fluorescein isothiocyanate (FITC)[15]. The concentration of AFGPs in the microfluidic channel was determined using the fluorescence intensity, which was calibrated prior to the experiments (Figure S1). The irreversibility of AFGP binding to ice was tested by performing solution exchange experiments using microfluidic devices. Details of the experimental setup can be found in the supplementary information and were described elsewhere[16]. In a typical experiment, double-distilled water was injected into the microfluidic channel and the temperature was lowered until the liquid froze. The temperature was then increased to melt the bulk ice and an IR laser was used to melt all other ice crystals except a single ice crystal with a diameter of ø~30-50 μm. Then an AFGP solution was injected into the microfluidic channel, and the remaining single ice crystal was melted to form an even smaller crystal (ø~20 - 30 μm). The temperature was then lowered to 0.02 – 0.06 ºC below the melting point of the crystal, and the crystal grew into a hexagonal bipyramid.

After a few minutes, the AFGP solution around the crystal was then replaced with water and the fluorescence intensity in the solution and on the ice crystal was monitored. Following exchange of almost all of the AFGP solution, the temperature was decreased at a fixed rate (0.075 °C/min) until a crystal burst was recorded.

Figure 1 presents the results of the solution exchange experiments for the large $AFGP_{1-5}$ (A-D) and small $AFGP_{7-8}$ (E-H) isoforms.

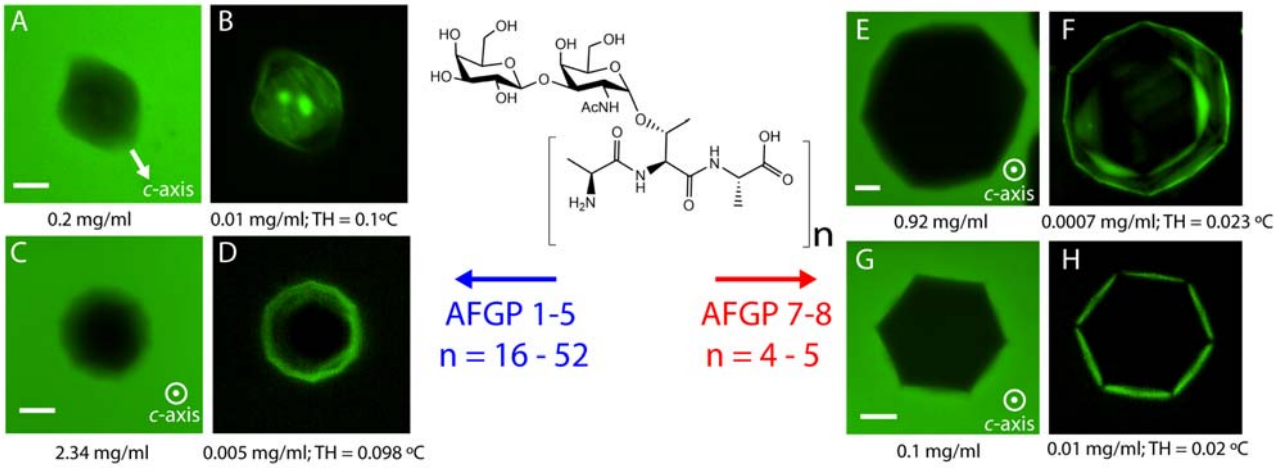

Figure 1 - Irreversible binding of large $AFGP_{1-5}$ (A-D) and small $AFGP_{7-8}$ (E-H) isoforms to ice. Images of ice crystals before (A, C, E, G) and after (B, D, F, H) the AFGP solution was exchanged with pure water. The AFGP concentration before and after the solution exchange is indicated, along with the measured TH activity. The inset shows the chemical structure of a typical AFGP repeat unit. Scale bar = 10 µm.

Experiments were performed using ice crystals that obtained a characteristic bipyramidal shape (thereby possess small basal surfaces) and were large enough (>20 µm) to remain in the microfluidic channel during the solution exchange (Figure 1A, B). Such crystals remained stable at 0.02 - 0.06 ºC below their melting point after the AFGP solution was removed. In other experiments, the ice crystals were oriented with their *c*-axis perpendicular to the microfluidic channel (Figure 1C-H), which physically inhibited growth of the basal plane[8], to which AFGPs cannot bind (Figure S2).

Both AFGP isoforms were found to bind irreversibly to ice, as demonstrated by the inhibition of ice growth at very low AFGP concentrations (<0.01 mg/ml) and at temperatures of >0.02 ºC below their melting point.

To demonstrate that the adsorbed AFGPs can inhibit ice growth in the near complete absence of the AFGPs in the surrounding solution, separate TH measurements of AFGP solutions were made at concentrations, that were the same as the residual concentrations, the latter determined by fluorescence intensity. Without solution exchange, the TH activity of $AFGP_{7-8}$ at 0.01 mg/ml was 0.007ºC, while after solution exchange the thermal hysteresis was 0.02 ºC at the residual concentration of 0.01mg/ml (Fig 1H). The TH activity of $AFGP_{1-5}$ at 0.01mg/ml without exchange was 0.03 ºC, which was only 33% of the 0.1ºC of hysteresis observed after solution exchange (Fig 1B). In another experiment (Fig 1C, D), the ice crystal was kept for 60 min at a supercooling of 0.06 ºC after the AFGP $_{1-5}$ solution was mostly replaced with water. No growth was observed during this period and the TH was 0.098 ºC at a residual concentration of 0.005 mg/ml (Fig. 1D). Altogether, these observations provide compelling evidence that AFGPs bind irreversibly to ice, and no desorption of proteins from the ice surface was evident at timescales up to an hour. After establishing that both AFGP isoforms bind irreversibly to ice, the adsorption rate of $AFGP_{1-5}$ to ice was measured. Knight and DeVries hypothesized that adsorption rates determine the TH of AF(G)Ps that cannot bind to basal surfaces[17]. To date, the only experimentally determined adsorption rate of an AF(G)P to ice was measured for AFP type III[7].

Adsorption rate measurements of $AFGP_{1-5}$ were obtained using a similar protocol as described previously[7]. These measurements were performed with AFGP concentrations from 0.01-0.2 mg/ml and 4-8 crystals were measured for each concentration (see SI text for further details). To fit the data, the following equations[7, 18-19] were used:

Eq. 1 $$\frac{d\theta}{dt} = k_{on}\,(1-\theta)c - \,k_{off}\theta$$

where θ is the surface coverage of AFGPs at time t, k_{on} and k_{off} are the adsorption and desorption constants, respectively, and c is the AFGP solution concentration in mg/ml. We assumed that AFGPs do not desorb from the surface, thus $k_{off} = 0$. The surface coverage θ is proportional to the fluorescence intensity on the ice surface, leading to equation 2, which is obtained by integration:

Eq. 2 $$I = I_{max}(1 - e^{-(\frac{t}{\tau})}$$

where I is the fluorescence intensity at time t, I_{max} is the maximum intensity obtained and τ is a time constant ($\tau = \frac{1}{k_{on}c}$). The adsorption rate k_{on} is the slope of the linear curve of the AFGP concentration vs. the inverse of the time constant, τ, and was determined to be k_{on} = 0.31 ± 0.034 mg/ml $^{-1}$ s $^{-1}$ (Figure 3). The adsorption rate for $AFGP_{1-5}$ is 50% faster than the adsorption rate of AFP type III[8]. To investigate further whether the adsorption rates of $AFGP_{1-5}$ determines the TH, 0.3 M of borate was added to the $AFGP_{1-5}$ solutions. Borate molecules are known to greatly reduce the TH activity of AFGP by forming complexes with the cis-hydroxyl groups of the disaccharide, thereby hindering AFGP binding to ice[20]. The adsorption rate of $AFGP_{1-5}$ in the presence of borate was found to be k_{on} = 0.11 ± 0.047 mg/ml $^{-1}$ s $^{-1}$, which is 33% of the adsorption rate of the pure $AFGP_{1-5}$. Hence, the diminished $AFGP_{1-5}$ adsorption rate in the presence of borate can explain the much lower, but still measurable, TH activity[20-21]. FTIR measurements[22] indicate that the secondary structure of $AFGP_{1-5}$ is not affected by the addition of borate (Figure S3). For $AFGP_{7-8}$, the adsorption rate could not be determined experimentally, as no clear accumulation on ice could be observed despite its irreversible binding to ice. This observation might seem a contradiction, which could be resolved assuming that $AFGP_{7-8}$ molecules adsorb slower to ice compared to $AFGP_{1-5}$ (as expressed by lower TH activity of the former), thus even at slow ice growth velocities (6 µm/sec) $AFGP_{7-8}$ is being overgrown by the advancing ice front, although some molecules are pushed away from the ice front into the solution (Figure S4A, B). When the $AFGP_{7-8}$ solution was replaced by water (Figure 1), or when the ice crystal was completely overgrown by bulk ice (Figure S4C and S5), ice-adsorbed $AFGP_{7-8}$ molecules were clearly

observed. Thus, the lack of observed accumulation of $AFGP_{7-8}$ on ice stems from slow adsorption rate that leads to engulfment, and by the high fluorescence signal from the solution that masks the adsorbed $AFGP_{7-8}$ molecules.

Arguably, the most debated topic is whether AFGPs bind irreversibly or reversibly to ice. We report conclusive evidence that both small and large AFGP isoforms bind irreversibly to ice. These findings are in line with results for the AFP type III from fish that also primarily targets the prism planes of ice. We propose certain criteria for moderate AF(G)Ps that target non-basal ice planes, which are part of a universal working mechanism. Criteria include (I) the capability to quickly bind to forming prism planes and (II) to prevent the growth of these planes and thereby minimizing the size of the basal planes, to which these proteins cannot bind.

The adsorption rate for $AFGP_{1-5}$ is higher compared to the globular AFP type III, which might suggest that at temperatures close to 0 °C, $AFGP_{1-5}$ has a defined secondary structure as previously suggested [10,23-24]. Our adsorption rate measurements further reveal an experimental link between TH activity and adsorption rate in the presence of borate, which is known to reduce the TH activity of AFGPs[20]. This effect stems from the significant reduction of the adsorption rate of $AFGP_{1-5}$ to ice. We suggest that borate binds to the disaccharides and that the resulting complex has a lower k_{on}, thus TH activity is reduced. This finding suggests that the AFGP-ice interaction occurs via the disaccharide sidechains rather than the hydrophobic groups[10] associated with the peptide backbone. In fact, a good match between the spacing of the hydroxyl groups on the sugars and the prism plane of ice does exist based on modeling of AFGP structures[25].

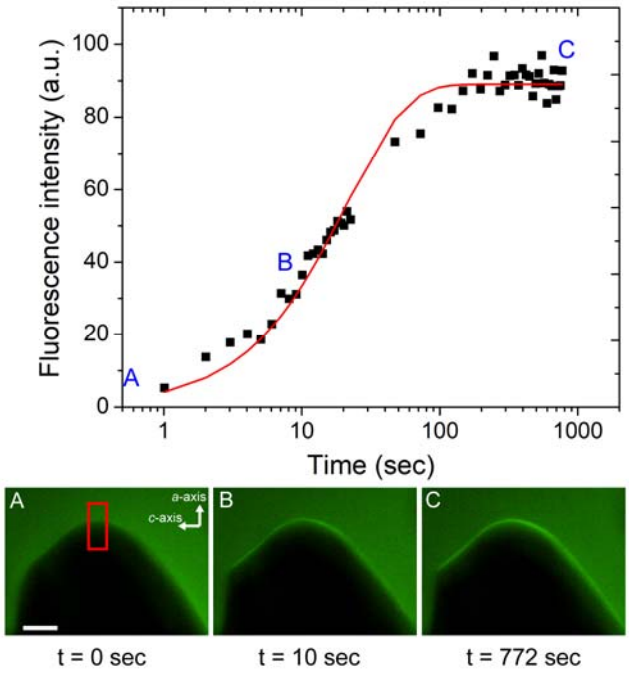

Figure 2 - Accumulation of $AFGP_{1-5}$ on non-basal surfaces. Measurements were initiated when melting of the ice crystal has ceased (A) and AFGPs adsorbed to the surface (B, taken 10 sec after A). The crystal was annealed at 0.01 °C below its melting point for 12 min (C) and the fluorescence intensity on the non-basal surfaces was recorded (maximum intensity inside the red rectangle in A). Scale bar = 15 µm.

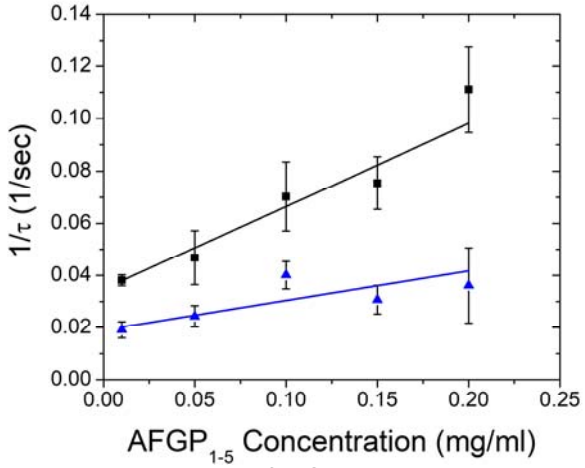

Figure 3 - Adsorption rates of $AFGP_{1-5}$ to ice. The dependence of the inversed time constant, τ, on $AFGP_{1-5}$ solution concentration was used to determine adsorption rates (k_{on}) of pure $AFGP_{1-5}$ (black squares) and $AFGP_{1-5}$ with 0.3 M borate (blue triangles). The slopes of the linear curves were k_{on} = 0.31 ± 0.034 mg/ml^{-1} s^{-1} for pure $AFGP_{1-5}$ and 0.11 ± 0.047 mg/ml^{-1} s^{-1} for $AFGP_{1-5}$ with borate. Each data point includes 4-8 ice crystals.

In summary, we report three important findings involving the interaction between AFGPs and ice surfaces; 1) both AFGP isoforms bind irreversibly to ice as indicated in the AFGP solution exchange experiments; 2) the adsorption rate of $AFGP_{1-5}$ is 50% faster than that of AFP type III and 3) the AFGP-borate complex can bind to ice, but its adsorption rate is decreases by 65% compared to pure AFGPs.

ASSOCIATED CONTENT

Supporting Information

Experimental setup.

Microfluidic solution exchange experiments

Adsorption rate measurements

Figure S1 – The fluorescence intensity calibration.

Figure S2 - Fluorescence intensity of the ice/water interface.

Figure S3 - Effect of borate on the secondary structure of $AFGP_{1-5}$.

Figure S4 and S5 – Ice growth dynamics in the presence of $AFGP_{7-8}$.

AUTHOR INFORMATION

Corresponding Author

*rdrori@yu.edu

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