The Crystal Structures of Man(α1–3)Man(α1-O)Me and Man(α1–6)Man(α1-O)Me in Complex with Concanavalin A*

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The crystal structures of concanavalin A in complex with Man(α1–6)Man(α1-O)Me and Man(α1–3)Man(α1-O)Me were determined at resolutions of 2.0 and 2.8 Å, respectively. In both structures, the O-1-linked mannose binds in the conserved monosaccharide-binding site. The O-3-linked mannose of Man(α1–3)Man(α1-O)Me binds in the hydrophobic subsite formed by Tyr-12, Tyr-100, and Leu-99. The shielding of a hydrophobic surface is consistent with the associated large heat capacity change. The O-6-linked mannose of Man(α1–6)Man(α1-O)Me binds in the same subsite formed by Tyr-12 and Asp-16 as the reducing mannose of the highly specific trimannose Man(α1–3)[Man(α1–6)Man(α1-O)Me]. However, it is much less tightly bound. Its O-2 hydroxyl makes no hydrogen bond with the conserved water 1. Water 1 is present in all the sugar-containing concanavalin A structures and increases the complementarity between the protein-binding surface and the sugar, but is not necessarily a hydrogen-bonding partner. A water analysis of the carbohydrate-binding site revealed a conserved water molecule replacing O-4 on the α1-3-linked arm of the trimannose. No such water is found for the reducing or O-6-linked mannose. Our data indicate that the central mannose of Man(α1–3)[Man(α1–6)]Man(α1-O)Me primarily functions as a hinge between the two outer subsites.

Lectins are carbohydrate-binding proteins involved in biological recognition processes. They occur in plants as well as in animals. Lectins mediate cellular recognition in the immune system, fertilization, cancer, infections, embryogenesis, and transport and targeting of glycoproteins (1). For plant lectins, a variety of different physiological functions have been proposed (2). They defend the plant against herbivorous animals (3, 4) and play a role in the legume-symbiosis (4, 5). Plant lectins are also valuable tools for biochemical, analytical, and therapeutic purposes (6). The crystal structures of many plant lectins have revealed how they specifically recognize their carbohydrate ligands (7). The structural basis of carbohydrate specificity is best understood for the legume lectins. These forms a group of proteins with a conserved subunit structure and spanning a wide range of carbohydrate specificities (8). The legume lectins recognize their ligands via a conserved monosaccharide-binding site and several distinct carbohydrate-binding subsites (8). Furthermore, the different quaternary structures that are adopted allow for the selection of multivalent ligands with different characteristic distances between their epitopes, adding an extra dimension to their specificity. This results, for example, in the formation of highly ordered and homogeneous cross-linked lattices between the lectin and multivalent carbohydrates (9).

ConA is the most extensively studied legume lectin. The crystal structures of the complexes of ConA with a series of carbohydrates have been solved: methyl-α-D-mannopyranoside (10) and methyl-α-D-glucopyranoside (11), Man(α1–3)[Man(α1–6)Man (which is the trimannoside ConA epitope of N-linked carbohydrates) (12, 13), the pentasaccharide GlcNAcβ1–2Man(α1–3)GlcNAcβ1–2Man(α1–6)Man (14), and the dimannoside Man(α1–2)Man(α1-O)Me (15). In this work, we describe the interactions of Man(α1–6)Man(α1-O)Me and Man(α1–3)Man(α1-O)Me, the two dimannoside parts of the highly specific ConA epitope Man(α1–3)[Man(α1–6)Man(α1-O)Me], with ConA. The M3M and M6M disaccharides are the epitopes recognized on the α1-3- and α1-6-linked arms, respectively, of certain high mannose and bisected hybrid-type sugars that are bivalent or monovalent for ConA (16). The M3M and M6M moieties of these sugars can be expected to bind in the same way as M3M and M6M in the crystal structures presented here. The M3M and M6M binding modes can, in principle, also occur for the trimannose M3M6M if its normal binding mode would be impaired. This is the case when a bisecting N-acetyl-D-glucosamine residue is β-linked to O-4 of the reducing mannose (17).

The thermodynamics of the binding of these specific sugars have been studied by different groups (18–26). We discuss the atomic interactions between the sugars and the lectin as we observe them in the crystal structure in relation to the measured enthalpic and entropic contributions to the binding.

EXPERIMENTAL PROCEDURES

Crystallization—ConA was purified by affinity chromatography on Sephacry as described for pea lectin (27). M6M and M3M were purchased from Toronto Research Chemicals and Sigma, respectively. Crystals of the complex of ConA with M6M were obtained in hanging drops after equilibration of a 10-μl drop composed of 5 μl of a ConA/sugar solution (7 mg/ml ConA and 2.75 mM M6M) and 5 μl of the bottom solution (7% Jeffamine M-600, 10% polyethylene glycol 8000, 50 mM NH₄COOH, 5 mM MgCl₂, and 25 mM phosphate buffer, pH 6.8) against 0.5 ml of the bottom solution. Diamond-shaped crystals of the complex
of ConA with M3M were obtained in hanging drops composed of 5 μl of the protein/sugar solution (5 mg/ml ConA and 5 mM M3M) and 5 μl of the bottom solution (14% polyethylene glycol 8000 and 35 mM potassium phosphate, pH 8) against 0.5 ml of the bottom solution.

Data Collection, Structure Solution, and Refinement—All data were collected on a MAR image plate using a Rigaku RU-200 rotating anode source. Processing of x-ray data and subsequent scaling were done with the HKL package (28). The conversion of intensities to structure factor amplitudes was done with the program TRUNCATE from the CCP4 package (29). Statistics of the x-ray data are given in Table I.

From a single M6M-ConA crystal, a total of 145° were recorded. The molecular replacement solution was found using AMoRE (30) (correlation coefficient = 0.36, R
<sub>merge</sub> = 0.56) using the ConA tetramer from Protein Data Bank entry 1ENQ. X-PLOR was used to refine the model (31). A random 10% reflection test set was kept separate from the reflections used for refinement for cross-evaluation by R<sub>free</sub>. Rigid body refinement reduced R<sub>merge</sub> to 0.35 with an R<sub>free</sub> of 0.34. Throughout the refinement, a bulk solvent correction was applied. For model building, the program TURBO-FRODO was used. After the positioning of the first monosaccharide unit in the monosaccharide-binding site and addition of the metal ions, positional and individual B-factor refinement led to a drop in R<sub>merge</sub> to 0.24 and in R<sub>free</sub> to 0.27. At this stage, electron densities were inspected, and the metal ions were added to the structures. No metal–ligand bond distance restraints were employed, and the metal–ligand bond distances were inspected, and the metal ions were added to the structures. No metal–ligand bond distance restraints were employed, and the metal ions were given zero charge. Water molecules were added only if 1) the water had at least one hydrogen-bonding partner; 2) the metal–ligand bond distance restraints were employed, and the metal–ligand bond distance was 2 Å; and 2) they share at least one contact with the protein. A water molecule was considered to be replaced by a sugar atom or vice versa when their mutual distance after superposition of the binding sites did not exceed 1.5 Å. Less conservative replacement, where this distance is between 1.5 and 2.0 Å, is still mentioned in Table IV. For a distance less than 1.5 Å, a structural water molecule (water 1 (12)) that makes hydrogen bonds with Asp-16 O-6-linked mannose (Fig. 2) and fewer interactions with the M3M6M (Table II). This results in a less tight binding of the M3M6M complex (Figs. 2 and 3). The conformations around the glycosidic linkages of M3M are very similar to what is seen in the M3M6M-ConA complex (Table II). The M3M α and φ angles enclose in the 2- or 3-kcal energy window of the energetically favorable region for the free disaccharide, but are a little outside the allowed predicted values for the M3M6M-ConA complex (33). This is probably due to the approach for docking the disaccharide in the carbohydrate-binding site of ConA, which did not allow for small structural adjustments in the protein.

Protein-Carbohydrate Interactions—The hydrogen bonds formed in the complexes of M3M6M, M6M, and M3M with ConA are presented schematically in Fig. 3. M6M and M3M bind with their O-1-linked mannose in the monosaccharide-binding site. This was expected because both the O-3 and O-6 sugar hydroxyl groups are involved in strong interactions in the monosaccharide binding of ConA (12, 22). The second mannose units of M3M and M6M bind in different subsites of the carbohydrate-binding site. Thereby, they approximately maintain the α1-6- and α1-3-glycosidic angles as found in the complex of ConA with M3M6M (Table II).

The binding pattern of M3M6M is comparable to what is seen in the M3M6M-ConA complex (Table II). The M3M α and φ angles enclose in the 2- or 3-kcal energy window of the energetically favorable region for the free disaccharide, but are a little outside the allowed predicted values for the M3M6M-ConA complex (33). This is probably due to the approach for docking the disaccharide in the carbohydrate-binding site of ConA, which did not allow for small structural adjustments in the protein.

Overall Structure and Carbohydrate Conformation—Both the M6M-ConA and M3M-ConA structures each contain one classical ConA tetramer (subunits A, B, C, and D) in the asymmetric unit. The ConA monomer consists of 237 amino acid residues and folds into a β-sandwich structure containing two sheets of six and seven β-strands, respectively, as well as a small third β-sheet (8, 32). There are no relevant structural changes outside the carbohydrate recognition sites in either of the two complexes. Residues 118 and 119, 150 and 151, and 161 and 162 are not visible in M3M and M6M, and residues 118–121 and 205–209 in M3M-ConA are less clearly determined by the electron density. The overall crystallographic B-factors of the two structures as well as the B-factors of the main chains, the side chains, the waters, and the sugars separately are presented in Table I.

The electron densities of M3M and M6M bound in the carbohydrate recognition site of ConA are shown in Fig. 1. The conformations of the M6M glycosidic linkages deviate slightly from the conformation observed in the complexed trisaccharose

| Space group | P<sub>2</sub><sup>1</sup> | P<sub>2</sub><sup>2</sup>, P<sub>2</sub><sup>1</sup>, P<sub>2</sub><sup>1</sup> |
|-------------|------------------|------------------|
| Unit cell   | a = 60.41 Å      | a = 69.51 Å      |
|             | b = 64.53 Å      | b = 117.03 Å     |
|             | c = 126.96 Å     | c = 120.92 Å     |
| β = 93.21°  |                 |                  |

| Contents of asymmetric unit | 1 tetramer | 1 tetramer |
|-----------------------------|-----------|-----------|
| Resolution (Å)              | 25.0 to 2.8 | 15.0 to 2.0 |
| Complete (%) (last shell)<sup>a</sup> | 99.9 (100.0) | 100.0 (99.8) |
| I<sub>o</sub>/I<sub>f</sub> (last shell)<sup>a</sup> | 17.1 (3.9) | 17.0 (6.3) |
| No. of unique reflections   | 195,361    | 498,686   |
| No. of measured reflections | 24,411     | 71,199    |
| Residual density            | 8.0        | 7.0       |
| R<sub>merge</sub> (last shell)<sup>a</sup> | 0.168 (0.603) | 0.082 (0.320) |
| R-factor (last shell)<sup>a</sup> | 0.164 (0.268) | 0.175 (0.234) |
| R<sub>free</sub>-factor (last shell)<sup>a</sup> | 0.257 (0.416) | 0.211 (0.276) |
| Ramachandran plot (%)       | 78.8       | 87.3      |
| Core region                 | 78.8       | 87.3      |
| Additional allowed          | 20.7       | 12.7      |
| Generously allowed          | 0.5        | 0.0       |
| Disallowed                  | 0.0        | 0.0       |
| r.m.s. <sup>2</sup> deviation from ideality |          |
| Bond length (Å)             | 0.013      | 0.010     |
| Bond angles (°)             | 1.826      | 1.712     |
| Dihedral angles (°)         | 27.393     | 27.558    |
| Improper angles (°)         | 1.699      | 1.556     |
| B-factors                   |            |
| Overall (Å<sup>2</sup>)      | 32         | 25        |
| Main/side/water/sugar (Å<sup>2</sup>) | 30/55/15/54 | 22/26/59/31 |
| PDB entry                   | 1QDO       | 1QDC      |

<sup>a</sup> Last shell: M3M, 2.9 to 2.8 Å; M6M, 2.1 to 2.0 Å. R<sub>merge</sub> = \( \sum_{hkl} I_{hkl}/(I_{hkl,\text{ave}}) - I_{hk1,i} = \sum_{hkl} I_{hkl}/I_{hk1,i} \) <br>
<sup>b</sup> r.m.s., root mean square; PDB, Protein Data Bank.
also present in the M6M structure. However, O-2 of the O-6-linked mannose and water 1 are within hydrogen-bonding distance (within 3.5 Å) in only one of the four sugar-binding sites of the asymmetric unit of M6M [Man(\(\alpha 1\rightarrow 3\))\(\alpha 1\rightarrow 3\)](\(\alpha 1\rightarrow 6\))Man(\(\alpha 1\rightarrow O\))Me in complex with ConA. The O-3-linked mannose of \([\text{Man(\(\alpha 1\rightarrow 3\))\(\alpha 1\rightarrow 3\)} \text{Man(\(\alpha 1\rightarrow O\))Me}] \text{ConA}\) covers the hydrophobic subsite neighboring the sugar-binding site (involving Tyr-12, Leu-99, and Tyr-100), in which also the aglycons of \(4\)-\(\alpha\)-D-mannopyranoside, \(4\)-\(\alpha\)-D-glucopyranoside (34), and \(4\)-\(\alpha\)-L-glucopyranoside (35) bind (Fig. 4a). The binding of the O-3-linked mannose of M3M involves no extra polar interactions compared with the monosaccharide, but depends merely on hydrophobic and van der Waals interactions (Table III). The electron density indicates that water 1 is also present in the M3M structure.

**Waters Involved in Carbohydrate Binding**—Prior to complexation, the polar groups of both the carbohydrate-binding site and the carbohydrate are extensively hydrated (36). Waters have to be released from both surfaces to allow the complex to form. In the absence of the sugar, waters can take in the positions of the sugar atoms and make the same hydrogen bonds with the protein (37). In this way, they can contribute directly to the properties of the protein by influencing its interaction with the sugar. In this context, Naismith et al. (10) observed that the waters that are expelled on binding of MeMan in the monosaccharide-binding site of ConA make similar hydrogen bonds with the protein as the sugar oxygen atoms O-4, O-5, and O-6 of MeMan with Asn-14 N-\(\text{d} 2\), Leu-99 N, and Tyr-100 N, respectively. A similar observation was made for the water molecules expelled from the surface of the carbohydrate-binding site of Erythrina coralloidendron lectin, a galactose/N-acetylgalactosamine-specific legume lectin (38). In this case, the positions of the key hydroxyls O-3 and O-4 of galactose are occupied by waters. The waters also make similar hydrogen bonds with the protein as O-3 and O-4 in the galactose complex. The atomic resolution structure of ConA revealed a water molecule directly above the aromatic ring of Tyr-12 that replaces C-6 of MeMan (39). The electron density indicates hydrogen

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**TABLE II**

| Carbohydrate conformation | \(\Psi\) \(\circ\) | \(\Phi\) \(\circ\) | \(\Omega\) \(\circ\) |
|---------------------------|----------------|----------------|----------------|
| M3M Average | 64 \(\pm 2\) | 121 \(\pm 2\) | -61 \(\pm 2\) |
| M3M | 66 \(\pm 2\) | 128 \(\pm 2\) | -57 \(\pm 2\) |
| M6M Average | 76 \(\pm 3\) | 128 \(\pm 2\) | -54 \(\pm 2\) |
| M6M | 70 \(\pm 2\) | 128 \(\pm 2\) | -54 \(\pm 2\) |

The glycosidic torsion angles \(\Psi\) and \(\Phi\) are as follows: \(\Psi = \phi(\text{O-5-C-1-O-1-C'-X})\) and \(\phi = \phi(\text{C-1-O-1-C'-X-C'-X+1})\); and for the \(\alpha 1\rightarrow 6\)-linkage, \(\omega = \phi(\text{O-1-C'-6-C'-5-O'-5})\) as defined (50). The values in the last column are the average values for the four crystallographically independent trimannosides in complex with ConA (Protein Data Bank entry 1CVN) with its variation.
bonding between this water and the aromatic ring of Tyr-12.

The question arises whether the mannose residues bound to the ConA subsites also expel water molecules from the protein surface. Therefore, the water structures of the complexes of ConA with M6M (Protein Data Bank entry 1QDC; 2.0-Å resolution, tetramer in de-asymmetric unit, 386 waters), MeMan (Protein Data Bank entry 5CNA; 2.0-Å resolution, tetramer, 533 waters), M3M6M (Protein Data Bank entry 10NA; 2.35-Å resolution, tetramer, 200 waters), and sugar-free ConA (Protein Data Bank entry 1NLS; 0.94-Å resolution, monomer, 318 waters) (39) were compared. The M3M-ConA complex was not included in this comparison because of its lower resolution. Fig. 5 shows the water molecules that are present in the binding site of sugar-free ConA. These were compared with those carbohydrate hydroxyls that are hydrogen bond donors and acceptors in the M3M, M6M, and M3M6M complexes (Fig. 5 and Table IV). Furthermore, the positions and hydrogen-bonding partners of the water molecules present in the binding sites of the complexes of ConA with MeMan, M6M, and M3M6M were compared (Table IV).

Upon M3M6M binding, a total of eight waters present in the sugar-free structure (waters 01–08) leave the carbohydrate-binding site (Fig. 5). In the monosaccharide-binding site, the key polar positions O-4, O-5, and O-6 are replaced by waters 01–03. Water 04 replaces the van der Waals contact of C-6 with the aromatic rings of Tyr-12 and Tyr-100 (39). Two waters, 05 and 06, are in the site where the reducing mannose of M3M6M binds. When a still larger oligosaccharide binds to ConA, the pentasaccharide GlcNAc-[β1–2]Man-[β1–3][GlcNAc-[β1–2]Man-[β1–6]Man (14), another conserved water molecule, is expelled. This water is found for both the M6M-ConA subunit A and MeMan-ConA structures in the empty subsites for the N-acetylglucosamine on the α1–6-linked arm. It makes the same hydrogen bonds with Ser-168 O-γ and Thr-226 O-γ1.

Water 1 is present in all sugar-containing structures with at least a monosaccharide bound and was first identified by Naismith and Field (12). Water 1 makes strong hydrogen bonds with the side chains of Arg-228, Asn-14, and Asp-16 (Fig. 3, a and b), but not with the sugar in the monosaccharide-binding site. In sugar-free ConA, water 1 is absent, and a salt bridge is formed between Arg-228 and Asp-16. On the other hand, the disruption of the salt bridge between Arg-228 and Asp-16 in sugar-free ConA is not required for sugar binding, as was previously proposed (21), and depends only on the crystal environment. The salt bridge is present in some complexes of ConA with sugars (including two of the four crystallographically independent subunits of MeMan-ConA and all four subunits of M3M6M-ConA), but is absent in the M3M-ConA and M6M-ConA structures. The conserved water 1 molecule fills a gap between the Arg-228 side chain and the monosaccharide-binding site. In M3M6M-ConA, water 1 is moreover hydrogen-bonded to O-2 of the reducing mannose. However, it makes a hydrogen bond with the O-6-linked mannose in only one of the four subunits of M6M-ConA. Whereas conserved water 1 leans against one side of the Arg-228 side chain, two more water molecules, waters 2 and 3 (Fig. 3b), support the Arg-228 side chain at the other side. Water 3 is present in all of the subunits of the M6M and MeMan complexes. Water 2 is present in three of four of the subunits of the MeMan and M6M complexes and also in the sugar-free structure. In both the methyl-α-1,6-glucose and the M3M6M complexes, these waters reorganize.

**DISCUSSION**

**M3M Binds in a Multipurpose Hydrophobic Subsite**—The O-3-linked mannose of M3M binds in a hydrophobic subsite formed by the side chains of Leu-99 and Tyr-100 neighboring the conserved monosaccharide-binding site (Fig. 4b). Oligosaccharide recognition via a subsite with a hydrophobic character is not unique to the M3M-ConA complex. It has also been recently observed in the complex of the seed lectin from Dolichos biflorus with the Forsmann disaccharide (40) and has
been predicted by molecular modeling for the fucose moiety of \( \text{Fuc(1\rightarrow2)Gal(1\rightarrow4)GlcNAc} \) binding to the *E. coralloidendron* lectin (41). The binding of the O-3-linked mannoside of M3M to a subsite formed by the hydrophobic side chains of Leu-99 and Tyr-100 is comparable to the binding of methylumbelliferyl covalently attached to glucose in 4'-methylumbelliferyl-\( \alpha \)-D-glucopyranoside (35) or the para-nitrophenyl groups of 4'-nitrophenyl-\( \alpha \)-D-mannopyranoside and 4'-nitrophenyl-\( \alpha \)-D-glucopyranoside (34). ConA-bound M3M and 4'-methylumbelliferyl-\( \alpha \)-D-glucopyranoside have very closely related conformations around their glycosidic linkages (Fig. 4a). Similarly, the increase in affinity by going from methyl-\( \alpha \)-D-mannopyranoside to M3M (a factor of 4) is equivalent to the one observed by going from methyl-\( \alpha \)-D-glucopyranoside to 4'-methylumbelliferyl-\( \alpha \)-D-glucopyranoside (42).

The binding of the O-3-linked mannoside of M3M to a dominantly hydrophobic surface as observed in our crystal structure agrees well with the published thermodynamic data. The study of Williams *et al.* (21), at pH 5.2, indicates a much larger change in heat capacity (\( \Delta C_p \)) upon binding of M3M (\( -110 \text{ cal mol}^{-1} \text{ K}^{-1} \)) compared with binding of MeMan (\( -60 \text{ cal mol}^{-1} \text{ K}^{-1} \)) or M6M (\( -40 \text{ cal mol}^{-1} \text{ K}^{-1} \)). This change in heat capacity is equal to the heat capacity change upon binding of M3M6M to ConA (\( -110 \text{ cal mol}^{-1} \text{ K}^{-1} \)). A large \( \Delta C_p \) indicates that the binding enthalpy is a function of temperature. Moreover, \( \Delta C_p \) is a term dominated by solvent effects. This mirrors the suggestion of Privalov and Gill (43) that the enthalpy change of hydrophobic hydration is a function of temperature. The contribution to \( \Delta C_p \) from the burial of hydrophobic surface is dominant over that from the burial of hydrophilic surface. Thus, the large \( \Delta C_p \) for the binding of M3M in is in agreement with M3M binding to a hydrophobic surface as seen in our crystal structure and with the release of water from this surface.

The dehydration of hydrophobic surfaces prior to complexation causes an increase in entropy (44). The favorable entropic effect results from the release of otherwise restricted water molecules into the bulk solvent (36, 44). This is due to the organization of water molecules in the vicinity of exposed apolar solute surfaces, which reduces their molecular degrees of freedom compared with the water molecules in the more dynamic bulk solvent. These water molecules cannot be seen in the protein crystal structures. In none of the ConA structures is interpretable electron density for a water molecule present at a distance closer than 4–5 Å to the hydrophobic surface formed by the side chains of Leu-99 and Tyr-100. Nevertheless, the positive entropic contribution to binding to the hydrophobic subsite is illustrated by the binding of phenyl-\( \alpha \)-D-glucopyranoside (42).

**Table III**

| M3M | M6M |
|-----|-----|
| C-1–Tyr-12 OH | C-3–Tyr-12 C-\( \zeta \), OH |
| O-1–Leu-99 C-\( \beta \), C-\( \gamma \), C-82 | O-3–Tyr-12 C-\( \zeta \), C-\( \gamma \), OH |
| O-1–Tyr-100 C-\( \epsilon \), C-\( \zeta \), C-82 | C-4–Tyr-12 C-\( \zeta \), C-\( \gamma \), OH |
| C-7–Leu-99 C-82 | O-4–Tyr-12 C-\( \epsilon \), C-\( \zeta \), C-\( \gamma \), OH |
| C-7–Tyr-100 C-\( \zeta \), C-\( \epsilon \), OH | O-4–Tyr-12 C-\( \epsilon \), C-\( \zeta \), C-\( \gamma \), OH |
| C-2–Tyr-12 C-\( \zeta \), OH | C-5–Leu-99 C-\( \beta \), C-\( \gamma \), C-82 |
| C-2–Leu-99 C-\( \beta \), C-\( \gamma \) | O-2–Tyr-12 C-\( \zeta \), OH |
| C-3–Leu-99 C-\( \beta \), C-\( \gamma \), C-82 | C-6–Leu-99 C-82 |
| C-4–Leu-99 C-82 | C-3–Leu-99 C-82 |
| O-5–Leu-99 C-82 | C-4–Leu-99 C-82 |
| C-5–Leu-99 C-\( \gamma \) | C-5–Leu-99 C-\( \gamma \) |

The absence of the carbohydrate. The conserved water \( 06 \) replaces O-4 of the mannose on the \( \alpha\)-3-linked arm of the specific epitope Man(\( \alpha\)-3)-Man(\( \alpha\)-6)Man(\( \alpha\)-1–4)Man(\( \alpha\)-1–3)

**Fig. 3.** Schematic presentation of Man(\( \alpha\)-3)[Man(\( \alpha\)-6)-Man(\( \alpha\)-1–4)Man(\( \alpha\)-1–3)]-Man(\( \alpha\)-O)Me (a), Man(\( \alpha\)-6)Man(\( \alpha\)-1–4)Man(\( \alpha\)-1–3)Man(\( \alpha\)-O)Me (b), and Man(\( \alpha\)-3)-Man(\( \alpha\)-6)Man(\( \alpha\)-1–4)Man(\( \alpha\)-1–3)Man(\( \alpha\)-O)Me (c) binding. Protein-carbohydrate distances are indicated. The positions of the key hydroxyls of Man(\( \alpha\)-3)[Man(\( \alpha\)-6)-Man in the site are invariably occupied by a water molecule in the absence of the carbohydrate. The conserved water \( 06 \) replaces O-4 of the mannose on the \( \alpha\)-3-linked arm of the specific epitope Man(\( \alpha\)-3)-Man(\( \alpha\)-6)Man(\( \alpha\)-1–4)Man(\( \alpha\)-1–3)Man(\( \alpha\)-O)Me. Three water molecules (waters 1–3) are conserved in the MeMan and Man(\( \alpha\)-6)Man(\( \alpha\)-1–4)Man(\( \alpha\)-1–3)Man(\( \alpha\)-O)Me structures and increase the complementarity between the monosaccharide-binding site and the sugar, rather than serve as hydrogen-bonding partners.
Man(α1–3)Man(α1-O)Me to ConA. The phenyl ring binds in the same hydrophobic subsite as the O-3-linked mannose of M3M (34) with an entropic gain compared with glucose (42). A factor counteracting the positive entropic effect is the loss of conformational flexibility around the glycosidic linkage. The orientation of the phenyl ring of 4'-nitrophenyl-α-D-mannopyranoside and 4'-nitrophenyl-α-D-glucopyranoside is determined by the crystal packing, leading to large differences in glycosidic angles between the four subunits of the asymmetric unit (34). For phenyl-α-D-glucopyranoside, the phenyl ring is still allowed to move after it is bound, to allow for the most favorable crystal packing. In contrast, the glycosidic linkages of M3M are very nearly identical in the four independent subunits. Thus, the entropic loss due to restriction of the glycosidic linkage is

![Fig. 4. Binding of Man(α1–3)Man(α1-O)Me in the hydrophobic subsite. a, superposition of M3M-ConA (light gray) and 4'-methylumbelliferyl-α-D-glucopyranoside (black). The O-3-linked mannose of M3M occupies the same subsite as the aglycon methylumbelliferyl of the substituted glucose. The conformations around the glycosidic linkages are similar. b, the hydrophobic character of the M3M subsite. M3M is shown as a ball-and-stick model. The van der Waals surface of ConA is colored according to residue properties: hydrophobic (brown), basic (blue), acidic (red), other polar residues (light green), or glycine (yellow). This figure was created using GRASP (49).](image)

![Fig. 5. Expulsion of water from the binding site of sugar-free ConA upon binding of M3M6M. Shown is a stereo figure of the superposition of waters 01–08 of sugar-free ConA on the M3M6M-ConA complex in the carbohydrate-binding site. This figure was created using BOBSCRIPT (48).](image)
probably higher for M3M than for sugars with a hydrophobic substituent.

According to Mandal et al. (19), the affinity of ConA for M3M is four times higher than for M6M. Both enthalpy (ΔH) and entropy (ΔS) values (10.7 compared with 8.4 kcal/mol and −4.5 compared with −3.1 kcal/mol) are higher for the former. M3M thus binds with a more favorable change in enthalpy than M6M; however, this is partly compensated for by a higher entropic cost compared with M6M. The higher entropic cost for M3M binding compared with M6M is apparently in contradic-

to the binding of the O-3-linked mannose of M3M to a
hydrophobic surface. Clearly, the differences in the thermodynamic parameters between M3M and M6M cannot be ex-
plained based on the crystallographic observations only.

Structural Basis for the Low Affinity of M6M—The triman-
nosyloligosaccharide of asparagine-linked glycoproteins,
M3M6M, is recognized by ConA with high affinity. It binds in
an extended sugar-binding site, with all three mannose units
making strong hydrogen bonds and van der Waals interactions
with the protein (Fig. 3a) (12). The outer mannose on the
α-1–6-linked arm of M3M6M binds in the monosaccharide-
binding site; the mannose on the α-1–3-linked arm binds to a
secondary site near Pro-13 and Thr-15; and the reducing man-

ose binds near the hydroxyl group of Tyr-12. The M6M-ConA

crystal structure shows the subsite for the O-6-linked mannose
of M6M to be similar to the one for the reducing mannose in
M3M6M (Fig. 3, a and b). However, ConA has no enhanced
affinity for M6M compared with MeMan (19). The binding of
M6M to ConA superficially resembles what is seen for the same
disaccharide portion of M3M6M, but the O-6-linked mannose
of M6M is a little more separated (0.8–1.4 Å) from the protein
surface than the reducing mannose in the M3M6M complex
(Fig. 2) due to different glycosidic angles (Table II). The num-
ber of hydrogen bonds made by the O-6-linked mannose of
M6M is reduced to a single one (O-4 with Tyr-12 OH). The
hydrogen bond of O-2 of the reducing mannose in the M3M6M
complex (Fig. 2a) with the conserved water 1 is absent in three
of the four ConA monomers of the asymmetric unit (Fig. 3b).

Tight interactions between the reducing mannose of M3M6M
and ConA seem to be established only through further stabili-
zation by the mannoside substituent on the O-3 hydroxyl of the
reducing mannose that can make extra interactions with Thr-
15, Pro-13, and Asp-16. It appears as if the second or reducing
mannose primarily serves as a hinge for bridging the two outer
mannosides of M3M6M, but does not by itself contribute to the
binding to a large extent.

| M3M6M | M6M | MeMan | Sugar-free | Protein ligands of sugar/water | Names |
|-------|-----|-------|------------|-------------------------------|-------|
| **Part I. Conserved waters important for the structure of the sugar-binding site** |
| Water 63 | 98 | 1 | Asn-14 N-ε2, Arg-228 N-η2 | Water 1 |
| **Part II. Waters leaving upon sugar binding** |
| O-4 | 252 (0.3) | Asp-208 O-62, Asn-14 N-62 | Water 01 |
| O-5 | 269 (1.3) | Leu-99 N | Water 02 |
| O-6 | 264 (1.3) | Asp-208 O-61, Tyr-100 N, Leu-99 N | Water 03 |
| O-6 | 375 (1.2) | Tyr-100 and Tyr-12 aromatic rings | Water 04 |
| **Part III. A water molecule interacting with the sugar in the monosaccharide site** |
| O-4 | 341 (1.4) | Thr-15 N, Pro-13 O, Thr-15 O-γ1 | Water 05 |
| C-7 of M3M | 445 (1.1) | Thr-100 OH | Water 06 |

All waters in this table are present in at least two of the four crystallographically independent subunits of the M3M6M (Protein Data Bank entry 1ONA), M6M (Protein Data Bank entry 1QDC), and MeMan (Protein Data Bank entry 5CNA) structures. Waters in the same row of the table are conserved among the different structures. The last column gives the names of the water molecules used in this paper. Waters 01–06 are waters present in the sugar-free structure (Protein Data Bank entry 1NLS). Part I shows waters that are conserved among some or all of the sugar·ConA complexes that are thought to be important for presenting a complementary binding surface to the sugar. Part II shows how waters can compensate for the absence of sugar atoms. The waters replace an M3M6M (first column) or M3M atom. The first four columns present water residue numbers from subunit A in the respective Protein Data Bank entries (except for water 229*, which is from subunit D of M6M). The distance (Å) of the replacing water molecule to the sugar atom position is written in parentheses next to the water residue number. Part III shows a water molecule occasionally interacting with O-2 of the sugar unit bound in the monosaccharide-binding site.
M6M makes one more sugar-protein hydrogen bond interaction (O-4 of the O-6-linked mannose with Tyr-12 OH) than the monosaccharide or M3M and buries a larger accessible surface area upon complexation (290 Å²) than M3M (276 Å²) or MeMan (228 Å²). This agrees with the slightly higher enthalpy change upon M6M binding compared with the monosaccharide MeMan. However, the decrease in enthalpy upon binding of the second mannose of M6M is compensated for entirely by a decrease in entropy and results in no gain of affinity compared with MeMan. This is in agreement with the finding that extra protein-ligand interactions and more buried surface area do not necessarily implicate a higher affinity (45).

The Role of Water in Carbohydrate Binding—Solvent reorganization is thought to account for 25–100% of the observed binding enthalpy (46), which makes the study of water structure in the sugar-binding site potentially interesting. However, in crystal structures, only the solvent molecules for which the position is well determined by the electron density can be evaluated for their importance in solvent reorganization. Moreover, differences in crystal packing lead to small differences in sugar-protein interactions as well as differences in visible water structure in the binding site. The flexibility of the sugars to adapt to a different packing environment results in different binding motifs that accidentally do or do not involve waters, in agreement with the dynamic character of bound carbohydrates. Water 9 (Table IV) has been shown to play a role in the realization of one of the binding motifs of mannose for ConA (11).

The primary function of some water molecules seems to be to help increase the fit between the protein and sugar, rather than to serve as specific hydrogen bond mediators (waters 1–3; see Fig. 3 and Table IV). Water 1 is a very good example of this. The presence of water 1 in all ConA structures with at least a monosaccharide bound, the lack of hydrogen bonds between water 1 and the sugar in the monosaccharide-binding site, the lack of hydrogen bonding of water 1 to O-2 of the O-6-linked mannose of M6M, and the gap between Arg-228 and the sugar if water 1 would not be present indicate that the main role of this water molecule is to give the protein-binding surface a shape complementary to the sugar.

Key polar groupings have been defined as the hydroxyl groups of an oligosaccharide which are outstandingly essential to complex formation as judged from the binding of deoxy analogues (47). In the absence of the oligosaccharide, they are replaced by water molecules. The water molecules then compensate for the lack of the polar sugar-protein interaction by sharing the same protein hydrogen-bonding partners as the sugar atoms (10). Not only polar interactions are compensated for. The C-6 atom of MeMan has been recently found to be replaced by a water in the sugar-free ConA structure (see water 04 in Table IV) (39). There is a clear correlation between the importance of the particular sugar-protein interaction and its replacement by a water molecule in the absence of the sugar atom. In our study, we found that only one more water molecule of the sugar-free structure (water 06 in Table IV) conservatively replaces a sugar atom of the highly specific ConA epitope M3M6M. The water replaces O-4 of the α-1,3-linked mannose, and it stays in this position when MeMan or M6M binds. This is in sharp contrast to the rest of the water structure that changes significantly upon MeMan or M6M binding. Remarkably, for the reducing mannose of M3M6M, no such conserved water is found. Water 4 in MeMan-ConA and water 05 in sugar-free ConA may compensate for the lack of O-3 or O-4, respectively, but their positions are not conserved (Table IV). This could indicate the minor importance of the direct interactions of this mannose with ConA, which agrees with the inefficient binding of the O-6-linked mannose of M6M to ConA as discussed above. It confirms the function of the reducing mannose of M3M6M as a hinge between two higher affinity subsites: the monosaccharide-binding site and the α-1,3-linked mannose subsite.

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Man(a1–3)Man(a1–O)Me / Man(a1–6)Man(a1–O)Me ConA Complexes