Glycoproteins

Antifreeze Glycoproteins: Elucidation of the Structural Motifs That Are Essential for Antifreeze Activity**

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Antifreeze proteins (AFPs) and glycoproteins (AFGPs) collectively abbreviated as AF(G)Ps are essential to the survival of many marine teleost fishes that reside in polar and subpolar waters where temperatures decline below the colligative freezing points of their body fluids.[1] Although the AF(G)Ps are markedly diverse in structure, they all function by binding to the surface of embryonic ice crystals to inhibit their growth. This binding results in a freezing point depression without an appreciable change in the melting point. The difference between the melting and freezing temperatures, termed thermal hysteresis (TH), is used to detect and quantify the antifreeze activity. In addition to effecting a thermal hysteresis, concentrated fish AF(G)Ps alter the morphology of the ice crystal into a hexagonal bipyramid.[2]

The AFGPs isolated from fish blood plasma consist of repeating tripeptide units $(Ala\text{-}Thr\text{-}Ala)_n$ with a disaccharide moiety (Galβ1-3GalNAcα1-) attached to each threonyl residue.[3] They range in molecular mass from approximately 33 kDa (50 repeating units) to 2.6 kDa (four repeating units).

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[**] This work was supported by the NEDO and the Ministry of Education, Science, and Culture. We also thank Ms. A. Maeda, Ms. H. Matsumoto, and Ms. S. Oka of the Center of Instrumental Analysis, Hokkaido University, for measuring elemental analyses and mass spectrometry studies.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

  DOI: 10.1002/ange.200353110

Although these AFGPs were the first antifreeze polypeptides to be described in detail, very little is known about their mechanism of action.[4] This contrasts with the considerable progress that has been made towards understanding the structure–function relationships of several of the AFPs.[5] This lack of progress is largely due to the structural complexity of the numerous isoforms of AFGP found in blood plasma and the ensuing difficulties in isolating sufficient quantities of pure material.[6] In addition, attempts to manufacture pure AFGP through the use of cell lines have not yet met with success. In light of these difficulties it would appear that chemical synthesis may be the only viable way to produce sufficient quantities of pure AFGP for detailed structure–function studies.

There have been a number of studies reporting the synthesis of AFGP-related compounds.[7] However, none of these compounds have been tested for antifreeze activity. This lack of success to date is largely because of the intrinsic difficulties involved in the construction of glycoproteins such as AFGPs that have relatively large molecular masses and contain a high density of sugar residues.

Recently, we have developed a strategy for the efficient chemical synthesis of sequential glycoproteins and have succeeded in synthesizing AFGPs (synthesized AFGPs are termed *sy*AFGPs) that are essentially identical to the naturally occurring AFGPs, and have the capacity to shape ice crystals into hexagonal bipyramids.[8] We now report on what we believe to be the structural motifs that are essential for the antifreeze activity of AFGPs, alter ice-crystal morphology and result in significant thermal hysteresis.

Antifreeze glycoproteins (*sy*AFGP$_{poly}$, **1**) were synthesized by directly polymerizing an unprotected glycopeptide macromonomer in the presence of diphenylphosphorylazide (DPPA) as a promoter.[8b] The weight-average molecular weight of **1** was estimated to be 6600 Da, which corresponded to 8–15 repeating units, and produced significant antifreeze activity (TH) (Figure 2C, b).

To determine the minimum number of tripeptide repeating units necessary for antifreeze activity, low-molecular-weight *sy*AFGPs were produced in a manner similar to that described for the *sy*AFGP$_{poly}$, **1**,[8b] but at a lower temperature and with a shorter reaction time. These *sy*AFGPs were then separated into homogeneous fractions of precise chain lengths by means of recycling preparative size-exclusion chromatography. As shown in Figure 1A, purification of up to seven repeating units was accomplished, and the calculated and observed mass for each glycopeptide was identical within experimental error (Figure 1C). The purity of each glycopeptide was demonstrated by using analytical reverse-phase (RP) HPLC (Figure 1B).

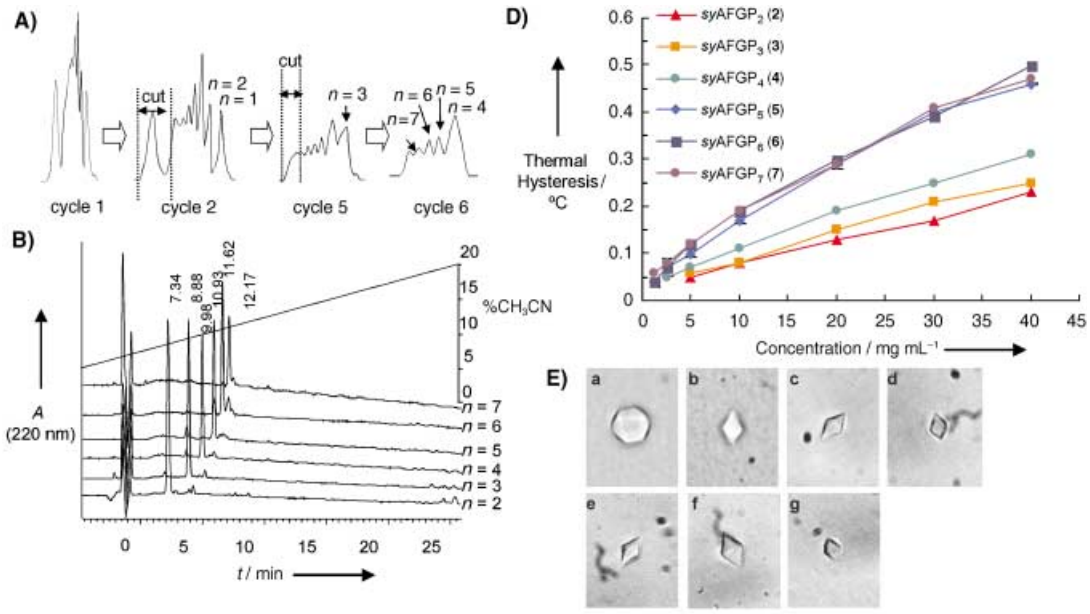
The relationship between thermal hysteresis activity, ice-crystal morphology, and synthetic AFGP chain length is presented in Figure 1D and E. Although high concentrations of the monomer *sy*AFGP$_1$, **1′**, exhibited no thermal hysteresis, it did produce hexagonal ice crystals (Figure 1E, a). This strongly suggests that the glycopeptide monomer is capable of some interaction with ice. All of the tripeptide *sy*AFGP polymers (**2**–**7**) displayed concentration-dependent thermal hysteresis and produced hexagonal bipyrimidal ice crystals. In addition there was a positive correlation between thermal hysteresis and chain length between two and five tripeptide repeating units (**2**–**5**). There was no increase in thermal hysteresis activity when the chain length increased from five–seven repeats (**5**–**7**), which suggests that the antifreeze activity of the *sy*AFGP was maximal at five repeats (Figure 1D). These results clearly demonstrate that a two-tripeptide *sy*AFGP is sufficiently large to control ice crystal growth.

To identify the structural motifs that are essential for antifreeze activity, a number of AFGP analogues were synthesized (Figure 2A) by using established methods.[9] The antifreeze activity of each of the polypeptides and analogues was evaluated by using a Clifton nanoliter osmometer and by examining ice crystal morphology (Figure 2B and C). Synthetic **1** produced hexagonal bipyramidal ice crystals, and exhibited a thermal hysteresis that was somewhat greater than that of the naturally occurring AFGP-8 (2.6 kDa). A lack of carbohydrate moiety (nonglycosylated polypeptide, **8**) resulted in the complete loss of thermal hysteresis and structuring of the ice crystal (Figure 2C, c). This result confirms those of earlier studies that indicate that the carbohydrate is essential for AFGP activity.[10]

The β-*O*-linked type glycoprotein **9** was designed to probe the importance of α-glycosidic linkage between GalNAc residue and threonyl side chain. Although the formation of the hexagonal ice crystal (Figure 2C, d) suggests weak interactions between the glycopeptide and the ice, a complete lack of thermal-hysteresis activity attests to the essential nature of this linkage.

Sialylated glycoprotein **10** was prepared by the enzymatic introduction of sialic acid at the C3 position of the galactose residue of *sy*AFGP$_{poly}$ **1**, as previously described.[8b] The results of this modification confirm those reported for a similar study with naturally occurring AFGP: the complete loss of thermal hysteresis[2c] (data not shown).[8b] There are two possible reasons for this result: 1) The introduction of the sialic acid residue destroys the AFGP conformation; 2) The sialic acid residue interferes with the ability of the glycoprotein to prevent ice growth.

Glycoproteins **11**–**15** were synthesized to examine the importance of the NHAc group at the C2 position of the carbohydrate moiety attached to threonine residue and the hydroxyl groups. Interestingly, the AFGP analogue that only contained the GalNAc residue **11** was fully functional, and had a capacity to form bipyramidal crystals and a thermal hysteresis that was slightly lower than that of **1** (Figure 2B and C, e). This result indicates that the terminal galactose residue is not necessary for activity. However, the apparent lower thermal-hysteresis activity of GalNAc **11** polypeptide suggests that the galactose residue may enhance the binding of AFGP with the ice surface. The LacNAc **12** polypeptide also inhibited growth of ice crystals (Figure 2C, f). However the thermal hysteresis values were considerably lower than those of **1** (Figure 2B), thus implying that the configuration of the sugar hydroxyl groups is important for the activity. AFGP analogues carrying Galβ1-3Gal **13** (data not shown),[8b] Gal **14** or Lac **15** did not show any thermal hysteresis, although they were able to produce hexagonal-like crystals (Figure 2C, g and h), which indicates the presence of weak interactions with



C)

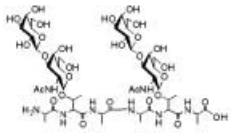
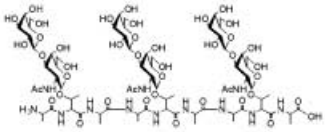
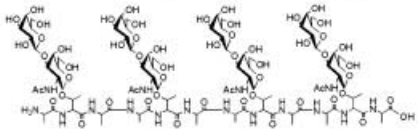
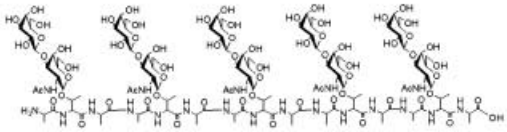
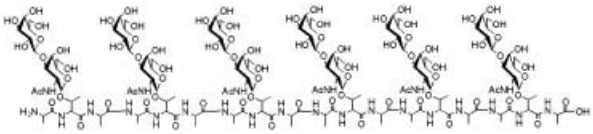
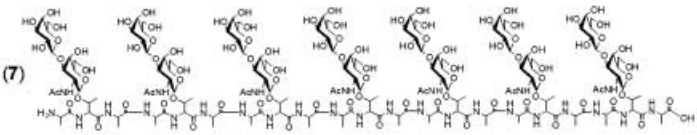
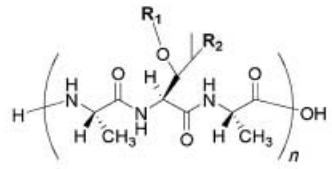
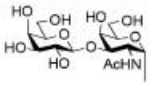
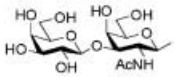
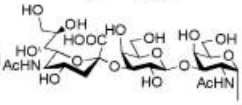
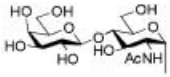
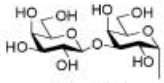
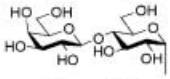
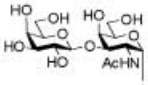
Codename	Structure	Calculated mass ([M + Na]+)	Observed mass
*syn*AFGP$_2$ (**2**)		1257.51	1257.29
*syn*AFGP$_3$ (**3**)		1865.76	1865.20
*syn*AFGP$_4$ (**4**)		2474.02	2473.82
*syn*AFGP$_5$ (**5**)		3082.27	3081.99
synAFGP$_6$ (**6**)		3690.53	3690.06
*syn*AFGP$_7$ (**7**)		4298.78	4298.00

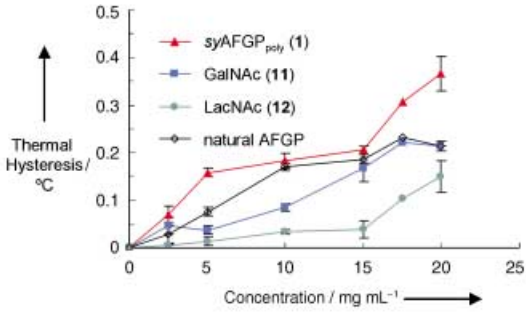
Figure 1. Relationship between molecular weight and antifreeze activity. A) Recycling size-exclusion-chromatography profile of synthetic AFGP. Synthetic AFGP was separated into a homogeneous fraction of discrete length by means of RP-HPLC on Shodex size-exclusion columns (OHpak SB-2002.5×2-and OHpak SB-2003) with water as an eluent. B) RP-HPLC profiles of **2**–**7**. The right-hand axis gives the percentage of acetonitile in 0.1% aqueous trifluoroacetic acid (%CH_3CN). C) Structure and MALDI-TOF mass spectrometric analysis of *sy*AFGPs. D) Thermal hysteresis activity of **2**–**7**, measured by means of a Clifton nanolitre osmometer (Clifton Technical Physics, Hartford, NY).[15] E) The ice-crystal morphologies in the presence of a) **1′** (40 mg mL^{-1}), b) **2** (40 mg mL^{-1}), c) **3** (10 mg mL^{-1}), d) **4** (10 mg mL^{-1}), e) **5** (10 mg mL^{-1}), f) **6** (10 mg mL^{-1}), g) **7** (10 mg mL^{-1}) in water were observed as described previously.[16] Photos were taken at −0.1 °C (b–g) or 0.0 °C (a).

A)



codename	R_1	R_2	Mn[a]	Mw[b]
*sy*AFGP$_{poly}$ (**1**)[8b]		CH_3	3 950	6 600
nonglycosylated polypeptide (**8**)	H	CH_3	1 690	2 400
β-*O*-linked glycoprotein (**9**)		CH_3	4 020	6 620
sialylated *sy*AFGP$_{poly}$(**10**)[8b]		CH_3	6 370	11 650
GalNAc (**11**)		CH_3	3 000	4 690
LacNAc (**12**)		CH_3	3 660	6 140
Galβ1-3Gal (**13**)[8b]		CH_3	4 100	5 480
Gal (**14**)		CH_3	3 180	4 230
Lac (**15**)		CH_3	4 700	7 000
ASA (**16**)		H	4 410	6 590

B)



C)

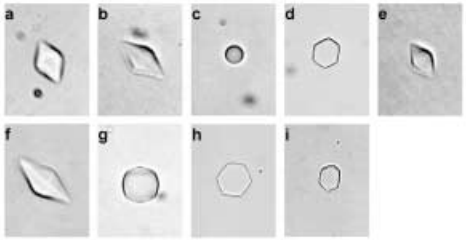


Figure 2. Structure–activity relationship of AFGP. A) AFGP analogues synthesized in this study. All compounds were used for the activity evaluation studies in polymeric form. B) Thermal hysteresis activity of synthetic AFGP. C) Ice-crystal morphology in the presence of a) natural AFGP, b) **1**, c) nonglycosylated polypeptide **8**, d) β-*O*-linked glycoprotein **9**, e) GalNAc **11**, f) LacNAc **12**, g) Gal **14**, h) Lac **15**, i) ASA **16**. Ice crystals were formed in the presence of 10 mg mL^{-1} glycoprotein in water. Photos were taken after 0.1 °C undercooling (a, b, e, f) or 0.0 °C (c,d, g–i).

ice. These results show that the acetamide (-NHCOCH$_3$) groups at the C2 position of the reducing end sugar residues are key functional groups required for the antifreeze activity. The Ala-Ser-Ala AFGP analogue **16**, was constructed to examine the importance of the γ-methyl groups on the Thr residue. This analogue exhibited no thermal hysteresis (Figure 2C, i), thus demonstrating that the γ-methyl groups of the Thr residues are also essential for activity.

From the evaluation of the activity discussed above, we conclude that the conformation of the glycoprotein is a key factor for antifreeze activity. Therefore, to study the relationship between the activity and the conformation, CD spectra of the synthetic (glyco)proteins in the far UV-absorption region were measured in aqueous solutions at neutral pH (Figure 3A–C). As reported previously, synthetic AFGP **1** gave a curve similar to that of natural AFGP[8b] thus providing evidence of the presence of polyproline type II (PPII) helix (Figure 3A, b).[4c,11] Similar spectra were also obtained in cases of active two-model glycoproteins **11** and **12** (Figure 3B, e and f), while Galβ1-3Gal **13** (data not shown),[8b] Gal **14**, Lac **15** (Figure 3B, g and h), β-Linked glycosides **9**, and nonglycosylated polypeptide **8** (Figure 3A, d and c) exhibited CD spectra typically found from the polypeptides of a disordered structure.[11c] It is reasonable to assume that acetamide group is a fundamental functional group for the construction of the specific structure of AFGPs. Interestingly, it was suggested that the Ser-replaced compound **16** seemed to contain a large α-helical structure (Figure 3B, i). In addition, sialylated glycoprotein **10**, which showed no antifreeze activity, still retains the similar secondary structure as AFGP (data not shown).[8b] Therefore, it seems that the terminal sialic acid residues introduced at the C3 position of the galactose residues might inhibit the successful adsorption of hydroxyl groups onto the surface of ice lattice. Alternatively, even small changes in the conformation might provide a drastic effect on the activity.

In the same manner, the chain-length dependency of the conformation of AFGP was also examined by use of **2**–**7** as

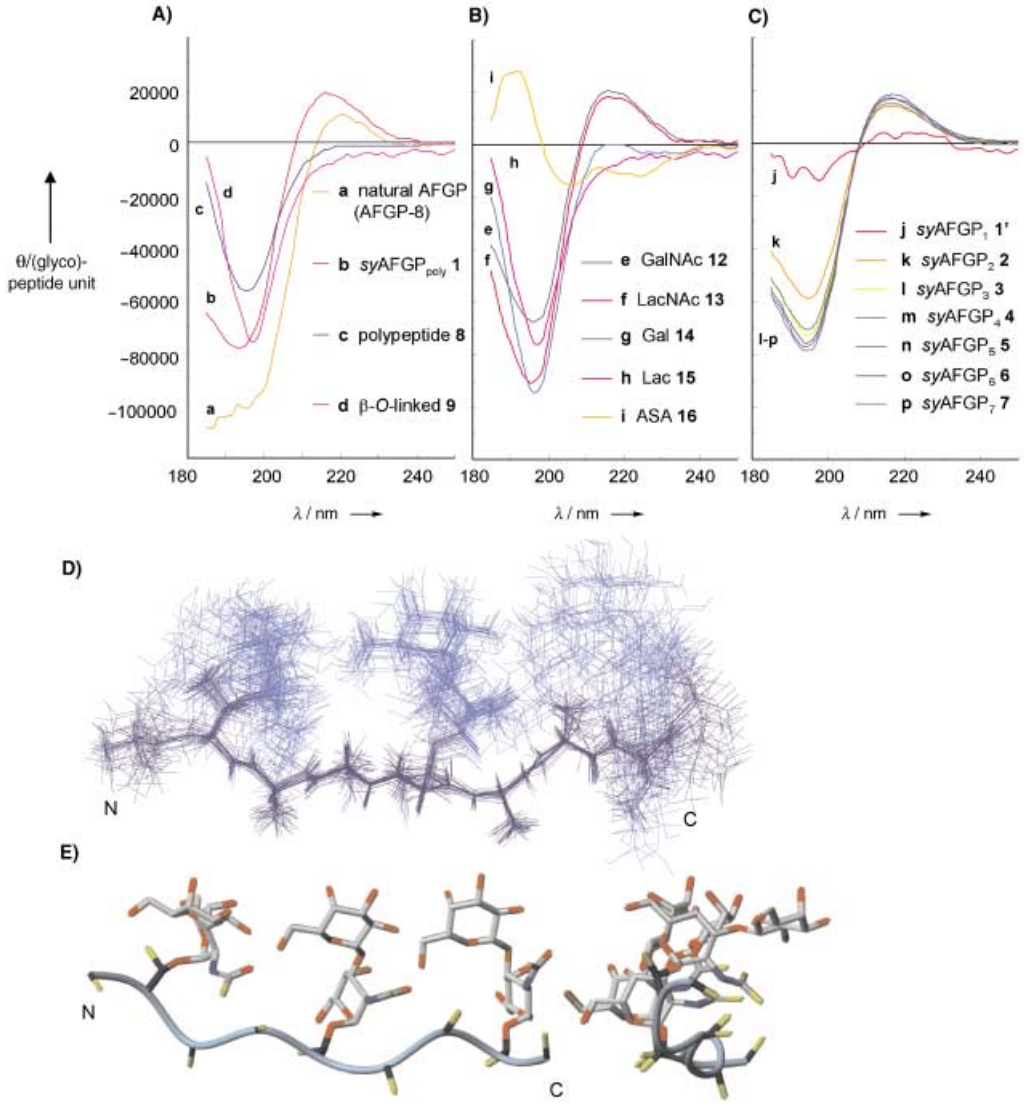


Figure 3. Structural analysis of synthetic compounds. A)–C) CD spectra of synthetic compounds. All curves were measured at 4 °C in water (0.1 mg mL^{-1}) in 1 mm path length quartz cells on a JASCO J-820 spectropolarimeter at 4 °C. D) Superposition of the 25 lowest energy structures of *sy*AFGP$_3$ calculated from NMR-based constraints. The peptide backbone is navy, and the carbohydrate moieties are royal blue. E) Structure closest to the average of the 25 best calculated models for **3**: yellow, methyl carbon; white, carbon in carbohydrate; gray, carbon in peptide side chains except for methyl carbon; blue, nitrogen; red, oxygen; the N- and C-terminal ends are identified. These images were generated by the program MOLMOL.[13]

shown in Figure 3C. The CD spectral curves of active compounds, **2**–**7**, are almost the same, thus indicating that they form virtually the same secondary structure. In contrast, **1**′ showed no characteristic Cotton effect, which suggests that the chain length of *sy*AFGP$_1$ is not long enough for the formation of a specific conformation. These results are consistent with that obtained in the activity-evaluation study, and they concluded that two repeating units (M_w = 1.2 kDa) is the minimum required to form the specific conformation and induce the successful interaction with the diffuse ice/water interface.

From the above results, we conclude that the specific activity of AFGP is derived from its intrinsic conformation. A number of local structures have been proposed for AFGPs, such as three-hold left handed helix similar to PPII helix,[4c,11b] and γ-turn.[4f] However, the X-ray crystal structure for AFGP has not yet been obtained, presumably because of the difficulty in obtaining a good crystal of a molecule that contains a high density of carbohydrates. Similarly, NMR spectroscopic methods have not succeeded in giving a definite structure because of the difficulties in assigning the NOEs of the molecule due to the repeating structure in AFGPs, which leads to significant spectral overlap. Consequently, these studies concluded that AFGPs do not adopt a rigid, well-defined structure although maybe a part of the molecule adopts a preferred conformation in solution.[4g,h]

The smallest glycopeptide found in nature is a 14-residue glycopeptide that contains four disaccharides (AFGP-8, AAT*AAT*PAT*AAT*PA: *glycosylated residue), and this fact made the conformational analysis difficult. As discussed

above, we have demonstrated that even *sy*AFGP$_2$ (AT*AAT*A: * glycosylated residue) has the antifreeze activity, and forms almost the same conformation as the larger *sy*AFGPs. These results encouraged us to examine the precise structure of AFGPs by use of *sy*AFGPs with short chain lengths, which should enable us to perform complete assignment of the spectrum. Thus, we choose *sy*AFGP$_3$ (AT*AAT*AAT*A: * = glycosylated residue) as a target for the structural analysis, which should make it easier to find the structural characteristics of AFGP compared with *sy*AFGP$_2$. Fortunately, we completed the sequence-specific assignments of *sy*AFGP$_3$ by using a combination of NOESY and COSY in both 9:1 H_2O/D_2O and D_2O, in which the spectral overlapping reduced significantly (see Supporting Information for details).

On the basis of the complete NMR assignment, we performed the structural calculations by using the program CNS with restraints[12] to determine the 3D solution structure of **3**. The statistical analysis of NMR restraints and a summary of structure statistics are given in Table 1 (see Supporting Information for NOE assignments, average glycosidic torsion angles for calculated models, and Ramachandran plots). An initial set of 50 structures was calculated by using the restraints, and a family of 25 accepted 3D structures was selected with the lowest potential energies. Figure 3D represents the superposition of the backbone coordinates for 25 converged structures, and the structure closest to the average of the 25 is shown in Figure 3E. Surprisingly, **3** appeared to form a well-defined structure, despite the absence of long- or medium-range constraints. As estimated from CD spectral analysis, the peptide backbone of **3** folds into a left handed helix in which three disaccharide moieties are on the same side of the molecule, thus constructing a hydrophilic face. In addition, it newly appeared that Ala–CH_3 groups and acetyl methyl groups in the GalNAc residues were clustered into one face of the molecule, forming a hydrophobic face. This amphiphatic structure of AFGP is similarly constructed in type I AFP, an amphiphatic single α-helix with consecutive alanines.[14] Taking into account of the results of CD experiments, one can imagine that a significant portion of larger AFGP also constructs a left handed helix. Again AFGP should have a more ordered structure than was previously estimated.[4g,h] Although detailed mechanism of action is unclear, the amphiphatic nature is presumed to be crucial for the AFGP specific activity.

Based on the structural results, we surmise that it is likely that antifreeze activity by AFGPs strongly depends on the presence of a specific-ordered helix similar to a PPII helix, and polymeric AFGP requires three key motifs such as *N*-acetyl group at C2 position of the reducing hexosamines, α-configuration of the *O*-glycosidic linkages between sugars and peptide chain, and γ-methyl group of the threonyl residue. We have also demonstrated that the antifreeze activity of AFPG was found even in the case of a dimer (**2**, $M_w = 1.2$ kDa). Further investigations that are aimed at elucidating the mechanism of action, and examining the potential use in clinical applications of AFGPs are under way and the results will be reported in due course.

Received: October 17, 2003 [Z53110]
Published Online: January 22, 2004

Table 1: Statistical analysis of NMR restraints and computed structure for **3**.

Summary of restraints and structural statistics	
Restraints	
Distance restraints[b]	
total	236
intraresidue	
peptide	26
glycan	97
sequential	
peptide	28
glycan	26
peptide to glycan	
within the same glycosylated residue	34
glycans on other peptide residues	25
dihedral restraints	
peptide backbone	5
peptide sidechain	3
glycans	3
Structural Statistics	
Average potential energies (kcal mol^{-1})[c]	
E_{total}	33.26 ± 0.30
E_{bonds}	1.15 ± 0.04
E_{angle}	16.28 ± 0.10
E_{impr}	3.87 ± 0.07
E_{VDW}[d]	0.54 ± 0.08
E_{NOE}[d]	5.99 ± 0.29
E_{cdih}[d]	0.00065 ± 0.00058
RMSD from idealized geometry	
bonds [Å]	0.0021 ± 0.00004
angles [°]	0.96 ± 0.0032
impropers [°]	0.63 ± 0.0056
Pairwise RMSD of 25 structures (Å)	
global region	
backbone atoms	0.69 ± 0.25
all heavy atoms	1.85 ± 0.81
(2–8) + T2GalNAc + T5GalNAc + T8GalNAc	
backbone atoms	0.39 ± 0.18
All heavy atoms	1.38 ± 0.62

[a] All energies and root mean square values were calculated by using the programs CNS1.1[12] and MOLMOL,[13] respectively. [b] See Supporting Information for details. [c] E_{impr}, E_{VDW}, E_{NOE}, and E_{cdih} are the energy of improper torsion angles, the van der Waals repulsion energy, the square-well NOE potential energy, and the dihedral potential energy, respectively. [d] The force constants for the calculations of E_{VDW}, E_{NOE}, and E_{cdih} were 4.0 kcal mol^{-1} Å^{-4}, 50 kcal mol^{-1} Å^{-1}, and 200 kcal mol^{-1} rad^{-2}, respectively.

Keywords: carbohydrates · computer chemistry · glycopeptides · glycoproteins

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