A Structure between Concanavalin A and Methyl-3,6-di-O-(α-d-mannopyranosyl)-α-d-mannopyranoside Reveals Two Binding Modes*

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The structure of concanavalin A in complex with the trimannose methyl-3,6-di-O-(α-d-mannopyranosyl)-α-d-mannopyranoside has been determined in a novel space group. In three of the four subunits of the concanavalin A tetramer, the interactions between the protein and the bound saccharide are essentially identical to those reported previously by other authors (Naismith, J. H., and Field, R. A. (1996) J. Biol. Chem. 271, 972–976). In the fourth subunit, however, the α1-3 linkage has a different conformation, resulting in a different part of the α1-3-linked mannose interacting with essentially the same surface of the protein. Furthermore, significant differences are observed in the quaternary associations of the subunits compared with the saccharide-free structures and other carbohydrate complexes, suggesting that the concanavalin A tetramer is a rather flexible entity.

Concanavalin A was the first legume lectin to be isolated (1), sequenced (2–4), and to have its three-dimensional structure determined by x-ray crystallography (5, 6). Concanavalin A belongs to the glucose/mannose-specific lectins (7) and was shown to have its highest binding affinity for methyl-3,6-di-O-(α-d-mannopyranosyl)-α-d-mannopyranoside (8). These results were confirmed by NMR dispersion studies of Brewer and co-workers (9–11), who further postulated an extended binding site for the trisaccharide and different conformational changes induced in the protein upon binding of methyl-3,6-di-O-(α-d-mannopyranosyl)-α-d-mannopyranoside and larger N-linked carbohydrates compared with the binding of simple monosaccharides such as methyl-α-d-mannopyranoside.

Nevertheless, the first view of a carbohydrate bound to concanavalin A in a specific manner was obtained only in 1989, when the structure of a concanavalin A-methyl-α-d-mannopyranoside complex was presented (12). Today, the details of the trimannose binding to concanavalin A have been unraveled by Naismith and Field (13) in a crystal form with a unit cell different from the structure discussed by us. The structure of concanavalin A complexed with methyl-α-d-glucopyranoside in a cubic crystal has been solved (14) and co-crystallization with methyl-α-d-arabinofuranoside (15) has been reported. Also, the structures of a number of other legume lectins and their carbohydrate complexes have been determined (16–19). On the other hand the thermodynamics of concanavalin A-carbohydrate interactions have been addressed in several recent studies and have been the subject of considerable debate (20–23). A refined structure of concanavalin A in complex with the specific trisaccharide methyl-3,6-di-O-(α-d-mannopyranosyl)-α-d-mannopyranoside is here compared with the recently published structure of Naismith and Field (13).

EXPERIMENTAL PROCEDURES

Purification of concanavalin A, crystallization of its complex with the methyl-3,6-di-O-(α-d-mannopyranosyl)-α-d-mannopyranoside, cryo-cooling and data collection have been reported elsewhere (24). Crystals belong to space group P2₁, unit cell parameters a = 59.83 Å, b = 62.84 Å, c = 125.92 Å, β = 93.87° and contain a complete concanavalin A tetramer in the asymmetric unit. Data within 15.0 and 2.3 Å were collected at 100 K from a single crystal with a FAST area detector on an Enraf-Nonius rotating anode source operated at 40 kV and 90 mA. Due to technical problems, the data collection had to be abandoned prematurely, resulting in a data set that is 71.3% complete between the resolution limits of 10 and 2.35 Å. Further attempts to flash-freeze other crystals have systematically failed, while at room temperature the crystals decayed too rapidly to allow data collection. Therefore, the structure was solved and refined using the data collected at 100 K. Molecular replacement using AMORE (25) with the tetrameric concanavalin A-methyl-α-d-mannopyranoside complex as the starting model (Protein Data Base entry 5CNA (26)), followed by rigid body refinement using reflections between 10 and 4 Å resolution, gave a solution with an R-factor of 0.323 and a correlation coefficient of 0.743. The structure was refined using X-PLOR (27) and monitored using the Rfree value (28), and the geometrical quality of the model was checked with PROCHECK (29) after each run of refinement cycles. Non-crystallographic symmetry restraints with a weight of 500 kcal mol⁻¹ Å⁻² were used throughout the refinement. Powell minimization and restrained B-value refinement resulted in a drop of the R-factor from 0.40 to 0.28, with the Rfree being 0.33.

At this stage, the metal ions and four trimannose molecules were fitted into the electron density with FRMOD (30). A simulated annealing refinement was then carried out, and the structure was rebuilt using standard protocols (31, 32). The final model, containing the complete concanavalin A tetramer, 201 water molecules, and four trimannose molecules, has a final R-value of 0.221 and a Rfree of 0.282 for all data between 10.0 and 2.4 Å. Further details on the refinement and data collection are given in Table I.

RESULTS AND DISCUSSION

Saccharide Binding—Overall, the structure presented here closely resembles the structure described by Naismith and Field (13) in a different spacegroup, but at a similar resolution. The atom numbering scheme of methyl-3,6-di-O-(α-d-mannopy-

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1 The abbreviation used is: trimannose, methyl-3,6-di-O-(α-d-mannopyranosyl)-α-d-mannopyranoside.
TABLE I
Data collection and refinement of the concanavalin A-trimannose complex

| Unit cell | a = 59.83 Å | b = 62.84 Å | c = 125.92 Å |
|-----------|-------------|-------------|-------------|
| β         | 93.87°      |             |             |
| Space group | P2₁         |             |             |
| Resolution | 8.0–2.35 Å  |             |             |
| Number of observed reflections | 44422       |             |             |
| Number of unique reflection | 28287       |             |             |
| Data completeness | 73.0%        |             |             |
| Rsymm (last shell) | 0.065 (0.197) |     |             |
| Crystallographic R-factor (last shell) | 0.221 (0.292) |     |             |
| Free R-factor (last shell) | 0.252 (0.382) |     |             |

Average B-value
- Main chain atoms: 17.27 Å²
- Side chain atoms: 19.42 Å²
- Solvent atoms: 19.05 Å²
- Trimannose molecules: 27.49 Å²
- Total: 15.90 Å²

Root mean square deviation on
- Bond lengths: 0.012 Å
- Bond angles: 1.73°
- Dihedrals: 26.52°
- Improper: 1.53°

Ramachandran plot quality
- Most favourable: 83.8%
- Additionally allowed: 16.2%
- Generously allowed: 0.0%
- Disallowed: 0.0%

TABLE II
Possible hydrogen bonds between the protein and the trimannose

| Donor   | Acceptor | Monomer | I  | II | III | IV |
|---------|----------|---------|----|----|-----|----|
| 1→6-Linked mannose | O4A       | Asp206  | OD2 | 2.50| 2.61| 2.54| 2.51|
|         | O6A       | Asp206  | OD1 | 3.48| 3.01| 2.93| 3.23|
|         | Asn14 N   | O4A     |     | 3.12| 3.14| 2.92| 3.17|
|         | Leu29 N   | O5A     |     | 3.09| 2.95| 2.94| 3.09|
|         | Leu29 N   | O6A*    |     | 2.56| 2.88| 2.98| 2.87|
|         | Tyr150 N  | O6A     |     | 2.57| 2.99| 3.05| 2.92|
|         | Arg228 N  | O3A     |     | 2.72| 3.25| 2.77| 2.96|
| Reducing core mannose | Tyr12 OH  | O4B†    |     | 2.81| 2.39| 2.80| 3.27|
|         | O2B       | Asp166  | OD2 | 3.10| 3.21| 3.00| 2.89|
|         | O2B       | Water   |     | 2.90| 2.80| 3.23| 3.33|
| 1→3-Linked mannose | Thr15 N   | O4C     |     | 3.01| 3.30| 3.40| 3.30|
|         | Thr15 N   | O3C     |     | 3.41| 2.98| 3.19| NP†|
|         | Tyr15 OG1 | O3C*    |     | 3.47| 2.73| 3.08| NP|
|         | Thr15 OG1 | O4C*    |     | NP  | NP  | NP  | NP  |
|         | Asp16 N   | O4C     |     | 2.81| 2.78| 2.97| NP|
|         | Asp16 N   | O6C     |     | NP  | NP  | NP  | NP  |
|         | Tyr12 OH  | O3C*    |     | NP  | NP  | NP  | NP  |

- Weak hydrogen bond, the angle D-H...A approaching 80°.
- The assignment of donor and acceptor is here arbitrary.
- NP†, not present.

TABLE III
Carbohydrate conformation

| Torsion angle | I      | II     | III    | IV     | I      | II     | III    | IV     |
|---------------|--------|--------|--------|--------|--------|--------|--------|--------|
| ϕₐ           | 49     | 71     | 75     | 65     | 63     | 65     | 69     | 65     |
| ϕₐ           | -176   | -175   | -170   | -177   | -170   | -174   | -169   | -169   |
| ωₐ           | -42    | -53    | -63    | -42    | -44    | -40    | -51    | -45    |
| ψₐ           | 88     | 67     | 71     | 133    | 67     | 64     | 65     | 69     |
| ϕₐ           | 107    | 125    | 119    | 126    | 127    | 119    | 131    | 124    |

FIG. 1. Atom numbering scheme for the trimannose molecule. The α1→6-linked mannose is labeled as A, the core methyl-α-D-mannose as B, and the α1→3-linked mannose as C. The glycosidic torsion angles ϕ, ψ, and ω are also indicated. ϕₐ = (O5A-C1A-O1A-C6B), ψₐ = (C1A-O1A-C6B-C5B), and ωₐ = (O1A-C6B-C5B-O5B), ψₐ = (O5C-C1C-O1C-C5B), and ψₐ = (C1C-O1C-C3B-C4B).
Concanavalin A-Trimannoside Interactions

ranosyl)-α-D-mannopyranoside is shown in Fig. 1. The trimannoside molecules bind with their α1→6 arm in the previously described monosaccharide binding site, while the reducing core mannose and the α1→3-linked terminal mannose bind in an extended cleft formed by residues Tyr12 to Asp16. The interactions between the protein and the bound saccharides are summarized in Table II. In three of the four monomers, the binding mode of the trimannoside to concanavalin A is very similar to the one described by Naismith and Field (13). The only interaction different between the predominant sugar conformation in our structure and the sugar conformation observed in the structure of Naismith and Field is between the hydroxyl group of Thr15 and O4C. In the fourth monomer, significant differences are observed in the conformation of the bound saccharide, resulting in a different set of interactions between the α1→3 terminal mannose and the protein, as shown in Table II.

It is of interest that despite the different conformation around the α1→3 linkage, the same protein residues are involved in the interactions, but with different partners on the carbohydrate side. While in the “major” conformation, O3C of the α1→3-linked terminal mannose interacts with the main chain oxygen of Pro13 and with OG1 of Thr15, in the alternative or “minor” conformation, these protein atoms form hydrogen bonds with O4C. Similarly, the interaction between the main chain NH of Asp16 and O4C is in the minor conformation substituted by an interaction with O6C. A weak interaction between O4C and the main chain NH of Thr15 seems to be present in both conformations, while the minor conformation has an additional hydrogen bond between the side chain of Tyr12 and O3C. These alternative binding schemes seem to be related to the “diamond-like” nature of carbohydrates, which was shown previously to be responsible for the similarities and differences between mannose and galactose binding in legume lectins (17) and to the binding of glucose and galactose in the Escherichia coli glucose/galactose and L-arabinose-binding proteins, respectively (33, 34).

Saccharide Conformation—The conformation of the trimannose, as described by its ϕ, ψ, and ω torsion angles (see Fig. 1 for a definition) is given in Table III next to the values obtained from the structure of Naismith and Field. All four independently refined trisaccharides have their α1→6 linkage in a conformation close to the major minimum in the potential energy maps (ϕ = 70°, ψ = −170°, ω = −60°) as calculated by Imberty et al. (35) and are also in agreement with the data on the conformations of Man α1→6 Man glycosidic linkages in solution as determined by NMR spectroscopy (36).

In the case of the α1→3 linkage, the situation is different: three of the four saccharides have a conformation close to the global minimum around ϕ = 70° and ψ = 100° (predominant conformation), while for the sugar bound to monomer IV, the conformation is close to a secondary minimum around ϕ = 150° and ψ = 150° (second conformation) also predicted by Imberty et al. (35). Examples of the electron densities for the predominant and the second conformation of the trimannose molecules are shown in Fig. 2. The predominant conformation of the correspond to the major conformation and thick bonds to the minor conformation as present in the final refined structure. Fo-Fc difference density is shown at a 3σ level. Positive difference density is shown as continuous chicken wire and negative as dashed chicken wire. The map was calculated after replacing the trimannose molecule bound to monomer IV by an equivalent molecule, but with a conformation identical to the one found in monomer III and subjecting that structure to 20 cycles of restrained B-value refinement and 40 cycles Powell minimization. C, stereo view of the electron density for the trimannose molecule bound to monomer IV calculated in the same way as described above for monomer III.
Concanavalin A-Trimannoside Interactions

Hydrogen bonds between the two concanavalin A dimers upon tetramer formation

| Donor        | Acceptor          | Distancesa |
|--------------|-------------------|------------|
| Ser108 OG    | Asp96 OD1         | 2.57       |
| His121 NE2   | Ser108 OG         | 2.90       |
| Lys116 NZ    | Glu192 OE2        | 2.93       |
| His121 NE2   | Asn131 OD1        | 2.84/3.04/3.03/2.68 |
| Lys114 NZ    | Glu192 OE2        | 3.06/2.97  |

a Hydrogen bonds were calculated using HBPLUS (40).
b Calculated from zinc-substituted concanavalin A (entry 1ENR, Bouckaert et al. (41)).
c Calculated from entry 5CNA (Naismith et al. (26)).
d Calculated from entry 1CNA (Naismith and Field (13)).
e Values for monomers I, II, III, and IV, respectively.

Saccharide is congruous with the one observed in the structure of Naismith and Field (13). Interestingly, the two different conformations resulted in the same hydroxyls interacting with the protein. Since it is unusual for a molecule that is specifically bound to a protein to show two distinct conformations, the possibility of misinterpretation of the electron density map and model bias was investigated by the use of simulated annealing-omit (Fo - Fc) maps and by refining the sugar bound to monomer IV in both conformations. These tests strongly suggested that the trimannoside bound to monomer IV has indeed a conformation different from those bound to the other three monomers (Fig. 2C). Despite the convincing electron density for this situation, no satisfying explanation could be found as why this is the case. In fact, the trimannoside molecule with the alternative conformation does not seem to be involved in extensive crystal packing interactions, the closest symmetry-related atoms being at 5.5 Å from the sugar atoms. In this way it does not appear to be prevented from adopting the same conformation as the other three trimannose molecules in the asymmetric unit. A similar case of different conformations of a bound ligand within a same crystal structure is found in the two dimers of the asymmetric unit of galectin-1 in complex with a biantennary octasaccharide of the complex type. Several crystal forms (monoclinic, trigonal, or hexagonal) have appeared to arise from the selection of different low energy sugar conformations on crystallization (37). A synthetic cysteine proteinase inhibitor E64-c in complex with papain was found in different binding modes in two different crystal forms (38). However in none of these cases could evidence for the implication of the diversity of ligand binding modes in specificity and function be given.

Quaternary Structure—A remarkable feature of our structure is the rather large movement of the subunits relative to each other within the tetramer. Such movements were first discovered in the methyl-α-D-mannopyranoside-concanavalin A complex (26) and are even more pronounced in our trimannose-concanavalin A complex. In the native concanavalin A structure, the four subunits are related by a crystallographic 222 symmetry. In the lower symmetry spacegroups of the saccharide complexes, the subunits have the freedom to rotate with respect to each other. The two dimers, each formed by the joining of the six-stranded back β-sheets of the monomers of concanavalin A into a continuous 12-stranded back β-sheet, turn away from their orthogonal back sheet to back sheet positioning. This leads to a larger overlap between the subunits and consequently to an expansion of the tetramer. In going from the sugar-free structure to the trimannose-concanavalin A complex, the dimer over dimer rotation within the tetramer is 5.5°, while this rotation is only 3° for the methyl-α-D-mannose-concanavalin A complex. Moreover, there is a significant stretching of the dimer: an outwards tilt of 2.4° occurs between the monomers I and II or III and IV (1° in the mannoside complex) in the canonical dimers. A similar tendency is observed in the trimannosyl complex of Naismith and Field (13). The small movements of the subunits within the two canonical dimers have no effect on the intradimer hydrogen bonding pattern (data not shown). The somewhat larger movements of the two dimers within the tetramer, however, disturb the interdimer hydrogen bonds: only one interaction is conserved in all four tetramers listed in Table IV. In the methyl-α-D-mannose-concanavalin A complex, this is compensated by an alternative network of hydrogen bonds and salt bridges, but no such compensation is seen in either of the two trimannose complexes.

It is unlikely that these subunit movements are a direct result from the binding of the saccharide, as the monomers themselves seem to be quite rigid. Williams et al. (20) reported a curved Scatchard plot (and a corresponding Hill coefficient of 1.3) only for the trimannose binding and not for monosaccharide binding or binding of any of the two disaccharide virtual complexes. The finding has been questioned by Brewer and co-workers (22). In a more recent study, Chervenak and Toone (23) argued that the non-linearity in Scatchard plots of the concanavalin A-trimannose interaction is real and temperature-dependent, suggesting a
dynamic origin of this apparent co-operativity rather than allosteric changes in protein conformation upon carbohydrate binding. It is therefore unlikely that the observed subunit movements are of allosteric origin. Most likely they are due to the different crystal environments. We consider this observation nevertheless relevant, as it indicates a certain flexibility of the concanavalin A tetramer in solution. The fact that these subunit movements are allowed to occur within the tetramer may have importance in the ability to release strain in the formation of cross-linked networks of lectins and oligosaccharides, as described by Mandal and Brewer (39).

On the contrary, the dynamic co-operativity of concanavalin A can be more readily explained by movements of the side chains of $\text{Arg}^{228}$, $\text{Tyr}^{100}$, and $\text{His}^{205}$. When comparing the trimannose structure with the native structure of concanavalin A and with the complex with $\text{Me-$\alpha$-D-mannopyranoside}$ (Fig. 3), it is seen that monosaccharide binding mainly involves movements of the side chains of $\text{Arg}^{228}$ and $\text{Tyr}^{100}$. In the trimannose complex, the conformation of Arg$^{228}$ is similar to that in the mannose complex, but the orientation of the side chain of $\text{Tyr}^{100}$ and also of $\text{His}^{205}$ in the neighboring loop seems to be closer to those in the saccharide-free structure. The Asp$^{15}$ side chain has the same conformation as in the native structure, making hydrogen bonding interactions to a water molecule (Wat in Fig. 3) that is hydrogen-bonded to both $\text{Asn}^{14}$ ND2 and to O2B of the trimannose. In contrast, it is turned away from the binding site in two of the four monomers of the Me-$\alpha$-D-mannopyranoside complex. Furthermore, there seems to be a slight movement (0.5 Å) of the loop from Pro$^{13}$ to Ile$^{17}$ and also of the loop Ser$^{204}$-Ala$^{207}$, that is not seen when comparing the saccharide-free structure with the mannose complex (Fig. 3). This movement, although small, seems to be necessary to correctly fit the $\alpha 1$–$3$-linked mannose in the binding site.

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