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Sweet entanglements – protein:glycan interactions in two HIV-inactivating lectin families

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Abstract

Structures and sugar binding by members of two lectin families, CVNH and OAAH, were determined to elucidate the basis for recognition of high-mannose glycans on the HIV envelope glycoprotein gp120. We solved NMR solution and/or crystal structures for several lectins and delineated their carbohydrate specificity by array screening and direct NMR titrations. Both families recognize different epitopes on high-mannose glycans, namely Man α (1–2)Man units at the ends of the D1 and D3 arms and α 3, α 6-mannopentaose at the central branch point of Man-8 or Man-9 for CVNH and OAAH lectins, respectively.

Introduction

Lectins, carbohydrate-binding proteins, are central players in numerous important cellular functions, such as cell-cell adhesion, trafficking of glycoproteins, glycosylation and many more (Sharon, 2008; Sharon and Lis, 1989, 2004). Unfortunately, for a large number of events, the underlying carbohydrate-mediated mechanisms are not fully elucidated, given their intricate nature. As an example of the complexity involved, one only needs to consider eukaryotic protein glycosylation such as human mucin-type O-glycosylation: this is carried out by a highly sophisticated enzymatic machinery composed of 20 different isoenzymes (Hassan et al., 2000; Ten Hagen et al., 2003).

Compared to oligonucleotides or polypeptides, the combinatorial possibilities of oligosaccharides are several orders of magnitude greater. Two possible anomeric configurations (α/β), different linkages (1–2, 1–3, 1–4, 1–6), different ring sizes (pyranose/furanose), as well as diverse branching points are possible; in addition, modification through acetylation, phosphorylation and sulfation also occurs (Herget et al., 2008; Marino et al., 2010; Richards and Lowary, 2009; Varki, 2009). Thus, an enormous level of diversity can potentially be explored in protein-carbohydrate interactions.

Structure and glycan binding of CV-N and single-domain CVNHs

CV-N is a small (11 kDa) virucidal lectin originally identified in aqueous extracts from the cyanobacterium *Nostoc ellipsosporum* during screening for anti-HIV activities (Boyd et al., 1997). The sequence of CV-N comprises two tandem repeats, each containing ~50 amino acids and two pairs of disulphide-bonded cysteines. The structure of CV-N exhibits pseudosymmetry with two distinct domains, A and B (Fig. 1A) (Bewley et al., 1998; Yang et al., 1999). One sugar binding site was identified on each domain by NMR titration experiments (Barrientos and Gronenborn, 2002; Barrientos et al., 2006; Matei et al., 2008; Shenoy et al., 2002): a shallow cleft on domain A and a somewhat deeper pocket in domain B, with both sites separated by ~40 Å at opposite ends of the molecule (Fig. 1B). The anti-HIV activity of CV-N is mediated via interactions between the protein and α (1–2) linked

mannose units on the terminal arms of the branched Man-8 and Man-9 structures on gp120 (Barrientos et al., 2003; Botos et al., 2002; Shenoy et al., 2002; Shenoy et al., 2001).

The NMR solution structure of the CV-N-Man α -(1 $\rightarrow$ 2)-Man α complex was solved using protein and carbohydrate at 1:1 and 1:2 molar ratios (Bewley 2001). Intra- and inter molecular NOEs for the protein alone and for Man α -(1 $\rightarrow$ 2)-Man α in the complex were extracted from 3D ^{15}N separated and ^{12}C filtered NOESY and 3D ^{12}C filtered/ ^{13}C separated NOESY spectra, and the complex structure was calculated using a procedure where backbone and non-interfacial side chains of CVN were fixed, while the interfacial side chains of the protein and Man α -(1 $\rightarrow$ 2)-Man α were free to move, subject to the restraints (Bewley 2001).

As part of a project aimed at understanding structure-function relationships in a family of CV-N homologous proteins, collectively designated as CVNHs, NMR solution structures of *T. borchii*, *C. richardii*, *N. crassa* and *G. zeae* CVNHs were determined (Fig. 1C–1F, respectively). These proteins were selected as representative members of the CVNH family, residing on different branches of the phylogenetic tree. (Koharudin et al., 2008; Matei et al., 2011; Percudani et al., 2005). All proteins exhibit the same fold and the overall structures resemble that of the founding member of the family, CV-N, albeit with noteworthy differences in loop conformation and detailed local structure. In addition, a number of distinct features are present in these related sequences and structures. For example (Fig. 1G), in fungal but not fern homologs, Cys residues corresponding to the two S-S bonds of CVN are absent and equivalent S-S bonds to those in CV-N (between C8-C22 and C58-C73) are only found in CrCVNH (between C9-C23 and C59-C74), with an additional disulphide bond between C3 and C102. For the other three proteins, TbCVNH, NcCVNH and GzCVNH, no S-S bridges are observed. In TbCVNH and GzCVNH, only one Cys residue corresponding to C22 of CV-N is conserved. The other Cys residues are replaced by S7 (CV-N C8), S/A59 (CV-N C58) and P/L75 (CV-N C73). One Cys is present at position 72 in TbCVNH and position 101 in GzCVNH. However, given that no possible interacting Cys side chains are found spatially close to position 58 or 101, no disulphide bond can be formed. In NcCVNH, all corresponding Cys are replaced by hydrophobic residues (Ala at positions 7/61 and Leu at positions 25/82).

Extensive carbohydrate binding studies were conducted by glycan-array and NMR screening and the ligand binding sites on all four proteins were identified by chemical shift perturbation studies (Koharudin et al., 2008; Matei et al., 2011). The number and location of binding sites vary for the four proteins and different ligand specificities exist (Fig. 1A – 1F). In CrCVNH, like in CV-N, two carbohydrate binding sites are present, one each on domain A and B, whereas TbCVNH contains only a single binding site on domain A. Both, NcCVNH and GzCVNH, possess a single binding site on domain B. CV-N and NcCVNH interact with several high mannose glycans, while TbCVNH, binds only tetra- and penta-mannose. Unique among the examined proteins, TbCVNH was found to also bind linear, mannose-lacking sugars made up of glucose only, or a combination of glucose, galactose and N-acetyl glucosamine connected by β -linkages. Thus, TbCVNH is the most promiscuous lectin in the CVNH family.

Structure and glycan binding of a multi-domain CVNH

Based on their domain organization, CVNHs have been grouped into three categories: type I proteins contain either single or multiple CVNH domains, each composed of tandem-sequence repeats; type II are multi-domain proteins which, in addition to the CVNH domain sequence, also contain an MS8 domain of unknown function; and type III proteins comprise interrupted CVNH domains, in which another domain sequence is inserted between

individual repeats in a single CVNH domain sequence (Percudani et al., 2005). For type III proteins, it was not clear, *a priori*, whether the characteristic fold of the CVNH domain would be retained, given the interruption in the sequence by the insertion.

The rice blast fungus *Magnaporthe oryzae*'s genome encodes a protein, containing a type III CVNH lectin, in which a LysM domain is inserted into the single CVNH module (Percudani et al., 2005). LysM domains are found in many modular bacterial and fungal enzymes involved in cell wall degradation and the domain was originally described for a lysozyme from the *Bacillus* phage phi 29 (Garvey et al., 1986; Saedi et al., 1987). Two nine-residue-long linkers connect the end of the first CVNH repeat to the N-terminal residue of the LysM domain, while the other joins the C-terminus of the LysM domain to the first amino acid of the second CVNH repeat. The NMR solution structure of MoCVNH-LysM revealed that the two domains are flexibly connected, lacking a fixed orientation between them (Koharudin et al., 2011) (Fig. 2A). The architecture of the individual domains closely resembles those of previously determined structures of CVNH and LysM domains, and the connections exhibit mobile, random-coil conformations. The fact that the domains behave as two independent units, rather than a single one, was evidenced by several pieces of data: (i) no NOEs were observed between residues in the linker regions and either of the two domains, and (ii) heteronuclear NOE relaxation data for the linker residues (hetNOE ~ 0.13 to 0.51) indicate substantial motion, compared to residues located in either domain, and (iii) the correlation times of the domains in the linked construct are very similar to those in their isolated counterparts.

MoCVNH-LysM binds oligo-mannose (via the CVNH domain) and oligo-N-GlcNAc (via the LysM domain) carbohydrates with millimolar and micromolar affinities, respectively (Koharudin et al., 2011) (Fig. 2B). The latter are the building blocks of chitin, a major constituent of fungal cell walls. In addition, binding of the peptidoglycan component N-GlcNAc- β -(1,4)-N-MurNAc is also observed (Fig. 2C). This strongly suggests that a chitin-related and oligomannose-containing polysaccharide may be the physiological ligand of this dual-domain lectin.

Structure and glycan binding of members of the OAAH family

Another anti-HIV lectin, named *Oscillatory Agardhii* Agglutinin (OAA) was recently discovered in the cyanobacterium *Oscillatory Agardhii* (Sato and Hori, 2009; Sato et al., 2000). Its gene sequence permitted placement into a phylogenetic tree composed of eight other homologous hypothetical proteins (Sato and Hori, 2009; Sato et al., 2007). We determined crystal structures of the OA lectin as well as that of a homolog from *Pseudomonas fluorescens*. The overall architecture of OAA and PFA is a compact β -barrel made up from a continuous ten-stranded, anti-parallel β -sheet (Fig. 3A–3B, respectively). Each of the amino acid sequence repeats folds into five β -strands, β 1 to β 5, (colored in grey and green for OAA and PFA, respectively, and β 6 to β 10 (colored in light blue and purple for OAA and PFA, respectively). The two sequence repeats are connected by a very short linker, comprising residues G67-N69 (colored in orange).

The linkers connecting strands β 2 and β 3, and β 7 and β 8, respectively, cross at the top or the bottom of the barrel and the first two β -strands of each sequence repeat (β 1- β 2 and β 6- β 7) and the next three β -strands (β 3- β 4- β 5 and β 8- β 9- β 10) are positioned on opposite sides of the barrel (Fig. 3A–3B). As a result, β 1 and β 2 in the first sequence repeat are located in the barrel between the β -strands β 7 and β 6 from the second repeat on one side and strands β 10, β 9 and β 8 on the other side. Similarly, strands β 3, β 4 and β 5 of the first repeat are flanked by β 8 and β 6, respectively, of the other sequence repeat. The swap of β -strands between the two sequence repeats creates an almost perfect C2 symmetric arrangement,

rendering the conformation of the five β strands in each sequence repeat extremely similar. This can be easily appreciated from the backbone atomic r.m.s.d. values of 0.66 and 0.70 Å for the OAA and PFA repeats, respectively.

After resonance assignments for both proteins were complete, glycan binding was directly tested by ^1H - ^{15}N HSQC NMR titrations. $\alpha 3, \alpha 6$ -mannopentaose binding is in slow exchange on the chemical shift scale (new bound resonances appear), suggesting relatively tight binding. Titration data sets were recorded for ^{15}N -labeled OAA and PFA, permitting for unambiguous delineation of the glycan binding sites on the proteins. In both proteins, site 1 is formed by loops between β -strands from the N- and C-terminal ends of the polypeptide (between $\beta 1/\beta 2$ and $\beta 9/\beta 10$), and site 2 comprises residues in the middle of the chain in loops between $\beta 4/\beta 5$ and $\beta 6/\beta 7$ (Fig. 3C–3F).

Initial efforts aimed at crystallizing a complex between OAA and Man-9 or $\alpha 3, \alpha 6$ -mannopentaose (either by soaking or co-crystallization), were unsuccessful due to the presence of N-cyclohexyl-3-aminopropane-sulfonic acid (CAPS) in the crystallization buffer that resulted in two bound CAPS molecules in the crystal structure of OAA (Fig. 3G). Changing crystallization conditions and omitting CHAPS, eventually yielded new crystals of both apo- and $\alpha 3, \alpha 6$ -mannopentaose-bound OAA in different space groups. The sugar binding pockets of site 1 and site 2 on OAA are in the same place as identified by NMR and exhibit very similar conformations (Fig. 3H). They reside between the loops on the surface of the protein. The M3 α (1–6)M4' disaccharide unit of the $\alpha 3, \alpha 6$ -mannopentaose is most deeply imbedded in the protein (Fig. 3I–3J), with the M4 α (1–3)M3 disaccharide also inside the binding pocket, while the M5' α (1–3)[M5'' α (1–6)]M4' trisaccharide unit is pointing towards solvent. The pyranose ring of M3 in the M4 α (1–3)M3 unit is stacked on top of the indole ring of tryptophan side chains, W10 in site 1 and of W77 in site 2, while the pyranose ring of M4 is inserted between two arginines side chains. The pyranose ring of M5' is flanked by residues in the loop connecting strands $\beta 1$ – $\beta 2$ and $\beta 6$ – $\beta 7$ in site 1 and 2, respectively, while the pyranose rings of M5'' and M4' are flanked by residues in the $\beta 9$ – $\beta 10$ and $\beta 4$ – $\beta 5$ loops for site 1 and 2, respectively.

Comparing the carbohydrate binding sites in the apo-structure and the sugar-bound X-ray structures revealed that in the free protein, the orientation of the peptide bond between W10 and G11 (binding site 1) and W77 and G78 (binding site 2) is flipped, with the carbonyl oxygens of W10 and W77 pointing in opposite directions. Upon sugar binding, the conformation in binding site 1 is essentially unchanged, i.e. the protein is already in a “bound” conformation, even in the absence of sugar, while $\alpha 3, \alpha 6$ -mannopentaose binding to site 2 changes the orientation of the W77/G78 peptide plane from the “free” to the “bound” conformation. We, therefore, conducted extensive NMR studies at different temperatures, including measurements of relaxation data for the free and bound protein; essentially identical values for the equivalent residues were obtained, indicating that the loops associated with sugar binding are equally flexible in both sites. In addition, we explored different temperatures for X-ray data collection, since differences between the NMR and X-ray results could have been caused by ‘freezing out’ conformations at cryogenic temperatures in the crystal. None of our data suggested that in the cryogenic X-ray structure the bound conformation was fortuitously selected. We, therefore, conclude that both loop regions in the free protein are flexible and that crystal packing effects around the carbohydrate binding site 1 forces the conformation of the $\beta 1$ – $\beta 2$ loop into the conformation seen in the carbohydrate-bound structure.

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Figure 1. Structures and sequence alignment of CVNH family members

(A) – (B) Structure (A) and location of two sugar binding sites on CV-N (B). (C) – (F) Solution structures and location of sugar binding sites for four new members of the CVNH family; TbCVNH (C), CrCVNH (D), NcCVNH (E), and GzCVNH (F). Note that domain A is colored in white for all structures while domain B is colored in blue, purple, light green, light blue, and light magenta for CV-N, TbCVNH, CrCVNH, NcCVNH, and GzCVNH, respectively. (G) Amino acid sequence alignment for five members of the CVNH family studied in our laboratory. The first and second sequence repeats are displayed in the upper and lower panel, respectively. Black brackets indicate the location of the disulphide bridges between cysteines in CV-N and all corresponding residues in the other sequences are boxed.

Figure 2. Structure and carbohydrate specificity of MoCVNH-LysM

(A) Ribbon representation of the full-length MoCVNH-LysM structure. Domains A and B of the CVNH domain are colored in white and light yellow, respectively. The LysM domain is colored in blue. The two flexible linkers are colored orange. (B) Interaction of MoCVNH-LysM with Mana(1–2)Man. The ^1H - ^{15}N HSQC spectra without and with excess Mana(1–2)Man at the final point in the titration (~51 molar excess) are colored in black and cyan, respectively. Apparent binding constants (K_d) of 5.23 ± 0.42 mM and 15.13 ± 1.13 mM were extracted from the binding isotherms for sub-domains A and B, respectively, using resonances of four residues in each sub-domain (inserts). (C) Interaction of MoCVNH-LysM with the (N-GlcNAc)5 pentasaccharide. The ^1H - ^{15}N HSQC spectra without and with excess (N-GlcNAc)5 pentasaccharide are colored in black and magenta, respectively. Similar to (B), an apparent K_d was extracted from the binding isotherms of five bound amide resonances, yielding a value of $\sim 28 \pm 3$ μM .

Figure 3. Overall architecture and carbohydrate binding specificity of OAA and PFA
 (A) and (B) Crystal structures of OAA and PFA determined at 1.55 and 1.70 Å, respectively. The first five β -strands, β 1 to β 5, are colored in grey and green, and the next β -strands, β 6 to β 10, are colored in light blue and purple for OAA and PFA, respectively. The two sets of five β -strands are connected by a short linker, comprising residues G67-N69 in both structures (colored in orange). (C) – (F) ^1H - ^{15}N HSQC spectra of OAA (C) and PFA (D) and chemical shift mapping of $\alpha,3,6$ -mannopentaose binding to OAA (E) and PFA (F). The titrations were carried out using 0.040 mM OAA or PFA in 20 mM NaAcetate, 20 mM NaCl, 3 mM NaN₃, 90/10% H₂O/D₂O (pH 5.0), 25°C. (G) and (H) X-ray structures of CAPS-bound OAA (G) and $\alpha,3,6$ -mannopentaose-bound OAA (H), determined at 1.20 and 1.60 Å, respectively. (I) and (J) Surface representations of OAA's carbohydrate binding sites 1 and 2, respectively, with the bound $\alpha,3,6$ -mannopentaose depicted in stick representation. The β strands from the first and second sequence repeats are colored in white and light blue, respectively. Protein residues directly interacting with the sugar in each binding site are labeled by single-letter code and the sugar rings of the carbohydrate are labeled as in the Man-9 structure.