Mannose-binding proteins (MBPs) are C-type animal lectins that recognize high mannosyl oligosaccharides on pathogenic cell surfaces. MBPs bind to their carbohydrate ligands by forming a series of Ca\(^{2+}\) coordination and hydrogen bonds with two hydroxyl groups equivalent to the 3- and 4-OH of mannos. In this study, the determinants of the orientation of sugars bound to rat serum and liver MBPs (MBP-A and MBP-C) have been systematically investigated. The crystal structures of MBP-A soaked with monosaccharides and disaccharides and also the structure of the MBP-A trimer cross-linked by a high mannosyl asparaginyl oligosaccharide reveal that monosaccharides or α1-6-linked mannos bind to MBP-A in one orientation, whereas α1-2- or α1-3-linked mannos binds in an orientation rotated 180\(^\circ\) around a local symmetry axis relating the 3- and 4-OH groups. In contrast, a similar set of ligands all bind to MBP-C in a single orientation. The mutation of MBP-A His\(^{189}\) to its MBP-C equivalent, valine, causes Man\(_{1}\)-3-Man to bind in a mixture of orientations. These data combined with modeling indicate that the residue at this position influences the orientation of bound ligands in MBP. We propose that the control of binding orientation can influence the recognition of multivalent ligands. A lateral association of trimers in the cross-linked crystals may reflect interactions within higher oligomers of MBP that are stabilized by multivalent ligands.

High mannos structures present on cell surfaces represent important recognition elements in the immune response. Serum mannose-binding proteins (MBPs)\(^1\) are C-type lectins that function in immune surveillance by recognizing high mannos structures present on pathogenic organisms such as bacteria and fungi (1). MBPs trigger cell killing by activating the complement pathway, leading to opsonization or direct lysis of the target cells (2–5). The ability of these proteins to distinguish foreign from self through the recognition of unique carbohydrate structures thereby contributes to host defense independent of an antibody response. Rodents and some other mammals (6) express two related MBPs in the serum and the liver in which the rat are designated MBP-A and MBP-C, respectively (7). Both proteins can fix complement, although the biological role of the liver-associated MBP is unclear at present.

Each MBP consists of a cysteine-rich N-terminal domain followed by a collagenous region, an α-helical “neck” and a C-terminal carbohydrate recognition domain (CRD) (7). The CRD mediates recognition of target cells, and the collagenous domain interacts with MBP-associated serine proteases that trigger the downstream complement response (8). MBP polypeptides are assembled into trimeric building blocks that contain collagenous triple helices. Trimer formation requires the presence of the neck region, which associates into an α-helical coiled-coil structure (9, 10). Disulfide bonds formed between cysteine-rich domains of MBP-A mediate higher order oligomerization of the trimeric building block, a property associated with more efficient complement fixation (8, 11).

MBPs have a broad carbohydrate specificity concordant with their need to recognize a variety of pathogenic cell surfaces. This specificity includes D-mannose, N-acetyl-D-glucosamine, and L-fucose (12). The common feature of these sugars is the presence of vicinal equatorial hydroxyl groups in the stereochemistry of the 3- and 4-OH groups of D-mannose, and these sugars are referred to herein as “Man-type” ligands. The structural basis of MBP carbohydrate specificity has been investigated by high resolution x-ray crystallographic analysis of rat MBP-A (13) and MBP-C (14). The MBP CRD structure consists of a compactly folded domain that contains a series of loops stabilized by two Ca\(^{2+}\). Carbohydrate binding occurs through direct coordination of one of the Ca\(^{2+}\), which is designated the principal Ca\(^{2+}\), and hydrogen bond interactions with side chains of amino acids that also serve as ligands for this Ca\(^{2+}\), thereby forming an intimately linked ternary complex of protein Ca\(^{2+}\) and sugar (Fig. 1).

Similar to most lectins, MBPs display only weak affinity for monovalent sugar ligands, with dissociation constants \(K_d\) in the millimolar range (12, 15), but bind avidly to target cell

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\(^1\) The abbreviations used are: MBP, mannose-binding protein; CRD, carbohydrate-recognition domain; NCS, noncrystallographic symmetry; MPD, 2,4-methylpentanediol; CNS, crystallography NMR system.

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High mannose structures present on cell surfaces represent important recognition elements in the immune response. Se-
surfaces. This property allows discrimination between foreign cells and host cells, both of which display Man-type ligands on their surfaces. The trimeric structures of fragments of rat and human MBPs comprising the CRD plus α-helical neck regions reveal that the sugar binding sites on the trimer are spaced too far apart to bind to different branches of typical vertebrate high mannose oligosaccharides (9, 10). However, they are presumably able to bind multivalently to the dense, repetitive arrays of Man-type ligands present on bacterial and fungal cell surfaces.

The CRDs of MBP-A and MBP-C share 56% amino acid sequence identity, and x-ray analysis has shown that they have very similar structures (14). The CRDs also show similar specificity for monosaccharide ligands, but differ in their affinities for oligosaccharides. MBP-C binds best to the trimannosyl core structures of N-linked carbohydrates, whereas MBP-A preferentially binds to terminal sugars of oligosaccharide chains (16). Lee and co-workers (17, 18) have reported that the linear trisaccharide Man1–2Man1–6Man reveals that the sugar binding sites on the MBP-A and MBP-C observed in these structures is that the orientation of the sugar ring is reversed in the two cases (Fig. 1). To assess whether this difference is related to the fine specificity of these two proteins, we have determined high resolution crystal structures of MBP-A and MBP-C bound to various carbohydrate ligands. The results indicate that a single residue in the binding site can influence the mode of ligand binding, which is likely to impact on the ability of the protein to interact with oligosaccharides. We also report the structure of trimeric MBP-A cross-linked by an oligosaccharide and discuss the implications of this structure for multivalent cell surface recognition and complement activation.

EXPERIMENTAL PROCEDURES

Materials—The O-methylglucosides of mannos, N-acetylgalactosamine and fucose, and also Man1–2Man, Man1–3Man, and Man1–6Man were obtained from Sigma. Man, GlcNAcAsn, Man1–3Man, GlcNAc–1–2Man1–3[GlcNAc–1–2Man1–3Man–1–2Man1–6Man] were purchased from V-Labs, Inc. Man1–2Man1–6Man and the bivalent glycopeptide N-Ac-Tyr-Asp-Gly-Gly-NH-(CH2)6-O-Man2 were gifts from Dr. Reiko Lee (Johns Hopkins University, Baltimore, MD).

Protein Constructs, Expression, and Purification—A trimeric fragment of MBP-A, designated c-MBP-A (10), and a dimeric fragment of MBP-C (14) were expressed, purified, and crystallized as described previously. The His189Val mutant of MBP-A was generated by site-directed mutagenesis using the MBP-A cDNA was altered to GTG at positions 658–660. The sequence of the MBP-A cDNA was altered to GTG at positions 658–660. The His189Val double mutant of MBP-A was created by combining restriction fragments from the two singly mutated expression plasmids. The mutant proteins were expressed and purified as for the wild-type proteins.

Preparation of Ligand-soaked Crystals and Data Collection—Crystals of the wild-type and mutant MBP-A trimer and MBP-C dimer were grown at 20–22°C by hanging drop vapor diffusion. MBP-A crystals were prepared by mixing equal volumes of 12 mg ml−1 MBP-A in 10 mM NaCl and 10 mM CaCl2 with reservoir solution consisting of 8–13% polyethylene glycol 3350 or 8000, 100 mM Tris-Cl, pH 7.4, 10 mM NaCl, 12 mM CaCl2, and 2 mM NaN3. For data collection, crystals were adapted into 2,4-methylpentanediol (MPD) by serial transfer into 30% MPD at 15–20% MPD at 10–15-min intervals. Sugar ligands were included in the last two cryopreservation solutions at the concentrations indicated in Table I. MBP-C crystals were grown by mixing equal volumes of 12–20 mg ml−1 protein in 10 mM Tris-Cl, pH 7.5, and 12 mM CaCl2 with reservoir solution containing 11–14% polyethylene glycol 8000, 100 mM Tris-Cl, pH 7.4, 100 mM NaCl, 12 mM CaCl2, and 2 mM NaN3. The crystals were adapted into MPD by serial transfer into synthetic mother liquor consisting of the reservoir solution and 0.5, 10, 15, 20, and 25% MPD at 10–15-min intervals. Sugar ligands were included in the last two cryopreservation solutions at the concentrations indicated in Table II. The crystals were flash-frozen in a 100 K nitrogen gas stream for data collection.

Data were measured on an RAXIS-III imaging plate detector mounted on a rotating Cu anode x-ray generator operating at 4.5 kV. 100% data collection on MBP-A and MBP-C of data were measured with typical exposures of 60–75 min.2 image. For MBP-C, 100–120° of data were measured with exposures of 15–20 min.1.5 image. Data were integrated and scaled with Denzo and Scalepack (19). Data collection statistics are presented in Tables I and II.

Structure Solution and Refinement of Ligand-soaked Complexes—In all cases, the crystals were sufficiently isomorphous to the unliganded MBP-A (10) or MBP-C (14) structures to permit direct solution by rigid body refinement. All water and other solvent molecules were removed as the alternate side chain conformations. Ca2+ and the refined individual temperature factors were retained in the starting models. All refinement calculations were performed in crystallography NMR system (20). Before any refinement commenced, a subset of the data was used to refine the test set for the calculation of the free R factor. The oligosaccharide structures (MBP-A trimer or MBP-C dimer) were first refined as a single rigid body, and then the individual protomers were used as rigid bodies at 4 Å resolution. The resolution was then moved to 2.8 Å where another round of rigid body refinement of the protomers was run before switching to standard positional refinement. Refinement proceeded in several rounds of increasing resolution and consisted of alternate positional and individual temperature factor refinement. An overall anisotropic temperature factor (22) and bulk solvent correction (23) were applied throughout. For the first four structures of each protein, water molecules were independently identified in Fo – Fc maps and refined. The water molecules common to the four structures were then identified to generate a common set that could be added to subsequent structures without repicking them individually (275 for MBP-A, 192 for MBP-C). Use of these sets allowed the remaining waters and other solvent molecules to be found rapidly and the structures to be completed. Refinement statistics are presented in Tables I and II.

Cryocrystallization and Structure Determination of an MBP-A Trimer-Man, GlcNAcAsn Complex—Hanging drop vapor diffusion at 20°C was used to prepare crystals of the MBP-A trimer-Man, GlcNAcAsn complex. A 5 mM Tris-Cl, pH 8.0, 10 mM NaCl, 20 mM CaCl2, and 1 mM Man5GlcNAcAsn was mixed with an equal volume of reservoir solution containing 10% (w/v) polyethylene glycol 8000, 100 mM Tris-Cl pH 8.0, 10 mM NaCl, 20 mM CaCl2, and 2 mM NaN3. The oligosaccharide was required for the growth of the crystal, and crystallization was sensitive to the precise ratio of protein to sugar. Grow in two habits: hexagonal plates and hexagonal rods with missing corners. The latter form was used for structure determination. A single crystal of dimensions 0.50 × 0.35 × 0.15 mm2 was transferred directly to a containing the reservoir plus 20% MPD for <1 min and then flash-cooled in a 100 K nitrogen stream. Data were measured on an RAXIS-III imaging plate detector mounted on a Cu-rotating anode operating at 4.5 kilowatts and equipped with a graphite monochromator. The crystal-to-detector distance was 250 mm, and the detector was swung 10° off axis. A He-filled diffused beam path was mounted between the crystal and detector. The exposure times were 150 min2 oscillation. The data were processed with Denzo and Scalepack. The postrefined unit cell is a = 61.80 Å, b = 255.56 Å, c = 61.79 Å, and β = 119.6°. The diffraction spots could be predicted and integrated on a hexagonal lattice, but the data reduction indicated that the space group is monoclinic, P21, (Robs = 4.4%, Rfree = 5.4% in the supergroup P21/c, Robs = 52.4%). The final data set had a Rsym of 4.4% (50–2.5 Å) and 16.3% (2.93–2.80 Å) with 83.0% > 3σI (60.7% last shell). Data were measured an average of 1.9 times (1.4 × last shell). The data are 94.4% complete overall (last shell 75.9%).

Based on the typical protein partial specific volume of 1.21 ml/g, the asymmetric unit could contain between 1 and 4 trimers. A cross-rotation function calculated using data in the resolution range of 2.8–2.5 Å revealed a 3-fold noncrystallographic symmetry (NCS) axis oriented 18° from the b axis and 60° from the a axis, and three 2-fold NCS axes perpendicular to the 3-fold axis. The structure was solved by molecular replacement using the MBP-A trimer (10) with Ca2+, solvent molecules, and alternate conformations removed as a search model. All molecular replacement calculations were performed using X-FLOR (24). A cross-rotation function calculated using data in the resolution range of 3.0–2.5 Å revealed a 3-fold noncrystallographic symmetry (NCS) axis oriented 18° from the b axis and 60° from the a axis, and three 2-fold NCS axes perpendicular to the 3-fold axis. The structure was solved by molecular replacement using the MBP-A trimer (10) with Ca2+, solvent molecules, and alternate conformations removed as a search model. All molecular replacement calculations were performed using X-FLOR (24).
of 10–4 Å and a maximum Patterson integration radius of 45 Å produced six solutions of approximately the same height that were significantly higher than any other solution (a range of the six solutions 6.2–6.7 standard deviations (σ) over the mean, next solution 4.9 σ over the mean). Patterson-correlation refinement (25) of the top 20 crossrotation function peaks was carried out in two steps for each solution, first using the trimer as a rigid body and then using the three protomers as individual rigid bodies. The six most significant solutions from the crossrotation function remained as such after this procedure. The six peaks fell into two groups of three. Within each group, the three peaks are related by the 3-fold NCS, and the two groups are related by the 2-fold NCS, implying that the asymmetric unit contains two trimers (64% solvent content). Translation searches (10–4 Å) run separately on the two trimers gave one significant peak (10.3 σ, next peak 2.9 σ over the mean). After placing one trimer, the second trimer was placed with respect to the first in a one-dimensional search along the y axis calculated for the four possible choices of origin. This procedure gave a single solution 8.9 σ (next peak 2.6 σ). The two placed trimers were initially refined as separate rigid bodies, and then as six individual protomers, first using data from 10–4 Å and then using data from 10–2.9 Å. The R-value at the end of this procedure was 0.385. The trimers are packed to give a hexagonal unit cell geometry, but because the trimer axes are tipped 18° off of the β axis, the space group is P21, rather than its supergroup P61.

Refinement of the structure was performed in CNS using positional and individual temperature factor refinement. The trimers do not obey perfect NCS, and free R value tests indicated that there was no advantage in imposing NCS restraints. After the first round of refinement, most of the ManGlcNAcAsn could be built into the model. The final model comprises 894 amino acids, 14 sugar residues (only the terminal sugars are visible in one of the three copies; in the others, the six mannose residues are visible), and 18 Ca2+.

RESULTS

Structure of Monosaccharide Complexes with MBP-A—The previously determined structure of the MBP-A CRD complexed with ManGlcNAcAsn (13) defined the binding of terminal mannose groups to the CRD through the 3- and 4-OH groups (Fig. 1, left). Each of these OH groups forms a coordination bond with the principal Ca2+ and two hydrogen bonds with amino acids that are also Ca2+ ligands. The CRDs in these crystals are cross-linked by the oligosaccharide, precluding experiments in which other ligands are soaked into the crystal. The crystals of the homologous MBP-C CRD grow in the absence of sugar and were used to examine the structures of a series of cognate monosaccharides (14). Surprisingly, the orientation of monosaccharides bound to MBP-C is reversed relative to MBP-A (14) (Fig. 1A, right). Although the monosaccharides are asymmetric structures, the two orientations can be generated by rotation around a local 2-fold symmetry axis that relates the equatorial 3- and 4-OH groups of mannose or their stereochemical equivalents. In this paper, we define the orientation observed in the MBP-A-ManGlcNAcAsn (i.e. with the 3-OH of mannose or its equivalent bound to Glu185 and Asn187) as orientation I (Fig. 1, left) and the reverse as orientation II in which the 4-OH occupies this position (Fig. 1, right).

Unlike the MBP-A CRD bound to ManGlcNAcAsn, the trimeric fragment containing the neck and CRD crystallizes in the absence of sugar (10). Therefore, the crystals of the trimeric fragment were used to visualize the binding of Man-type ligands to MBP-A. The original structure determination of this fragment employed 25% glycerol as a cryoprotectant (10). In that structure, a molecule of glycerol was bound at the Ca2+ site through vicinal OH groups in a manner identical to that observed in sugars bound to MBPs. Therefore, high concentrations glycerol or any other compound containing vicinal OH groups cannot be used as cryoprotectants, because they would compete with sugar ligands for binding. The compound MPD, which does not have vicinal OH groups, was found to be an effective cryoprotectant. A “native” sugar-free structure of the trimeric MBP-A fragment was determined in MPD (Table I). The protein structure is identical to that observed in the presence of glycerol. At the binding site, two water molecules occupy the positions taken by the 3- and 4-OH groups of mannose as previously observed in MBP-C (14), producing an eightcoordinated Ca2+ (Fig. 2A).

The α-methyl-O-glycosides of d-mannose, N-acetyl-d-glucosamine, and L-fucose as well as β-methyl-O-1-fucose were soaked into trimeric MBP-A crystals in the presence of MPD, and their structures were determined (Table I). As expected, the bound sugars use the same set of Ca2+ coordination and hydrogen bonds as seen in previous structures of MBP-A and MBP-C. Unexpectedly, however, α-Me-O-Man binds in orientation II (Fig. 2B), the same orientation observed in monosaccharide complexes with MBP-C. In two of the protomers, α-Me-O-GlcNAc binds in orientation I (Fig. 2C), and in the other, it binds in orientation II (Fig. 2D).

The structures of the two fucose anomers bound to MBP-A are of interest given the -4-fold differences in their affinity for MBP-A (15). As shown in Fig. 2, E and F, both anomers bind to MBP-A in the same orientation as fucose binds to MBP-C with the 2- and 3-OH groups coordinating to Ca2+. The mutation of His189 to alanine reduces the discrimination to ~2-fold (15). In the present structures, the anomeric oxygen of the α-anomer contacts this residue (Fig. 2E), whereas it does not in the β-anomer. These data suggest that interactions of the α-anomer with His189 ring contribute to the binding energy and can lead to anomeric discrimination.

Oligosaccharide Binding to MBP-A and to MBP-C—The difference between the orientations of monosaccharides and the terminal mannoses of ManGlcNAcAsn bound to MBP-A raised the possibility that compatibility with crystal lattice formation by the cross-linked complex might be a factor in determining the orientation of the terminal sugars in that structure. To eliminate the potential effect of such lattice con-
Ligand Orientation in Mannose-binding Proteins

The ligand concentration in the soak solution was 200 mM. The space group is C2 with approximate unit cell constants a = 79.0 Å, b = 85.3 Å, c = 98.3 Å, β = 106° with one trimer in the asymmetric unit. Numbers in parentheses are for last shell. rmsd, root-mean-square deviation.

| Ligand          | Resolution (last shell) | R<sub>merge</sub> | % complete | Average redundancy | R<sub>free</sub> | Bond length r.m.s. | Angle r.m.s. | Ramachandran plot |
|-----------------|-------------------------|-------------------|------------|-------------------|-----------------|-------------------|--------------|------------------|
| Native (MPD)    | (2.01–1.95)             | 0.045             | 82.1       | 95.4              | 2.8             | 0.234             | 0.198       | 0.007            | 1.4             | 89.5%            |
| αMe-O-Man       | (2.01–1.95)             | 0.029             | 55.3       | 83.9              | 2.5             | 0.241             | 0.207       | 0.007            | 1.3             | 89.5%            |
| αMe-O-GlcNAc    | (2.07–2.00)             | 0.052             | 78.7       | 98.6              | 2.8             | 0.249             | 0.212       | 0.007            | 1.3             | 89.2%            |
| αMe-O-Fuc       | (1.96–1.90)             | 0.050             | 89.0       | 97.3              | 2.6             | 0.224             | 0.189       | 0.007            | 1.3             | 90.2%            |
| βMe-O-Fuc       | (2.07–2.00)             | 0.044             | 85.4       | 98.2              | 3.0             | 0.229             | 0.200       | 0.008            | 1.4             | 90.5%            |
| Manα1–3Man      | (2.00–2.00)             | 0.047             | 87.1       | 98.8              | 2.9             | 0.231             | 0.198       | 0.009            | 1.4             | 89.7%            |
| Manα1–3Man (H189V mutant) | (1.94–1.90) | 0.063             | 85.7       | 94.6              | 2.9             | 0.252             | 0.223       | 0.008            | 1.4             | 90.0%            |
| Manα1–3Man (H189V/F2087V mutant) | (2.05–2.00) | 0.046             | 82.9       | 93.2              | 2.5             | 0.232             | 0.201       | 0.008            | 1.3             | 89.0%            |

<sup>a</sup> R<sub>merge</sub> = \[\sum_{i=1}^{N} \frac{|I_i(h) - \langle I(h) \rangle|}{\sum_{i=1}^{N} I_i(h)}\], where \(I_i(h)\) = observed intensity and \(\langle I(h) \rangle\) = mean intensity obtained from multiple measurements.

<sup>b</sup> R<sub>free</sub> = \[\frac{1}{N_{\text{free}}} \sum_{i=1}^{N_{\text{free}}} |F_i| - |F_c|\], where \(|F_i|\) = observed structure factor amplitude and \(|F_c|\) = calculated structure factor amplitude for the working and test sets, respectively.

<sup>c</sup> As defined in Procheck (26).

Given the apparent difference between binding of monosaccharides and oligosaccharides to MBP-A, it was of interest to determine whether such differences also occur in the interaction of MBP-C with various ligands. The crystal structures of MBP-C soaked with various oligosaccharides (Table II) reveal that Manα1–3Man binds to MBP-C in orientation II as do larger oligosaccharides including the linear trisaccharide Manα1–3Man (Table I). In each case, the terminal sugar is bound in orientation II (Table III). Therefore, unlike the MBP-A ligand binding site, the MBP-C site does not appear to be sensitive to the nature of the glycosidic substituent of the terminal sugar.
what difficult to detect small populations of another orientation bound at a given site, but it is clear that one orientation predominates in each case. As noted above, the C3 methyl group of Ile207 contacts C6 of the bound mannose in orientation I. To assess whether this residue contributes to the orientational preference, the double MBP-A mutant His189 Val/Ile207 Val was prepared, crystallized, and soaked with Manα1-3Man (Table I). The double mutant also binds in a mixture of orientations, although in this case a mixture of orientations is observed in two of the protomers and only orientation I is seen in the third protomer.

The structural comparisons presented here are derived from crystals that were cryopreserved in solutions containing 25% MPD. Data measured from MBP-C crystals soaked in Manα1–3Man but cryopreserved in 1.3 M l-rhamnose, which can be used as a cryoprotectant because its vicinal hydroxyl groups do not have the correct stereochemistry for binding at the principal Ca2+ site and therefore does not compete with the ligand of interest, show that the ligand binds in a mixture of orientations I and II.2 These observations are consistent with the notion that the orientational preferences arise from restrictions in accessible conformational space rather than outright steric exclusion. In principle, the conformational equilibrium and orientational preference could be affected by changes in water structure and bulk properties such as the lower dielectric environment of a 25% MPD solution or the higher viscosity of a concentrated l-rhamnose solution. The necessity of cryopreserving the crystals prevents systematic comparison in the presence of a more physiological buffer. Nonetheless, the comparisons done under identical conditions clearly indicate that particular amino acids in the binding site can confer real differences in orientation depending on the nature of the glycosidic substituent.

**Structure of a Cross-linked MBP-A Trimer**–Further insight into the binding of oligosaccharides to MBP-A was obtained by examining crystals of the trimeric MBP-A fragment grown in the presence of the asparaginyl oligosaccharide Manα6GlcNAc2Asn. The crystal structure was determined at 2.8 Å resolution. The asymmetric unit of the crystals contains two trimers that are cross-linked by the oligosaccharide (Fig. 4A). The trimers are arranged in hexagonally packed sheets in the crystal with the sheets cross-linked by the oligosaccharide (Fig. 4B). A single trimer is cross-linked to three different trimers in the opposing sheet, i.e. each protomer of a trimer is cross-linked to one protomer from three different trimers.

The cross-linking Manα6GlcNAc2Asn oligosaccharide is well ordered in two of the three protomers, with all six mannose residues visible (Fig. 4C). The Manα1–2Man and Manα1–6Man branches bridge trimers of MBP-A in the lattice. The Manα1–2Man is bound in orientation I as observed in the structure of the same oligosaccharide bound to the MBP-A CRD (13). The Manα1–6Man branch, which was not bound in the previous structure, is bound here in orientation II (Table III and Fig. 4). The α1–6 linkage places the terminal mannose residue in this branch one bond further from the penultimate residue and allows greater conformational flexibility due to the additional ω-torsion angle. Modeling indicates that the presence of His189 is unlikely to influence the accessible conformations of the α1–6 linkage with the terminal sugar in the binding site. Thus, the terminal sugar on this branch appears to behave like methyl glycosides.

**DISCUSSION**

MBPs do not show substantial affinity differences in binding to monosaccharides compared with disaccharides and higher

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K. Ng, K. Drickamer, and W. Weis, unpublished observations.
Ligand Orientation in Mannose-binding Proteins

The ligand concentration in the soak solution is indicated in the first column. The space group is P2₁2₁2₁ with approximate unit cell constants a = 60.6 Å, b = 75.3 Å, c = 57.6 Å with one dimer in the asymmetric unit. Numbers in parentheses are for last shell. rmsd, root-mean-square deviation.

| Ligand | Resolution (last shell) | Data collection | Refinement |
|--------|-------------------------|----------------|------------|
|        | Å                       | Rsym a | % > 3σI | % complete | Average redundancy | Rmerge b | Rfree b | Bond length rmsd | Angle rmsd | Ramachandran plot: most favored/disallowed c |
| Mana₁–3Man (200 mM) | 1.74 | 0.065 | 83.6 | 99.9 | 9.4 | 0.235 | 0.213 | 0.007 | 1.4 | 93.0/0.0 |
|                     | (1.77–1.74) | (0.271) | (54.8) | (99.1) | (7.0) |
| Trimmannosyl core | 1.80 | 0.068 | 75.2 | 94.8 | 3.5 | 0.259 | 0.212 | 0.006 | 1.3 | 91.8/0.0 |
| (100 mM) | I | (1.86–1.80) | (0.111) | (36.2) | (81.8) | (1.8) |
| High affinity linear trimannose | 1.95 | 0.068 | 75.3 | 99.9 | 3.7 | 0.242 | 0.210 | 0.006 | 1.3 | 92.9/0.0 |
| (100 mM) | (1.92–1.85) | (0.252) | (48.0) | (99.9) | (3.7) |
| GlcNAc-terminated core | 1.90 | 0.064 | 75.1 | 97.4 | 3.7 | 0.239 | 0.198 | 0.006 | 1.3 | 93.9/0.0 |
| (70 mM) | | (1.97–1.90) | (0.183) | (51.4) | (91.2) | (2.5) |
| Bivalent Man-terminated glycopeptid e | 1.80 | 0.048 | 79.4 | 97.1 | 3.1 | 0.243 | 0.211 | 0.006 | 1.3 | 93.4/0.0 |
| (50 mM) | | (1.86–1.80) | (0.156) | (50.8) | (89.5) | (2.4) |

See Table I.

a O-methyl glycosides. For αMe-O-GlcNAc, orientation II is seen in protomer C, and orientation I is seen in the other two.
b See Table I.
c See Ref. 14.
d Disaccharide.
e In Man₆GlcNAc(Asn).

TABLE III
Summary of sugar-binding orientation at the principal Ca²⁺ site in MBP-A and MBP-C

The two orientations are illustrated in Fig. 1.

| Wild-type proteins | Monosaccharides b | Monosaccharides b |
|-------------------|-------------------|-------------------|
| Monosaccharides a | II                | II                |
| Mana₁–3Man c,d     | I                 | Mana₁–3Man c,d    |
| Mana₁–2Man d       | II                | Trimannosyl core b,d |
| Mana₁–6Man d       | II                | High affinity linear trimannosyl d |
|                   |                   | GlcNAc-terminated core d |
|                   |                   | Bivalent Man-terminated glycopeptide d |

Mutants f

| Mana₁–3Man c,d (H189V) | A: II | B: I | C: II |
|-------------------------|-------|------|-------|
|                        |       |      |       |
| Mana₁–3Man c,d          | A: I (50%), II (50%) | B: I | C: I (50%), II (50%) |

Oligosaccharides (12, 15, 17). The local symmetry of the 3- and 4-OH groups of mannose and the lack of steric occlusions suggests that Man-type ligands should be able to bind to MBPs in either binding orientation shown in Fig. 1. It would seem entropically advantageous for the ligand to be able to bind in either orientation. The crystal structures presented here are at resolutions sufficient to allow visualization of mixtures of binding orientations at a given site at least to ~25% occupancy. The data clearly show that the wild-type MBP sites usually interact with a particular ligand in only one of the orientations. The physical basis for this selectivity is not apparent from the structures.

Small differences in binding energy have been reported for oligosaccharides. Table II shows binding to MBP-C with Mana₁–2Man₆OMe and Mana₁–2Man₆OMe showing increases in the free energy of binding relative to mannose of 0.6 and 1.2 kcal mol⁻¹, respectively (17). These data were interpreted as being indicative of a second binding site on MBP-C that is absent in MBP-A, which does not show these differences. However, the structure of Mana₁–2Man₆OMe bound to MBP-C does not reveal a second binding site, because only the terminal mannose residue interacts with the surface of the protein. In previous studies, a second binding site was observed in a MBP-C CRD crystal soaked in 1.3 M α-methylmannoside, but in this case the aglycon points into the structure (14). Modeling indicates that this site would not be able to accommodate a larger substituent at the glycosidic position, so it is very unlikely that this represents a binding modality for real oligosaccharides.

The differences in the binding of MBP-A and MBP-C to oligosaccharides may be attributed to the less restrictive nature of the MBP-C site, which might allow a more facile accommodation of the nonterminal portions of these ligands. For example, in both Mana₁–2Man₆OMe and Mana₁–2Man₆OMe, the penultimate sugar has free 3- and 4-OH groups, which could form the typical Ca²⁺ coordination and hydrogen-bonding scheme at the binding site. Indeed, we have seen evidence for this mode of binding in soaks of both MBP-A and MBP-C crystals with Mana₁–2Man and Mana₁–6Man (data not shown). If there were two equally probable ways of

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binding to the site using one of the two sugars in these compounds, an entropic gain of about 0.4 kcal mol\(^{-1}\) would be expected. Multiple binding modes, combined with differences in the conformational space available to the ligands in MBP-A and MBP-C, might explain the observed differences in the binding of MBP-C to monosaccharide and oligosaccharide ligands. It should be noted, however, that the studies demonstrating higher affinity binding of the oligosaccharides to MBP-C employed a trimeric MBP-C fragment (17, 18) so that some of the differences could arise from differences in the arrangement of CRDs in MBP-C and MBP-A.

Given that MBPs do not show substantial energetic differences in binding to a monosaccharide or disaccharide, what advantage might be conferred by the ability of the binding site to dictate the orientation of the bound ligand? There are two reasonable possibilities, either or both of which could apply in particular systems. The first possibility is that there are in fact small differences in binding energy, such as those seen for MBP-C, that give rise to significant energetic differences when the interaction is multivalent. The second possibility, illustrated in Fig. 5, is that the specificity for a particular binding orientation might affect the ability of an oligomeric lectin to interact simultaneously with different branches of an oligosaccharide. An oligosaccharide is shown with two nonreducing terminal sugars (gray and white hexagons). The carbohydrate-recognition domains of a trimeric lectin are shown as ovals. The primary interaction of the shaded monosaccharide residue and the shaded protein monomer in two different orientations would result in different secondary interactions of the unshaded units. On the left, the interaction of the gray monosaccharide residue with the gray protein monomer places the white sugar away from other binding sites. On the right, the gray sugar binds in an orientation that places the white sugar away from other binding sites. Note that this orientation effect could apply equally well to binding to primary and secondary sites within the same protomer.

FIG. 4. Structure of the cross-linked complex between the MBP-A trimer and Man\(_6\)GlcNAc\(_2\)Asn. A, arrangement of molecules in the crystal lattice showing a side view of three asymmetric units with adjacent trimers packed laterally in sheets and cross-linked by the sugar. The carbohydrate is shown in yellow, and each asymmetric unit is shown in a different color. B) top view of cross-linked trimers in the lattice. The cross-linking of one trimer to three neighbors in the lattice is evident. C, electron density map (\(F_o - F\), omit map, 3\(\sigma\)) and model of the observed Man\(_6\). The principal Ca\(^{2+}\) of the two cross-linked protomers is shown as a cyan sphere. D, a view of the Man\(_6\)(1,6)Man branch terminus bound to MBP-A. The view is approximately that of Figs. 18 and 3.

FIG. 5. Illustration of how specificity for a particular binding orientation might affect the ability of an oligomeric lectin to interact simultaneously with different branches of an oligosaccharide. An oligosaccharide is shown with two nonreducing terminal sugars (gray and white hexagons). The carbohydrate-recognition domains of a trimeric lectin are shown as ovals. The primary interaction of the shaded monosaccharide residue and the shaded protein monomer in two different orientations would result in different secondary interactions of the unshaded units. On the left, the interaction of the gray monosaccharide residue with the gray protein monomer places the white terminal sugar in a position in which it is able to interact with another protomer of the lectin. On the right, the gray sugar binds in an orientation that places the white sugar away from other binding sites. Note that this orientation effect could apply equally well to binding to primary and secondary sites within the same protomer.
tional space available, because some of the entropic penalty of binding could be paid by the interaction at the first protomer. It seems probable that these mechanisms will be of importance in many multivalent lectin-ligand interactions.

In the case of MBP-A, biological specificity comes from the ability of the trimer to bind multivalently only to the dense arrays of Man-type ligands present on foreign cell surfaces, but not to high mannosyl oligosaccharides present on the host cell (10). This discrimination arises from the architecture of the trimer, in which the interaction of the CRD with the coiled-coil neck domain sets the carbohydrate-binding sites 53 Å (rat) or 45 Å (human) apart. This distance is much greater than the distance between terminal mannosyl residues on vertebrate high mannosyl oligosaccharides, so MBP-A cannot bind to these structures with high avidity. The role of binding site orientation and its influence on multivalent binding of MBP-A to large arrays of sugar on pathogen surfaces is not clear at present.

Serum MBPs are found as higher oligomers organized into “bouquet” structures with the N-terminal regions of the trimers cross-linked together (11). It would be expected that the multiple trimers would provide another level of increased avidity in the recognition of the target cell surface. The lateral association of trimers seen in the crystal (Fig. 4) creates a flat two-dimensional array of trimer binding sites, an arrangement consistent with the notion that multiple trimers can interact simultaneously with arrays of glycans on pathogen surfaces. Viewed in this way, one layer of the crystal would represent a dimensional array of trimer binding sites, an arrangement and its influence on multivalent binding of MBP-A to large arrays of sugar on pathogen surfaces is not clear at present.

Complement activation by MBPs requires interaction with MBP-associated serine proteases. Much of the MBP in serum is cross-linked together (11). It would be expected that the multiple trimers could interact simultaneously with arrays of glycans on pathogen surfaces. Viewed in this way, one layer of the crystal would represent a cell surface presenting mannosyl-terminated oligosaccharides for recognition by MBP (Fig. 4, red layer), with the opposing layer representing multiple trimers in the bouquet (Fig. 4, green layer). There may also be flexibility in the trimer as evidenced by the clostridin sensitivity of the boundary between the collagenous and a-helical neck regions (10), such that the trimers in the bouquet can adapt to the various molecular landscapes presented by the target.

Complement activation by MBPs requires interaction with MBP-associated serine proteases. Much of the MBP in serum is cross-linked together (11). It would be expected that the multiple trimers could interact simultaneously with arrays of glycans on pathogen surfaces. Viewed in this way, one layer of the crystal would represent a dimensional array of trimer binding sites, an arrangement and its influence on multivalent binding of MBP-A to large arrays of sugar on pathogen surfaces is not clear at present.

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REFERENCES

1. Weis, W. I., Taylor, M. E., and Drickamer, K. (1998) Immunol. Rev. 163, 19–34
2. Ikeda, K., Sannoh, T., Kawasaki, N., Kawasaki, T., and Yamashina, I. (1987) J. Biol. Chem. 262, 7431–7440
3. Kuhlman, M., Jäner, K., and Ezekowitz, R. A. B. (1989) J. Exp. Med. 169, 1733–1745
4. Super, M., Levensky, R. J., and Turner, M. W. (1990) Clin. Exp. Immunol. 79, 144–150
5. Tenner, A. J., Robinson, S. L., and Ezekowitz, R. A. (1995) Immunol. 3, 485–493
6. Moges, T., Ota, T., Tauber, A. I., and Sastry, K. N. (1996) Glycobiology 6, 543–550
7. Drickamer, K., Dordal, M. S., and Reynolds, L. (1986) J. Biol. Chem. 261, 6878–6886
8. Wallis, R., and Drickamer, K. (1999) J. Biol. Chem. 274, 3580–3589
9. Sheriff, S., Chang, C. Y., and Ezekowitz, R. A. B. (1994) Nat. Struct. Biol. 1, 789–794
10. Weis, W. I., and Drickamer, K. (1994) Structure 2, 1227–1240
11. Wallis, R., and Drickamer, K. (1997) Biochem. J. 325, 391–400
12. Lee, R. T., Ikukawa, Y., Fay, M., Drickamer, K., Shao, M.-C., and Lee, Y. C. (1991) J. Biol. Chem. 266, 4810–4815
13. Weis, W. I., Drickamer, K., and Hendrickson, W. A. (1992) Nature 360, 127–134
14. Ng, K. K.-S., Drickamer, K., and Weis, W. I. (1996) J. Biol. Chem. 271, 663–674
15. Iobst, S. T., Wormald, M. R., Weis, W. I., and Drickamer, K. (1994) J. Biol. Chem. 269, 15505–15511
16. Childs, R. A., Feizi, T., Yuen, C.-T., Drickamer, K., and Quesenberry, M. S. (1993) J. Biol. Chem. 268, 20770–20777
17. Lee, R. T., and Lee, Y. C. (1997) Glycoconj. J. 14, 357–363
18. Quesenberry, M. S., Lee, R. T., and Lee, Y. C. (1997) Biochemistry 36, 2724–2732
19. Otwinowski, Z., and Minor, W. (1997) Methods Enzymol. 276, 307–326
20. Brünger, A. T., Adams, P. D., Clore, G. M., Gros, P., Grosse-Kunstleve, R. W., Jiang, J.-S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. Sec. D 54, 905–921
21. Brünger, A. T. (1992) Nature 355, 472–475
22. Sheriff, S., and Hendrickson, W. A. (1987) Acta Crystallogr. Sec. A 43, 118–121
23. Jiang, J.-S., and Brünger, A. T. (1994) J. Mol. Biol. 243, 100–115
24. Brünger, A. T. (1992) X-FOR, Version 3.1, Yale University, New Haven, CT
25. Brünger, A. T. (1990) Acta Crystallogr. Sec. A 46, 46–57
26. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 283–291
27. Imberty, A., Gerber, S., Tran, V., and Perez, S. (1990) Glycoconj. J. 7, 27–54
28. Petrescu, A. J., Petrescu, S. M., Dwek, R. A., and Wormald, M. R. (1999) Glycobiology 9, 343–352
29. Woods, R. J., Pathiason, A., Wormald, M. R., Edge, C. J., and Dwek, R. A. (1998) Eur. J. Biochem. 258, 372–386
30. Chen, C. B., and Wallis, R. (2001) J. Biol. Chem. 266, 25894–25902
Orientation of Bound Ligands in Mannose-binding Proteins: IMPLICATIONS FOR MULTIVALENT LIGAND RECOGNITION
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