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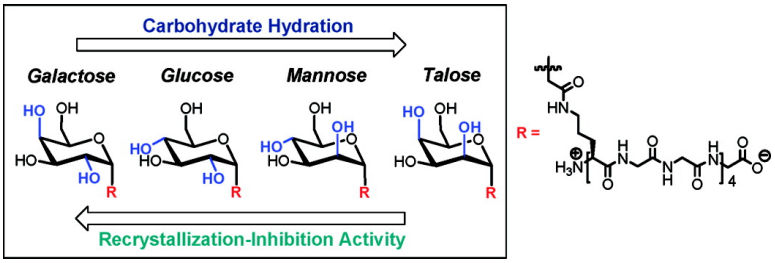

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The Importance of Hydration for Inhibiting Ice Recrystallization with C-Linked Antifreeze Glycoproteins

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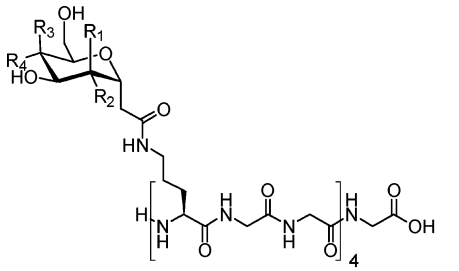
The role of hydration in modulating solution conformation, molecular recognition and biological activity of oligosaccharides, proteins, and nucleotides is widely recognized.[1] However, this effect is often neglected when investigating many biological processes,[2] such as the mechanism by which biological antifreezes inhibit the growth of ice. Antifreeze glycoproteins from Teleost fish have the unique ability to prevent in vivo ice growth in organisms inhabiting subzero environments. Consequently, these organisms are protected against cryo-injury and death. An understanding of the structural attributes essential for inhibiting the recrystallization of ice (antifreeze activity) is of current interest as it will allow for the design of novel cryoprotectants useful for the preservation of cells, tissues, and organs.[3]

Recently, we have investigated the relationship between carbohydrate configuration and recrystallization-inhibition (RI) activity in functional C-linked AFGP analogues. Toward this end, we prepared a series of C-linked AFGP analogues incorporating D-galactose, D-glucose, D-mannose and D-talose hexoses **1**−**4** (Figure 1).



1: Gal R_1=H;R_2=OH;R_3=OH;R_4=H
2: Glc R_1=H;R_2=OH;R_3=H;R_4=OH
3: Man R_1=OH;R_2=H;R_3=H;R_4=OH
4: Tal R_1=OH;R_2=H;R_3=OH;R_4=H

Figure 1. Structure of C-linked antifreeze glycoprotein analogues.

The four C-linked AFGP analogues **1**−**4**, were prepared as reported previously[4] and analysis for antifreeze activity was performed.[4a,5] C-Linked AFGP analogues **1**−**4** did not show any thermal hysteresis (TH) activity. However, analogue **1** did exhibit weak dynamic ice shaping indicating that this compound had the ability to interact with the ice lattice.[4a] Analogues **1**−**4** were assessed for their ability to function as inhibitors of recrystallization (Figure 2). All samples were compared to a solution of phosphate buffered saline (PBS) which was used as a negative control for inhibition of recrystallization. A solution of AFGP-8 isolated from Gagus *ogac* was generously provided by AquaBounty Farms as a positive control. Each solution was tested at three different concentrations to rule out any nonspecific RI effects.[5b] The *Y*-axis in Figure 2 represents the mean largest grain size (MLGS) where bars smaller than PBS indicate recrystallization-inhibition activity. The D-mannose and D-talose analogues (**3** and **4**, respectively) exhibited very weak RI activity with MLGS values similar to PBS, the negative control. D-Glucose analogue **2** exhibited moderate RI activity while D-galactose analogue **1** was the most potent analogue with a MLGS value of 0.00354 mm^2 at 5.54 × 10^{-6} M.

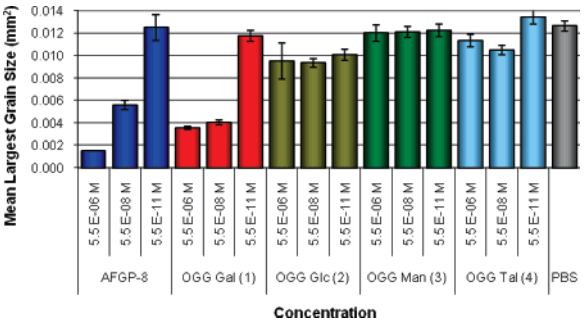


Figure 2. Mean largest grain size of AFGP-8, C-linked analogues (**1**−**4**), and PBS standard at −6.4 °C after 30 min anneal time. Error bars indicate SEM.

These results verify that the carbohydrate configuration is very important for RI activity. Clearly, RI activity is maximized when the C4 hydroxyl group is axial and all other hydroxyl groups are in an equatorial position. Differences in hydration for each carbohydrate residue may explain the observed trend. Galema et al. have studied the hydration of various monosaccharides using molecular dynamics simulations and kinetic experiments as well as density and ultrasound measurements.[6] Consequently, the partial molar volumes and isentropic partial molar compressibilities of many commercially available hexoses have been determined. In general, these values relate to the volume of space occupied by a molecule upon hydration by water and quantify the "compatibility" of a carbohydrate with the three-dimensional hydrogen-bonded network of bulk water. Monosaccharide pyranoses differing in the hydroxyl group stereochemistry at C2 and C4 show a relatively wide range of partial molar compressibilities. The molar compressibilities of D-talose, D-mannose, D-glucose, and D-galactose are shown in Figure 3 where hexoses with low molar compressibility values exhibit the poorest fit in the three-dimensional hydrogen-bonded network of water.[6a]

Unfortunately, the hydration of C-linked carbohydrate derivatives has not been studied in detail. It is well recognized that C-linked carbohydrate derivatives adopt many more conformations than their O-linked analogues[7] but are still able to adopt the key "biologically active" conformations in vitro ensuring they are just as active or in some instances more active than their O-linked counterparts.[8] Furthermore, conformational analyses of C-linked and O-linked oligosaccharides suggest that these two substrates can adopt identical conformations as intramolecular hydrogen bonds play a significant role in dictating conformation.[8c,9] Intramolecular hydrogen-bond cooperativity also has an effect on the structuring of water as shown by Dashnau et al.[10] These studies indicate that the stereochemistry of the C4 hydroxyl is crucial in forming intramolecular hydrogen bonds. In addition, the relative stereochemistry at C4 and

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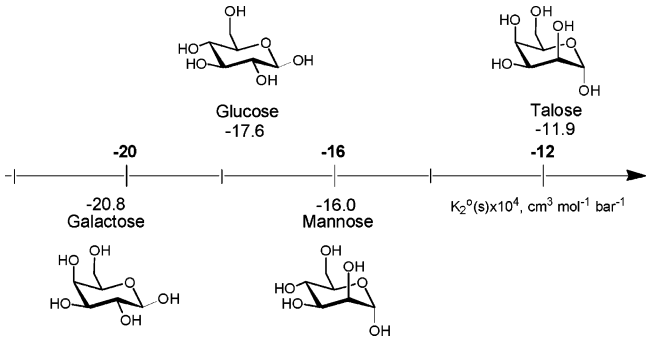

Figure 3. Partial molar compressibilities of monosaccharides in aqueous solution at 298 K ($K_2°(s) \times 10^4$, cm^3 mol^{-1} bar^{-1}). Values are given for dominant conformer in solution.[6a]

C2 modulates the magnitude of hydration.[6b,10] Stereochemistry at the anomeric and C3 positions has been shown to not affect hydration significantly,[10] and thus the hydration characteristics between an O-linked pyranose and a C-linked pyranose is not expected to differ.

Our data (Figure 2) indicate that the compatibility of a hexose with the three-dimensional hydrogen-bonded network of water is inversely proportional to recrystallization-inhibition activity. Talose-containing AFGP analogue **4** exhibits no RI activity (Figure 2). On the basis that D-talose would have the best "fit" into the three-dimensional hydrogen-bonded network of water (compared to the other hexoses), this analogue would demand the least re-organization of bulk water for hydration and thus the least amount of energy. In contrast, D-galactose analogue **1** would have the lowest molar compressibility value and hence, would exhibit the poorest fit into the three-dimensional hydrogen-bonded network of water. Consequently, the energetic cost to incorporate this analogue into the three-dimensional hydrogen-bonded network of water would be the largest.

Recrystallization is defined as the formation of larger crystals at the expense of smaller ones, and its mechanism has been dealt with extensively in the metallurgical literature.[11] With respect to ice, larger crystals are formed by the addition of bulk water molecules to a transitional domain between the ice/water interface known as the quasi-liquid layer (QLL)[12,13] and subsequently from the QLL into the ice lattice. According to IR studies by Sadtchenko et al., the thickness of the QLL is inversely proportional to temperature, and at −6 °C (the temperature at which our RI assay is performed), the thickness of the quasi-liquid layer is ∼1 nm, which equates to about only three monolayers of water.[12] The consequence of such a thin layer means that a carbohydrate residue with low molar compressibility embedded in this region will have a very pronounced affect on the ordering of the bulk water/QLL interface. Because RI activity is inversely proportional to hydration, we believe that recrystallization-inhibitors (biological antifreezes or our C-linked AFGP analogues) may function by disturbing the highly ordered structure of supercooled water. The net result of perturbing the ordered structure of supercooled water would be an inhibitory effect on ice growth as the transfer of a water molecule from bulk water to the QLL and the ice lattice would be high in energy.

C-Linked AFGP analogue **1** possesses custom-tailored antifreeze activity in that it has potent RI activity and no TH activity. We know that the dynamic ice shaping observed with C-linked AFGP analogue **1** is a property unique to biological antifreezes[4a] and that dissolving the sample in PBS negates any false positive effects in the RI assay.[5b] Consequently, the observed antifreeze activity is authentic. Some of our other C-linked analogues have similar activity profiles, suggesting this class of molecules represents a novel lead structure for the design of new cryoprotectants.[5c] The conformations of analogues **1**−**4** were examined by circular dichroism spectroscopy (CD) and indicate that the glycopolymers are highly flexible in solution. This is consistent with the conformation of native AFGP.[14,15] A high degree of flexibility in the polypeptide may be an important factor for RI activity in that it would allow the glycoprotein to move easily through the concentrated solution of solute during the freezing process.

In summary, we have demonstrated that the configuration of the carbohydrate moiety in C-linked AFGP analogues is extremely important and modulates recrystallization-inhibition activity. It seems likely that differences in hydration for each C-linked pyranose alter the compatibility of the carbohydrate moiety with the three-dimensional hydrogen-bonded network of supercooled bulk water. Consequently, the energy associated with transferring a water molecule to the ice lattice changes and can result in inhibition of ice growth. These results emphasize the importance of additional studies to further elucidate the role of hydration in carbohydrates, peptides, and proteins and its subsequent affects on conformation, recognition, and activity in biological systems. The influence of different polypeptide backbones in **1**−**4** on RI activity, cytotoxicity profiles, and effectiveness as cryoprotectants will be reported in due course.

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Supporting Information Available: Experimental procedures, synthetic schemes and spectral data (NMR and CD). This material is available free of charge via the Internet at http://pubs.acs.org.

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