The Xenograft Antigen Bound to *Griffonia simplicifolia* Lectin 1-B₄

**X-RAY CRYSTAL STRUCTURE OF THE COMPLEX AND MOLECULAR DYNAMICS CHARACTERIZATION OF THE BINDING SITE***

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The shortage of organs for transplantation into human patients continues to be a driving force behind research into the use of tissues from non-human donors, particularly pig. The primary barrier to such xenotransplantation is the reaction between natural antibodies present in humans and Old World monkeys and the *Ga*(1–3)*Gal* epitope (xenograft antigen, xenoa antigen) found on the cell surfaces of the donor organ. This hyperacute immune response leads ultimately to graft rejection. Because of its high specificity for the xenograft antigen, isolectin 1-B₄ from *Griffonia simplicifolia* (GS-1-B₄) has been used as an immunodiagnostic reagent. Furthermore, haptens that inhibit natural antibodies also inhibit GS-1-B₄ from binding to the xenoa antigen. Here we report the first x-ray crystal structure of the xenograft antigen bound to a protein (GS-1-B₄). The three-dimensional structure was determined from orthorhombic crystals at a resolution of 2.3 Å. To probe the influence of binding on ligand properties, we report also the results of molecular dynamics (MD) simulations on this complex as well as on the free ligand. The MD simulations were performed with the AMBER force-field for proteins augmented with the GLYCAM parameters for glycosides and glycoproteins. The simulations were performed for up to 10 ns in the presence of explicit solvent. Through comparison with MD simulations performed for the free ligand, it has been determined that GS-1-B₄ recognizes the lowest energy conformation of the disaccharide. In addition, the x-ray and modeling data provide clear explanations for the reported specificities of the GS-1-B₄ lectin. It is anticipated that a further understanding of the interactions involving the xenograft antigen will help in the development of therapeutic agents for application in the prevention of hyperacute xenograft rejection.

The major barrier to xenotransplantation (1) is a hyperacute immune response (2), in which Gal(1–3)Gal (xenograft antigen) present on the surface of non-primate tissues triggers the rejection from human transplant recipients (3–5). The ubiquitous presence of anti-Gal(1–3)Gal antibodies in humans, Old World monkeys and apes is paralleled by the absence of Gal(1–3)Gal on the cell surfaces of those species (6). The natural antibodies attack the surface endothelial cells leading to complement activation and organ death. Several approaches to this problem have been considered (1), including inhibition of the anti-Gal(1–3)Gal antibodies, induction of tolerance to the xenoa antigen (7), and transgenic alteration of the Gal(1–3)Gal epitope present on the cell surfaces of the donor species (8, 9). To date, only transient suppression of the anti-Gal(1–3)Gal immune response has been achieved (1). Overcoming xenograft rejection has become increasingly important due to the huge demand for organ transplants; a study in 1998 estimated that the demand had increased by 100% over the period 1990 through 1998 (1).

*Griffonia simplicifolia* lectin-1 (GS-1) is a carbohydrate-binding glycoprotein that is isolated from the seeds of the African leguminous shrub. As in many other legume lectins, GS-1 relies on the presence of divalent metal cations for its carbohydrate-binding activity (10). GS-1 is a mixture of five tetrameric isolectins that vary in their content of A and B subunits (11). The A subunit was found to bind strongly to both GalNAc and Gal residues, while favoring GalNAc (12). Competitive binding studies have shown that the GS-1 isolectin composed of four B subunits (GS-1-B₄) has a high affinity for the Gal(1–3)Gal sequence (12, 13). Therefore, GS-1-B₄ has found application as an immunodiagnostic reagent in studies of the xenograft antigen. Furthermore, inhibitors of the interaction between anti-Gal(1–3)Gal antibodies and the xenograft antigen also inhibit carbohydrate binding to GS-1-B₄ (14–16). Notably, the binding of Gal(1–3)Gal to GS-1-B₄ appears to be determined primarily by the presence of the terminal α-galactosyl residue; other linkages may be tolerated, as long as they contain a terminal Gal residue. Thus, GS-1-B₄ is not a perfect model for the natural antibodies; however, it does provide an opportunity to gain detailed insight into the mechanism of recognition of the xenoa antigen. To determine the mechanism for the observed specificities, as well as to obtain the first structure of the xenoa antigen bound to a protein, we have de-

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The atomic coordinates and structure factors (code IHQL) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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‡ The abbreviations used are: GS-1, *G. simplicifolia* lectin-1; AMBER, assisted model building and energy refinement; CNS, crystallography & NMR system; CRD, carbohydrate recognition domain; Gal, t-galactopyranosyl; GLYCAM, glycosides and glycoproteins in AMBER; MD, molecular dynamics; NCS, noncrystallographic symmetry; RCSB, Research Collaboratory for Structural Bioinformatics; SANDER, simulated annealing and energy refinement; TIP3P, transferable intermolecular potential −3 point.

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determined the x-ray crystal structure of the GS-1-B4 Gal(1–3)Gal complex.

A number of structural studies have been reported for the xenograft antigen and related oligosaccharides (12, 17–21). These studies used both computational and NMR spectroscopic methods to determine the solution conformation. Earlier computational studies employed adiabatic energy mapping to predict low energy conformations for the Gal(1–3)Gal linkage. More recently, both gas-phase Monte Carlo and molecular dynamics (MD) simulations have been employed to examine ligand flexibility (19, 21). To determine the extent to which water mediates the conformational properties of the ligand, the present study employed MD simulations of the free ligand in explicit water at atmospheric pressure and room temperature. These long 10-ns simulations are extremely computer-intensive, however, they are able to predict with accuracy the influence of solvation and binding interactions on the conformational and dynamic properties of carbohydrates (22). To examine the influence of protein binding on ligand dynamics, as well as to obtain a complete spatial and temporal picture of the interaction, 2- to 5-ns MD simulations of the bound complex, were also performed with explicit water. These simulations provide additional insight into the structural significance of bound waters, seen to mediate the carbohydrate-protein interaction in the x-ray structure.

EXPERIMENTAL PROCEDURES

Crystallization, Diffraction Data Collection, and Structure Solution by Molecular Replacement—The crystallization, x-ray diffraction data collection, and molecular replacement for the GS-1-B4 complex with Gal(1–3)Gal-OMe are described in detail elsewhere (23).

| Table I Diffraction data analysis, space group P2₁2₁2₁ |
|---|---|---|
| Parameter | Crystal I | Crystal II | Combined |
| a (Å) | 111.00 | 111.42 | 111.21 |
| b (Å) | 51.32 | 51.12 | 51.28 |
| c (Å) | 76.91 | 77.22 | 77.11 |
| Resolution (Å) | 30.00–2.65 | 20.00–2.25 | 20.00–2.20 |
| Complete crystal | 2.77–2.65 | 2.35–2.25 | 2.30–2.20 |
| Completeness (%) | 97.4/95.9 | 85.4/85.2 | 96.7/83.8 |
| Completeness I/σ(I) > 3 (%) | 83.2/59.2 | 72.5/55.6 | 76.9/48.4 |
| Rsym (%) | 6.9/28.0 | 5.0/24.9 | 14.2/41.6 |

Structure Refinement—Data collected from two crystals from different crystallization drops where used in the structural refinement. The combined data set was obtained by merging individual integrated reflection files using SCALEPACK of the HKL software suite (24). Of 22,043 observed (23,055 theoretical) reflections between 20- and 2.2-Å resolution, 1500 were set aside as test observations (25). The CNS suite of programs (26), with a maximum likelihood target function (27), was used throughout the entire process of refinement. After two rounds of independent rigid-body refinement of the two instances of the search model polypeptide chain (RCSB ID: 1GSL) (28), the NCS transformation matrix between the two molecules was determined. The resultant operators were used in the application of NCS constraints in the initial stages of refinement. Real-space density fitting was performed using O (29). After the inclusion of two metal ions (30) and carbohydrate chains, NCS constraints were removed and replaced by gradually decreasing restraints. Prior to PDB submission (as RCSB ID: 1HQL) (31) the model quality was assessed using PROCHECK (32).

Molecular Dynamics—The SANDER (33) module of AMBER 5.0 (34) was utilized in conjunction with the PARM98 parameter set for proteins and the GLYCAM (35) parameter set for glycosides and glycoproteins. A single subunit of the GS-1-B4 x-ray crystal structure IHQL was protonated with INSIGHTII (36), and a 25-Å droplet containing 1389 TIP3P waters (37) was placed around O3 of the non-reducing end of the disaccharide (Galα, residue 243). Initially, the solvent positions were optimized with 9000 steps of steepest descent, followed by 1000 steps of conjugate gradient, energy minimization. This was followed by a period of simulated annealing, during which the solvent was heated to 300 K over 20 ps, held at 300 K for 60 ps, before being cooled to 5 K over an

FIG. 1. The biological tetramer of GS-1-B4 extends over two asymmetric units containing one Galα(1–3)Galβ-OMe molecule in the binding site of each subunit of the lectin (left, Rasmol). Ramachandran plot of the refined complex model (right, Procheck).

FIG. 2. Packing of GS-1B₄ subunits inside the crystallographic unit cell (Rasmol image).
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RESULTS AND DISCUSSION

**X-ray Data Reduction and Structure Refinement**—The structure of the complex was solved by molecular replacement using diffraction data from 8 to 4 Å, from only one crystal (Crystal I), resulting in the placement of two instances of the search model polypeptide in the asymmetric unit. However, the subsequent refinement proved challenging. Apart from a 25-residue N-terminal sequence (38), no further sequence information was available for GS-1-B$_4$. Although the related lectin GS-4 has 12 of its 27 N-terminal amino acid residues in common with GS-1-B$_4$, a loop in this region of the chain contains 3 additional residues (28).

**Table II**

| Root mean square deviation from ideal geometry |
|-----------------------------------------------|
| Bond lengths                                  |
| 0.007 Å                                       |
| Bond angles                                   |
| 1.2°                                          |
| Dihedral angles                               |
| 26.4°                                         |
| Improper angles                               |
| 0.75°                                         |
| Coordinate error (cross-validated)            |
| Luzzati                                       |
| 0.27 Å (0.32 Å)                               |
| Sigma A                                       |
| 0.21 Å (0.24 Å)                               |
| Temperature factors                           |
| Mean                                          |
| 38.9 Å$^2$                                     |
| Wilson                                        |
| 42.6 Å$^2$                                     |
| Ramachandran plot, residues in:               |
| Most favored regions                          |
| 84.8%                                         |
| Additional allowed regions                    |
| 14.7%                                         |
| Generously allowed regions                    |
| 0.5%                                          |

**Table III**

| Crystal | Free sugar | Complex |
|---------|------------|---------|
|         |            | 2-ns    | 5-ns    |
| $\Phi$  | $-31.8$    | $-44.2$ (14) | $-37.7$ (14) | $-35.7$ (10) |
| $\Psi$  | $-15.9$    | $-24.7$ (18) | $-21.8$ (16) | $-15.0$ (14) |

**Fig. 3.** Schematic representation of metal coordination in GS-1-B$_4$. The metal binding site of GS-1-B$_4$, closely resembles that reported for GS-4, where the side chains of Glu$^{139}$, Asp$^{133}$, Asn$^{135}$, Asp$^{140}$, and His$^{145}$ four water molecules, and the carbonyl oxygen of Trp$^{133}$ directly interact with the metal ions.

**Fig. 4.** Interactions at the carbohydrate binding site of GS-1-B$_4$. Distances shown are in Å and represent the mean between the values of the two monomers in the asymmetric unit.

**Fig. 5.** Glycosidic torsion angles of the free ligand in water determined over the 10-ns MD simulation. A, B, C, and D refer to previously identified conformations for this disaccharide (20).

misplaced regions. This could be attributed to the uncertainty surrounding the number of residues and the types of side chains to be fitted in areas with low correlation between electron density and model coordinates.

A second set of diffraction data, this time extending beyond 2.2 Å resolution, was collected using a crystal (Crystal II) grown in a separate experiment under the conditions described for Crystal I (29). This data set was less complete than that for Crystal I, for resolutions lower than 2.65 Å, apparently due to increased crystal mosaicity and ensuing rejection of overlapping reflections. Applying the parameters used in the molecular replacement for the Crystal I data, these data failed to provide a structure solution using the CNS program. Consequently, several strategies were considered for scaling data from both crystals into a combined data set to use the additional higher resolution data during refinement. Ultimately, the combined set was obtained by scaling together individual integrated reflection files from DENZO (24).
Although Table I highlights significant discrepancies between the data from both crystals, the procedure resulted in a data set that proved to be of sufficient quality for successful refinement by alternating slow-cool simulated annealing and real-space model rebuilding. Initially, refinement was confined to one of the polypeptide chains in the asymmetric unit, and coordinates for the second chain were generated by strict application of NCS operators. At this stage, difference density clearly indicated the position of two metal ions. Unlike the commonly observed presence of a Ca$^{2+}$ and a transition metal cation combination (30) two calcium cations were employed initially in the refinement. This decision was based on a study of GS-1 metal dependence (10) and our failure to detect significant amounts of Mn$^{2+}$ from sequencing of fragments from CNBr2 digests and published N-terminal sequence (38), unpublished data derived proved to be 25.5% ($R_{	ext{crys}} = 26.6\%$). Lastly, the addition of crystallographic water atoms and substitution of Mn$^{2+}$ in a metal analysis of a GS-1-B$_4$ solution at this point, the quality of the electron density permitted significant amounts of Mn$^{2+}$ to one of the polypeptide chains in the asymmetric unit, and residues of the $N$-glycan on residue Asn$^{27}$. NCS constraints were replaced by restraints when the crystallographic residual had been improved to 25.5% ($R_{	ext{crys}} = 26.6\%$). Lastly, the addition of crystallographic water atoms and substitution of Mn$^{2+}$ for one Ca$^{2+}$ in each subunit, both based on difference map density, produced a preliminary model. In the absence of the complete GS-1-B$_4$ amino acid sequence, the model was based on the published N-terminal sequence (38), unpublished data derived from sequencing of fragments from CNBr2 digests and sequences of homologous *Griffonia simplicifolia* lectins. This model was updated when the complete sequence became available (40).

**Description of the Biologically Active Tetramer**—The asymmetric unit consists of two single chain subunits A (not to be confused with the A-type subunit of GS-1) and B. Subunits A and B are related by a non-crystallographic 2-fold axis oriented roughly perpendicular to the 6-stranded “back” $\beta$ sheet, common in legume lectin monomers (30). However, unlike the case in the “canonical” dimer found in concanavalin A (41), the two subunits do not arrange to form a large 12-stranded sheet. Rather, the strands composed of residues 4–11, 239–231, and 69–76 appear to align as extensions of strands 69–231, and 4–11 in the other subunit, respectively. Aromatic residues such as Trp$^{13}$ and Phe$^{78}$ and non-polar side chains of Ala$^{10}$ and Leu$^{231}$ exhibit the closest contacts with the peptide chain in the other subunit. Application of the crystallographic symmetry-based transformation $(1 - x, -y, z)$ to the atomic coordinates of subunits A and B generated subunits A* and B*, respectively. Interestingly, the mode of association observed between A and A*, as well as between B and B*, resembles that in the GS-4 dimer (28), with a nearly perpendicular alignment of the strands in the $\beta$ sheets at the interface (Figs. 1 and 2).

**Metal Binding Site**—Based on a published biochemical study of the metal dependence of GS-1 (10), Ca$^{2+}$ was the only diva-

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**Table IV**

*Hydrogen bonding interactions between *Gala* residue and the GS-1-B$_4$ lectin*

| Donor atom | Acceptor atom | 2-ns run $d$ | 5-ns run $d$ | X-ray $d$ |
|------------|---------------|-----------|-----------|--------|
| Glu$^{106}$ NH | *Gala* O3 | 3.2 (0.2) | 3.2 (0.2) | 3.1 |
| Asn$^{134}$ N$^{\gamma}$H1 | *Gala* O3 | 3.5 (0.3) | 3.5 (0.3) | 3.1 |
| Gly$^{221}$ NH | *Gala* O4 | 3.7 (0.2) | 3.5 (0.2) | 3.4 |
| Asn$^{222}$ NH | *Gala* O4 | 3.3 (0.2) | 3.4 (0.2) | 4.1 |
| Asn$^{222}$ NH | *Gala* O5 | 3.3 (0.2) | 3.8 (0.2) | 3.4 |
| Asn$^{222}$ NH | *Gala* O6 | 3.6 (0.2) | 3.5 (0.2) | 3.1 |
| Asn$^{223}$ NH | *Gala* O6 | 3.1 (0.1) | 3.3 (0.4) | 2.8 |
| Asn$^{223}$ N$^{\gamma}$H1 | *Gala* O6 | 3.5 (0.2) | 3.5 (0.2) | 3.9 |
| Gala O2H | Glu$^{106}$ Oe1 | 3.5 (0.3) | 3.4 (0.4) | 2.7 |
| Gala O3H | Asp$^{88}$ O81 | 3.0 (0.2) | 2.9 (0.1) | 2.6 |
| Gala O3H | Asp$^{88}$ O82 | 3.2 (0.2) | 3.2 (0.2) | 3.5 |
| Gala O4H | Asp$^{88}$ O81 | 3.8 (0.2) | 3.8 (0.1) | 3.3 |
| Gala O4H | Asp$^{88}$ O82 | 2.9 (0.1) | 2.8 (0.1) | 2.6 |

* $d$ in Angstroms, standard deviation in parentheses, and percent hydrogen bond occupancies in boldface.

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2 E. van Damme, personal communication.

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**Fig. 6.** Hydrogen bonding interactions between the *Gala* residue and the CRD (carbohydrate recognition domain) of the GS-1-B$_4$ lectin from the 2-ns MD simulation. All distances are the mean values, given in A.

**Fig. 7.** Hydrogen bonding interactions involving the complexed water from the 2-ns MD simulation. Boldface lines represent interactions present in both conformations. The dashed lines represent interactions taking place only in configuration E, and the wavy lines represent interactions taking place only in configuration W.
lent metal ion added to the protein buffer used in the crystal-
ization of GS-1-B4 (23). Consequently, both metal sites were 
treated as being occupied by Ca$^{2+}$ ions in the initial stages of 
refinement. Significantly shorter bond distances to surround-
ing protein side-chain atoms and some remaining 
refinement. Significantly shorter bond distances to surround-
bound to the protein sample even during isolation. Although 

electron density permitted the modeling of the core GlcNAc 
superior continuity when compared with that on A. Electron 
observed on both subunits, but the density on subunit B shows 
edensity suggests glycosylation at residue Asn 27. It is 
treatment as being occupied by Ca$^{2+}$ ions (28, 42).

$N$-Glycosylation—Native GS-1-B$_4$ is a glycoprotein (10), and 
electron density suggests glycosylation at residue Asn$^{57}$. It is 
observed on both subunits, but the density on subunit B shows 
superior continuity when compared with that on A. Electron 
density permitted the modeling of the core GlcNAc$^{1}$– 
$4$GlcNAc sequence; however, even in case of the B subunit, 
density for the carbohydrate residues is weak and does not 
cover all atoms.

The Xenograft Antigen in the Carbohydrate Binding Site of 
GS-1-B$_4$—The terminal $\alpha$-galactosyl residue (Gala) of the 
xenograft antigen is represented by well-contoured electron 
density in the CRDs of both the A and B subunits. Interactions 
between the side chain of Asp$^{89}$ with hydroxyl groups HO-3 and 
HO-4, as well as between the side chains of Asn$^{135}$ and the 
amide nitrogen of Glu$^{106}$ with HO-3 (Fig. 1), are paralleled by 
similar interactions involving residues Asp$^{89}$, Asn$^{135}$, and 
Gly$^{107}$ in the complex between GS-4 and the Lewis b human 
blood group determinant (28). Additional contacts can be found 
between the backbone amides of residues Asn$^{222}$ and Asn$^{223}$ 
and HO-6. Residues Gly$^{105}$ and Glu$^{106}$ distinguish GS-1-B$_4$ 
from a variety of other legume lectins, in which a Gly-Gly 
sequence is highly conserved in this region, thus the 
interaction between the side chain of Glu$^{106}$ and hydroxyl groups 
HO-2 and HO-3 of the Gala residue is noteworthy.

Significant density for the $\beta$-methyl galactosyl residue (Gal$\beta$) 
is only seen in the B subunit. This residue is situated well 
above the protein surface. Notably, in the ligand bound to 
subunit B, the Gal$\beta$ residue is found in close proximity to the 
loop region extending from residues 61 through 69 of a mole-
cule of subunit A, which is generated by a crystallographic 
symmetry operation. This presumably restricts the mobility of 
the carbohydrate ligand and, therefore, improved its contribu-
tion to diffraction (Figs. 3 and 4 and Table II).

| Donor atom | Acceptor atom | Distance$^a$ (Å) |
|------------|---------------|-----------------|
| Wat$^{96}$ OH1 | Gal$\beta$OMe O2 | 2.0 (0.2) 40 |
| Wat$^{96}$ OH1 | Gal$\beta$OMe O3 | 2.7 (0.3) 11 |
| Wat$^{96}$ OH1 | Glu$^{106}$ Oe1 | 3.4 (0.5) 8 |
| Wat$^{96}$ OH1 | Gal$\beta$OMe O2 | 3.6 (0.4) 5 |
| Wat$^{96}$ OH2 | Glu$^{106}$ Oe2 | 3.3 (0.5) 13 |
| Wat$^{96}$ OH2 | Asn$^{56}$ Oe1 | 3.0 (0.2) 5 |
| Wat$^{96}$ OH2 | Gal O2 | 2.2 (0.3) 26 |
| Wat$^{96}$ OH2 | Gal$\beta$OMe O2 | 3.0 (0.2) 31 |
| Wat$^{96}$ OH2 | Gal$\beta$OMe O3 | 3.2 (0.2) 48 |
| Trp$^{122}$ Ne1H1 | Wat$^{96}$ O | 2.9 (0.2) 77 |
| Asn$^{56}$ N$\overline{3}$H2 | Wat O | 3.1 (0.2) 96 |
| Gal$\beta$OMe O4H | Wat$^{96}$ O | 3.5 (0.4) 24 |
| Asn$^{56}$ N$\overline{3}$H2 | Wat$^{96}$ O | 3.7 (0.2) 4 |

$^a$ In Angstroms, standard deviation in parentheses, and percent hydrogen bond occupancies in boldface.

Free Ligand—Throughout the 10-ns MD simulation in water, the 
glycosidic torsion angles in Gala(1–3)Gal$\beta$OMe showed only brief, relatively localized transitions from the 
equilibrium conformation. The $\Psi$ angle showed increased flex-
ibility relative to the $\Phi$ angle, which is consistent with other 
$\alpha$-linkages (20) and with earlier predictions that the Gala(1– 
3)Gal linkage is relatively flexible (see Fig. 3). The major con-
formation present is shown in Table III and as conformation B 
in Fig. 3. This conformation was predicted to be the lowest in 
energy and has been found experimentally to be the most 
populated in solution in related oligosaccharides (41, 20, 21, 
18). Two additional minor conformations were found and are 
referred to as A and D, (nomenclature consistent with a previ-
ous conformational energy map calculated for this linkage) 
(20). An additional higher energy theoretical conformation (C) 
(20) was not populated during our simulation. Overall, the 
average $\Phi$ and $\Psi$ angles determined by the MD simulation
remained close to those of the ligand in the x-ray crystal structure of the complex. Therefore, it may be concluded that GS-1-B₄ recognizes the lowest energy conformation of Galα(1–3)GalβOMe, in which Φ adopts a conformation expected on the basis of the exo-anomeric effect (44) (Fig. 5 and Table III).

Bound Galα(1–3)GalβOMe Conformational Analysis—The average Φ and Ψ angles from the 2-ns MD simulation were in good agreement with the x-ray data. The analysis was halted at 2 ns, because Wat₅₆ dispersed out of the CRD at just over 2 ns. According to the x-ray data from subunit B, this water participates in a bridge between the ligand and the protein and may be of importance in stabilizing the protein-ligand complex. For comparison, a 5-ns MD simulation was performed, in which Wat₅₆ was restrained in the x-ray position. The longer simulation revealed Φ and Ψ angles that were more rigid than observed in the 2-ns MD simulation, suggesting that an indirect result of restraining the water was to attenuate the mobility of neighboring residues.

In both the 2- and 5-ns MD simulations a rotation around the C5–C6 bond of the Galα residue occurred. The transition occurred after ~100 ps in the longer run and 500 ps in the shorter run. This transition resulted from the formation of a new interaction between O₆ of the Galα residue and O₃ of the Galβ residue at the expense of interactions between O₆ and Asn residues 222 and 223. A weak interaction, involving the N₈₂ atom of Asn²²² and O₆ was maintained throughout the simulation, in contrast to interactions involving the backbone amide atoms of Asn²²² and Asn²²³, which were broken during the transition. The ability of N₈₂ to maintain contact was most likely facilitated by the flexibility of the side chain, in comparison to the more rigid backbone. This result indicates more flexibility in the ligand than might be expected on the basis of epitope mapping studies, which have shown that substituents at the O₆ position in Galα decrease the affinity (12).

Hydrogen Bonding Analysis—Because the MD simulations include hydrogen atoms, it is possible to include them in an analysis of hydrogen bond properties, such as donor acceptor assignments, and hydrogen bond occupancies. In the calculation of occupancies, hydrogen bonding interactions were assumed to be present if the participating heavy atoms were ≤4 Å apart, and the angle formed between the heavy atoms and the donating hydrogen was ≤60°, as defined in the CARNAL module of AMBER 5.0. The corresponding standard deviations for the inter-atomic positions were calculated only when the requirements for hydrogen bond occupancy were fulfilled. Therefore, typically strongest hydrogen bonds have the highest occupancies, the smallest standard deviations, and the shortest heavy atom separations. The dependence on hydrogen position results in an analysis that is more sensitive than that based on the x-ray data, which relies solely on the heavy atom separation, with a separation of ≤3.2 Å being characterized as moderately strong, and a separation from 3.2 to 4.0 Å being indicative of a weak, hydrogen bond (45). The MD data provide considerable additional insight into the dynamic or fluxional nature of these interactions (Fig. 6 and Table IV).

In the crystal structure, the distance between Glu¹⁰⁶ Oe₁ and the oxygen atom of hydroxyl group HO-2 in Galα is exceptionally short, with a heavy atom separation of 2.6 Å. In both MD simulations of the complex this interaction lengthened to a value of ~3.4 Å. Similarly, the interactions between HO-3 and HO-4 of Galα with the carboxylate group of Asp⁸⁸ display extremely close contacts in the x-ray structure (2.6 Å), but lengthened to a more common value of ~2.9 Å in the MD simulations. The extent to which this illustrates the limitations of the x-ray data, versus a genuine difference between solution and crystalline environments, is unclear and may only be resolved with collection of a high resolution data set (Fig. 7 and Table V).

The only difference between the two simulations of the complex was the treatment of the bound water. As a result, interactions involving Wat₅₆, which are shown in Table V, are quite different in each simulation of the complex. Wat₅₆ populates two positions, denoted E and W (referring to coordination to Glu¹⁰₆ or Trp¹³²), during the 2-ns simulation and are illustrated in Fig. 4. Although Wat₅₆ and the carboxylate of Glu¹⁰₆ are tightly coordinated in configuration E, the interaction has a low overall occupancy due to the fact that the W configuration is present for the majority of the time. In the W configuration two new interactions with Wat₅₆ form, involving HO-2 of Galβ and Ne₁ of Trp¹³². Therefore, these small occupancies illustrate a dynamic, but not necessarily weak, interaction. This suggests that the water is mobile in the binding site, consistent with the absence of electron density for Wat₅₆ in subunit A. Therefore, the positional constraints employed in the 5-ns MD simulation may yield misleading results in the statistical analysis of properties dependent on this water molecule. This raises a considerable question regarding the role played by this water in the binding mechanism (Figs. 8 and 9).

Lectin Specificity—The binding site consists of a deep cavity, which accommodates only the first residue of the disaccharide (see Fig. 7). Modeling indicated that epimerization of C4 in the terminal Galα residue (Galα → Galβ) would result in the loss of a strong interaction between hydroxyl group HO-4 and Asp⁸⁸ O₂. Similarly, epimerization of C2 in the Galα residue would result in the loss of two interactions, namely with Galα₂-Glu¹⁰₆ Oe₁ and Galα O₂-Wat₅₆. Each of these observations is consistent with experimental data that show this lectin to have the highest binding affinity for oligosaccharides characterized by terminal Galα residues (12). Furthermore, modeling indicated that alteration of the α-(1–3) linkage to a β-(1–3) linkage would be sterically unfavorable, due to close contacts formed between the reducing end of the disaccharide and Trp¹³². This is
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also consistent with experimental data that revealed GS-1-B4 to have a much stronger affinity for Galc conjugated to human serum albumin than for the corresponding Galβ conjugate (12, 46).

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