Carbohydrate Specificity and Salt-bridge Mediated Conformational Change in Acidic Winged Bean Agglutinin

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Structures of two crystal forms of the dimeric acidic winged bean agglutinin (WBAII) complexed with methyl-α-D-galactose have been determined at 3.0 Å and 3.3 Å resolution. The subunit structure and dimerisation of the lectin are similar to those of the basic lectin from winged bean (WBAI) and the lectin from *Erythrina corallodendron* (EcorL). The conformation of a loop and its orientation with respect to the rest of the molecule in WBAII are, however, different from those in all the other legume lectins of known structure. This difference appears to have been caused by the formation of two strategically placed salt bridges in the former. Modelling based on the crystal structures provides a rationale for the specificity of the lectin, which is very different from that of WBAI, for the H-antigenic determinant responsible for O blood group reactivity. It also leads to a qualitative explanation for the thermodynamic data on sugar-binding to the lectin, with special emphasis on the role of a tyrosyl residue in the variable loop in the sugar-binding region in generating the carbohydrate specificity of WBAII.

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Introduction

Specific recognition between proteins and carbohydrates is of prime importance in many biological processes such as cell-cell communication, host-pathogen interactions, targeting of cells, cancer metastasis, growth and differentiation. Due to their ability to discriminate subtle variations in carbohydrate and glycoconjugate structures, lectins have become valuable tools in different areas of biological and medical research (Sharon & Lis, 1989; Van Damme *et al*., 1997; Lis & Sharon, 1998). Legume lectins are a model system of choice to study the molecular basis of these recognition events because of their easy availability and remarkable diversity in carbohydrate specificity and oligomerization despite strong sequence conservation. Crystallographic studies on legume lectins have provided invaluable information on the structural aspects of lectin-carbohydrate recognition and specificity (Rini, 1995; Weis & Drickamer, 1996; Drickamer, 1997; Loris *et al*., 1998; Vijayan & Chandra, 1999; Bouckaert *et al*., 1999a).

The seeds of winged bean, *Psophocarpus tetragonolobus*, contain two lectins of similar molecular weights, which differ in their isoelectric points and blood group specificity. The basic lectin WBAI has a pI > 9.5 and binds to human blood group A and B antigens and not to the O antigenic determinant. The crystal structure of WBAI solved and refined at 2.5 Å resolution revealed details of the lectin-monosaccharide interactions at the atomic level, and provided a structural rationale for its blood group specificities (Prabu *et al*., 1998). Winged bean acidic lectin, WBAII, an N-glycosylated homodimeric lectin with a molecular mass ~54,000 Da, has a pI ~ 5.5 and exhibits stronger affinity towards O-type erythrocytes, through specific binding to the terminally monofucosylated H-antigenic determinant (Patanjali *et al*., 1988; Acharya *et al*., 1990), than to A and B types.

Abbreviations used: WBAII, winged bean acidic agglutinin; WBAI, winged bean basic agglutinin; conA, concanavalin A; DGL, *Dioclea grandiflora* lectin; GS4, lectin IV from *Griffonia simplicifolia*; EcorL, *Erythrina corallodendron* lectin; PNA, peanut agglutinin; DBL, *Dolichos biflorous* seed lectin; GlcNAc, *N*-acetylglucosamine; Fuc, fucose; Man, mannose; GalNAc, *N*-acetylgalactosamine.

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Legume lectin-carbohydrate interactions primarily involve four loops in the carbohydrate binding region, three of which remain largely invariant (Young & Oomen, 1992). The interactions involving the fourth loop (loop D) varies substantially and is believed to be responsible for the differences in specificities of legume lectins (Sharma & Surolia, 1997). This loop is shorter in WBAII than that in WBAI and elucidation of the role of this loop in generating the differences in specificities between WBAII and WBAI is one of the main objectives of the structural analysis of the WBAII-methyl-α-D-galactose complex. We also attempt to correlate the structural information on the novel binding site of WBAII with the available thermodynamic data on carbohydrate binding to WBAII.

Figure 1. WBAII dimer in Form I, showing the N-linked sugars, metal ions and bound carbohydrate in one subunit. This Figure and Figures 2, 4 and 5 were made using MOLSCRIPT (Kraulis, 1991).

Results and Discussion

Structure of the subunit, glycosylation and unique disposition of a loop

WBAII crystallised in two different space groups, *R*3 (Form I) and *C*2 (Form II), in the presence of methyl-α-D-galactose. One dimer of the lectin in the asymmetric unit of Form I and four in that of Form II provide altogether ten copies of the WBAII subunit. According to the gene sequence (EMBL code: AJ250242), each subunit of WBAII consists of 240 amino acid residues. However, the electron density could not account for two residues Ser-Asn following Pro112. These two residues do not exist in other legume lectins as well. Therefore, residues following Pro112 were numbered sequentially, leaving out these two residues. The electron density maps indicated three other variations from the reported sequence at positions 28, 101 and 120 where Asn, Gln and Phe side chains have been fitted instead of Ser, Gly and Leu, respectively. This could be a consequence of the presence of closely related isolectins.

The secondary and tertiary structures of WBAII are similar to those of other legume lectins with major deviations restricted to the loops. The structure of the dimeric lectin, as observed in Form I, is illustrated in Figure 1. Each subunit contains a six-stranded back β-sheet, a seven stranded curved front β-sheet, a third smaller β-sheet which plays a role in holding the two larger β-sheets together (Banerjee *et al*., 1996) and loops in addition to a Mn^{2+} and Ca^{2+}. The ten crystallographically independent subunits in the two crystal forms have the same structure. Unless otherwise specified, subunit A in Form I is used in the discussion of the structure. Among the legume lectins of known three-dimensional structure, WBAI and EcorL exhibit the highest sequence homology with WBAII, the sequence identity being 62 and 54 %, respectively.

The plant specific heptasaccharide (Manα6(Manα3)(Xylβ2)Manβ4GlcNAcβ4-(L-Fucα3) GlcNAcβ) is covalently bound to the side-chain of Asn76 in the third sheet (Figure 1). Clear electron density exists for the three terminal sugar rings (Manβ4GlcNAcβ4GlcNAcβ) of the heptasaccharide in both the subunits of Form I. In Form II, density appears only for the two terminal sugars (GlcNAcβ4GlcNAcβ) in three of the four dimers. O6 of the terminal GlcNAcβ residue makes a good hydrogen bond with Gln224 OE2 in all subunits. The conformational angles at the N-glycosylation site and about the glycosidic bonds of the N-linked saccharide have values comparable to those observed in other available crystal structures (Christlet *et al*., 1999; Petrescu *et al*., 1999).

A distinct structural feature of WBAII lies in the movement of the 35-45 loop towards the front of the molecule in WBAII with an r.m.s.d. of about 6 Å in C^{α} positions compared to its position in other lectins of known structure (Figure 2). In the latter, this loop is close to the strand which is glycosylated only in WBAII. However, modelling studies and energy calculations did not indicate any influence of glycosylation on the disposition of the loop. A close examination of the structure indicated that the change in the conformation of the 35-45 loop is caused by the formation of salt bridges between the side-chains of Glu32 and Lys35 and the side-chains of Lys45 and Asp104 (Figure 3). These are broken when the loop is moved to its position in the other legume lectins. Residues 45 and 104 have opposite charges only in WBAII. The situation in relation to the other salt bridge is subtler. There are several other legume lectins in which residue 32 is glutamic acid and 35 is a basic residue. However, in every such lectin, residue 22 is aspartic acid. Here, the basic side-chain of 35 can, and indeed often does, form a salt bridge with Asp22 with a loop conformation observed in the other legume lectins. However, in WBAII, residue 22 is glutamine. Therefore, Lys35 and the loop change conformation to that observed in the crystal structure to form a salt bridge

Figure 2. Superposition of the C^{α} traces of legume lectins conA, GS4, WBAI, EcorL, PNA, DBL, lentil lectin, soybean agglutinin, phytohemagglutinin L & WBAII. WBAII is shown in black.

with the side-chain of Glu32, which is in the close proximity.

Dimerisation

The glycosylation site in WBAII, as in WBAI and unlike in EcorL, is far away from the inter-subunit interface in the ''canonical'' dimer originally found in concanavalin A (conA) (Hardman & Ainsworth, 1972). Glycosylation, therefore, does not prevent the lectin from forming a canonical dimer. However, WBAII like WBAI, forms a ''handshake'' type of dimer observed in homologous EcorL, demonstrating again that the variability in quaternary association in legume lectins is caused by factors intrinsic to the protein itself (Prabu *et al*., 1998, 1999). The inter-subunit hydrogen bonds present at the interface are Arg72 NH1—O Val186, Arg72 NH2—O Val186, Asn175 ND2—OG1 Thr188, Lys166 NZ—O Thr188. Three of these direct hydrogen bonds are found in EcorL and WBAI dimer interfaces as well. The surface area buried (Connolly, 1983) on dimerisation is similar in the three lectins. The amino acid residues at the dimer interface are largely conserved in them. A completely buried water molecule at the interface on the 2-fold axis of the dimer which is conserved in both WBAI and EcorL is absent in WBAII. This is presumably the effect of the substitution of a histidine residue, which bridges the water molecule in EcorL and WBAI, by an asparagine residue in WBAII.

The primary binding site

Clear density for the bound methyl-α-D-galactose is seen in one subunit of Form I and all subunits of Form II. The interactions between the lectin and the sugar in the two forms are illustrated in Figure 4. The hydrogen bonds involving Asp88, Glyl06 and Asn129 are conserved in all legume lectins. The other protein-carbohydrate hydrogen bonds in WBAII are those of Tyr215 N and Gln216 NE2, both belonging to loop D (214-221), with the galactose O4 and O6 respectively. In addition, a water bridge connects Gal O6 to Gln85 O. This water-mediated interaction is present in the EcorL-sugar complexes while O6 is directly hydrogen bonded to NE2 of the equivalent histidyl residue in WBAI. Again, as in other legume lectins, the side-chain of an aromatic residue, Phe127 in WBAII, stacks against the sugar ring. A unique feature of the primary sugar binding site of WBAII is the stacking of an aromatic residue, Tyr215, in loop D against the sugar. In fact, the monosaccharide is sandwiched between the side-chains of Phe127 and Tyr215.

Another interesting feature of the sugar binding site is Asnl07 in loop B. In all other legume lectins except peanut agglutinin (PNA), where it is Thr in a longer loop, the equivalent position is occupied by an aromatic or a large hydrophobic residue. The mostly conserved aromatic residue at this position, along with a conserved Trp131 and the main-chain atoms of 106 (WBAII numbering) forms a hydrophobic pocket adjacent to the primary site, described by Hamelryck *et al*. (1999) as a multi-purpose binding pocket, involved in interactions with hydrophobic sugar residues or substituents. That PNA has no affinity for GalNAc appeared to be in consonance with this suggestion (Ravishankar *et al*., 1999). However, the binding affinity of WBAII for GalNAc is five times that of Gal in spite of the presence of an Asn residue at 107 instead of an aromatic residue (Srinivas *et al*., 1998). Simple modelling studies show that the acetamido group in the WBAII-binding site has hydrophobic interactions with Trp131. In addition, the oxygen atom in it could form a water bridge with Asn107. This additional interaction perhaps compensates for the loss of hydrophobic interactions resulting from the replacement of the aromatic residue at this position.

In WBAI, steric hindrance with its long loop D results in a ten times lower affinity of the lectin to the β-methyl substituent of galactose compared to

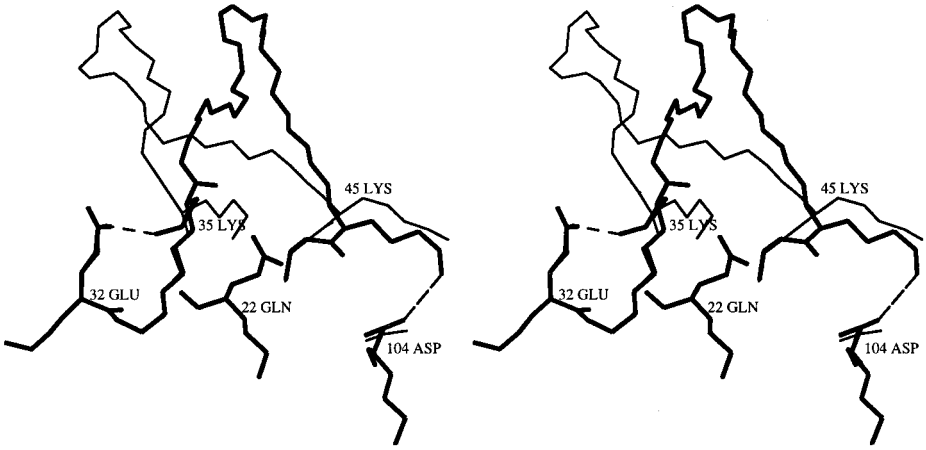

Figure 3. Stereo view of loop 35-45 and its neighbourhood in WBAII (thick lines) and its conformation as in other legume lectins (thin lines). Side-chains of only residues involved in salt bridges are shown.

that of the α-substituent (Prabu *et al*, 1998; Khan *et al*., 1986). As a consequence, the lectin does not recognise the O blood group substance in which the galactose is β-linked to the rest of the oligosaccharide. Loop D in WBAII is shorter. However, the Tyr215 appears to offer some steric resistance to the β-methyl substituent resulting in the lectin's lower affinity to it compared to the α-substituent (Srinivas *et al*., 1998). However, the resistance is mild enough to be more than compensated by the interactions of the sugar moiety β-linked to galactose as in O blood group substance.

Oligosaccharide binding and the role of Tyr215

An attempt is made here to relate the available thermodynamic data on WBAII (Srinivas *et al*., 1998, 1999) with its structure in a qualitative manner, as has been done for conA, (Bradbrook *et al*., 1998; Moothoo & Naismith, 1998; Moothoo *et al*., 1999) and EcorL (Moreno *et al*., 1997), with emphasis on comparative affinities rather than on absolute values. For this purpose, the models of H-type II trisaccharide (Fucα2Galβ4GlcNAcβ) belonging to the two conformational families (hereafter referred to as conformations I and II) suggested by Imberty *et al*. (1995) were separately docked into the sugar binding site of WBAII and EcorL, and the complexes were refined using energy minimisation. The binding sites of the complexes are shown in Figure 5(a) and (b). Interaction energy and shape complementarity between the lectin and oligosaccharide and the hydrophobic surface area buried on association in the energy minimised models are given in Table 1. In the complexes involving WBAII, the interaction energy clearly favours that with conformation I of the oligosaccharide. The differences in the other parameters between the two models are marginal. Both the models have the same hydrogen bonds involving the central galactose residue. However, two hydrogen bonds, Fuc O2 ... ND2 Asn129 and GlcNAc O7 ... NE2 Gln216 and a strong hydrophobic interaction between Fuc 6-CH3 with Tyr215 exist only

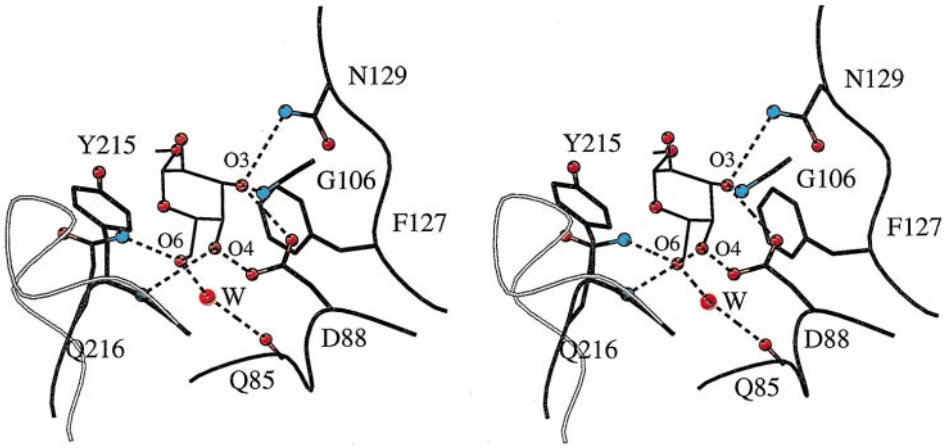

Figure 4. Sugar-binding site of WBAII (thick lines) with the bound methyl-α-D-galactose molecule (thin lines). Also shown is loop D of WBAI in grey.

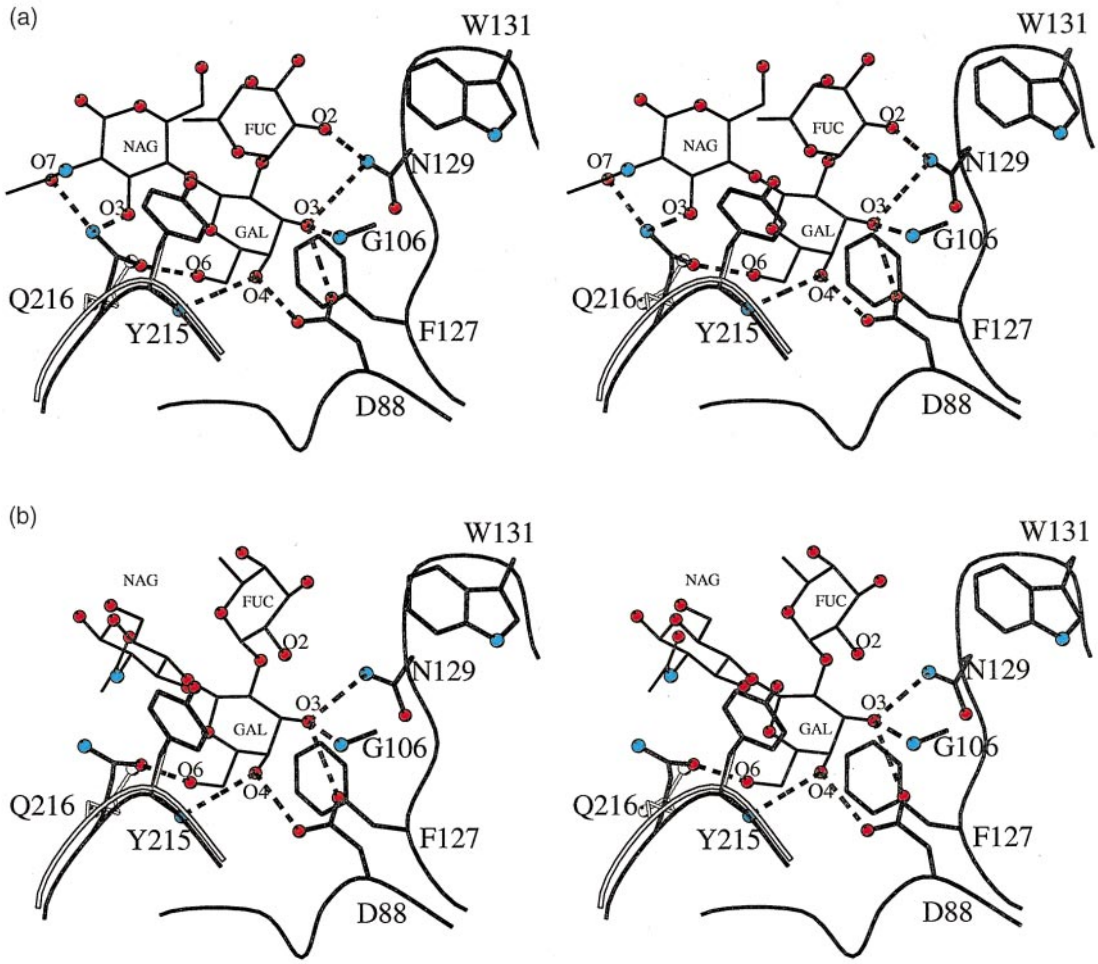

Figure 5. (a) Stereo view of the interactions of H-type II trisaccharide in conformation I with protein atoms in WBAII. Loop D of EcorL is shown in grey. (b) Stereo view of the interactions of H-type II trisaccharide in conformation II with protein atoms in WBAII. Loop D of EcorL is shown in grey.

in the model involving conformation I (Figure 5(a) and (b)). Furthermore, the internal energy of the oligosaccharide conformers is such that the probabilities of occurrence of conformation I and II are in the ratio 95:5 (Imberty *et al*., 1995). Therefore, only models involving conformation I are considered below.

Thermodynamic measurements (Srinivas *et al*., 1999) show much reduced affinity of 3O-Gal, 4O-Gal and 6O-Gal deoxy analogues of the H-type II oligosaccharide to WBAII, confirming the role of the primary hydrogen-bonding interactions. The 2-deoxy fucosyl congener binds with a tenfold reduced binding affinity while binding is altogether abolished in the corresponding 2-methoxy congener. The model involving conformation I shows that the hydrogen bond with the fucosyl residue is lost when it is deoxygenated at the 2′ position while a methoxy group at the same position has severe steric contacts with Trp131. The binding of the 6-nor analogue is also highly entropic indicating the hydrophobic nature of the interactions of the rest of the fucosyl residue with the lectin. The binding is still higher when the methyl group is present, as required by the model involving conformation I. Also the 85-fold stronger affinity of 2-fucosyllactose compared to that of 2-fucosylgalactose (Srinivas *et al*., 1999) implies an extended binding site in WBAII The hydrogen bonds with Gln216 and the van der Waals and hydrophobic interactions with Tyr215 involving GlcNAc in the model is in qualitative conformity with this conclusion.

Lectin-carbohydrate interactions are generally enthalpically driven and exhibit enthalpy-entropy compensation (Toone, 1994; Chervenak & Toone, 1995). Srinivas *et al*. (1999), have commented on the relative predominance of the entropic factor in the energetics of binding of WBAII to fucosylated saccharides and also noted a distinct change in heat capacity upon binding, not yet observed in any other lectin-sugar interaction. The model of the complex provides a qualitative rationale for this unique property of the lectin. The prominent role of Tyr215 in generating this property becomes further evident when the sugar-binding site of WBAII is compared with those of EcorL (Shaanan *et al*., 1991; Elgavish & Shaanan, 1998) and WBAI

Table 1. Buried non-polar surface area (Å^2), interaction energy (kcal/mol) and shape complementarity in the complexes formed by docking H-type II trisaccharides in two conformations (I & II) in the binding sites of WBAII and EcorL

	WBAII		EcorL	
Conformation	I	II	I	II
Non-polar surface area buried (protein)	134	136	163	145
Non-polar surface area buried (sugar)	135	143	126	141
Interaction energy	−109	−56	−114	−62
Shape complementarity	0.75	0.72	0.66	0.65

(Prabu *et al*., 1998; Manoj *et al*., 1999b). The H-type II oligosaccharide cannot bind to WBAI on account of the steric clash of the GlcNAc residue with large loop D of the lectin. Also, the lectin does not have a pronounced pocket for the fucosyl residue. Though the sugar-binding sites of WBAII and EcorL are very similar, there are important differences in side-chains (Figure 6). The major difference as far as sugar binding is concerned, between the two lectins is Tyr215, which is an Ala in EcorL. When the H-type II oligosaccharide is similarly docked into the two binding sites and minimised, the sugar buries 61 Å^2 of the hydrophobic surface area of the Tyr215 in WBAII while the hydrophobic surface area of the Ala buried in EcorL is 37 Å^2. The relative affinity of 2-fucosyllactose to EcorL is four times that of galactose (Surolia *et al*., 1996). The corresponding number is 500 in the case of WBAII. There is a tyrosyl residue in EcorL at a position corresponding to Gly105 in WBAII, but unlike in Tyr215 in WBAII, its aromatic ring does not interact with the sugar molecule. In fact, the presence of a tyrosyl residue at 215 and the possibility of a water bridge involving Asn107, referred to earlier, provide a qualitative rationale for the trend in the enhancement of the affinity of WBAII for the fucosyl derivative. In similar studies, shielding of hydrophobic surface has been invoked as the prime reason for the fourfold increased binding affinity of Man(α1-3)Man to conA compared to α-D-mannose (Bouckaert *et al*., 1999b) and the high affinity of *Dolichos biflorus* seed lectin (DBL) for the disaccharide GalNAc(α1-3)GalNAc (Hamelryck *et al*., 1999). Also, a single amino acid residue has been shown to be responsible for the difference in the binding affinities of a biantennary complex carbohydrate to DGL and conA (Rozwarski *et al*., 1998). The above observations also demonstrate the importance of crucial side-chains in sugar binding, which provides a basis for site-directed mutagenesis to achieve enhanced or novel specificities.

Materials and Methods

Crystal structure analysis

WBAII was purified and crystallised as a complex with methyl-α-D-galactose in two forms as described previously by Kortt (1985) and Manoj *et al*. (1999a). Intensity data were collected on a MAR imaging plate system and were processed and scaled using the programs DENZO and SCALEPACK (Otwinowski, 1993). Details of data processing are given in Table 2. The structures of both the crystal forms were solved uneventfully with molecular replacement techniques (Rossmann & Blow, 1962; Rossmann, 1972) using the program AMoRe (Navaza, 1994). A unique solution for the dimer in Form I was obtained using the coordinates of the dimeric basic lectin (WBAI) from winged bean (Prabu *et al*., 1998; RCSB Protein Data Bank code 1WBL), as the search model. The structure of Form II was solved using the partially refined coordinates of the dimer of Form I. The structure of Form I was refined first with X-PLOR (Brünger, 1992) and then with CNS (Brünger *et al*., 1998) while only CNS was used in the refinement of Form II. The refinement in both the cases involved simulated annealing using the ''mlf'' target (Adams *et al*., 1997) as a step. Non-crystallographic restraints and grouped temperature factors were used in all steps. The Form II structure was built using an electron density map that was averaged over all the subunits in the asymmetric unit with the program RAVE (Kleywegt & Jones, 1994). At every rebuilding step, the stereochemistry of the model was carefully examined using the Ramachandran map (Ramachandran *et al*., 1963) and appropriate databases

Table 2. Data on crystals of WBAII

	Form I	Form II
Space group	*R*3	*C*2
Cell parameters		
a (Å)	182.1	135.4
b (Å)	182.1	127.3
c (Å)	45.0	140.0
β (deg.)	-	95.9
No. of dimers asymmetric unit	1	4
Solvent content (%)	53.8	55.7
Data collection		
Resolution (Å)	3.0	3.3
Last shell (Å)	3.10-3.0	3.42-3.30
Number of observed reflections	47,085	69,174
Number of unique reflections	11,152	32,900
Completion (%)	98.9 (98.2)	93.1 (93.6)
R_{merge} (%)[a]	10.7 (44.2)	11.8 (38.1)
Number of reflections with $I > 2\sigma I$ (%)	83.6 (55.4)	73.0 (41.8)

[a] $R_{merge} = \Sigma|I_i - \langle I\rangle|/\Sigma\langle I\rangle$. Values for the last shell are given within brackets.

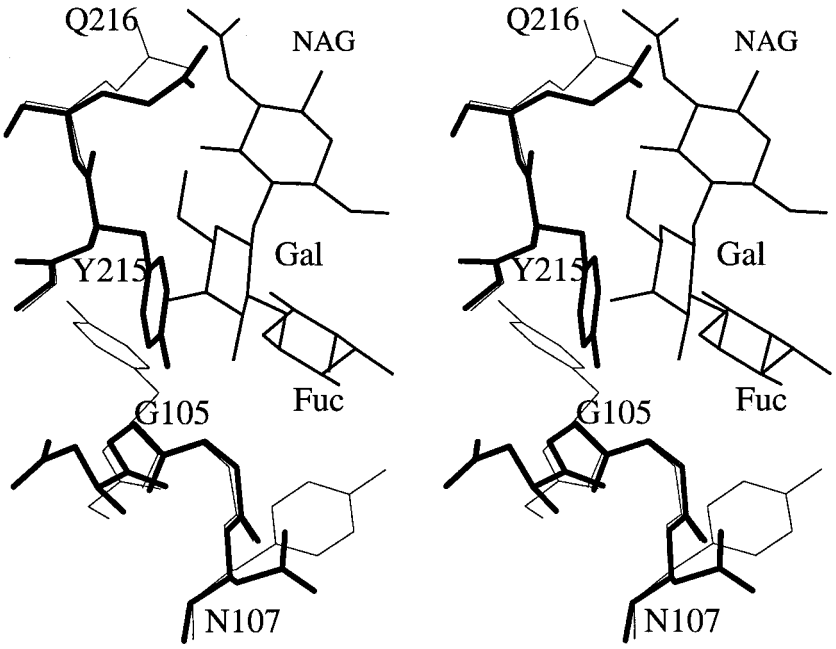

Figure 6. Stereo view of the superposition of loop B and loop D of WBAII (thick lines) and EcorL (thin lines). The modelled trisaccharide is also shown.

(Jones *et al*., 1991; Zou & Mowbray, 1994). During the final stages of refinement the model was checked using omit maps (Bhat & Cohen, 1984; Vijayan, 1980). Details of the refinement and model statistics are listed in Table 3. A conventional Luzzati plot (Luzzati, 1952) yields an error of ~0.32 Å and ~0.36 Å in atomic coordinates of Form I and Form II, respectively.

Table 3. Refinement and model statistics

	Form I	Form II
Resolution range (Å)	20.0-3.0	20.0-3.3
Final *R*-factor[a]	0.198	0.204
Final R_{free}	0.251	0.240
NCS model (Restrained)	2-fold	8-fold
B-factor model	Grouped (mc/sc)	Grouped (mc/sc)
Number of protein atoms	3432 (234 residues)	14,933 (236 residues)
Number of carbohydrate atoms	91	272
Number of metal ions (Ca^{2+}, Mn^{2+})	4	16
Number of solvent atoms	41	80
Average *B*-factors (Å^2)	46.2	47.4
rmsd from ideal values[b]		
Bond lengths (Å)	0.008	0.009
Bond angles (deg.)	1.5	1.6
Improper torsion angles (deg.)	26.6	26.9
Residues (%) in Ramachandran plot[c]		
Core region	84.0	83.2
Additionally allowed region	15.2	16.2
Generously allowed region	0.8	0.6
Disallowed region	0.0	0.0
Outliers[d]	4.1	4.8

[a] $R = \Sigma||F_o| - |F_c||/\Sigma|F_o|$; R_{free} calculated in the same way but for a subset of reflections that is not used in the refinement; No σ cutoff was applied.
[b] Deviations from ideal geometry parameters as defined by Engh & Huber (1991).
[c] As calculated by PROCHECK (Laskowski *et al*., 1993).
[d] As defined by Kleywegt & Jones (1996).

Docking of oligosaccharide ligands

The final refined subunit A of the WBAII-methyl-α-D-galactose complex in Form I was used for docking the oligosaccharide ligand after removing the N-linked oligosaccharide and water molecules, except those coordinated to the metal ions. Models of the terminal trisaccharide of the H-type II histo-blood group were constructed for docking purposes using dihedral angles around the glycosidic bonds of the Galβ1-4GlcNAc and Fucα1-2Gal disaccharides given by Imberty *et al*. (1995). The trisaccharides were then positioned into the binding site by superimposing the galactose rings in the crystal structure and in the trisaccharide. Subsequently, a 5 Å water shell was generated around the model of the complex using the Biosym package INSIGHT-II©. Hydrogen atoms were generated and the model was subjected to conjugate gradient energy minimisation using CNS (Brünger *et al*., 1998) employing harmonic restraints of 30 kcal/molÅ^2 and 5 kcal/molÅ^2 to all C^α atoms and side-chain atoms, respectively. The same protocol was followed for docking of the trisaccharides into the EcorL-binding site. The program MSROLL (Connolly, 1983) was used to calculate buried surface area. The CNS package (Brünger *et al*., 1998) was used for computing interaction energy and shape complementarity was computed using the method of Lawrence & Colman (1993).

RCSB Protein Data Bank accession cordinates

Coordinates and structure factors have been deposited in the RCSB Protein Data Bank: 1F9K and r1F9Ksf for the Form I structure; 1FAY and r1FAYsf for the Form II structure.

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