Carbohydrates

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Random-Coil:α-Helix Equilibria as a Reporter for the Lewis^X–Lewis^X Interaction**

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Weak multivalent interactions are now recognized as key to many biological processes.[1] Since the discovery of carbohydrate–carbohydrate interactions (CCIs),[2] studies of this phenomenon have now linked CCIs (both *cis*- and *trans*-CCIs) to critical biological recognition events, such as cell signaling and adhesion, fertilization, and metastasis.[3] CCIs are intrinsically weak so their study and quantification at the monovalent level is a significant challenge that represents the focus of this Communication.

Previous work has relied on macroscopic or multivalent systems including synthetic polymers,[4] micelles and vesicles,[5] glycosylated nanoparticles,[6] and Langmuir–Blodgett monolayers.[7] These studies have generally (though not exclusively) focused on the biologically important Lewis^X–Lewis^X (Le^X–Le^X) interaction.[4–8] As a result, a number of factors important in CCIs are now apparent, and these include multivalent (Velcro-like) presentation of carbohydrates on a surface; a requirement for polyamphiphilic surfaces associated with the hydraphobic effect;[9] and, in certain cases, roles for both divalent metal cations (e.g. Ca^{2+}) and ionic (charge) effects. While multivalency effectively amplifies CCIs, the complexity of such macroscopic systems makes mapping the individual impact of component carbohydrate (CHO) units and their associated molecular features difficult to define.

To achieve a more detailed picture of CCIs, while recognizing the inherent challenge of studying this phenomenon in isolation (i.e. outside of a multivalent environment),[10] we have evaluated the ability of a conformationally dynamic system to report on a weak, attractive CCI based on Le^X–Le^X. Random-coil:α-helix equilibria displayed by alanine-rich peptides in aqueous solution, where helix content is highly sensitive to small changes in the free energy of helix formation, provide an attractive, effective and potentially versatile vehicle for this purpose (Figure 1a). The requisite peptides are readily accessible, and helix content can be measured accurately by circular dichroism (CD) spectroscopy. We posit that with two CHOs ligated at specified positions (Figure 1b) on the peptide backbone, perturbation of this highly sensitive equilibrium to a more helical state would indicate the presence of an attractive (i.e. stabilizing) CCI, thereby providing a means of studying this phenomenon in comparative isolation, outside of a multivalent environment.

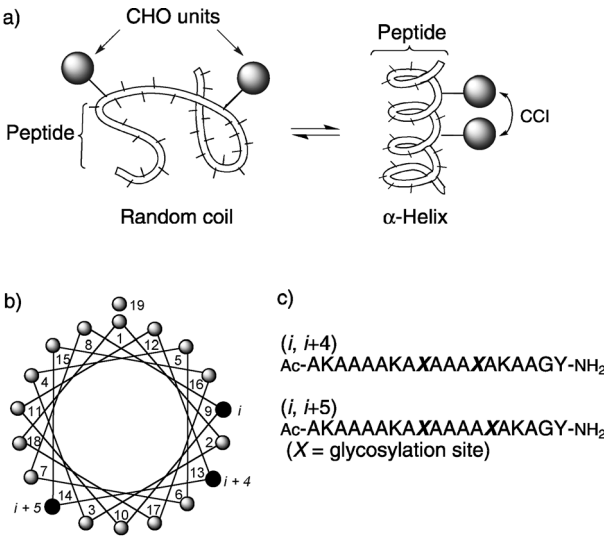

Figure 1. a) Schematic of random-coil:α-helix equilibrium for detection of CCIs. b) Helical wheel diagram showing functionalized *i*, *i*+4, and *i*+5 sites. c) General peptide sequences used in this study.

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tration) and orthogonally protected Lys units provided the two necessary glycosylation sites. The N- and C-termini were capped with acetyl and amide groups, respectively, to remove charges and stabilize the helix. Peptides were designed to be close to 50% helical in order to be maximally sensitive (given the weak nature of the interaction to side-chain interactions being pursued) involving the CHO guests. Modified Lifson–Roig helix–coil theory[11] was used to predict α-helix contents for the isolated peptides in aqueous solution, using parameters for interior and N-cap positions of helices.[12] Given the α-helical repeat of 3.6 amino acids per turn (Figure 1b) glycosylation sites were spaced *i*, *i*+4 to locate two CHO moieties close in space, and to maximize the population of (and preference for) the helical component in the presence of a stabilizing CCI.

General controls were provided by *i*, *i*+5 variants since this relationship provides no stabilizing interaction within an α-helix (see Figure 1b); other controls/benchmarks were also employed as discussed below. Specifically, we have used these sequences to probe the Le^X–Le^X interaction, and to validate the applicability and veracity of the coil:helix approach to detecting and studying CCIs. The introduction of a functionalized C_3 linker based on Le^X thioglycoside **1**[13] was achieved (see Supporting Information), and provided Le^X thiol **2a** and disulfide **2b** (Figure 2); both were used for peptide ligation. The same C_3 linker unit was common to all the CHO-based controls employed in this study.

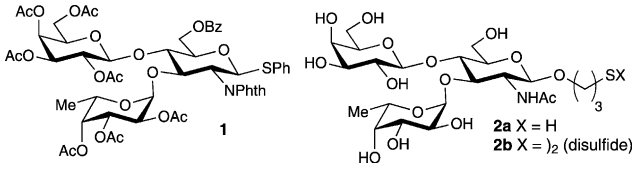

Figure 2. Le^X thioglycoside **1**, Le^X thiol **2a** and disulfide **2b**.

Solid-phase Fmoc protocols provided a series of underivatized and *N*-ε-acetyl peptides **3a–d** and **4a–c**, respectively. In addition, and using the same C_3 linker, monoLe^X peptide **5a**, guest bisglycopeptides **5b** and **5c** incorporating two Le^X trisaccharide units, and the corresponding monosaccharide (glucose) **6a/b** and disaccharide (lactose) **7a/b** variants were prepared. CD data were obtained as an average of 30 spectra at five different concentrations and were performed in triplicate to determine experimental repeatability. The intensity of the CD signals at 222 nm indicated that all peptides tested had a helical component in the range of 37–54% (Table 1, Figure 3 and Figure 4, and Supporting Information), consistent with the original peptide design.

Participation of higher-order aggregates was excluded as no concentration dependencies were observed within the range 12.5–200 μM; this is important as peptide aggregation would interfere with the detection of a monovalent, isolated CCI.

The control systems were further calibrated. Peptides carrying free (i.e. positively charged) lysine at *i*, *i*+4 and *i*, *i*+5

Table 1: Helicities of peptides: controls and glycopeptides variants.

Control peptides (***X*** = K)			% Helicity[a]
3a	*i*, *i*+4	Ac-AKAAAAKA***X***AAA***X***AKAAGY-NH_2	38
3b	*i*, *i*+5	Ac-AKAAAAKA***X***AAAA***X***AKAAGY-NH_2	41
3c	*i*, *i*+4	Ac-AKAAAAKA***E***AAA***X***AKAAGY-NH_2	54
3d	*i*, *i*+5	Ac-AKAAAAKA***E***AAAA***X***AKAAGY-NH_2	39
Acetylated control peptides (***X*** = KAc)			
4a	–	Ac-AKAAAAKA***X***AAAKAKAAGY-NH_2	52
4b	*i*, *i*+4	Ac-AKAAAAKA***X***AAA***X***AKAAGY-NH_2	52
4c	*i*, *i*+5	Ac-AKAAAAKA***X***AAAA***X***AKAAGY-NH_2	51
Le^X glycopeptides (***X*** = $KCOCH_2S(CH_2)_3OLe^X$)			
5a	–	Ac-AKAAAAKA***X***AAAKAKAAGY-NH_2	51
5b	*i*, *i*+4	Ac-AKAAAAKA***X***AAA***X***AKAAGY-NH_2	54
5c	*i*, *i*+5	Ac-AKAAAAKA***X***AAAA***X***AKAAGY-NH_2	49
Other controls (***X*** = $KCOCH_2S(CH_2)_3$O-Glc **6a/b** or O-Lac **7a/b**)			
6a	*i*, *i*+4	Ac-AKAAAAKA***X***AAA***X***AKAAGY-NH_2	40
6b	*i*, *i*+5	Ac-AKAAAAKA***X***AAAA***X***AKAAGY-NH_2	48
7a	*i*, *i*+4	Ac-AKAAAAKA***X***AAA***X***AKAAGY-NH_2	37
7b	*i*, *i*+5	Ac-AKAAAAKA***X***AAAA***X***AKAAGY-NH_2	49

[a] CD spectroscopy was performed at 5°C (pH 7.0; 10 mM MOPS buffer) with helicity values calculated using $f_h = (\theta_{obs} - \theta_{coil})/(\theta_h - \theta_{coil})$.[14] Experimental errors, based on multiple scans and replicates (see Supporting Information), were all within the range 0.4–1.4%.

(controls **3a** and **3b**) had similar and relatively low (38% and 41%, respectively) helical contents.[15] To demonstrate the sensitivity of the peptide reporter to a stabilizing side-chain interaction, we used a known[16] glutamate-to-lysine interaction: the *i*, *i*+4 peptide **3c** displayed a higher helix content (54%) than the *i*, *i*+5 **3d** (39%), confirming the ability of a stabilizing effect to enhance helix content in the peptide reporter system used here.[17]

The *N*-ε-Ac-K (mono- and two bisacetylated) variants **4a–c** relate more closely to the Le^X glycopeptides **5a–c** in structural and charge terms and offered an important control set; we suggest that N-capped **4a–c** are more relevant to this study as controls than the free lysine **3a/b** variants. Monoacetylated **4a**, the *i*, *i*+4 and *i*, *i*+5 bisacetylated peptides **4b** and **4c**, respectively, and the monoLe^X glycopeptide **5a** displayed similar levels of helicity (51–52%, Table 1), indicating that these substitutions (both NAc and monoglycosylation) do not affect significantly the coil:helix distribution.

Comparison of the two key bisglycosylated Le^X glycopeptides **5b/c** showed that *i*, *i*+4 **5b** had a higher helical content than *i*, *i*+5 isomer **5c** (54% vs. 49%). Although small, the increase in helicity associated with the *i*, *i*+4 isomer **5b** (5% vs. **5c** compared against 1% for **4b** vs. **4c**), together with the trend associated with two other glycopeptide controls **6a/b** and **7a/b** (see below), indicates the presence of a stabilizing carbohydrate–carbohydrate interaction.

The differences in helical content observed are small, though we suggest significant based on the effect associated with other glycopeptides controls. Both *i*, *i*+4 and *i*, *i*+5 isomers of mono- and disaccharide bisglycopeptides **6a/b** and **7a/b** incorporating glucose (Glc) and lactose (Lac, Galβ1-4-Glc) moieties, respectively, were synthesized and coil:helix distributions determined. In each case, and in marked

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contrast to the bisLe^X glycopeptides **5b/c**, the *i*, *i*+4 variants (**6a** and **7a**) displayed a lower helical content than the corresponding *i*, *i*+5 controls (**6b** and **7b**); the *i*, *i*+4 monosaccharide **6a** and disaccharide **7a** controls were 8% and 12% less helical than the *i*, *i*+5 variants **6b** and **7b** respectively (Figure 3).[18]

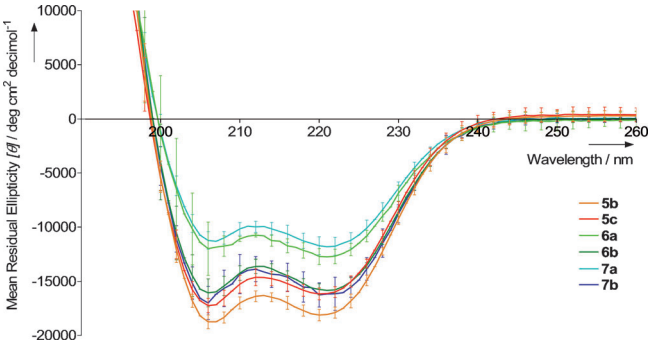

Figure 3. CD spectroscopy of glycopeptides **5b/c**, **6a/b**, and **7a/b** showing error to two standard deviations.

This demonstrates that the introduction of a mono- then disaccharide moiety (i.e. glyco-based controls possessing a steric, and an escalating steric demand, but no associated stabilizing interaction) results in an increasing destabilization of the helical state. Also noteworthy is that the three bisglycosylated *i*, *i*+5 (i.e. non-interacting, Figure 1b) peptides **5c**, **6b** and **7b**, all have closely similar helix contents at approximately 49% suggesting that isolation of the CHO units from each other has been achieved. These data, and in particular the trend, shown schematically in Figure 4, of a decrease in helical content associated with mono- and disaccharide substrates (**6a** and **7a**) compared to the increase observed for the (trisaccharide) Le^X–Le^X glycopeptide **5b** supports the conclusion that this peptide reporter system is responding to a weakly attractive, stabilizing and single (monovalent) CCI.

Helix contents can be accurately predicted from helix–coil theory, provided that parameters for the residues present are known.[11b] This has been carried out for the glycopeptides discussed here, to provide the free energies associated with adding carbohydrate units to the helix and the free energies of the side-chain interaction energies.[19] This indicates the Le^X–Le^X interaction (associated with *i*, *i*+4 **5b**) stabilizes the helix by approximately 0.5 kcal mol^{-1}, compared to the other *i*, *i*+4 bisglycopeptides **6a** and **7a**.

Given the role reported for Ca^{2+} within Le^X-based carbohydrate interactions,[2,3] the effect of Ca^{2+} on the helix preference of **5b** was also examined. Using either a large excess of Ca^{2+} (167 mM with 100 μM of **5b** in 10 mM MOPS buffer at pH 7.0) or under titration conditions (50 μM to 10 mM Ca^{2+}), we observed no significant difference in helical content of **5b**.[20] This lack of a Ca^{2+} effect is interesting but not without precedent[21] and, as articulated earlier by Penadés et al.,[8a] draws attention to the limited understanding currently available as to the precise mechanism by which Ca^{2+} mediates CCIs.

While reflecting the inherently weak nature of CCIs, the differences in helical content and trend observed (Table 1 and Figure 4) are consistent with observation of a monovalent and favorable carbohydrate–carbohydrate interaction. This, in turn, supports the ability of a dynamic random-coil:α-helical peptide system to provide a qualitative read-out of CCIs. Given that peptide synthesis and carbohydrate ligation are straightforward, this reporter, validated here with Le^X–Le^X, offers a new means of probing the molecular level details of a weak but fundamentally important biological interaction.

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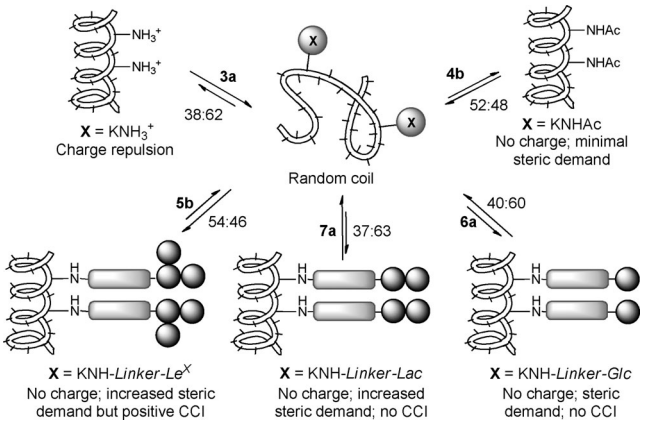

Figure 4. Trends in helical content observed between free lysine **3a** and *N*-Ac **4b** controls and mono-, di-, and trisaccharide-based glycopeptides (**6a**, **7a**, and **5b**, respectively). Ratios shown are for helical:random coil in each of the relevant *i*, *i*+4 substrates.

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[17] Thermal denaturation of key peptides (**3c/d** and **5b/c**) was also performed. Analysis was complicated because the random coil:helix equilibrium is not a two-state system, but a dynamic ensemble of many conformations. Apparent T_M values were observed and the differences, though small, were consistent with the equilibrium CD experiments (see Supporting Information).

[18] We suggest that, given the differences in structure of the side-chain moieties, it is more meaningful to compare helicity values within a series (i.e. *i*, *i*+4 vs. *i*, *i*+5) rather than rely solely on absolute values between difference controls/glycopeptides.

[19] In modified Lifson–Roig theory, these parameters are preferences for helix interior (w), N-cap (n), C-cap (c) helix initiation (v) and *i*, *i*+4 side-chain interactions (p).[11] In the peptides discussed here, the relevant parameters are $w(\mathrm{Ala}) = 1.70$, $w(\mathrm{Lys}) = 1.00$, $w(\mathrm{Gly}) = 0.048$, $w(\mathrm{Tyr}) = 0.48$, $n(\mathrm{acetyl}) = 5.9$, $n(\mathrm{Ala}) = 1.0$, $n(\mathrm{Lys}) = 0.72$, $n(\mathrm{Gly}) = 3.9$, $c(\mathrm{All}) = 1.0$ and $v = 0.048$;[12b] Peptide **4c** contains a KAc residue, with unknown helix-coil parameters. We can safely assume that $n = 1$ for all novel residues, as a helix is highly unlikely to initiate at a substituted position in the middle of the sequence, so uncertainty in this N-cap parameter makes very little difference to the predicted helix contents. This leaves only one unknown in peptide **4c**, namely w(KAc). Fitting the experimental helix content of 51% for **4c** gives $w(\mathrm{KAc}) = 0.89$ and hence the free energy of transfer of KAc from the unfolded state to a helix interior is $-RT\ln(0.89) = 0.06\ \mathrm{kcal\,mol^{-1}}$. The error range in the experimental helix content of **4c** is 49% to 53%. Fitting to these values gives a range of 0.83 to 0.95 for w(KAc). Similar analyses gives the following helix interior preferences for the glycosylated side-chains: $w(\mathrm{KCOCH_2S(CH_2)_3OLeX}) = 0.83$, range 0.78 to 0.89, $\Delta G = -RT\ln(0.83) = 0.10\ \mathrm{kcal\,mol}$; $w(\mathrm{KCOCH_2S(CH_2)_3O\text{-}Glc}) = 0.80$; range 0.75 to 0.86, $\Delta G = -RT\ln(0.80) = 0.12\ \mathrm{kcal\,mol^{-1}}$; $w(\mathrm{KCOCH_2S(CH_2)_3O\text{-}Lac}) = 0.83$, range 0.78 to 0.89, $\Delta G = -RT\ln(0.83) = 0.10\ \mathrm{kcal\,mol^{-1}}$. All of these side-chains are therefore weakly destabilizing to the helix. In peptides with *i*, *i*+4 side-chain interactions, the only unknown is now the side-chain interaction parameter, p. Fitting the experimental helix content of 52% for peptide **4b**, and using w(KAc), gives $p(\mathrm{KAc\ to\ KAc}) = 1.18$, range 1.04 to 1.34. The KAc to KAc interaction thus weakly stabilizes the helix by $-RT\ln(1.18) = -0.09\ \mathrm{kcal\,mol^{-1}}$. Similarly, for **5b** $p(\mathrm{KCOCH_2S(CH_2)_3OLe^X\ to\ KCOCH_2S(CH_2)_3OLe^X}) = 1.54$, range 1.35 to 1.77, $\Delta G = -RT\ln(1.54) = -0.23\ \mathrm{kcal\,mol^{-1}}$; for **6a** $p(\mathrm{KCOCH_2S(CH_2)_3O\text{-}Glc\ to\ KCOCH_2S(CH_2)_3O\text{-}Glc}) = 0.69$, range 0.61 to 0.78, $\Delta G = -RT\ln(0.69) = 0.20\ \mathrm{kcal\,mol^{-1}}$; and for **7a** $p(\mathrm{KCOCH_2S(CH_2)_3O\text{-}Lac\ to\ KCOCH_2S(CH_2)_3O\text{-}Lac}) = 0.53$, range 0.46 to 0.60, $\Delta G = -RT\ln(0.53) = 0.34\ \mathrm{kcal\,mol^{-1}}$.

[20] Given the sensitivity of the conformational preferences of the peptide backbone to cations, these titration experiments were carried out under conditions of comparable ionic strength. In addition, the effect of Na^+ on the helix preference of **5b** was examined and no significant difference in helicity was observed between Ca^{2+} (167 mM; $I = 0.5$) and Na^+ (500 mM; $I = 0.5$). Full details of these titration studies are provided in the Supporting Information.

[21] The interaction between Le^X moieties with and without Ca^{2+} in a variety of circumstances has been described and those regions of Le^X that interact with a divalent cation have been proposed. Methods used include NMR spectroscopy,[8c,13,22] MS and computational studies,[23] and models representing both *trans*- and *cis*-CCIs have been reported.[24] However, no Le^X–Ca^{2+} interactions have been detected by NMR spectroscopy in pure water, only in aqueous MeOH, MeOH or DMSO or related mixtures. A direct interaction between Le^X units (in the absence of Ca^{2+}) has been reported,[13] but see also Ref. [22d]. Importantly, Penadés et al. identified a Ca^{2+}-independent Le^X–Le^X interaction using atomic force microscopy (AFM)[8a] while observing a Ca^{2+}-dependent effect using analogously glycosylated gold nanoparticles (AuNPs).[6a]

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