X-ray Crystal Structure of the Soybean Agglutinin Cross-Linked with a Biantennary Analog of the Blood Group I Carbohydrate Antigen[†,‡]

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ABSTRACT: Soybean agglutinin (SBA) (*Glycine max*), which is a tetrameric GalNAc/Gal-specific lectin, has recently been reported to form unique, highly organized cross-linked complexes with a series of naturally occurring and synthetic multiantennary carbohydrates with terminal GalNAc or Gal residues [Gupta, D., Bhattacharyya, L., Fant, J., Macaluso, F., Sabesan, S., & Brewer, C. F. (1994) *Biochemistry 33*, 7495−7504]. In order to elucidate the nature of these complexes, the X-ray crystallographic structure of SBA cross-linked with a biantennary analog of the blood group I carbohydrate antigen is reported. The structure reveals that lattice formation is promoted uniquely by the bridging action of the bivalent pentasaccharide (β-LacNAc)$_2$Gal-β-R, where R is $-O(CH_2)_5COOCH_3$ and the β-LacNAc moieties are linked to the 2 and 6 positions of the core Gal. The structure of SBA complexed with the synthetic biantennary pentasaccharide has thus been determined by molecular replacement techniques and refined at 2.6 Å resolution to an *R* value of 20.1%. The crystals are hexagonal with a $P6_422$ space group, which differs significantly from that of crystals of the free protein. In the structure, each monomeric asymmetric unit contains a Man_9 oligomannose-type chain at Asn 75, with only the first two GlcNAc residues visible. The overall tertiary structure of the SBA subunit is similar to that of other legume lectins as well as certain animal lectins. However, the dimer interface in the SBA tetramer is unusual in that only one complete peptide chain is sterically permitted, thus requiring juxtapositioning of one C-terminal fragmented subunit together with an intact subunit. Association between SBA tetramers involves binding of the terminal Gal residues of the pentasaccharide at identical sites in each monomer, with the sugar cross-linking to a symmetry-related neighbor molecule. The cross-linking pentasaccharide is in a conformation that possesses a pseudo-2-fold axis of symmetry which lies on a crystallographic 2-fold axis of symmetry of the lattice. Hence, the symmetry properties of the bivalent oligosaccharide as well as the lectin are structural determinants of the lattice. The results are discussed in terms of multidimensional carbohydrate−lectin cross-linked complexes, as well as the signal transduction properties of multivalent lectins.

The oligosaccharide moieties of cell surface glycoproteins and glycolipids are receptors for lectins, hormones, toxins, antibodies, and viral proteins (Wright, 1992). Thus, lectin−carbohydrate interactions have been demonstrated to be involved in a variety of biological processes including mediation of cellular interactions (Lasky, 1992), protein targeting to cellular compartments (Ashwell & Harford, 1982), adhesion of leukocytes to endothelial cells (Sharon, 1993), and host−pathogen interactions (Sharon & Lis, 1989). Plant lectins have been particularly useful in the study of the molecular recognition properties of cellular oligosaccharides (Goldstein & Poretz, 1986) due to their ability to detect subtle variations in the structure of carbohydrates (Shanaan et al., 1991) as well as their broad distribution and ease of isolation (Sharon, 1993). Plant lectins have been important tools for *in vitro* targeting of carbohydrate receptors in eukaryotic cell systems (Goldstein & Poretz, 1986) and have been extensively employed in the classification of cells on the basis of their cell surface glycoproteins and glycolipids, as well as in blood typing, lymphocyte mytogenic stimulation, and cell agglutination assays (Brandley & Schnaar, 1986).

Although many of the best characterized lectins are members of the leguminous family, their role in plant physiology is still a matter of controversy. Evidence suggests that lectins may be involved in infection of leguminal root hairs by *Rhizobia* species, which are symbiotic and essential for the conversion of nitrogen into ammonia in such plants (Diaz et al., 1989). With few exceptions, all legume seeds have been found to contain lectins (Einspahr et al., 1986). While these proteins may differ in carbohydrate specificity and quaternary structure, they share several biochemical properties. Primary structure comparisons of approximately 20 lectins demonstrate that they possess a high degree of sequence homology, displaying over 20% invariant residues, most of which are involved in binding of sugar moieties and in metal ion coordination (Sharon, 1993). Legume lectins generally possess a single carbohydrate binding site per

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subunit, as well as tightly bound Ca^{2+} and Mn^{2+} ions (or other transition metal ions) which are required for their saccharide binding activity (Sharon, 1993). As can be expected from the high sequence homology, X-ray crystallographic studies of concanavalin A (ConA)[1] (Hardman & Ainsworth, 1974), pea lectin (Einspahr et al., 1986), favin (Reeke & Becker, 1986), *Lathyrus ochrus* lectin I (Bourne et al., 1992), and peanut lectin (Banerjee et al., 1994) reveal that the the metal binding sites are in the same locations, as are the carbohydrate binding sites which are adjacent to the Ca^{2+} sites (Sharon & Lis, 1990). In addition, the subunits of these lectins have nearly the same polypeptide fold, despite the fact that the ConA and peanut lectin monomers are comprised of a single subunit while the other three have two polypeptide chains in each monomer. This conserved folding pattern has also been recently reported for several animal lectins including the human serum amyloid protein (Emsley et al., 1994) and two members of the 14 kDa Gal-specific animal lectins (Lobsanov et al., 1994; Liao et al., 1994). Thus, it appears that certain plant and animal lectins share important conserved structural features that are required for their biological activities.

Legume lectins, like many animal lectins, also exist in multimeric forms. The monomeric subunits dimerize in a similar fashion in favin (Reeke & Becker, 1986), ConA (Hardman & Ainsworth, 1974), pea lectin (Einspahr et al., 1986), and *L. ochrus* lectin I (Bourne et al., 1994), forming an extended 12-stranded β-sheet. The dimeric lectin from *Erythrina corallodendron* is different, with an arrangement of subunits in the shape of a "handshake" (Shaanan et al., 1991). The latter configuration appears to be due to the location of the the covalently linked sugar which is proposed to prevent the formation of the extended sheet. The same explanation has been given for the absence of the conserved 12-stranded β-sheet in the structure of the dimer of lectin IV of *Griffonia simplicifolia* (Delbaere et al., 1990). The dimers of ConA and peanut lectin further associate into tetramers, which is not the case for the other three structures whose dimers conform to the 12-stranded β-sheet structure.

The precise mechanism(s) underlying lectin-induced cellular responses such as mitogenesis remain(s) unclear. However, binding and cross-linking of carbohydrate receptors (glycoproteins and glycolipids) by lectins such as ConA and the soybean agglutinin (SBA) (*Glycine max*), which is a GalNAc/Gal-specific tetrameric glycoprotein, appear to activate specific signal transduction pathways (Nicolson, 1976; Lis & Sharon, 1991). In this regard, many legume lectins have been shown to bind and cross-link specific branched-chain oligosaccharides to form highly ordered, unique cross-linked lattices. Furthermore, the specificity of carbohydrate−lectin interactions appears to be greater in the cross-linked complexes than in the corresponding soluble complexes. For example, quantitative precipitation studies of binary mixtures of a series of asparagine-linked (N-linked) oligomannose-type glycopeptides with the Glc/Man-specific lectin ConA indicate that each glycopeptide forms its own unique, homopolymeric cross-linked complex with the lectin (Bhattacharyya et al., 1988). Similar studies have also been carried out with mixtures of complex-type oligosaccharides and several Gal-specific lectins including SBA (Bhattacharyya & Brewer, 1992). The unique stability of homogeneous lectin−carbohydrate cross-linked complexes is due to the presence of long-range order in the complexes (Bhattacharyya et al., 1989). Indeed, it has been possible to observe by electron microscopy the presence of lattice patterns in the cross-linked complexes of certain lectin−carbohydrate mixtures. For example, negative stain electron microscopy has shown that isolectin A from *Lotus tetragonolobus* (LTL-A), which is a tetrameric fucose-specific glycoprotein, forms a two-dimensional lattice in the presence of difucosyllacto-*N*-neohexaose, a naturally occurring biantennary fucosyl oligosaccharide (Bhattacharyya et al., 1990; Cheng et al., 1994). This pattern is different from those of two other cross-linked complexes of LTL-A with structurally related fucose oligosaccharides (Bhattacharyya et al., 1990). Similarly, SBA, which cross-links and precipitates with a variety of N-linked complex-type and branched-chain glycolipid-derived carbohydrates possessing terminal Gal or GalNAc residues, shows the formation of highly organized patterns for many of these complexes by electron microscopy (Gupta et al., 1994). Quantitative precipitation studies, together with preliminary X-ray crystallographic data, indicated formation of unique cross-linked complexes between SBA and isomeric oligosaccharides related to the blood group I antigen in glycoproteins and glycolipids (Gupta et al., 1994).

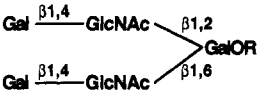

FIGURE 1: Structure of the 2,6-pentasaccharide. The aglycon moiety R is $-(CH_2)_5COOCH_3$. The Gal residue is in the β-anomeric configuration.

Since the biological activities of many lectins are related to their multivalent binding activities, it is important to determine not only the structural basis of their binding specificities with carbohydrates but also their cross-linking interactions with multivalent oligosaccharides. In this regard, the observation that SBA forms unique crystalline cross-linked complexes with a series of blood group I antigen derivatives makes this an ideal system to investigate (Gupta et al., 1994). SBA is a widely used multivalent lectin with important biological properties, including its ability to induce mitogenicity in lymphocytes (Novogrodsky & Katachalski, 1973) and to localize carbohydrate receptors on the surface of normal and transformed cells [cf. Sharon (1983)]. The lectin is a tetrameric glycoprotein of M_r 120 000 (Lotan et al., 1974) with one sugar binding site per monomer, as well as one Mn^{2+} and Ca^{2+} per subunit.

In the present study, we present the three-dimensional structure of SBA cross-linked with a bivalent pentasaccharide related to the blood group I antigen. The results provide insight into the specificity of lectin−carbohydrate cross-linked complexes, including the importance of the symmetry properties of both the carbohydrate and the lectin.

MATERIALS AND METHODS

The 2,6-pentasccharide shown in Figure 1 and the isomeric 2,4-pentasaccharide were synthesized as previously described

[1] Abbreviations: SBA, lectin from soybean (*Glycine max*); ConA, lectin from jack bean (*Canavalia ensiformis*); *L. ochrus*, lectin I from *Lathyrus ochrus* seeds; *E. corallodendron*, lectin from *Erythrina corallodendron* seeds; *G. simplicifolia*, lectin IV from *Griffonia simplicifolia* seeds; N-linked, asparagine-linked; LacNAc, Galβ(1,4)-GlcNAc; NMR, nuclear magnetic resonance. All sugars are in the D-configuration.

(Sabesan et al., 1992). The purity of the oligosaccharides was confirmed by ^{1}H NMR. Native SBA was purified as previously described (Bhattacharyya et al., 1988).

Crystallization. The SBA−2,6-pentasaccharide cross-linked complex was crystallized by the hanging drop vapor diffusion method using 3 μL of lectin solution (34 mg/mL SBA; 1.13 mM) and 755 μM sugar in 0.1 M HEPES, pH 7.2, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 0.15 M NaCl, which were mixed with 3 μL of precipitant solution (same buffer, except that the salt concentration was 0.5 M) on a silanized cover slip which was inverted and sealed above 700 μL of the precipitant solution. Single crystals of up to 1.0 mm in length on each edge could be grown in this way at 4 °C within 1 week. The unit cell dimensions are $a = b = 144.9$ Å, $c = 109.4$ Å, and $\alpha = \beta = 90°$, $\gamma = 120°$. Crystals of the SBA−2,4-pentasaccharide complex were also obtained by employing the same method and identical mother liquor. The unit cell parameters for the latter crystals are $a = b = 143.6$ Å, $c = 105.9$ Å, and $\alpha = \beta = 90°$, $\gamma = 120°$. Both cells have the same space group, $P6_422$. The asymmetric unit of both crystal types contains one SBA monomer complexed to one LacNAc arm of the pentasaccharide and one Man_9 chain. Both crystal forms contain approximately 70% solvent.

Data Collection and Processing. All X-ray diffraction data were collected using Cu Kα radiation (wavelength = 1.5418 Å) with a Siemens area detector coupled to a Rigaku RU-200 rotating-anode generator. The data were processed with the XENGEN software package [Siemens Analytical X-ray Instruments, Inc., Madison, WI (Howard et al., 1987)]. For the SBA−2,6-pentasaccharide crystal data, there were 17 287 unique data points to 2.6 Å (81% complete), with a mean $I/\sigma(I)$ value of 11.95 [$I/\sigma(I) = 2.1$ in the last resolution shell] and an R_{sym} value of 0.126 on intensity. The statistics and the structure of the SBA−2,4-pentasaccharide complex will be reported elsewhere.

Structure Solution. The crystal structure was determined by molecular replacement techniques. A single monomer of the pea lectin dimer (Einspahr et al., 1986) was used in the rotation and translation searches for the SBA complex, as implemented in the X-PLOR manual (Brunger, 1990). The model contained 1769 atoms comprising residues 1−179 (α-chain) and 189−234 (β-chain) [numbering based on the pea lectin cDNA clone (Higgins et al., 1983)]. The rotation function yielded a unique solution, employing data between 12.0 and 4.5 Å resolution. The same range was used to solve the translation function, which yielded the correct solution at 14.4σ, with the next peak at 4.8σ.

Structure Refinement. Rigid body refinement of the pea lectin monomer, rotated and translated according to the molecular replacement solutions, yielded an R-factor of 0.42 for the X-ray diffraction data between 8 and 2.4 Å resolution and with $F > 1\sigma$. The model was then assigned the correct sequence by replacing residues with the model building program TOM (Jones, 1985). Further improvements of the agreement between model and diffraction data were achieved by using positional refinement and simulated annealing molecular dynamics (X-PLOR) (Brunger, 1988, 1990). The R-factor of 0.31, found after 30 cycles of positional refinement of data from 8.0 to 2.4 Å resolution, decreased to 0.24 upon simulated annealing of the model from 3000 to 300 K in the same resolution range. At this point, the structure of jack bean ConA complexed to a single mannose residue (Derewenda et al., 1989) aided in clarifying certain loops, as well as the metal binding sites and the site for the binding of the terminal GlcNAc residue of the noncovalent sugar.

During the course of refinement, omit difference electron density maps [$2|F_o| - |F_c|(\phi_c)$ and $|F_o| - |F_c|(\phi_c)$ calculations] were used extensively. For these maps, the region of interest was eliminated from the model, and either conventional refinement or simulated annealing refinement (X-PLOR) of the model was performed before phases were calculated. Difference electron density maps calculated at this stage revealed clear density for a monosaccharide residue in the carbohydrate binding site of the monomer, as well as two density profiles typical of metal ions. Several rounds of positional refinement and simulated annealing with X-PLOR, followed by manual model building, were carried out to improve the model. Refinement of the structure was subsequently continued with TNT (Tronrud et al., 1988), and 39 water molecules were added. Several cycles of temperature factor refinement were also performed. A final crystallographic R-factor of 20.1% was obtained, and the structure displays rms values of 0.022 Å and 2.3° for bonds and angles, respectively.

RESULTS AND DISCUSSION

Carbohydrate and Metal Binding Sites of the SBA Monomer. The structure of the SBA monomer contains 234 of the total 253 residues and is similar to that of other legume lectins as expected from their high sequence identity (Sharon & Lis, 1990). The polypeptide fold consists of two separate sheets of antiparallel β-strands; there are six strands in the first β-sheet, which is referred to as sheet I in ConA (Hardman et al., 1972) (Figure 2, light blue). The other β-sheet (sheet II of ConA) is composed of seven antiparallel strands, the outer two of which compose the support of the metal binding region (Figure 2, dark blue).

The electron density is weak in two specific areas of the map. These areas include residues 112−119, which consist of a loop on the surface of the molecule that connects β-strands VI and VII, and the carboxyl terminus, after residue 234. Weak electron density in this region may reflect either the flexibility of the carboxyl terminus of the protein in the crystal or C-terminal truncation, as described in a later section. Figure 3 is the Ramachandran plot of the ϕ, ψ angles for the refined backbone of the SBA monomer. All but four residues fall within the allowed regions of low conformational energy. Two of these residues are glycines, another is Asn 136, which is a loop on the surface of the protein, and the last one is involved in a cis-peptide bond (Asp 88) which is conserved among many lectins.

As in other legume lectins (Sharon & Lis, 1990), the carbohydrate binding site of SBA is a depression on the protein surface which shares residues with the Ca^{2+} binding site. The Gal and GlcNAc residues of one arm of the pentasaccharide are well ordered in the refined electron density map, and the β(1,4) bond between the terminal Gal and GlcNAc and the β-linkage(s) between GlcNAc and the central Gal are also visible (Figure 4). The aliphatic aglycon moiety is not visible in the electron density and is assumed to be relatively mobile and without specific contacts. Van der Waals interactions with the side chains of Phe 128, Ile 216, Leu 214, Ala 105, and Ala 87 as well as six hydrogen bonds to protein atoms and to a bound water hold the

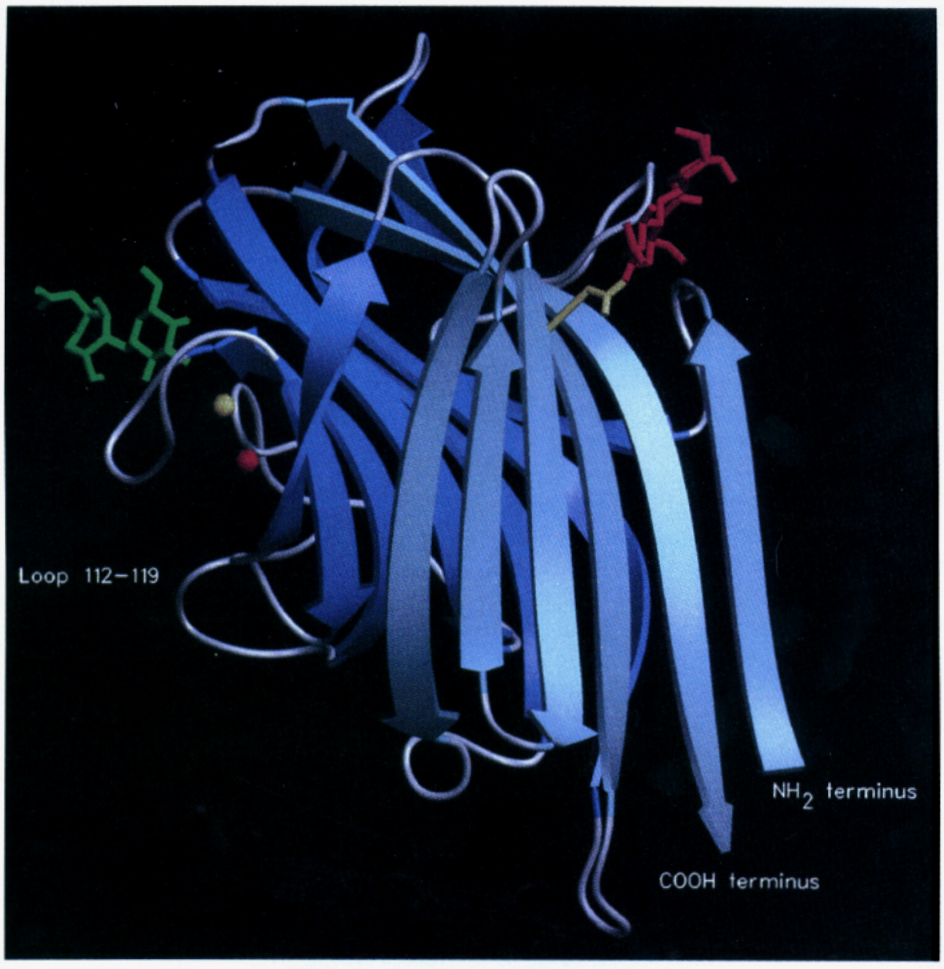

FIGURE 2: Schematic ribbon diagram of the SBA structure with bound Ca^{2+} (yellow), Mn^{2+} (red), one LacNAc arm of the noncovalent sugar (green), and the two GlcNAc moieties of the Man_9 chain (red) covalently bound to Asn 75 (yellow). The view looks to β-sheet I (light blue), with the seven β-strands composing β-sheet II in darker blue, in the back. The figure was made with the graphics program SETOR (Evans, 1993).

terminal Gal in the binding site (Table 1). These hydrogen bonds include those to ND2 of Asn 130, the main-chain carbonyl of Ala 105, OD2 of Asp 88, OD1 of Asp 88, and NH of Leu 214 which, along with the hydrophobic side chains of Ala 87 and Phe 128, occupy similar relative positions in the combining sites of other legume lectins and constitute the framework necessary for binding sugars (Reeke & Becker, 1986). In addition to a hydrogen bond with the 6-OH of the Gal moiety, Asp 215 forms two hydrogen bonds with the 3-OH and the *N*-acetyl moiety of the GlcNAc of the pentasaccharide, thus indicating extended binding site interactions with the protein. Hemagglutination inhibition measurements show that the 2,6-pentasaccharide binds as well as LacNAc to SBA (Gupta et al., 1993).

SBA is known to possess approximately 40-fold enhanced affinity for GalNAc relative to Gal (Pereira et al., 1974; Hammarstrom et al., 1977). This enhanced affinity has recently been shown to be due to more negative enthalpy of binding of GalNAc (D. Gupta and C. F. Brewer, unpublished results). Analysis of the structure of the bound complex of SBA with the 2,6-pentasaccharide suggests that replacement of Gal by GalNAc would allow the *N*-acetyl group of GalNAc to form a hydrogen bond with the side chain of Asp 88.

The Ca^{2+} and Mn^{2+} sites are also highly conserved in legume lectins. In SBA, the ions are approximately 4.5 Å apart and occupy sites in which Asp 126 and Asp 133 each contribute both carboxyl groups as ligands, one to each ion. The bonding coordination of Mn^{2+} is hexahedral, being liganded not only to the aforementioned carboxylates but also to OE1 of Glu 124. The Mn^{2+} also interacts with N2 of the imidazole ring of His 138 and two water molecules, as predicted from electron spin-echo envelope modulation studies (McCracken et al., 1991). The Ca^{2+} has hexahedral coordination, being further stabilized by interactions with the side-chain amide moiety of Asn 130, the carbonyl of Phe 128, and two water molecules within the cavity.

In ConA, one of the water ligands to Ca^{2+} is also hydrogen bonded to OD2 and the main-chain oxygen atom of Asp 208. The proximity of these two protein atoms to the binding site is a result of the cis-peptide bond between residues 207 and 208. In SBA, the cis-peptide bond, which is equally present in LOLI (80/81), pea lectin, and favin (81/82), lies between residues Ala 87 and Asp 88. In the structure of legume lectins, the cis-peptide bond is believed to be important for stabilization of the Ca^{2+} binding site (Einspahr et al., 1986), and it has been proposed that metal binding induces trans–cis isomerization of this peptide bond (Brown et al., 1977; Reeke & Becker, 1986).

SBA is an unusual glycoprotein in that it possesses only one type of N-linked carbohydrate, a $Man_9(GlcNAc)_2$ chain, per monomer (Dorland et al., 1981). The $Man_9(GlcNAc)_2$ chain has recently been demonstrated to be covalently linked to Asn 75 by electrospray mass spectrometry analysis of a

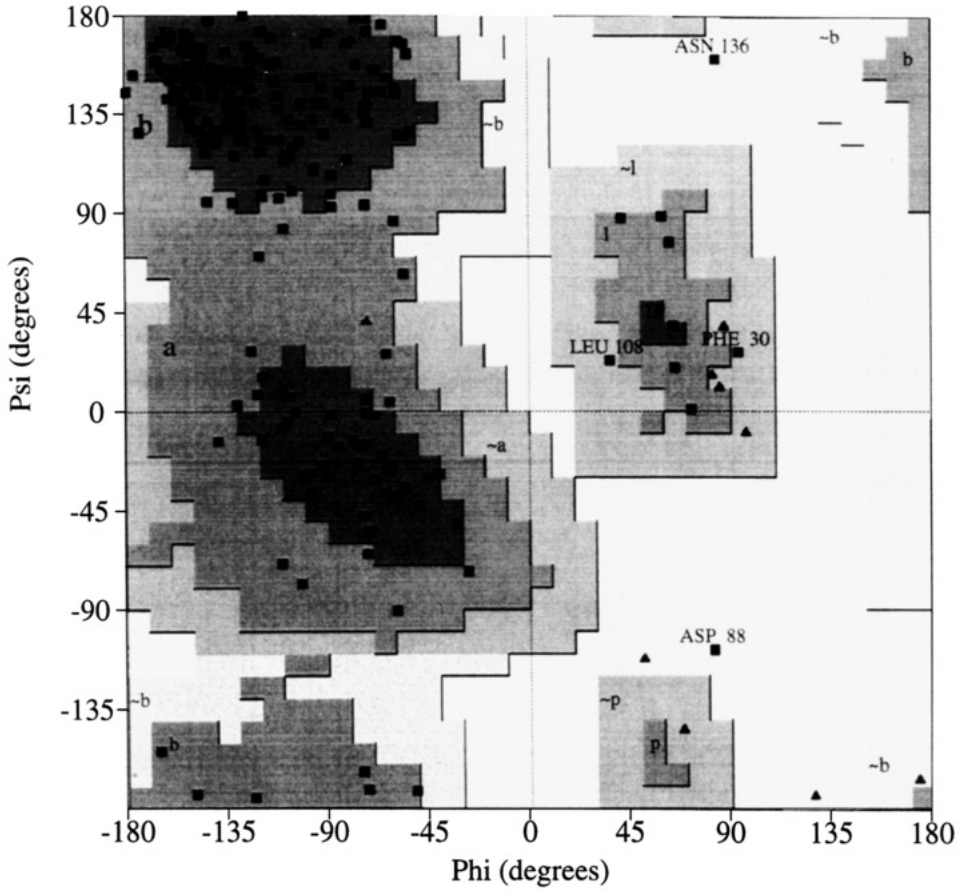

FIGURE 3: Ramachandran plot for the refined SBA structure with bound 2,6-pentasaccharide, Man_9, Ca^{2+}, and Mn^{2+}. The ϕ and ψ angles of all residues are in the "allowed" regions of low energy, with the exception of Asp 88 (which is involved in the cis-peptide bond) and Asn 136 (a surface loop residue).

FIGURE 4: The cross-linking sugar region of the electron density map with $|F_o| - |F_c|$ and the calculated phase angles as coefficients computed by using the program TOM (Jones, 1985). Four amino acid residues which contact with the carbohydrate ligand, namely, Asp 215, Asp 88, Asn 130, and Phe 128, are shown.

Table 1: Sugar−Protein Interactions

type of interaction	sugar residue	protein residue	distance (Å)
noncovalent sugar	O-4 Gal	OD1 Asp 88	3.1
	O-4 Gal	NH Leu 214	3.2
	O-3 Gal	OD1 Asp 88	2.9
	O-3 Gal	OD2 Asp 88	3.1
	O-3 Gal	O Ala 105	3.4
	O-3 Gal	ND2 Asn 130	3.4
	O-6 Gal	OD1 Asp 215	2.5
	O-6 Gal	CD1 Ile 216	3.3
	O-3 GlcNAc	OD1 Asp 215	2.4
	O-3 GlcNAc	OD2 Asp 215	3.5
	N-Z GlcNAc	OD2 Asp 215	3.4
	C-3 Gal	CE2 Phe 128	3.5
	C-5 Gal	CZ Phe 128	3.5
	C-6 Gal	CD1 Ile 216	3.5
	C-6 Gal	OD1 Asp 215	3.0
covalent sugar	O-6 GlcNAc 1	O Asn 9	2.8
	O-5 GlcNAc 1	ND2 Asn 75	2.6
	N-2 GlcNAc 1	ND2 Asn 75	1.9
	C-1 GlcNAc 1	CB Asn 75	2.7
	C-1 GlcNAc 1	CG Asn 75	2.5
	C-2 GlcNAc 1	CG Asn 75	3.2

peptidase digest (Tang et al., 1994). The four $Man_9(GlcNAc)_2$ chains on SBA have been shown to bind to ConA and form 2:1 and 1:1 ConA:SBA cross-linked complexes, depending on the relative ratios of the two molecules in solution (Khan et al., 1991). In addition, evidence has been provided for an essential role of the $Man_9(GlcNAc)_2$ chain(s) in the folding and assembly of SBA (Nagai & Yamaguchi, 1993; Nagai et al., 1993).

The orientations of the four $Man_9(GlcNAc)_2$ chains in the SBA tetramer are shown in Figure 5 (in white). Only the two terminal GlcNAc residues are well resolved in the map, with the core Man residue barely detectable. The values of the ϕ, ψ glycosidic bond torsion angles for the GlcNAcβ-(1−4)GlcNAc linkage (where ϕ = O-5, C-1,O-4′,C-4′ and

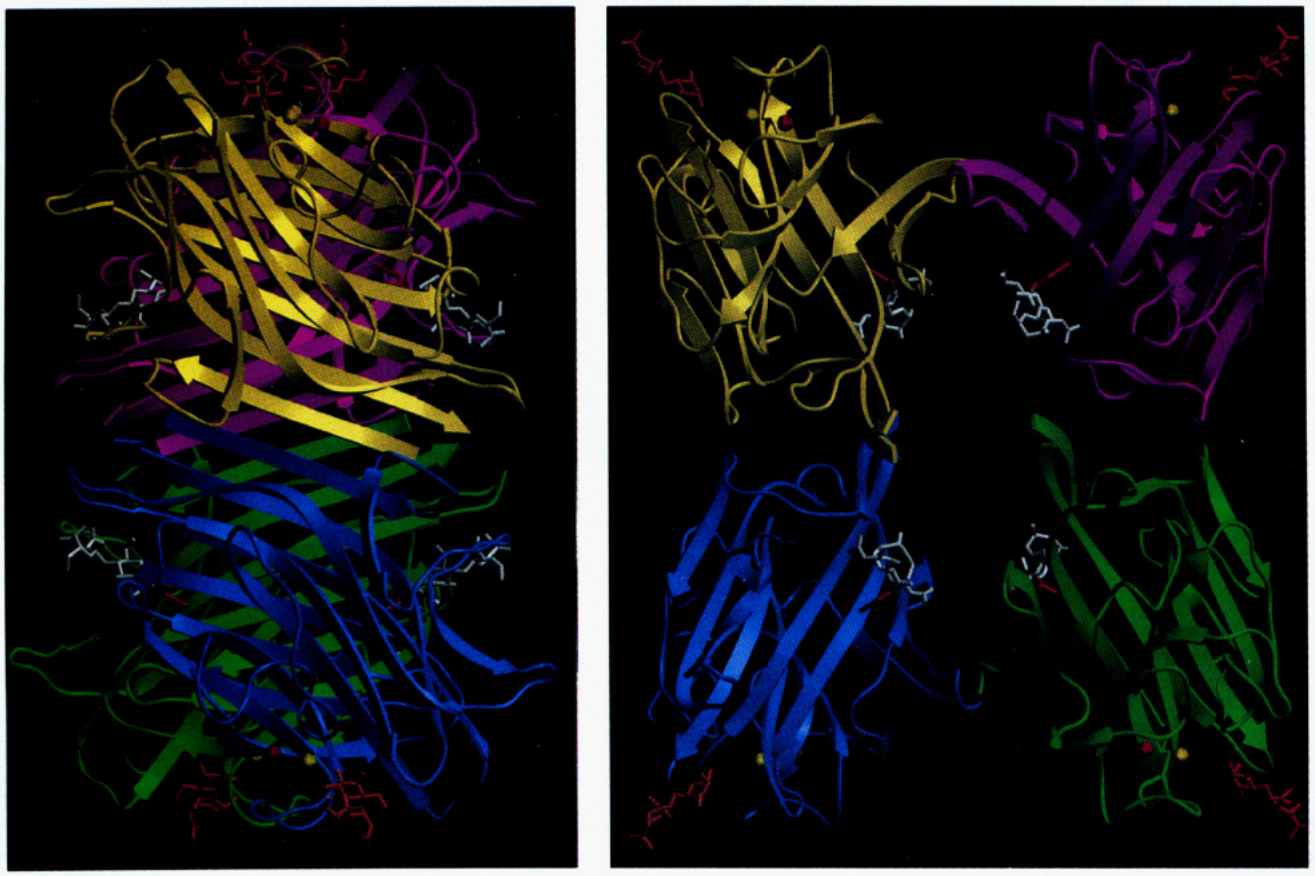

FIGURE 5: (a, left) Schematic ribbon diagram of two SBA dimers which compose the tetramer from the perspective of the z axis. The angle between the two 12-stranded β-sheets (one sheet: yellow and blue subunits; second sheet: green and pink subunits) is 33°. The Man_9 chains (white), covalently bound to Asn 75 (red), are pointing away from the dimer−dimer interface. The noncovalent sugars (orange) are positioned to cross-link symmetry-related molecules which are not shown. The Ca^{2+} (yellow) and Mn^{2+} (red) ions in each monomer are shown, near the noncovalent sugar binding sites. The figure was created with the program SETOR (Evans, 1993). (b, right) Schematic ribbon representation of the SBA tetramer from the perspective of the x axis of the unit cell (a 90° rotation from the panel a; the color scheme is the same). Association of dimers to form a tetramer promotes the formation of an "interdimer space" which is filled with solvent molecules and the carboxyl-terminal peptides of each respective subunit. The extra electron density which accommodates the carboxyl-terminal peptides is located between the yellow and pink monomers, as well as between the blue and green monomers. This view also clarifies the binding mode of the 2,6-pentasaccharide, which binds to the same site in all four molecules and cross-links to neighboring subunits by binding to the identical site. The image was generated with the program SETOR (Evans, 1993).

ψ = C-1, O-4, C-4′, C-5′) are −86°, −93°. The side chain of Asn 75 (in red), which makes the covalent bond with the terminal GlcNAc residue, projects toward the solvent-accessible region, away from the dimer−dimer interface. The N-linked GlcNAc residue interacts with the protein via hydrophobic and H-bonding with Asn 75 and via a H-bond with Asn 9 (Table 1). These carbohydrate−protein interactions may influence the folding properties of the protein, as previously suggested (Nagai & Yamaguchi, 1993; Nagai et al., 1993).

SBA "Dimer". The subunit present in the asymmetric unit of SBA is related by a molecular 2-fold axis to form the complex which resembles the ConA dimer (Hardman & Ainsworth, 1972). By analogy, we will refer to it as the SBA dimer, even though no dimeric form of the protein has been observed in solution. The two subunits of the dimer are joined such that their β-sheets I align to form a 12-stranded sheet spanning one face of the dimer (Figure 5a: the yellow and blue subunits form one dimer; the green and pink subunits form the second one). Six main-chain hydrogen bonds are formed between the first β-strands of the two subunits at the dimer interface (residues Thr3 and Ser#7, Ser#7 and Thr3, Ser5 and Ser#5, Thr#3 and Ser7, and Ser7 and Thr#3, where # represents the amino acid in the second subunit). Several other intersubunit contacts which involve hydrogen bond formation, both side chain to side chain or side chain to main chain, occur within the interface. These contacts include NH of Ala1 to OD1 of Asn#9, NH of Ala1 to ND2 of Asn#9, NH of Ser5 to OG of Ser#5, NH of Ser#5 to OG of Ser5, ND2 of Asn9 to NH of Ala#1, and OD1 of Asn9 to NH of Ala#1.

Although the 2-fold symmetric assembly of subunit β-sheets to form a 12-stranded antiparallel β-sheet is similar to that relating subunits in the dimers of several other lectins such as the pea lectin (Einspahr et al., 1986), ConA (Hardman & Ainsworth, 1972), favin (Reeke & Becker, 1986), and lectin I from *L. ochrus* (Bourne et al., 1994), this arrangement differs from the one displayed by the dimeric association of peanut lectin monomers (Banerjee et al., 1994), as well as the dimers of the lectin from *E. corallodendron* (Shaanan et al., 1991) and lectin IV of *G. simplicifolia* (Delbaere et al., 1990). The latter two proteins are glycosylated, and covalent interactions with sugar residues have been suggested to cause their divergent modes of dimerization. In the peanut lectin structure, the 12-stranded β-sheet does not exist; instead, the two flat six-stranded β-sheets are related by local 2-fold axes inclined at 73° angles with respect to the molecular 2-fold axis

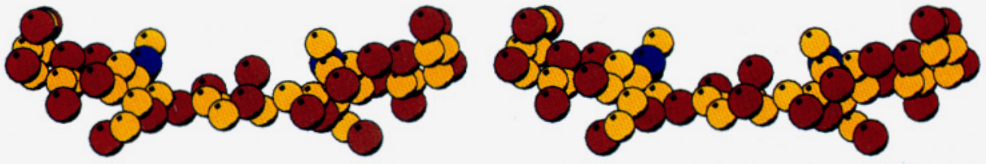

FIGURE 6: Stereo diagram of the 2,6-pentasaccharide in CPK representation in the cross-linked complex with SBA. For clarity purposes, the "core" Gal residue is shown in one of two nonsymmetric orientations, while the outer two LacNAc residues are related to each other by 2-fold symmetry. The $\beta(1,6)$ arm of the oligosaccharide is bound in the $\omega = -60°$ (gg) conformation, where ω is the dihedral angle defined by O-5, C-5, C-6, and O-6. The core Gal residue was manually positioned into the structure for visualization purposes and is not derived from the electron density map. The 6-O of the core Gal residue is artificially compressed in the structure. The figures were generated with the program MOLSCRIPT (Kraulis, 1991).

(Banerjee et al., 1994). Consequently, the six-stranded β-sheets are further apart in peanut lectin than in SBA. Peanut lectin is not glycosylated, indicating that it is not the presence of covalent interactions with sugar monomers which regulates the dimeric association of lectins (Banerjee et al., 1994). The structure of SBA further confirms this observation, since SBA is a glycosylated protein whose dimeric structure has not been affected by the covalent interaction with the Man chain.

A number of legume lectins including the peanut agglutinin (Thibault et al., 1993), *Dolichos biforus* lectins DB58 from the leaves, and the seed lectin (Carter & Etzler, 1975; Schnell et al., 1994) as well as SBA undergo posttranslational modification to give rise to C-terminal truncated subunits in addition to the intact subunits which are present in the proteins. Electrospray ionization mass spectrometry studies have identified four C-terminal truncated subunits with 1−13 amino acids missing from the C-terminal end, as well as the intact subunit in three isolectins of SBA (Mandal et al., 1994). The three isolectins were composed of two intact and two truncated subunits in different combinations. Unlike the *Dolichos biforus* lectin in which the truncated subunits are not active (Schnell et al., 1994), both intact and C-terminal fragmented subunits of SBA are active in their sugar binding activities. More recently, electrospray ionization time-of-flight mass spectrometry studies have shown that isolectin 1 from SBA consists of ~90% heterodimers comprising the tetramer, with each heterodimer composed of one intact (α) chain and one C-terminal fragmented (β) chain missing 13 residues (Tang et al., 1994). Refolding studies have indicated that homotetramers of SBA (i.e., those containing only full-length protein) are not formed, whereas heterotetramers comprised of intact and truncated subunits are capable of refolding (Brewer, unpublished results). These results suggest that SBA, along with certain other C-terminal fragmented lectins including the *Dolichos biforus* lectin (Etzler, personal communication), requires formation of heterotetramers in the active proteins. The molecular basis of this structural requirement is apparent in the SBA−2,6-pentasaccharide complex, which reveals electron density in the region of the dimer-dimer interface which is large enough to accommodate only one carboxyl-terminal peptide. From the perspective of Figure 5b, this density is located between the yellow and pink monomers, as well as between the blue and green monomers. Since there is one molecule of SBA in the asymmetric unit, this heterogeneous C-terminal density is averaged over all of the molecules in the crystal and therefore cannot be interpreted. Hence, each intact SBA subunit must face a truncated subunit in this region, which is the reason why the tetramers are composed of two intact and two truncated subunits.

SBA Tetramer. Figure 5b shows the tetramer formed by cross-linking of SBA monomers along the x axis of the unit cell. Although the asymmetric unit of SBA in the $P6_422$ space group is monomeric, the active SBA tetramer is generated by symmetry. Unlike ConA, the SBA tetramer does not dissociate into two dimeric species at more acidic pH values. Its tetrameric assemblage, however, is much like the one displayed by ConA, consisting of a dimer of dimers in which the 12-stranded β-sheet of each so-called dimer faces each other. The stability of SBA as a tetramer may be explained by the fact that there is a greater number of main-chain to side-chain contacts in SBA within the region of dimer−dimer contact, which is involved in the formation of the tetramer, than in the structures of ConA and pea lectin (the latter being a dimer). These contacts may be responsible for the compactness of the SBA tetramer, thus not allowing for disassemblage of the tertiary structure.

Conformation of the 2,6-Pentasaccharide in the Complex. Figure 6 illustrates the conformation of the repeating 2,6-pentasaccharide units in the crystal of SBA. The pentasaccharide possesses a pseudo-2-fold axis of symmetry relating the two LacNAc moieties and is primarily bound to the carbohydrate binding sites of two symmetry-related molecules via its terminal Gal moieties. Thus, a single pentasaccharide molecule binds between different subunits of neighboring SBA tetramers and constitutes an integral component of the crystal lattice. Although the core Gal residue of the 2,6-pentasaccharide is not well defined in the electron density map because of its asymmetry, the two LacNAc arms of the carbohydrate are well defined and establish the conformation of the cross-linking oligosaccharide in the lattice. The values of the ϕ, ψ glycosidic bond torsion angles [defined for the Gal$\beta(1-4)$GlcNAc linkage as ϕ = O-5, C-1, O-4′, C-4′ and ψ = C-1, O-4, C-4′, C-5′] are −95°, −137°, which are similar to those reported for LacNAc bound to the 14 kDa bovine spleen S-lectin (Liao et al., 1994). The rotationally flexible $\beta(1,6)$ arm of the oligosaccharide must be in the $\omega = -60°$ (gg) conformation, where ω is the dihedral angle defined by O-5, C-5, C-6, and O-6 [cf. Bock et al. (1992)] in order to achieve the observed relative orientations of the two LacNAc arms. In this conformation, the two LacNAc arms are symmetrically related by a crystallographic 2-fold axis through the core Gal residue. Interestingly, the $\omega = -60°$ (gg) conformation of the 2,6-pentasaccharide is the least energetically favorable of the three allowed rotamer populations around the $\beta(1,6)$ arm, disfavored by ~1−2 kcal/M (Sabesan et al., 1992). This implies that the symmetry requirements of the lattice and crystal packing constraints dominate the conformational preference of the cross-linking oligosaccharide. The results also demonstrate a bound conformation of a branched-chain

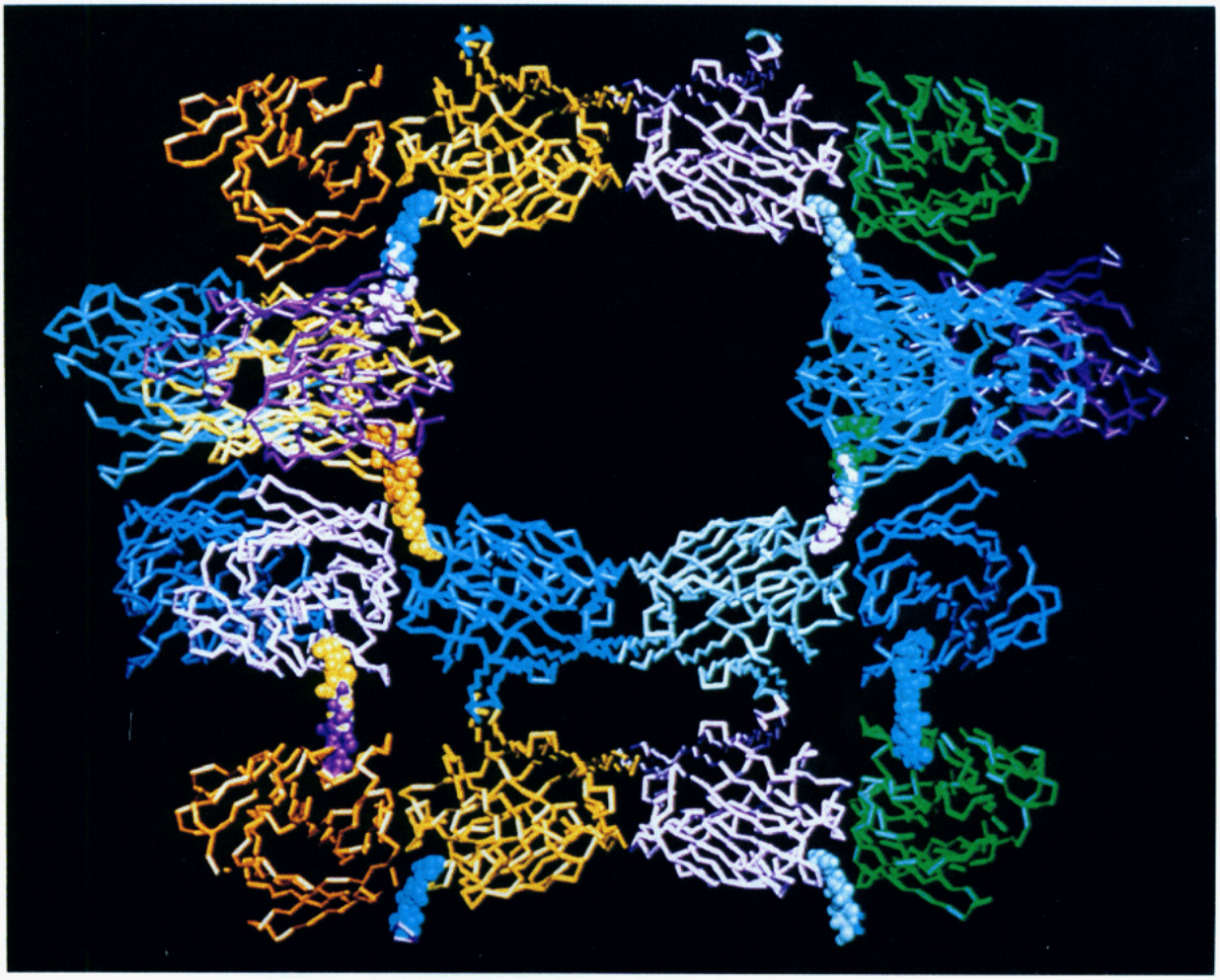

FIGURE 7: Lattice figure of the SBA−2,6-pentasaccharide complex, from the perspective of the *x* axis. Protein molecules are shown as trace models, with each tetramer represented in four colors. The cross-linking sugar molecules are shown as CPK models along the *z* axis, with each half of a single molecule shown in two colors. The aglycon moiety is not shown. One SBA tetramer in the bottom center of the figure is shown cross-linked to adjacent tetramers (structures not completely shown). One arm of the carbohydrate binds to a specific site on each subunit of a tetramer and then cross-links to a neighboring tetramer by binding to the corresponding site on another monomer.

oligosaccharide possessing one of several possible internal symmetry relationships relating the outer binding epitopes (Gupta et al., 1994).

Lattice Structure. Analysis of the crystal lattice also allows identification of a 6-fold axis of symmetry which forms the basis of the structural network. A lattice is thus generated with pentasaccharide molecules cross-linking neighboring SBA subunits (Figure 7). Salient features of the lattice are the absence of a high degree of strong stabilizing protein−protein contacts and the formation of large, solvent-filled cavities. The SBA−2,6-pentasaccharide crystals are quite large, attaining lengths of 3−4 mm, but all crystals tested diffracted to a maximum of 2.5 Å. This is not surprising since these crystals contain a high level of solvent (approximately 70%). These observations indicate that lattice formation is, for the most part, a carbohydrate-driven event, which is why this crystal complex possesses a different space group and unit cell dimension from that of the crystalline free protein (space group *C*2; unit cell dimensions $a = 118.6$ Å, $b = 88.9$ Å, $c = 165.9$ Å, $\beta = 103.0°$) (Shannon et al., 1984).

LTL-A is a tetrameric fucose binding legume lectin with a primary sequence similar to that of SBA (Goldstein & Poretz, 1986). It has been reported to form unique cross-linked complexes with three biantennary fucosyl oligosaccharides which are observable by electron microscopy (Bhattacharyya et al., 1990). Cheng et al. (1994) have analyzed the structure of one of the complexes involving difucosyllacto-*N*-neohexaose, a 3,6-branched carbohydrate with a core Gal residue, using a combination of electron microscopy, X-ray diffraction, computer simulation of electron micrographs, and molecular model building. The resolution of the X-ray data was approximately 10 Å. The lattice was found to be two-dimensional, with the bivalent saccharide cross-linking individual monomers of adjacent tetramers in the lattice, similar to that of the SBA−2,6-pentasaccharide complex. On the basis of the symmetry of the lattice and the structure of the lectin (assumed to be similar to that of ConA), it was concluded that the topography of LTL-A around the monomer−monomer interface cross-linked by the oligosaccharide was self-complementary and that this interface along with the saccharide existed on a nearly 2-fold axis of symmetry between adjacent protein molecules. The limited resolution of the data prevented determination of whether there were any protein−protein interactions across the monomer−monomer interface, in addition to that of the cross-linking oligosaccharide. In the present study, it is clear from the three-dimensional lattice of the SBA−2,6-pentasaccharide complex that the oligo-

saccharide provides the majority of the interactions between cross-linked tetramers of SBA.

The lattice structure of the SBA−2,6-pentasaccharide complex differs from those of two other recently described carbohydrate−lectin cross-linked complexes. Wright (1992) recently reported the crystal structure of a cross-linked complex of the wheat germ agglutinin isolectin 1 (WGA1) complexed with a tryptic sialoglycopeptide fragment from glycophorin A which contains the O-linked tetrasaccharide NeuNAc-α2,3-Gal-β1,3-(α2,6-NeuNAc)Gal-Nac-α1-O-Thr. The WGA1−glycopeptide complex is asymmetrical in the number of binding sites used in the protein as well as the position of the cross-linking carbohydrate in the lattice. In addition, the complex possesses bound saccharide that is involved in carbohydrate−carbohydrate interactions rather than carbohydrate−protein cross-linking interactions, and there are substantial protein−protein contacts in the lattice. The SBA−2,6-pentasaccharide complex, on the other hand, is completely symmetrical, and there is little evidence of substantial protein−protein interactions.

Recently, Weis et al. (1992) reported the crystal structure of the mannose binding protein A from rat cross-linked with a Man_6 glycopeptide. In this case, the protein was a proteolytic dimeric fragment that was cross-linked with the divalent N-linked glycopeptide. The structure of this complex therefore involves divalent carbohydrate−protein interactions along one dimension and predominantly protein−protein contacts along the other dimensions. Thus, the structure of this cross-linked complex differs substantially from the one in the present study.

The ability of a series of blood group I related isomeric oligosaccharides, including the 2,6-pentasaccharide, to induce different cross-linked lattices with SBA has been reported (Gupta et al., 1994). It is apparent that the structural properties of both the carbohydrate and lectin in the present study are responsible for the formation of the highly organized SBA−2,6-pentasaccharide cross-linked complex. We have also obtained crystals and solved the structure of SBA in the presence of the 2,4-pentasaccharide. Crystals were obtained under the same conditions as for the SBA−2,6-pentasaccharide complex, and data were collected in the same fashion to approximately the same resolution. Although the refinement of the SBA−2,4-pentasaccharide complex is yet incomplete, it is clear that the differences in unit cell parameters between the SBA−2,4-pentasaccharide and SBA−2,6-pentasaccharide complex crystals are directly related to differences observed in lattice structure. For the SBA−2,4 pentasaccharide complex, crystals are of the space group $P6_422$, with unit cell parameters $a = b = 143.6$ Å, $c = 105.9$ Å, $\alpha = 90°$, $\beta = 90°$, $\gamma = 120°$ (the length of the c axis is approximately 3 Å shorter than the length of the same axis in the SBA−2,6-pentasaccharide crystal). This difference in c axis length is reflected in the lattice structure, in which the distance between the galactose O3 hydroxyl atoms of neighboring symmetrical monomers is 25.34 Å in the 2,6-pentasaccharide and 21.71 Å in the 2,4-pentasaccharide. These results suggest a molecular basis for the formation of homogeneous carbohydrate−protein cross-linked complexes, namely, the formation of highly symmetrical lattices whose dimensions are highly dependent on the specific multivalent cross-linking carbohydrate as well as the symmetry properties of both the carbohydrate and lectin.

CONCLUSIONS

The present findings, in addition to providing the first structure of a tetrameric glycoprotein plant lectin, also provide a molecular basis for a new source of specificity in multivalent carbohydrate−lectin interactions, namely, the formation of unique homogeneous cross-linked lattices even in the presence of mixtures of the molecules. These interactions appear to be important in lectin-mediated aggregation events involving cellular recognition and signal transduction processes in both plants and animals. For example, the mitogenic activities of both plant and animal lectins appear to require aggregation of their respective glycoconjugate (glycolipids and glycoproteins) receptors. Our findings indicate that the carbohydrate moieties of specific glycoconjugate receptors can be cross-linked by multivalent lectins into homoaggregates. The immunomodulatory activities of lectins may be related, as in the activation by ConA and SBA of the T cell receptor and its closely associated proteins [cf. Liscastro et al. (1993)]. Most C-type lectins possess multimeric structures (Drickamer & Taylor, 1993) and are therefore potentially capable of forming cross-linked complexes with specific multivalent carbohydrate ligands which may be important for their biological activities. Lastly, since the driving force for the formation of homoaggregates of multivalent lectins with carbohydrates is solely thermodynamic, other multivalent ligand−macromolecular systems must possess the same properties. The only requirement is that one of the two interacting molecules must be greater than divalent so that two- or three-dimensional noncovalent cross-linked lattices form and crystalline-type packing constraints exist. The crystal structure of the SBA−2,6-pentasaccharide complex thus represents a structural model for lectin−carbohydrate multidimensional clustering *in vivo* and a common thermodynamic mechanism for selectively aggregating a dispersed population of multivalent receptors in biological systems for activation through clustering.

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