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Antifreeze Glycoprotein Activity Correlates with Long-Range Protein−Water Dynamics

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Abstract: Antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs) enable the survival of organisms living in subfreezing habitats and serve as preservatives. Although their function is known, the underlying molecular mechanism was not understood. Mutagenesis experiments questioned the previous assumption of hydrogen bonding as the dominant mechanism. We use terahertz spectroscopy to show that antifreeze activity is directly correlated with long-range collective hydration dynamics. Our results provide evidence for a new model of how AFGPs prevent water from freezing. We suggest that antifreeze activity may be induced because the AFGP perturbs the aqueous solvent over long distances. Retarded water dynamics in the large hydration shell does not favor freezing. The complexation of the carbohydrate *cis*-hydroxyl groups by borate suppresses the long-range hydration shell detected by terahertz absorption. The hydration dynamics shift toward bulk water behavior strongly reduces the AFGP antifreeze activity, further supporting our model.

Antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs) enable the survival of organisms living in subfreezing habitats[1,2] and serve as preservatives. We use terahertz spectroscopy to show that antifreeze activity is directly correlated with long-range collective hydration dynamics. The influence of AFGPs on the collective hydration dynamics is found to be more pronounced at low temperatures. We propose a mechanism in which AFGPs act as a carrier for carbohydrates to maximize their hydration shell. Hydration water has a depressed freezing point compared to the bulk. The complexation of the carbohydrate *cis*-hydroxyl groups by borate suppresses the long-range hydration shell detected by terahertz absorption and also strongly reduces the AFGP antifreeze activity, further supporting our model.

AFPs and AFGPs function by lowering the freezing transition temperature, but not the melting point (equilibrium freezing point). The separation of the freezing temperature and the melting point is referred to as a thermal hysteresis. They operate in a noncolligative manner and are effective at molar concentrations 300−500 times lower than those of salts.

We recently showed that carbohydrates[3] have long-range dynamical hydration shells, proportional in size to the number of carbohydrate−water hydrogen bonds. Here we use terahertz absorption spectroscopy to show that the dynamical hydration shell of AFGP increases in size as the freezing point is approached.

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AFGPs comprise one of four structural classes of AFPs.[4,5] They consist of repeating $(Ala\text{-}Ala\text{-}Thr)_x$ units, with x ranging from 4 to 50. The repeats show little sequence variation,[4] and their structure is intrinsically disordered.[6] To date, AFGPs cannot be expressed and post-translationally modified in cell lines using molecular biology approaches, an aspect which prevents extensive biophysical studies. Short oligomers (tetra- and octamers) have been prepared using solid phase peptide synthesis from monomer building blocks, respectively.[7] Low yields and extensive purification by HPLC limit the application of AFGP routine synthesis for biophysical analysis although new synthetic strategies have been developed recently and involve e.g. native chemical ligation and cysteine desulfurization.[8]

Chemical polymerization has been used to investigate structural motifs essential for antifreeze activity.[9] The three structural motifs important for activity involve (1) the *N*-acetyl group at position C2, (2) the *O*-glycosidic linkages between sugars and the peptide chain, and (3) the γ-methyl group at the threonine residue (Figure 1). The mechanism by which these structural motifs functionally affect overall antifreeze activity is unknown. Compared to native AFPs, AFGPs exhibit high flexibility and do not have a well-defined structure.[10] Due to the lack of surface structure it is unlikely that AFGPs interact with growing ice crystals by defined local molecular interactions. Zepeda et al.[11] proposed an alternative mechanism in which the glycoprotein interacts with a solvated ice surface, but this still does not explain how AFGPs can be active at such low concentrations. An even longer-range effect on surrounding water appears likely.

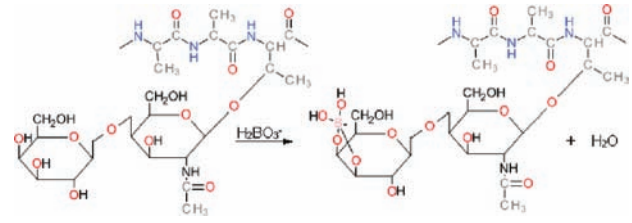

Figure 1. Inactivation of AFGP by sodium borate.

We purified antifreeze glycoproteins from the antarctic notothenioid toothfish, *Dissostichus mawsoni*, consisting of $(Ala\text{-}Ala\text{-}Thr)_x$ sequence repeats ($x \approx 40$). The disaccharide β-D-galactosyl-(1→3)-α-*N*-acetyl-D-galactosamine is joined to the hydroxyl oxygen of the Thr residue through a glycosydic linkage (Figure 1). The protein yielded a maximum extrapolated hysteresis of 1.7 °C (Figure 2). In 0.3 M sodium borate buffer, the hysteresis was reduced to 0.25 °C, as measured with a nanoliter osmometer Clifton nanoliter cryoscope. Reversible borate inactivation by complexation of borate with the *cis*-hydroxyl groups of the AFGP galactose units is known from previous structure-based[5] studies.

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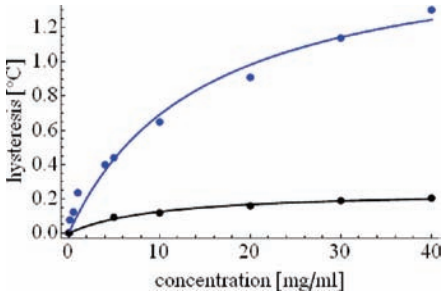

Figure 2. Hysteresis of AFGP (blue) and AFGP in 0.3 M borate buffer (black), as a function of AFGP concentration in water. The data were fitted to the Langmuir equation $(\Delta T/\Delta T_{max}) = (c/c_{1/2})/[(c/c_{1/2}) + 1]$, where $c_{1/2}$ is the AFGP concentration at half-maximum hysteresis $(1/2(\Delta T/\Delta T_{max}))$. T_{max} is 1.7 °C for the active form of AFGP and 0.25 °C for the inactivated form.

We studied the ability of AFGPs to influence long-range collective water dynamics occurring on the subpicosecond time scale. We used terahertz (THz) spectroscopy that is specifically sensitive to collective sub-ps hydration dynamics around proteins and carbohydrates.[12] We previously showed that water within the dynamical hydration shell around proteins and carbohydrates absorb more strongly than bulk water[3] ("THz excess").

Using our p-germanium laser spectrometer operating in a double-beam configuration for background subtraction,[12] we directly measured the change of intermolecular water network vibrations induced by the AFGPs. We analyzed the THz radiation transmitted through AFGP sample solutions in one channel of the THz setup. As reference we used the same cell filled with water and measured the transmitted THz radiation. By Beer's law we determined the $\Delta\alpha$ as the difference in the integrated THz absorbances of the AFGP sample solution (α_{sample}) and of the aqueous reference ($\alpha_{reference}$) which was between 2.4 and 2.7 THz: $\Delta\alpha = \alpha_{sample} - \alpha_{reference}$. THz absorbance measurements were performed at low-humidity (<8%) and under temperature stabilized conditions ($T \pm 0.05$ K, where T is the respective experimental temperature).

We varied protein concentration, temperature, and borate concentration. In Figure 3, any deviation from a linear dependence of $\Delta\alpha$ is attributed to a dynamical hydration shell.[3] At 20 °C, this THz excess is ca. 6 cm^{-1} with a maximum at AFGP concentrations of ~12 mg/mL. At 5 °C, the THz excess is even more pronounced (~10 cm^{-1}) and peaks at lower AFGP concentrations of ~4 mg. Larger hydration shells overlap at lower protein concentration, causing the peak,[12] and here we estimate an increase in hydration shell size from ca. 20 Å to ca. 35 Å from 20 to 5 °C. Thus at physiological conditions effectively all water is perturbed.

The observation of a long-distance modification of water dynamics at low temperatures is remarkable because our previous THz studies showed optimal protein−hydration water coupling at temperatures well above room temperature. The extensive low-temperature modulation of hydration dynamics is indicative of optimized coupling between fast water dynamics and AFGP near freezing temperature.

In contrast, addition of borate to the glycoprotein solution eliminates the THz excess (Figure 3): At 20 °C, we even observe a decrease in $\Delta\alpha$ indicating a less flexible water network ($\Delta\alpha$ decreases with temperature). Borate thus has a significant effect on the long-range hydration shell around the glycoprotein at low and high temperature. Borate also greatly reduces the antifreeze activity (Figure 2), proving that modulation of long-range hydration dynamics is directly correlated with antifreeze activity (Figure 2).

Mutagenesis experiments questioned the previous assumption of hydrogen bonding as the dominant mechanism.[13,14] Our results

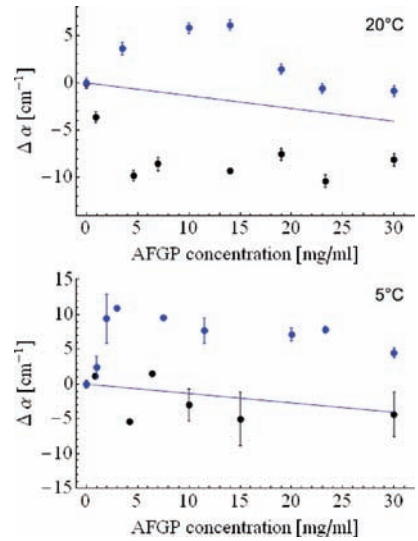

Figure 3. Difference $\Delta\alpha$ in THz absorbance (integrated between 2.4 and 2.7 THz) of AFGP dissolved in water relative to a water reference. Upper panel: 20 °C. Lower panel: 5 °C. Active AFGP shows a concentration-dependent excess of THz absorbance that shifts to lower protein concentration at the lower temperature (blue data points). Addition of borate (black data points) eliminates the THz excess. Error bars are reported at ± standard deviation determined by statistics due to multiple independent measurements. The blue line represents the expected linear THz absorption decrease due to water displacement by AFGP.

provide evidence for a new model of how AFGPs prevent water from freezing. We suggest that antifreeze activity may be induced because the AFGP perturbs the aqueous solvent over long distances. Retarded water dynamics in the large hydration shell does not favor freezing. Borate complexes the glycoprotein and shifts the hydration dynamics towards bulk water behavior, thus reducing antifreeze activity.

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