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Carbohydrate- π Interactions: What Are They Worth?

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Abstract

Protein-carbohydrate interactions play an important role in many biologically important processes. The recognition is mediated by a number of noncovalent interactions including an interaction between the α -face of the carbohydrate and the aromatic side chain. To this end, this interaction has been studied in the context of a β -hairpin in aqueous solution, in which the interaction can be investigated in the absence of other cooperative noncovalent interactions. In this β -hairpin system both the aromatic side chain as well as the carbohydrate was varied in an effort to gain greater insight into the driving force and magnitude of the carbohydrate- π interaction. The magnitude of the interaction was found to vary from -0.5 to -0.8 kcal/mol, depending on the nature of the aromatic ring and the carbohydrate. Replacement of the aromatic ring with an aliphatic group resulted in a decrease in interaction energy to -0.1 kcal/mol, providing evidence for the contribution of CH- π interactions to the driving force. These findings demonstrate the significance of carbohydrate- π interactions within biological systems and also demonstrate its utility as a molecular recognition element in designed systems

Introduction

Many biological processes, including bacterial cell wall recognition, viral and bacterial infections, and fertilization, rely on carbohydrate-protein interactions.^{1, 2} Additionally, glycosylation as a posttranslational modification affects the hydration and conformation of a protein.^{3, 4} Due to its significance in biology, understanding the driving force for binding carbohydrates in water is an active area of research.⁵⁻⁷ In addition to hydrogen bonding, a common feature of carbohydrate binding proteins is the interaction of the α -face of the carbohydrate with the face of an aromatic side chain (Figure 1).^{8, 9} Carbohydrate- π interactions have been investigated through a variety of analytical techniques including NMR, IR, molecular modeling, and X-ray.¹⁰⁻¹⁶ The Simons group utilized IR and modeling to examine the interaction.¹⁷ Jimenez-Barbero et al have used NMR and modeling to examine the driving force for binding of oligosaccharides to the hevein domain and variations thereof.¹⁸⁻²⁰ These studies indicate that the carbohydrate- π interactions are important to the recognition of carbohydrates and that the interaction is dependent on the electronic nature of the aromatic group. However, there is limited experimental data investigating the favorable contribution of carbohydrate- π interaction in isolation.^{15, 16} Given the importance of carbohydrate recognition in biology, a better understanding of the role of carbohydrate- π interactions is warranted.

Previously, we reported an attractive interaction between a tryptophan diagonally cross-strand from a tetraacetylglucoserine which stabilized the folding of a β -hairpin.²¹ Examining the proton NMR shifts of the carbohydrate protons demonstrated that the interaction was primarily through the α -face of the carbohydrate and the face of the Trp sidechain, suggesting a

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carbohydrate- π interaction. Upfield shifting and NOE data is consistent with the geometry shown in Figure 1b. The magnitude of the interaction was found to be -0.8 kcal/mol, which is greater than the magnitude of a Lys-Trp cation- π interaction measured in the same model system.²² However, when the acetyl groups on glucose were removed, the interaction between the carbohydrate and tryptophan was lost. The reduction was attributed to the increased desolvation cost of the unprotected glucose.

To further investigate the efficacy of an isolated carbohydrate- π interaction in aqueous solution, two series of peptides were synthesized and studied. In the first series, aromatic or aliphatic residues (**X**) were incorporated in close proximity to a tetraacetylglucoserine, Ser(Ac₄Glc) on the face of a β -hairpin (Figure 2). In the second series, Trp was kept constant and the nature of the carbohydrate (**Z**) was varied.

Results and Discussion

Design

The 12-residue sequence used in this study is based on peptides previously described, in which a stabilizing carbohydrate- π interaction between a Trp at position 2 and tetracylated glucose at position 9 was explored.²¹ Several key features have been maintained including: a +3 charge to provide solubility and discourage aggregation; an Asn-Gly turn nucleator sequence; and a hydrophobic cluster on both the HB face of the hairpin (Val-3, Val-5, and Ile-10) and the NHB face (X-2 and Leu-11). Aromatic amino acids and carbohydrates were placed in positions 2 (**X**) and 9 (**Z**) respectively. These positions have been shown to allow diagonal cross-strand interaction and provide ample room to allow for the bulky side chains.²²⁻²⁵ The glycosylated series were synthesized per literature methods and introduced into the peptide chain as Fmoc-protected amino acids (see Supporting Information).^{21, 26-31}

Characterization of Structure

β -Hairpin structure characterization was accomplished by a number of NMR measurements described below, including: carbohydrate chemical shifts, α -hydrogen (H_α) chemical shifts; glycine splitting; and cross strand NOEs. NMR spectroscopy provides insight into the geometry of the interaction, as the carbohydrate protons are shifted upfield when in close proximity to the face of the aromatic sidechains due to ring current effects.³² The extent of downfield shifting of H_α relative to random coil is an indicator to the extent of β -sheet conformation at each position along the strand. Downfield shifting of H_α by > 0.1 ppm is indicative of β -hairpin formation.³³ The fraction folded at each residue can be determined by comparing the observed H_α chemical shifts to those in the unfolded state and in the fully folded state (obtained from an unfolded control peptide and a cyclic control peptide, respectively; see Experimental Procedures).^[Syud, 1999 #30] Alternatively, glycine splitting, when compared to a cyclic control, acts as a global indicator of β -hairpin conformation.³⁴ Fraction folded values determined from H_α shifting and Gly splitting were generally in good agreement. Finally, long distance cross strand NOEs between side chains are consistent with β -hairpin structure and were observed for all peptides.

Variation of the Aromatic side chain (X)

To examine the role of the aromatic side chain, a series of peptides were synthesized in which Trp was replaced with other aromatic or hydrophobic side chains including 1-Nal, 2-Nal, Phe, and Cha (Figure 2) while maintaining the carbohydrate as S(Ac₄Glc).

1-Nal was substituted for Trp to investigate the significance of the NH group in Trp. In addition, 1-Nal has a greater surface area than Trp (1-Nal: 161Å² compared to Trp: 147Å²) and the electron density on the face of the ring is not as great (Figure 3). This substitution produced a

well-folded peptide which is as folded as WS(Ac₄Glc) within experimental error (Table 1 and Figure 4). The protons on the α -face (H1, H3, and H5) were all shifted upfield, indicating that the α -face packs against the aromatic face of 1-Nal (Figure 5), similar to the shifting observed for WS(Ac₄Glc). The protons H6 and H6' were also found to be shifted to a lesser extent, indicating that the exocyclic CH₂ interacts with the aromatic face. This interaction and geometry has precedent in galactose binding lectins. While the acetyl groups could not be assigned, the maximum upfield shift was ≤ 0.07 ppm (this is assuming that the methyl group peak which was farthest downfield in the control peptide is the farthest upfield in the β -hairpin). This is significantly less than the shifts of the protons of the α -face of the carbohydrate, which range from -0.6 to -1.2 ppm, and indicates that the acetyl groups play little to no direct role in the stabilizing interaction. NOESY NMR displayed long distance cross-strand interactions in the peptide indicating the peptide is properly folded in a β -hairpin structure. Strong NOEs were also observed between the carbohydrate and 1-Nal (Figure 6), although not as extensively as seen with Trp.

Because the β -sheet propensity of each amino acid influences the stability of the folded state, one cannot directly compare extent of folding of two peptides in which X has been varied and attribute differences exclusively to sidechain-sidechain interactions. To determine the energetic contribution of the diagonal sidechain-sidechain interaction alone, a double mutant cycle was completed.³³⁻³⁹ A double mutant cycle replaces two interacting side chains with two non-interacting side chains. A single mutation disrupts the interaction of interest but could additionally cause other changes to stability (i.e. β -sheet propensity, hydrogen bonding, etc). The double mutant corrects for these unintentional changes leaving only the noncovalent interaction of interest. In this study, X and Z were exchanged for Leu and Ser, respectively. Leu was chosen as it has a high β -sheet propensity that minimizes net loss of β -hairpin stability. Ser was chosen as it has a small polar side chain and that does not interact diagonally. The double mutant cycle reveals that the interaction of 1-Nal with S(Ac₄Glc) has a similar magnitude to the Trp-S(Ac₄Glc) interaction (Table 2).

2-Nal was substituted at the X position to determine the influence of orientation differences between 1-Nal and 2-Nal on the carbohydrate- π interaction. The results were similar to both 1-Nal and Trp, indicating that the carbohydrate interacts in a favorable manner with 2-Nal via stacking with the aromatic sidechain, despite the differences in orientation of the two sidechains (Table 1 and Figure 4). The upfield shifting of the peptide H α protons demonstrated that the β -hairpin conformation was well formed throughout the peptide with a similar stability as WS(Ac₄Glc) and 1-NalS(Ac₄Glc). Additionally, the carbohydrate shifts were similar in magnitude to those in the peptides with the other two large aromatic groups (Figure 5), namely, that the protons of the carbohydrates α -face are shifted significantly upfield indicating an interaction with the aromatic face. Long-distance NOEs between the carbohydrate's α -face and 2-Nal also support a stacking geometry (Figure 6). The magnitude of the interaction of 2-Nal with Ac₄Glc was also found to be similar to that of Trp and 1-Nal, as determined from the double mutant cycle (Table 2).

The situation changes when the smaller Phe was placed in the sequence at position 2. The percent folded was reduced from 85% to 57% (Figure 4 and Table 1). Phe is known to have a lower β -sheet propensity than Trp,⁴⁰[Smith, 1997 #121] but the double mutant cycle indicates that the loss in β -hairpin stability is due in part to a weakening of the carbohydrate- π interaction (Table 2). Additionally, the carbohydrate's α -face protons are not upfield shifted nearly as much as when X is a larger aromatic group. There are significantly fewer unambiguous NOEs between the Phe side chain and the face of the carbohydrate than between the larger aromatic side chains and the carbohydrates. The smaller changes in chemical shift of both the H α and the carbohydrate protons indicate a less folded hairpin and a less favorable carbohydrate- π interaction.

When the aromatic nature of the **X** sidechain is removed by replacing Phe with Cha, the stability of the hairpin is further reduced (Table 1), despite the fact that Cha has been shown to have a higher β -sheet propensity than Phe.⁴¹ A double mutant cycle indicates that the interaction of Cha with Ac₄Glc is weaker than that of Phe with Ac₄Glc (Table 2), despite the similar facial surface area. Unlike the aromatic peptides in this series, there are no unambiguous NOEs between the cyclohexane side chain and the carbohydrate (Figure 6). Since Cha is not aromatic, no shifting of the carbohydrate protons is observed.

To provide additional insight into the effect of the **X** group on the interaction with Ac₄Glc, we performed thermal denaturations on WS(Ac₄Glc), FS(Ac₄Glc), and ChaS(Ac₄Glc) by NMR.³³ Fitting of the data provides values for ΔH° , ΔS° , and ΔC_p for folding (Table 3). Since the only change in the peptide sequence is the **X** group at position 2, changes in the driving force for folding can be attributed to the role of that residue in stabilizing the folded state. One can see that for Trp and Phe folding is more enthalpically favorable than for Cha, which does not significantly interact with Ac₄Glc. This is consistent with an enthalpic driving force for the interaction of the carbohydrate with the aromatic ring, as has been observed in other systems,^{5, 42} and is suggestive of CH(δ^+)- π and dispersion forces as major contributors to the interaction.

Variation of the Carbohydrate

The carbohydrate was also varied while holding Trp constant and the impact on the sidechain-sidechain interaction was explored. Previous studies comparing Ac₄Glc and Glc suggested that Ac₄Glc formed a favorable carbohydrate- π interaction, but the desolvation cost appeared to be too high for Glc to interact favorably with Trp.[Kiehna, 2007 #85] Two additional acylated carbohydrates, Ac₄Gal and Ac₃GlcNAc (Figure 2) have been substituted for Ac₄Glc. Ac₄Gal investigates the effect of the stereochemistry at C4 on the carbohydrate- π interaction. Ac₃GlcNAc investigates the replacement of oxygen at C-2 with nitrogen. We also investigated the deprotected counterpart, GlcNAc, in which only the nitrogen at C-2 is acetylated. Lastly, we investigated Me₄Glc to further investigate the role of desolvation and determine the role (if any) of the acetyl groups.

The only difference between the Ac₄Glc and Ac₄Gal is the orientation of the alcohol at C-4 (axial vs. equatorial) (Figure 2). The binding sites of many galactose binding proteins (galectins) contain an aromatic residue which interacts with the “hydrophobic cluster” made up of C-4, C-5, and C-6 (Figure 7).⁴³ The upfield shifting of the 6/6' protons of Ac₄Glc in WS (Ac₄Glc) suggested that such an interaction at C-4, C-5, and C-6 may be feasible in the β -hairpin. Thus, we replaced Ac₄Glc with Ac₄Gal and investigated its interaction with Trp. There was only a small change in the fraction folded for WS(Ac₄Gal) relative to WS(Ac₄Glc), as measured by the glycine splitting and H α shifts (Table 4 and Figure 8). NOEs between the sugar and Trp indicate that the interaction occurs on the α -face of the sugar, as was seen for Ac₄Glc. Inspection of the carbohydrate chemical shifts reveals that the protons of the α -face (C-1, C-3, and C-5) are the most shifted relative to random coil but C-4 is not significantly shifted (Figure 9), indicating the same geometry as for WS(Ac₄Glc) rather than that seen in galectins. This may be due to conformational restrictions of the system rather than a specific preference for one geometry over the other. The extent of shifting at positions 1, 3, and 5 of Ac₄Gal is similar to that observed in Ac₄Glc, suggesting that the interaction with the α -face is equally as favorable. This is consistent with the interaction energy determined from double mutant cycles (Table 5), which is within error of that measured for Ac₄Glc.

Another common carbohydrate found in nature is GlcNAc. This carbohydrate has two distinctive features, namely that nitrogen replaces oxygen at C-2, and that the nitrogen is acetylated. Both of these differences change how the carbohydrate interacts with the face of the Trp. The tetraacylated sugar, Ac₃GlcNAc, was used to compare directly with Ac₄Glc. The

presence of the amide reduces the interaction relative to WS(Ac₄Glc): the fraction folded is reduced from 0.85 to 0.73 (Figure 8 and Table 4) and the double mutant cycle demonstrates that the interaction energy is reduced by 0.2 kcal mol⁻¹ (Table 5). However, the protons of Ac₃GlcNAc α -face are not nearly as upfield shifted as compared to WS(Ac₄Glc) (Figure 9). The proton at C-1 is the only one that is significantly shifted, suggesting a change in geometry due to the amide at C-2. This suggests that the energetic term from the double mutant cycle arises from a favorable interaction other than the carbohydrate-p interaction alone. Indeed, downfield shifting of the NH group of Ac₃GlcNAc (9.34 ppm) suggests that it may participate in a hydrogen bond. The Trp NH is not significantly shifted, however (10.16 ppm vs 10.11 ppm in an unfolded control peptide).

When the acyl groups are removed from the Ac₃GlcNAc the fraction folded only decreases slightly (0.73 vs. 0.70 for Ac₃GlcNAc and GlcNAc, respectively). However, the carbohydrate protons are not nearly as upfield shifted when compared to the other carbohydrates, with the greatest shifting occurring at C-6 (Figure 9). In fact, the chemical shifts of GlcNAc are similar to those of Glc, which did not display a significant interaction with Trp.²¹ There are several weak unambiguous NOEs between the α -face of the carbohydrate (Figure 10). The double mutant cycle indicates that the interaction energy for GlcNAc is comparable to Ac₃GlcNAc. Thus, it appears that some sort of a favorable interaction occurs, but via a different geometry than seen with other carbohydrates. However, NMR provides no evidence of hydrogen bonding involving either the GlcNAc NH (7.82 vs 7.88 ppm in the unfolded control peptide) or the Trp NH (10.17 vs 10.11 ppm in the unfolded control peptide).

We also investigated the peptide in which the acetyl protecting groups of Ac₄Glc were replaced with methyl groups to probe the role of desolvation and to determine if there is a specific influence of the acetyl groups. The peptide WS(Me₄Glc) is equally well folded as WS(Ac₄Glc) (Table 4, Figure 8), and exhibits numerous NOEs between the Trp residue and α -face of the sugar (Figure 10), indicating that Me₄Glc also forms a favorable interaction with Trp. Double mutant cycles indicate that the magnitude of the interaction is within error for Me₄Glc and Ac₄Glc. This appears to suggest that protection of the hydroxyl groups, and hence reduction of the desolvation cost, is indeed the primary difference between peptides WS(Ac₄Glc) and WS(Me₄Glc) relative to the unprotected glucose in WS(Glc). However, Ac₄Glc and Me₄Glc do not behave identically; peptide WS(Me₄Glc) does not demonstrate the same extent of upfield shifting of the carbohydrate protons at positions C-1, C-3, and C-5 as does peptide WS(Ac₄Glc) (0.4-0.6 ppm for peptide WS(Me₄Glc) versus 0.6-1.35 ppm for peptide WS(Ac₄Glc)), despite the similar stability of the β -hairpins. This may be due to competition of the methyl groups for interaction with the Trp, as the methyl groups are also upfield shifted by up to 0.25 ppm. Indeed, the magnitude of upfield shifting of the methyl groups is very similar to the shifting that Cuevas and coworkers observed in their study of carbohydrate- π interactions with Me₅Glc.⁴⁴ Thus, it appears that while Me₄Glc forms a favorable interaction with Trp, the interaction of the α -face of the sugar is not the only contributor to the interaction.

Discussion

The system described here has allowed for the systematic investigation of carbohydrate-aromatic interactions in the absence of other cooperative noncovalent interactions, such as hydrogen bonds, thus allowing for the quantification of the binding energy and an exploration of the factors that contribute to the interaction. Variation of the X sidechain provides significant insight into the nature and driving force of the carbohydrate-aromatic interaction. The similar interactions of Ac₄Glc with Trp, 1-Nal, and 2-Nal confirm that Ac₄Glc interacts primarily with the face of the aromatic ring and that any hydrogen bonding to the NH of Trp is a minor contributor to the interaction at best (≤ 0.1 kcal/mol). The fact that 1-Nal and 2-Nal interact similarly indicates that this model system has enough flexibility to optimize the interaction

when the orientation of the aromatic ring is varied. Comparison of Trp to Phe indicates that the surface area of the aromatic ring impacts the magnitude of the interaction (-0.8 kcal/mol for Trp versus -0.5 kcal/mol for Phe).

The interaction between carbohydrates and aromatic groups has been variously described in terms of the hydrophobic effect, dispersion forces, and CH- π interactions.^{20, 45, 46} Comparison of Phe versus Cha at position X indicates that the carbohydrate- π interaction is more favorable than an equivalent hydrophobic interaction between Ac₄Glc and an aliphatic sidechain (-0.5 kcal/mol for Phe versus -0.1 kcal/mol for Cha). This is similar to what has been seen in protein mutation studies, in which mutation of an aromatic residue in the binding pocket abolishes binding of the carbohydrate.^{20, 47} Because naturally occurring aliphatic amino acids have a different size and shape than an aromatic residue, the results from protein mutation studies have been difficult to attribute solely to the loss of aromaticity. Since Phe and Cha have the same facial surface area, the comparison within the β -hairpin model system is more direct, and clearly indicates that aromaticity influences the interaction energy. The preference for interaction of Ac₄Glc with Phe over Cha and the greater enthalpic driving force for folding of FS(Ac₄Glc) relative to ChaS(Ac₄Glc) points to CH(δ^+)- π interactions as a significant contributor to the driving force of the interaction, as cyclohexane is more polarizable than benzene, and so dispersion forces would be expected to be stronger with Cha than Phe.⁴⁸ Moreover, Cha is also more hydrophobic than Phe, arguing against the hydrophobic effect as the primary driving force for interaction. This is consistent with Jimenez-Barbero's finding that variation of the electronics of the aromatic ring influences carbohydrate binding in the hevein domain.²⁰

Variation of the carbohydrate provides insight into the balance of features that influence this interaction. Within the β -hairpin model system, it appears that interaction on the α -face of the carbohydrate is most favorable, even when another "hydrophobic" surface is present, as in Ac₄Gal. In the case of Ac₃GlcNAc and GlcNAc, the interaction energy decreases and the geometry of the interaction changes, likely because of the presence of the amide at C-2 which is expected to have stronger interactions with solvent than the corresponding ester.

A comparison of Ac₄Glc to Me₄Glc was made to address the roles of desolvation and electrostatics to the interaction. We have previously shown that Ac₄Glc interacts favorably with Trp, with an interaction energy of approximately -0.8 kcal/mol, but that removal of the acetyl groups results in loss of the favorable interaction. We attributed this to differences in desolvation cost, although the electron-withdrawing nature of the acetyl groups also results in differences in the partial charge on the α -face of the sugar between Ac₄Glc and Glc, as indicated by the electrostatic potential maps (Figure 11). Thus, we investigated the interaction of Me₄Glc with Trp because it has a similar electrostatic potential map to Glc, but its desolvation cost is significantly reduced. The observed stabilizing interaction ($\Delta\Delta G = -0.8$ kcal/mol) indicates that paying the desolvation cost is indeed enough to allow for a favorable carbohydrate- π interaction, and that there is nothing unique about the acetylated glucose. However, NMR shifts of Me₄Glc indicate that interaction of Trp with the α -face is reduced and that direct interaction with the polarized methyl groups is also occurring. Thus, a direct comparison of the role of electrostatics in the interaction of Trp with Ac₄Glc and Me₄Glc is not possible, as there are different contributors to the interaction energy. Nonetheless, the NMR data suggest that the weaker polarization of the α -face of the sugar may reduce the carbohydrate- π interaction and that the interaction between the methyl groups and Trp provide a compensating interaction.

Since the carbohydrate- π interaction is only observed when the hydroxyl groups are protected in this system, the question arises as to whether this interaction is significant in carbohydrate binding proteins, where the carbohydrate is unprotected. We have shown that the role of the

protecting groups is to desolvate the sugar to allow for interaction with the aromatic ring. Within a carbohydrate binding protein, hydrogen bonding groups are pre-organized for the same task. Thus, it appears that Nature uses cooperative interactions between the aromatic ring and hydrogen bonding groups to desolvate and bind the carbohydrate.⁴² Indeed, obtaining this sort of cooperative binding may be the primary challenge in designing synthetic receptors for carbohydrates in water.⁷

We have measured a wide range of noncovalent interactions within the same peptide model system, and so a direct comparison can be made between them. Surprisingly, the interaction energy between Ac₄Glc and Trp is larger than the cation- π interaction between Lys and Trp (-0.4 kcal/mol),^{22, 25} but is similar in magnitude to the interaction between KMe3 and Trp (-1.0 kcal/mol).²³ Thus, the carbohydrate- π interaction is a considerable interaction. In contrast, the interaction of Cha with Ac₄Glc is the same magnitude as its interaction with Lys (-0.1 kcal/mol).²²

Lastly, these studies also provide evidence for a novel method of influencing protein structure. In structural studies of glycosylated proteins and peptides, it has generally been found that stabilization of the folded state occurs because glycosylation rigidifies the peptide backbone, thereby destabilizing the unfolded state.⁴⁹⁻⁵⁵ In contrast, in the system reported here, incorporation of a carbohydrate- π interaction results in enthalpic stabilization of the folded structure through a specific interaction.

Conclusion

This study provides insight into the role of carbohydrate- π interactions in carbohydrate recognition by proteins. The energetic contribution of the carbohydrate- π interaction between the α -face of the pyranose ring and the face of an aromatic ring was found to range from -0.5 and -0.8 kcal mol⁻¹, and is dependent on the nature of both the aromatic ring and the carbohydrate. Of significance is the fact that a favorable interaction is only observed when the hydroxyl groups of the carbohydrate are protected, whether with acetyl groups or methyl groups. This implies a significant cost for desolvation of the sugar. However, NMR data suggests that the interaction of Ac₄Glc is more favorable than for Me₄Glc, which may imply an electronic tuning of the interaction. Moreover, the preferential interaction of the pyranose ring with the face of a phenyl group relative to a cyclohexyl ring suggests that CH(δ^+)- π interactions play a measurable role in the interaction. These studies provide a better physical understanding of the driving force behind the carbohydrate- π interaction as well as insight into their magnitude and significance relative to other noncovalent interactions which have been measured in the same model system. In addition to providing insight into the recognition of carbohydrates by proteins, we expect that the findings of this study will be useful in the development of new and improved receptors for carbohydrate recognition.

Experimental Section

Peptide Synthesis and Purification

All peptides were synthesized on Fmoc-PAL-PEG-PS amide resin using standard solid-phase protocols on a continuous flow Pioneer Peptide Synthesizer (Applied Biosystems). Fmoc-amino acids (4-6 equiv) were activated and coupled with 0.45M HBTU/HOBt in DMF. The following protecting groups were used: Arg(Pbf), Asn(Trt), Cys(Trt), Gln(Trt), Lys(Boc), Ser(tBu), Thr(tBu), Trp(Boc). Deprotection of the Fmoc groups was achieved with 2% piperidine, 2% 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in DMF. All peptides were acylated at the N-terminus using a 5% acetic anhydride/6% lutidine/DMF solution and amidated at the C-terminus. Peptide resin cleavage and deprotection was performed simultaneously by treatment with 95% trifluoroacetic acid (TFA)/2.5% triisopropylsilane (TIPS)/2.5% H₂O, for 2-3 hours

under nitrogen. The TFA was removed by distillation under vacuum. The crude peptides were precipitated with cold ether, extracted into water and lyophilized.

Crude peptides were then dissolved and purified by reverse phase HPLC using a Vydac C₁₈ semipreparative column. Peptides were eluted with a linear gradient of 95% H₂O /5% acetonitrile with 0.1% TFA (Solvent A) and 95% acetonitrile/5% water with 0.1% TFA (Solvent B) from 0-30% B. Peptides were detected by monitoring at 220 and 280 nm. Molecular weights were determined using ESI mass spectrometry. Disulfide bonds were formed by DMSO oxidation of purified peptides in PBS (pH 7.4). Peptides were then repurified by RP-HPLC.

NMR Spectroscopy

NMR samples were made to concentrations of 1-3 mM and analyzed on a Varian Inova 600 MHz instrument. Samples were dissolved in D₂O buffered with 10 mM acetate-d₃ pH 4.2 and referenced to DSS. NMR spectra were collected with between 8-64 scans using a 1.5 s presaturation. All 2D NMR experiments used pulse sequences from the chempack software including TOCSY, gCOSY, and ROESY. TOCSY and gCOSY experiments were performed with 4-8 scans in the 1st dimension and 256 in the 2nd dimension. ROESY experiments were performed with 32 scans in the 1st dimension and 256-512 in the 2nd dimension. All spectra were analyzed using standard window functions (sinebell and gaussian with shifting). Assignments were made using standard methods. Thermal denaturations were performed in duplicate in 5-10 degree increments. The temperature was calibrated with methanol and ethylene glycol standards using Varian macros.

Determination of Fraction Folded

To determine the chemical shifts of the fully folded state, 14-residue disulfide-linked analogues of peptides were synthesized with the sequence Ac-CRXVTVNGKZILQC-NH₂ where X = Trp, 1-Nal, 2-Nal, Cha, or Phe and Z = Ser(Ac₄Glc), Ser(Ac₄Gal), Ser(Ac₃GlcNAc), Ser(GlcNAc), and Ser(Me₄Glc) and characterized by NMR. To determine the chemical shifts of the unfolded state, a series of 7-residue peptides were synthesized and characterized. The sequences of these peptides were Ac-RXVTVNG-NH₂ (X = 1-Nal, 2-Nal, Cha, and Phe) and Ac-NGKZILQ-NH₂ (Z = Ser(Ac₄Gal), Ser(Ac₃GlcNAc), Ser(GlcNAc), and Ser(Me₄Glc)). The 7-mers with either Trp or Ser(Ac₄Glc) had previously been described.²¹ The fraction folded was determined from eq 1.

$$\text{Fraction Folded} = f = [\delta_{\text{obs}} - \delta_0] / [\delta_{100} - \delta_0] \quad (\text{eq 1})$$

where δ_{obs} is the observed chemical shift, δ_{100} is the chemical shift of the cyclic peptide and δ_0 is the chemical shift of the unfolded control peptides. The fraction folded as determined by glycine splitting was determined with the equation:

$$\text{Fraction folded} = \frac{\Delta\delta_{\text{Gly hairpin}}}{\Delta\delta_{\text{Gly cycle}}} \quad (\text{eqn 2})$$

Double Mutant Cycle

Double mutant cycles were performed to quantify the interaction between the series of carbohydrates and the sidechain X. Single mutant peptide in which Ser(Ac₄Glc), Ser(Ac₄Gal), Ser(Ac₃GlcNAc), Ser(GlcNAc), or Ser(Me₄Glc) were replaced by Ser and 1-Nal, 2-Nal, Phe,

and Cha were replaced by Leu. The double mutant contained both substitutions. The singly mutated peptides RWVTVNGKSILQ and RLVTVNGKS(Ac₄Glc)ILQ as well as the double mutant RLVTVNGKSILQ were previously reported.²¹ Difficulties arose in the synthesis of the cyclic RLVTVNGKS(Me₄Glc)ILQ control. The glycine splitting value of cyclic RLVTVNGKS(Ac₄Glc)ILQ control was used in its place. The energy of folding for each peptide was determined from the difference in chemical shift of the glycine hydrogens. The side chain interaction energy was then determined using equation 3.

$$\Delta\Delta G(X - Z) = \Delta G_{XZ} - \Delta G_{XS} - \Delta G_{LZ} + \Delta G_{LS} \quad (\text{eqn } 3)$$

Thermal Denaturation

Variable temperature NMR was used to perform the thermal denaturation experiments. A temperature range of 275 to 330K was explored in five-degree increments. The temperature was calibrated using methanol and ethylene glycol standards. The change in glycine chemical shift difference was used to determine the fraction folded at each temperature. The fraction folded of the peptide was plotted against temperature, and the curve was fitted with equation 4.

$$\text{Fraction Folded} = \frac{\exp(\frac{x}{RT})}{(1 + \exp(\frac{x}{RT}))}$$

$$x = (T[\Delta S_{298}^{\circ} + \Delta C_p \ln(\frac{T}{298})]) - [\Delta H_{298}^{\circ} + \Delta C_p(T - 298)] \quad (\text{eqn } 4)$$

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) Interaction between glucose and Trp 183 in the *E. coli* chemoreceptor protein (pdb: 2GBP).

⁹ (b) Interaction geometry for Trp and Ac4Glc in the context of a beta-hairpin peptide.²¹

Figure 2.
 β -hairpin structure and **X/Z** side chains.

Figure 3. Electrostatic potential maps of the sidechains at position **X** in the β -hairpin. (a) indole; (b) naphthalene; (c) benzene; (d) cyclohexane. Electrostatic potential maps were generated with MacSpartan: HF/6-31g*; Isodensity value = 0.02; range = -25 (red, electron rich) to 25 kcal/mol (blue, electron poor).

Figure 4. Fraction folded as determined at from $H\alpha$ chemical shifts. WS(AcGlc) values originally reported in Ref 19.

Figure 5.

(a) Upfield shifting of carbohydrate protons in peptides WS(Ac₄Glc), 1-NalS(Ac₄Glc), 2-NalS(Ac₄Glc), PheS(Ac₄Glc), and ChaS(Ac₄Glc). Conditions: 50 mM sodium acetate-*d*₄, pH 4.0 (uncorrected) at 298 K, referenced to DSS. WS(Ac₄Glc) values originally reported in ^{Ref 19}.

Figure 6. Unambiguous NOEs (red arrows) observed between the carbohydrate side chain and **X** (1-Nal, 2-Nal, Phe and Cha). Unambiguous NOEs are defined as that can be definitively assigned to a particular set of protons.

Figure 7.
A Trp-Gal interaction in the binding pocket of galectin.⁴³

Figure 8. Fraction folded as determined from $H\alpha$ chemical shifts. WS(Ac₄Glc) values were originally reported in Ref 19.

Figure 9. Upfield shifting of carbohydrate protons in peptides. WS(Ac₄Glc) values were originally reported in Ref 19.

Figure 10.

Unambiguous NOEs (red arrows) observed between the carbohydrate side-chain (Ac₄Gal, Ac₃GlcNAc, GlcNAc, and Me₄Glc) and Trp. Unambiguous NOEs are defined as that can be definitively assigned to a particular set of protons.

Figure 11. Electrostatic potential maps of the sidechains at position Z in the β -hairpin. (a) Ac₄Glc; (b) Glc; (c) Me₄Glc. Electrostatic potential maps were generated with MacSpartan: HF/6-31g*; Isodensity value = 0.02; range = -25 (red, electron rich) to 25 kcal/mol (blue, electron poor).

Table 1

Fraction Folded and ΔG° (folding) at 298 K for β -hairpins.^a

X	S(Z)	$\Delta\delta\text{Gly}$ (ppm) (± 0.005 ppm)	Fraction Folded (Gly splitting) ^b	Fraction Folded (H α) ^c	ΔG (folding) (kcal mol ⁻¹) ^d
Trp ^e	Ac ₄ Glc	0.484	0.85	0.83 (0.02)	-1.03
1-Nal	Ac ₄ Glc	0.484	0.86	0.83 (0.03)	-1.08
2-Nal	Ac ₄ Glc	0.471	0.83	0.81 (0.04)	-0.94
Phe	Ac ₄ Glc	0.325	0.57	0.51 (0.13)	-0.17
Cha	Ac ₄ Glc	0.242	0.45	0.42 (0.12)	0.12

(a) Conditions: 50 mM sodium acetate-*d4*, pH 4.0 (uncorrected) at 298 K, referenced to DSS.

(b) Error is ± 0.01 .

(c) H α fraction folded was determined from the average of the values for Val 3, Val 5, Lys 8, and Ile 10. The standard deviation is shown in parentheses.

(d) ΔG determined using glycine-splitting values; error is ± 0.05 kcal/mol.

(e) Previously reported in Ref 19.

Table 2

The Diagonal Interaction Energies Between Residues 2 and 9 as Determined by Double Mutant Cycles

X	S(Z)	$\Delta\Delta G$ (kcal mol ⁻¹) ^a
Trp ^b	Ac ₄ Glc	-0.8
1-Nal	Ac ₄ Glc	-0.7
2-Nal	Ac ₄ Glc	-0.7
Phe	Ac ₄ Glc	-0.5
Cha	Ac ₄ Glc	-0.1

^aThe error in the diagonal interaction energy is ± 0.1 kcal mol⁻¹^bPreviously reported in Ref 19

Table 3

Thermodynamic parameters for folding at 298 K obtained from thermal denaturation of the peptides.

Peptide	ΔH° (kcal mol ⁻¹)	ΔS° (cal mol ⁻¹ K ⁻¹)	ΔC_p (cal mol ⁻¹ K ⁻¹)
WS(Ac ₄ Glc) ^(a)	-5.9	-16.4	-112
FS(Ac ₄ Glc)	-4.23	-13.77	-77
ChaS(Ac ₄ Glc)	-2.96	-10.32	-88

^(a) Previously reported in Ref 19.

Table 4

β -Hairpin Fraction folded and ΔG (folding) at 298 K.

X	S(Z)	$\Delta\delta_{\text{Gly}}$ (± 0.005 ppm)	Fraction Folded (Gly splitting) ^b	Fraction Folded (H α) ^c	ΔG (folding) (kcal/mol) ^d
Trp ^e	Ac ₄ Glc	0.484	0.85	0.83 (0.02)	-1.03
Trp	Ac ₄ Gal	0.466	0.82	0.77 (0.04)	-0.90
Trp	Ac ₃ GlcNAc	0.421	0.73	0.66 (0.09)	-0.59
Trp	GlcNAc	0.392	0.70	0.64 (0.02)	-0.50
Trp	Me ₄ Glc	0.50	0.88	0.81 (0.01)	-1.18
Trp ^c	Glc	0.383	0.65	0.63 (0.02)	-0.37
Trp	OH	0.375	0.64	0.60 (0.01)	-0.34

(a) Conditions: 50 mM sodium acetate-*d*4, pH 4.0 (uncorrected) at 298 K, referenced to DSS.

(b) Error is ± 0.01 .

(c) H α fraction folded was determined from the average of the values for Val 3, Val 5, Lys 8, and Ile 10; the standard deviation is shown in parentheses.

(d) ΔG determined using glycine-splitting values; error is ± 0.05 kcal/mol.

(e) Previously reported in Ref 19.

Table 5

The diagonal interaction energies as determined by Double Mutant Cycles

X	Y	$\Delta\Delta G$ (kcal mol ⁻¹) ^a
Trp ^b	Ac ₄ Glc	-0.8
Trp	Ac ₄ Gal	-0.7
Trp	Ac ₃ GlcNAc	-0.6 ^c
Trp	GlcNAc	-0.5 ^c
Trp	Me ₄ Glc	-0.8 ^c

^aThe error in the diagonal interaction energy is ± 0.1 kcal mol⁻¹^bPreviously reported in Ref 19.^cAlthough the interaction energy for this mutant is similar to that of the Trp-Ac₄Glc interaction, the NMR data suggests that an interaction other than the carbohydrate- π interaction is contributing. See text for details.