Dynamics of the Hydration Water of Antifreeze Glycoproteins

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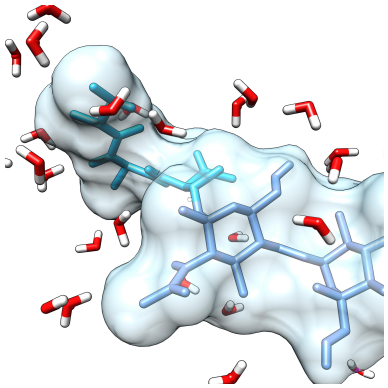
Abstract

Antifreeze glycoproteins (AFGPs) are unique proteins that inhibit the growth of ice by a mechanism that is still unclear. We study the dynamics of water in aqueous solutions of small and large isoforms of AFGPs using polarization-resolved femtosecond infrared spectroscopy. We find that a fraction of the water molecules is strongly slowed down by the interaction with the antifreeze glycoprotein surface. The fraction of slow water molecules scales with the size and concentration of AFGP, and is similar to the fraction of slow water observed for non-antifreeze proteins, both at room temperature and close to biologically relevant working temperatures. We observe that inhibiting AFGP antifreeze activity using borate buffer induces no changes in the dynamics of water hydrating the AFGP. Our findings support a mechanism in which the sugar unit of AFGP forms the active ice-binding site.

TOC graphic



Keywords

Antifreeze glycoproteins, water dynamics, infrared pump-probe spectroscopy, borate

Antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs) are classes of proteins that suppress ice crystal growth in organisms, thereby enabling their survival in freezing and subfreezing habitats.[1,2] The success of AF(G)Ps as an efficient protection against freezing can be witnessed by their wide distribution among biological kingdoms. AF(G)Ps from different species have evolved independently and show a great diversity in structures. Despite their structural heterogeneity, all AF(G)Ps are believed to work via an adsorption-inhibition mechanism in which the molecules recognize and irreversibly bind to embryonic ice crystals and prevent the macroscopic ice growth.[3]

Among all identified AF(G)Ps, the AFGPs play a unique role. AFGPs were the first antifreeze proteins discovered and are subject to considerably less structural variations than AFPs. A typical AFGP consists of the repeating tripeptide unit (alanyl-alanyl-threonyl) in which the secondary hydroxyl group of the threonine residue is glycosylated with the disaccharide β-D-galactosyl-(1,3)-α-D-N-acetylgalactosamine, as shown in figure 1. The molar mass of AFGPs varies between 2.6 and 33.7 kDa, which corresponds to 4 to 50 repetitions of the glycosylated tripeptide unit. The AFGP isoforms can show minor sequence variations and are typically grouped into size classes, with $AFGP_1$ representing the largest and $AFGP_8$ the smallest.

Interestingly, neither the solution structure nor the ice-binding site of AFGPs have been identified conclusively.[4,5] Another property of AFGPs that is far from being understood is their tremendous capacity to inhibit ice recrystallization (IRI), a process that is highly relevant for several industries and medical applications. AFGPs prevent the growth of large

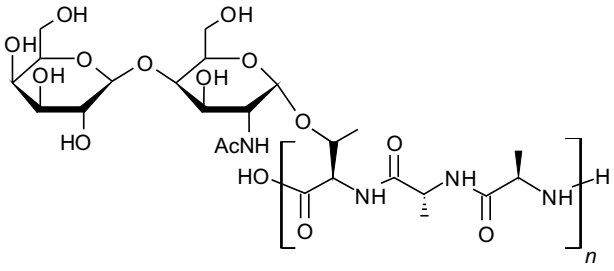


Figure 1: Chemical structure of a typical antifreeze glycoprotein (AFGP) repeat; $n = 4 - 50$.

ice crystals at the expense of small ones (i.e. Ostwald ripening) several magnitudes better than any other AFP or known ice recrystallization inhibitor.[6,7] AFPs and AFGPs have a high specific affinity for the solid phase of water over the liquid form that is usually present in vast excess. It is now widely accepted that the capacity of many AFPs to bind to ice involves the hydration shell of the AFPs as an active player. Direct experimental evidence for the involvement of water molecules in the mode of action of AFPs was found in the X-ray crystal structures of several AFPs[8,9] and in advanced spectroscopic studies,[10] which identified preordered "ice-like" water layers around the active ice-binding site (IBS) of AFPs, even at room temperature. These findings are consistent with the hypothesis that some AFPs bind to ice because their hydration shell provides a structural match to a particular ice crystal face. These findings were further confirmed by molecular dynamics simulation for most classes of AFPs.[11–14]

For AFGPs, the involvement of hydration water is less clear. A specialized hydration shell is not necessarily expected for the AFGPs, as the hydroxyl groups of the AFGP sugar moieties have a spatial orientation that appears to match to oxygens on the prism plane and thus bind directly to the ice lattice.[15] Early viscosity, translational diffusion, and NMR experiments showed that the amount of water affected by AFGPs is not significantly different from the amount of water affected by other glycoproteins.[5] However, terahertz spectroscopy[16] and MD simulations[17,18] provided evidence for a considerable long-range effect of AFGPs on the dynamics of hydration water that seems to correlate with antifreeze activity.

Here we study the hydration dynamics of large ($AFGP_{1-5}$) and small ($AFGP_{7-8}$) isoforms of antifreeze glycoproteins using polarization-resolved femtosecond infrared spectroscopy. This technique directly probes the picosecond reorientation dynamics of water molecules, via the decay of the anisotropy of the vibrational excitation. We perform experiments both at room temperature and at temperatures close to the biologically relevant working temperature of the glycoproteins. We also investigate the effect of the inhibitor borate on the interaction between AFGPs and water.

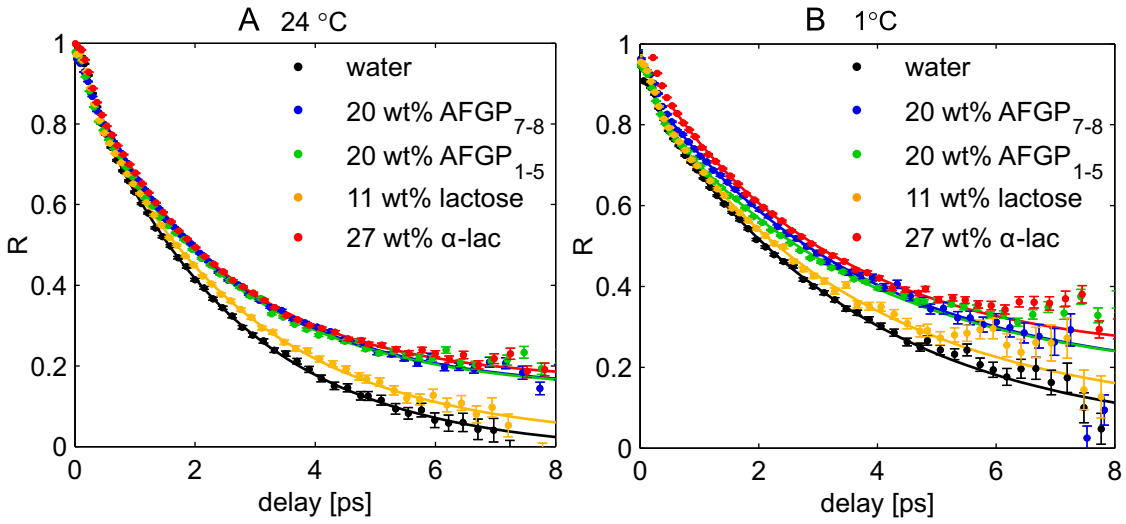


Figure 2: **Water reorientation dynamics in solutions of AFGPs, α-lactalbumin and lactose.** Anisotropy decay of the OD stretch vibration for isotopically diluted water, and for solutions of 20 wt% $AFGP_{7-8}$, 20 wt% $AFGP_{1-5}$, 11 wt% lactose and 27 wt% α-lactalbumin in isotopically diluted water, at 24 °C (A) and 1 °C (B). The solid lines are fits to a single exponential with an offset R_{slow}.

Figure 2A presents the measured anisotropy decay for water and aqueous solutions of $AFGP_{7-8}$, $AFGP_{1-5}$, lactose and α-lactalbumin at room temperature (24 °C). For pure water, the anisotropy decays exponentially with a time constant of 2.45 ± 0.15 ps. This means that water molecules reorient on a 2.45 ps timescale, as reported earlier.[19,20] Upon the addition of proteins to the solution, an additional slow component appears in the anisotropy decay. Since the time constant of this component is larger than our experimentally accessible time window of 8 ps, we can model this component as an offset. Hence, we fit the anisotropy decays measured for the different solutions with the following expression: $R(t) = R_0 e^{-t/\tau_{reor}} + R_{slow}$. The results are shown as solid lines in figure 2. For all AFGP solutions, we find that the time constant τ_{reor} of the exponential component stays within 2.45 ± 0.15 ps, which means that the water molecules that are not contained in the slow component reorient with the same rate as in bulk water. Using the experimentally determined slow fraction R_{slow}, and the protein concentration c, we can calculate the average number of slow hydroxyl groups per AFGP repeat unit N_{slow} . We find an average of 35 ± 6 slow hydroxyl groups per AFGP

tripeptide repeat for both the smaller and larger AFGPs, indicating that the local water dynamics are similar for the different AFGP isoforms.

The 35 ± 6 slow hydroxyl groups per AFGP repeat unit may represent water molecules being slowed down by the amino acid residues, water molecules slowed down by the sugar unit, and the response of the hydroxyl groups of the sugar unit itself. To get an estimate of the latter two contributions, we measured the water reorientation dynamics in solutions of the sugar lactose. Lactose contains the same β-D-galactopyranosyl group as the saccharides present in the AFGP sugar moieties, and its concentration is chosen such that it matches the concentration of AFGP sugar groups by weight. As seen in figure 2A, the anisotropy decay for the lactose solution shows only a moderate slowdown compared to bulk water; the slow water fraction is much smaller than the slow water fraction that is observed for AFGP solutions. This observation indicates that most of the water molecules that exhibit slow reorientation dynamics in AFGP solutions are not slowed down by the AFGP sugar groups, but rather by the AFGP peptide backbone. The reorientation time for the lactose solution is 2.65 ± 0.15 ps, which means that most water molecules reorient only slightly slower than in bulk water. The anisotropy offset observed for the lactose solutions corresponds to 12 ± 4 slowly reorienting hydroxyl vibrations, which can in part be associated with slow water molecules and in part be associated with the hydroxyl groups of the lactose itself. This result implies that the 35 ± 6 slow hydroxyl groups that are slowed down in their reorientation per AFGP repeat unit largely represent water molecules that are slowed down by the three amino acid residues of this unit.

We thus conclude that a total number of ∼23 hydroxyl groups are slowed down by the three amino acid residues (corresponding to ∼8 hydroxyl groups per residue, or ∼4 water molecules), and that the remaining 12 slow hydroxyl groups are associated with water slowed down by the sugar unit and the response of the sugar unit itself. It should be noted that the above description of the effect of sugars on the reorientation dynamics of water differs from the description we presented before.[21,22] The anisotropy decay we measure for solutions of

lactose is very similar to the anisotropy decay measured previously for other sugar solutions. However, here we used a simpler description of the anisotropic response to enable a direct comparison of the response of the sugar unit with that of the three amino acid residues of the AFGP repeat unit.

We also compare the slow reorientation component of AFGP solutions with that of other protein solutions. For the non-glycosylated protein α-lactalbumin, we find a reorientation time constant of 2.45 ± 0.15 ps, and an offset that corresponds to 16.5 ± 1.2 slowly reorienting hydroxyl groups per 3 amino acid residues, equivalent to 2.75 ± 0.2 slow water molecules per residue for the protein in its folded state, in agreement with previous studies.[20] In its unfolded state, 4.6 ± 0.5 water molecules are slowed down per amino acid residue of α-lactalbumin.[20] The latter number is close to the 4 water molecules per residue that are slowed down by the AFGP peptide backbone. The small difference can be explained from the somewhat larger average size of the amino acid side-chains of α-lactalbumin.

We further studied the water reorientation dynamics in the same solutions at 1 °C (fig. 2B), a temperature that is closer to the biologically relevant working temperature of AFGPs. At 1 °C, the anisotropy of pure water decays with a time constant of 3.5 ± 0.3 ps for water. This reorientation time constant stays the same for all solutions. The number of slow hydroxyl groups per repeat unit, presented in table 1, is clearly higher at 1 °C compared to 24 °C, but follows the same trend when comparing different protein and sugar solutions.

Table 1: Number of slowly reorienting hydroxyl groups N_{slow} per AFGP tripeptide repeat unit, per 3 amino acid residues (for α-lactalbumin) or per molecule (for lactose), at 24°C and 1°C.

	N_{slow} at 24°C	N_{slow} at 1°C
$AFGP_{7-8}$	35 ± 6	40 ± 6
$AFGP_{1-5}$	35 ± 6	38 ± 6
Lactose	12 ± 4	18 ± 6
α-lactalbumin	16.5 ± 1.2	24 ± 9
α-lactalbumin unfolded[20] (in 12 mol/kg urea)	28 ± 3	-
$AFGP_{7-8}$ + borate	35 ± 6	37 ± 6

Consequently, the effect of AFGPs on the dynamics of water depends on temperature in the same way as the effect of non-antifreeze proteins and sugars.

To investigate further whether the dynamics of hydration water are correlated with antifreeze activity, we inhibit the antifreeze activity by adding borate. Borate molecules can bind reversibly to diol-containing compounds such as sugars, and it has been suggested that borate interacts with AFGP by binding to the cis-hydroxyl groups of the β-D-galactopyranosyl group. This binding greatly reduces the antifreeze activity.[1,23] Figure 3 presents the anisotropy decay for solutions of the smaller isoform AFGP7-8 in water and in 0.3 M borate buffer, at 1 °C and 24 °C. At both temperatures, we find that the addition of borate does not change the anisotropy decay within the error bars. Thus we conclude that the loss of antifreeze activity upon addition of borate is not correlated with a change in dynamics of the water molecules hydrating AFGP.

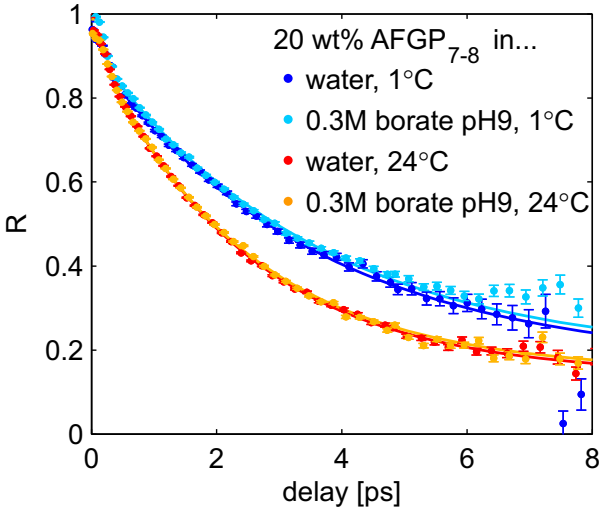


Figure 3: **Effect of borate on the water reorientation dynamics.** Anisotropy decay of the OD stretch vibration for solutions of 20 wt% $AFGP_{7-8}$ in isotopically diluted water and in isotopically diluted 0.3 M borate buffer at pH = 9.0, at 1 °C and 24 °C. The solid lines are fits to a single exponential with an offset R_{slow}.

Interestingly, it was not possible to accurately measure the anisotropy decay for the larger $AFGP_{1-5}$ in a borate buffer, as these solutions formed a gel. Gel formation is a clear indication of inter-protein interactions. These interactions likely arise from the formation of

borate cross-links between different AFGP sugar moieties, a mechanism that is supported by linear FTIR spectra of $AFGP_{1-5}$, presented in figure 4. The spectra exhibit peaks at 1645 cm^{-1} and 1555 cm^{-1} that correspond to the amide I and amide II vibrations, respectively, and that are sensitive to the hydrogen-bond configuration of the AFGP peptide backbone. These peaks do not change upon the addition of borate, indicating that the peptide conformation is similar. In contrast, the peaks associated with the vibrational modes of the sugar unit and borate change significantly. For $AFGP_{7-8}$ we observe a similar trend (see fig. S1), indicating that the smaller isoforms can form similar inter-protein cross links. We assume that the AFGP molecules in these solutions do not form a gel due to their significantly smaller size.

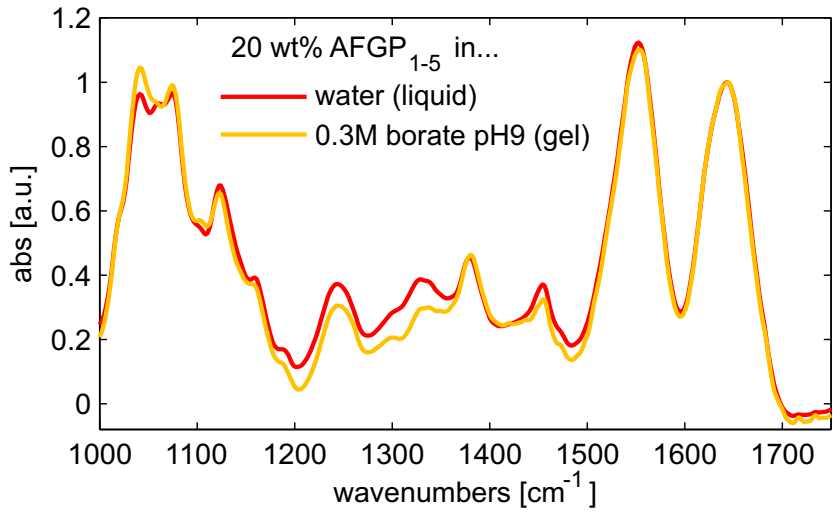


Figure 4: **Effect of borate on the FTIR spectrum of AFGP in aqueous solution.** Linear infrared spectra of 20 wt% $AFGP_{1-5}$ in isotopically diluted water (liquid) and in isotopically diluted 0.3 M borate buffer at pH = 9.0 (gel). The spectra are recorded at 24 °C and corrected for the water and buffer background, respectively.

Unravelling the hydration dynamics of AFGPs may provide important information on the nature of their ice-binding site and the origin of their extremely high IRI activity. Using polarization-resolved femtosecond experiments, we find that the dynamics of water near antifreeze glycoprotein surfaces are quite similar to the dynamics of water near other proteins and sugars, and that the majority of the slowly reorienting water molecules is slowed down by the AFGP peptide backbone. Our findings agree very well with early viscosity, translational

diffusion, and NMR experiments, which indicate that AFGPs affect a similar amount of water as non-antifreeze glycoproteins.[5] Our findings are also consistent with vibrational surface sum-frequency generation (VSFG) spectra of aqueous solutions of AFGP that also show no indication of ice-like or unusually structured water hydrating the AFGP (fig. S2). In principle, the reorientation dynamics of water molecules can be determined with MD simulations as well, and it would therefore be interesting to see such simulations on AFGPs and control substances in the future.

We further find that the local hydration dynamics of the smaller and larger AFGPs are almost identical. The antifreeze activity of $AFGP_{7-8}$, however, is known to be only 60% of the activity of larger $AFGP_{1-5}$ on a weight basis.[24] The larger and smaller AFGPs also show different ice shaping properties.[25] We thus find strong indications that the hydration water of AFGP is not of significant importance for its antifreeze activity. This finding is further supported by our observation that the well-known antifreeze glycoprotein inhibitor borate does not alter the dynamics of water hydrating the AFGP.

The effects of AFPs and AFGPs on the structure and dynamics of water have also been studied with THz absorption measurements.[16,26] It was observed that the addition of AFPs and AFGPs increases the terahertz absorption, and that for AFGPs this effect diminished upon the addition of borate. Based on the present measurements of the reorientation dynamics of water molecules hydrating AFGP, we conclude that the observed THz absorption change is probably not due to a change in the reorientation dynamics of the hydrating water molecules, but due to a change of the spectrum of the low-frequency vibrations of the hydration shell, induced by a structural change of the water surrounding AFGP or by the coupling between the intermolecular water vibrations and the low-frequency modes of sugar groups and borate ions.

Inactivation of AFGP is likely caused by a direct binding between the borate and the sugar unit.[1,24] This notion agrees with the observations of the linear FTIR spectra that indeed provide evidence for borate-sugar association. Strikingly, the borate ions cannot

only bind to one AFGP molecule, but are able to form inter- and intra-protein cross-links between AFGP molecules, which leads to gel formation at sufficiently high concentration. The formation of inter- and intra-protein links can also explain why the ice crystal growth behaviour of $AFGP_{1-5}$ in borate solutions is similar to that of $AFGP_{7-8}$: the binding and cross-linking by borate reduces the effective amount of sites of $AFGP_{1-5}$ that are able to interact with the ice.

Taken together, our observations are consistent with a direct ice-binding mechanism, with the AFGP sugar groups as the active binding site, as previously suggested.[1,23] Based on the differences in activity between the smaller and larger isoforms and the effect of borate binding on the activity of both of them, the number of available ice-binding sites per AFGP seems to play an important role. The high IRI activity of AFGPs compared to AFPs might thus be related to the existence of multiple ice-binding sites, located on the same flexible protein chain,[27,28] thereby making AFGPs particularly efficient in preventing the grain boundary migration that leads to the growth of large ice crystallites.[29,30]

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Supporting Information Available

Experimental methods; linear infrared spectra of $AFGP_{7-8}$ in water and in borate buffer; VSFG spectra of $AFGP_{1-5}$ and N-acetyl-D-galactosamine solutions.

This material is available free of charge via the Internet at `http://pubs.acs.org/`.

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