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The structure of the saccharide-binding site of concanavalin A

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A complex of concanavalin A with methyl α-D-mannopyranoside has been crystallized in space group $P2_12_12_1$ with a = 123.9 Å, b = 129.1 Å and c = 67.5 Å. X-ray diffraction intensities to 2.9 Å resolution have been collected on a Xentronics/Nicolet area detector. The structure has been solved by molecular replacement where the starting model was based on refined coordinates of an I222 crystal of saccharide-free concanavalin A. The structure of the saccharide complex was refined by restrained least-squares methods to an *R*-factor value of 0.19. In this crystal form, the asymmetric unit contains four protein subunits, to each of which a molecule of mannoside is bound in a shallow crevice near the surface of the protein. The methyl α-D-mannopyranoside molecule is bound in the C1 chair conformation 8.7 Å from the calcium-binding site and 12.8 Å from the transition metal-binding site. A network of seven hydrogen bonds connects oxygen atoms O-3, O-4, O-5 and O-6 of the mannoside to residues Asn14, Leu99, Tyr100, Asp208 and Arg228. O-2 and O-1 of the mannoside extend into the solvent. O-2 is hydrogen-bonded through a water molecule to an adjacent asymmetric unit. O-1 is not involved in any hydrogen bond and there is no fixed position for its methyl substituent.

Key words: concanavalin A/electron density map/methyl-α-D-mannopyranoside/saccharide binding

Introduction

The structure of concanavalin A, the saccharide-binding protein of the Jack bean (*Canavalia ensiformis*), has been studied since an I222 crystal of the saccharide-free protein was described (Greer *et al.*, 1970). Once the structure of concanavalin A had been solved (Edelman *et al.*, 1972; Hardman and Ainsworth, 1972) it seemed that solution of the structure of the saccharide-binding site would follow shortly. This expectation was not fulfilled; mainly because the I222 crystal dissolves on addition of saccharide. In 1976, Hardman and Ainsworth found where the saccharide bound by locating the iodine atoms in the complexes of two iodo-derivatives of glucose with concanavalin A in a $C222_1$ crystal at 6 Å resolution.

In solution, the minimal mol. wt. of concanavalin A is that of a dimer (Kalb and Lustig, 1968). Each of its identical subunits has one saccharide-binding site (Yariv *et al.*, 1968) and two distinct metal-binding sites: one for a transition metal ion and another for a calcium ion (Kalb and Levitzki, 1968). When the naturally occurring metals are removed from concanavalin A in acid, the protein no longer binds saccharides. Addition of the missing metals at the right pH restores saccharide binding (Yariv *et al.*, 1968). The specificity of saccharide binding has been reviewed by Goldstein (1976). Both six-membered pyranosides and five-membered furanosides bind at the same site. The epimeric methyl α-D-glucopyranoside and methyl α-D-mannopyranoside which bind with similar affinity have been used to study the saccharide-binding site (e.g. Dani *et al.*, 1981).

We present a 2.9 Å electron density map of the saccharide-binding site. It shows the stereochemistry of the mannoside–concanavalin A interactions clearly and explains why the metals are needed to bind the sugar. It also explains why $P2_12_12_1$ crystals form and are stable only with methyl α-D-mannopyranoside, and why this cannot be replaced by the glucoside.

Results

The protein mass of the asymmetric unit in the crystalline complex of concanavalin A with methyl α-D-mannopyranoside corresponds to a tetramer of chemically identical subunits of concanavalin A. Figure 1 shows one of the saccharide-binding sites superimposed on the electron density map. The pattern of axial and equatorial substituents of the methyl α-D-mannopyranoside fits well into the electron density, but there is no electron density for the methyl substituent of the anomeric oxygen. The van der Waals volume of the saccharide also fits the binding site. A hydrophobic patch, corresponding to C-5 and C-6 of the saccharide, is in contact with the ring atoms of Tyr12 and Tyr100. The oxygen atoms of the portion of the saccharide that lies within the site are all in contact with oxygen or nitrogen atoms of the protein.

The mannoside is bound in the C1 chair conformation with the plane of the ring oriented very nearly along a straight line through the two metal ions and extending out of the protein into the water (Figure 2). The distance from the centre of the ring to the calcium ion is 8.7 Å and to the transition metal 12.8 Å. C-4 of the saccharide ring is deepest in the cavity and C-1 is closest to the surface. Both O-1 and O-2 extend into the solvent.

A network of hydrogen bonds links oxygen atoms of the sugar to hydrogen donors and acceptors of the protein (Figure 2 and Table I). OH-6 is bonded to OD1 of Asp208, and the lone-pair of O-6 is bonded to the peptide NH's of Tyr100 and Leu99. These hydrogen bonds fix the C5–C6 bond in a staggered conformation. The ring oxygen O-5 is bonded through its lone-pair to the peptide NH of Leu99

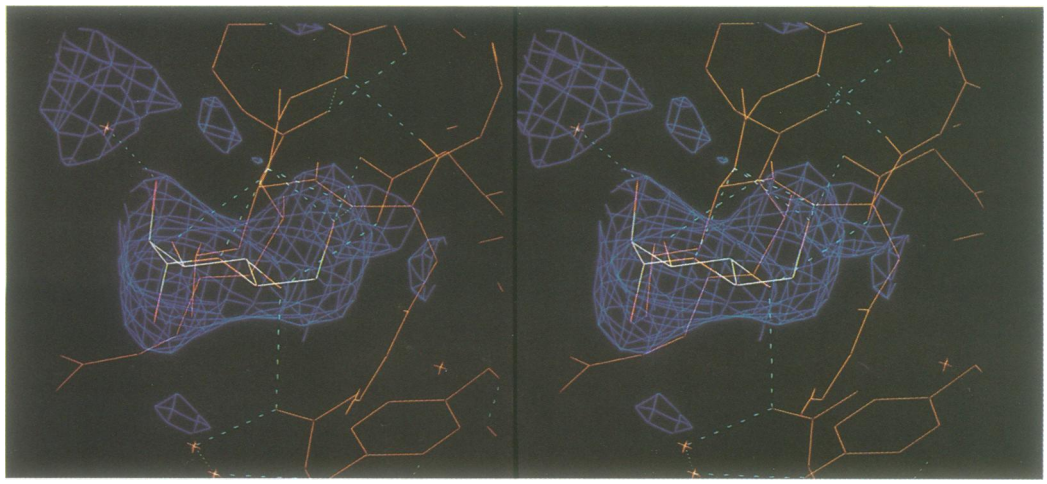

Fig. 1. A stereoscopic view of the saccharide-binding tie. The bound saccharide molecule is superimposed on the difference-electron density map (blue) together with the portion of the protein which constitutes the site. Hydrogen bonds are shown as broken lines. See Figure 2 for a detailed view of the hydrogen-bonding network. The methyl substituent of O-1 has not electron density and is not shown in the figure.

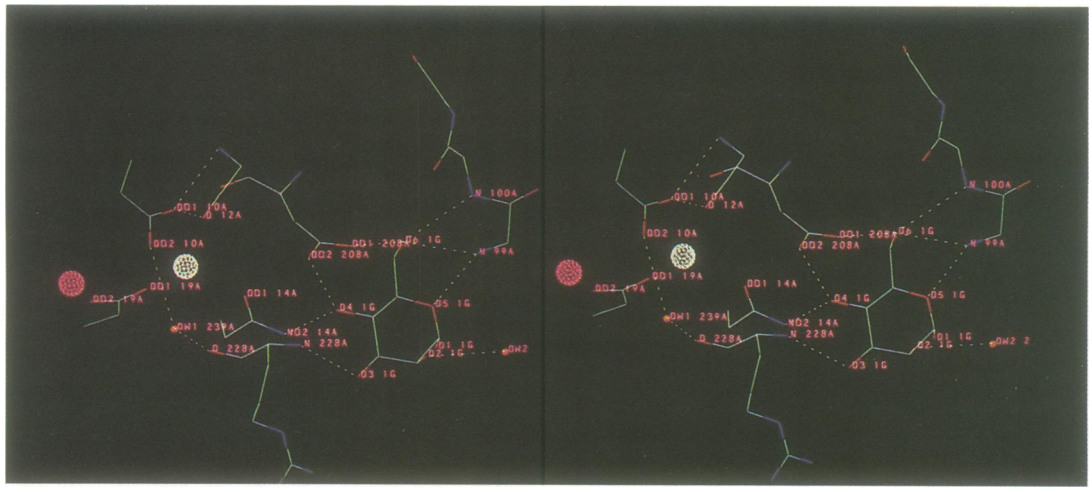

Fig. 2. A stereoscopic view of the saccharide-binding site showing the network of hydrogen bonds which stabilizes the interaction between the protein and the saccharide. Calcium and transition metal are shown as white and pink spheres. Bonds between the metal ions and their ligands are not shown in this figure for the sake of clarity.

Table I. The hydrogen-bonding scheme of the saccharide-binding site of concanavalin A

Hydrogen bonds			
Donor	Acceptor	D−A Distance (A)	Comments
O-4	Asp208 OD2	2.84	
O-6	Asp208 OD1	2.72	
Arg228 NH	O-3	3.15	Arg water-bridged to calcium
Asn14 ND2	O-4	3.24	OD2 is a calcium ligand
Leu99 NH	O-5	3.02	bifurcated H-bond
Leu99 NH	O-6	2.92	bifurcated H-bond
Tyr100 NH	O-6	3.25	Tyr ring carbons interact with C-5 and C-6 of MAN
O-2	WAT (external)	3.13	water bridges to Ser168 and Thr226 (Figure 3a) or Asp71 (Figure 3b) of an adjacent tetramer

which also forms a bifurcated hydrogen bond to O-6. OH-4 is bonded to OD2 of Asp208, and the lone-pain of O-4 is bonded to the amide NH of Asn14, thereby fixing the rotation about the C4−O4 bond. The lone-pair of O-3 is bonded to the peptide NH of Arg228, but there is no hydrogen acceptor within bonding distance of OH-3.

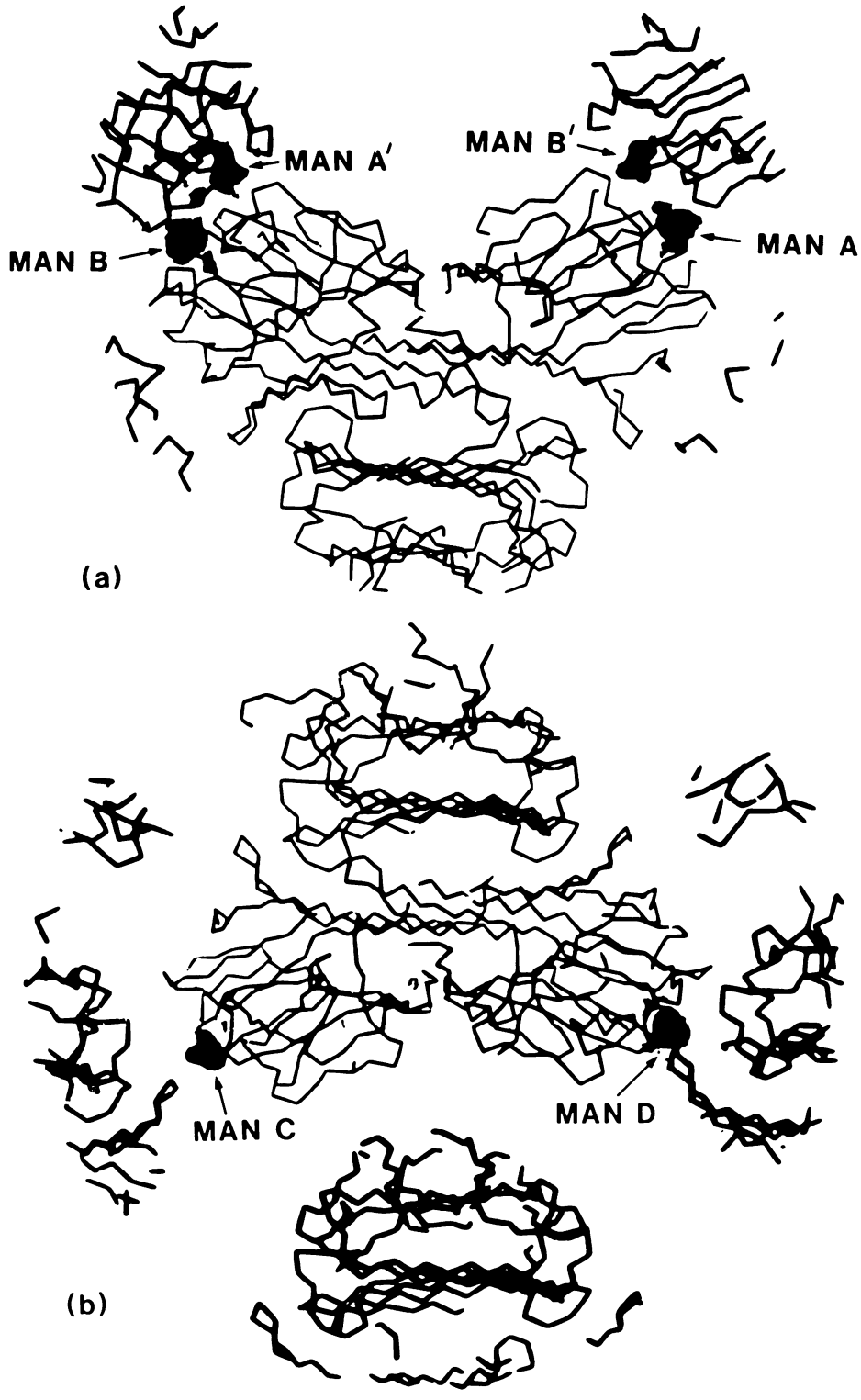

Fig. 3. The α-carbon backbone of a tetramer of the concanavalin A complex with methyl α-D-mannopyranoside and portions of the adjacent tetramers. (**a**) View in the *y*-direction showing the proximity of the bound mannoside molecules in one concanavalin A dimer to the mannoside bound to the adjacent tetramers. Each of the two pairs of proximate sites is related by a local pseudo-dyad axis normal to the *xz*-plane. (**b**) View in the *x*-direction showing the proximity of each of the bound mannoside molecules in the second concanavalin A dimer to the surface of an adjacent tetramer. Locations of bound mannoside are labelled MAN.

The hydroxyl group of C-2 is not hydrogen-bonded to the saccharide-binding site but linked through a water bridge to an adjacent subunit. These bridges link each tetramer to four adjacent ones. They fall into two distinct types. In the first type water forms bridges to Ser168 and Thr226 of the adjacent tetramer (Figure 3A); in the other it forms a link to Asp71 of the adjacent tetramer (Figure 3B). We find no hydrogen bond of O-1.

Two of the amino acid residues involved in the network of hydrogen bonds to the mannoside are also linked to the calcium ion (Figure 2). Asn14 is bonded directly to calcium via OD1, and Arg228 is indirectly bound to calcium by a

hydrogen bond from its peptide carbonyl to a water molecule which is one of the calcium ligands.

Discussion

Figures 1–2 show the mannoside to be bound in the C1 chair conformation, confirming the results of Brewer *et al.* (1973), but the carbon–manganese distances derived from studies of nuclear magnetic resonance relaxation were inaccurate and led to an incorrect positioning of the saccharide by Brewer *et al.* (1973) and by Villafranca and Viola (1974). The substituents of carbons 3, 4 and 6 are equatorial and that of carbon-2 axial. The α-anomeric substituent of carbon-1 is also axial and is directed out of the site and into the surrounding solvent, making no contact with the protein. The absence of electron density for the methyl substituent of O-1 suggests that it is free to rotate about the C1–O1 bond. This might explain why there is no hydrogen bond to the lone-pair of O-1, since its orientation would be random, but it is also true that the anomeric oxygen is a weak acceptor.

Like other saccharide-binding proteins, concanavalin A binds saccharide by forming hydrogen bonds with nearly all the donors and acceptors of the saccharide hydroxyls (see review by Quiocho 1986). Four of the sugar's seven hydrogen bonds are to backbone NH groups of the proteins and two are to amino acid residues that are bound to the calcium ion. OD1 of Asn14 is a direct ligand of calcium and Arg228 is hydrogen-bonded via its carbonyl oxygen to a water molecule which is a ligand of calcium. Van der Waals interactions also contribute. Tyr12 interacts hydrophobically with the mannoside and is bonded to calcium by its peptide carbonyl oxygen.

In the saccharide-free protein, Asp208 is hydrogen-bonded to one of the two water molecules which are ligands of calcium (Hardman *et al.*, 1982), but that water molecule is absent from the saccharide complex (Figure 2). It is not clear why binding of the saccharide should have this effect on the coordination of the calcium.

Tyr100, Asp208 and Arg228 take up different positions in the crystal structures of metal-containing and metal-free concanavalin A, which lacks a saccharide-binding site. Tyr12, which is in van der Waals contact with the saccharide together with Tyr100, is also displaced (Shoham *et al.*, 1979). It seems that calcium pulls the amino acid residues to the saccharide-binding site into the required positions, just as transition metals organize the amino acid ligands of the calcium-binding site (Shoham *et al.*, 1979). Binding of the saccharide should therefore stiffen the protein around the saccharide and the metal ions (cf. Sturtevant, 1977; Jacrot *et al.*, 1982).

$P2_12_12_1$ crystals dissolve if depleted of the mannoside, apparently because its C-2 hydroxyl group stabilizes them by cross-linking adjacent, symmetry-related subunits (Figure 3). This may be why $P2_12_12_1$ crystals do not survive exposure to the closely related methyl α-D-glucopyranoside. The glucoside, which binds to the saccharide-binding site with affinity similar to that of the mannoside, differs from the mannoside only in its configuration about C-2. If the glucoside were bound in the site like the mannoside, O-2 of the glucoside would not be well placed for cross-linking the asymmetric units in the $P2_12_12_1$ crystal. The glucoside complex of concanavalin A crystallizes in the cubic space-group I23 and does not survive exposure to the mannoside (Yariv *et al.*, 1987). It will be interesting to see if O-2 of the glucoside stabilizes the packing of the protein in the cubic crystal.

Our list of atomic coordinates for the concanavalin A–mannoside complex will be submitted to the Protein Data Bank (Bernstein *et al.*, 1977).

Materials and methods

The complex of concanavalin A with methyl α-D-mannopyranoside

The crystals were grown from stock solutions of 80–150 mg protein/ml by dialysis equilibration with a solution of the following composition: 0.05 M Pipes, 0.1 M $NaNO_3$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 2 mg/ml NaN_3 and 0.1 M methyl α-D-mannopyranoside (Pfanstiehl), pH 6.8. The crystal density was 1.165 g/ml and the protein content, determined from the weight of a cluster of drained crystals as described by Yariv *et al.* (1987), was 0.52 g protein/g crystal. The space-group is $P2_12_12_1$ as determined from symmetries and systematic absences observed in precession photographs. The unit-cell dimensions are a = 123.9 Å, b = 129.1 Å and c = 76.5 Å. The protein mass in the unit cell was calculated to be 392200 daltons which is equivalent to 98 100 daltons per asymmetric unit. This corresponds closely to one tetramer of concanavalin A subunits of 25 630 daltons each (Cunningham *et al.*, 1975). The crystal vol per unit of mol. wt, V_m, is 2.75 $Å^3$/dalton.

Stability of the crystal depends on the presence of methyl α-D-mannopyranoside. When crystals are transferred to the same solution from which they had grown but without mannoside, or with methyl α-D-glucopyranoside instead, they dissolve. A crystal was handled and mounted for X-ray analysis in contact with its mother liquor at the synchrotron. It diffracted to a resolution of 1.6 Å.

X-ray data were collected with a Nicolet/Xentronics Imaging Proportional Chamber using CuKα radiation from a sealed tube source equipped with a graphite monochromator. This system was commissioned and calibrated in some detail in order to minimize data errors and optimize data collection. A detailed statistical analysis of the data set for the mannoside complex of concanavalin A is given by Derewenda and Helliwell (1989) and only a brief summary is given here. Satisfactory resolution of spots was achieved with a crystal-detector distance of 17 cm. The detector chamber was positioned asymmetrically so that the high resolution edge was at 2θ = 14° corresponding to 2.9 Å resolution. The data were collected by rotation around three axes in frames of 0.25°. One crystal was used for measurement of the entire data set which took a total of 4 days. Exposure time was 400 s/frame with the generator operating at 50 kV and 35 mA and the global rate of data acquisition was ~5000 counts/s. A total of 95 408 observations was recorded corresponding to 23 599 unique reflections. The data set was completed to 3.0 Å resolution and included ~70% of the reflections between 3.0–2.9 Å. 3307 observations were discarded in the course of data reduction. The final merging R value on intensity for symmetry-related reflections was 0.054. The data collection software was that of Blum *et al.* (1987) and the data reduction software was that of Howard *et al.* (1987).

The starting atomic model

The starting point of our refinement was the coordinate file for saccharide-free (I222) concanavalin A from the Brookhaven Protein Data Bank deposited by Reeke *et al.* (1975). This structure had not been subjected to least squares refinement. Therefore, we collected a 2 Å data set on a saccharide-free I222 cyrstal of concanavalin A at the SERC Daresbury Synchrotron Radiation Source Station 7.2 (Helliwell *et al.*, 1982) and refined the model structure against these data by the restrained least-squares method of Hendrickson and Konnert (1979) The refinement converged to an R-factor of 0.24 from a starting value of 0.45. A complete description of the refinement and the refined structure will be published separately.

Structure solution

A self-rotation function calculated in spherical-polar coordinates (CCP4 Suite) confirmed the position of a non-crystallographic dyad axis observed in precession photographs of the hk0 zone. A strong peak (57% of origin peak) occurred at ω, ϕ, κ of 90°, 45°, 180°. We assumed the tetramer which constitutes the asymmetric unit to be arranged as a pair of dimers as in the saccharide-free I222 crystal (Reeke *et al.*, 1975). Such a dimer, constructed from the refined I222 coordinates described above and placed in a P1 cell of dimensions $a = b = c$ = 100 Å $\alpha = \beta = \gamma$ = 90°, was used

as our model for cross-rotation analysis. Structure factors calculated from the model were used with the observed structure factors between 10 and 4 Å resolution to compute a three-dimensional cross-rotation function in Eulerian space. A prominent peak, in excellent agreement with the self-rotation analysis, was observed at α, β, γ of 0°, 0°, 45°. This result was not unexpected in the light of the observation that the lengths of the $\alpha\beta$ face diagonals in the $P2_12_12_1$ cell correspond closely to the α and β cell edges of the I222 cell and that the y-dimension is nearly the same in the two cells. The calculation was repeated with a tetramer as the model structure and the main maximum was now located at α, β, γ of 0°, 0°, 50°. A suitably rotated tetramer was used with the *R*-factor minimization procedure, SEARCH (CCP4 Program suite, Daresbury Laboratory), to locate its position in the unit cell. Initially, a projection solution against 0k1 data only was searched for in 1A steps and a prominent ridge of low *R*-factor values was found for $y = 0.25$. A two-dimensional search in the xz-plane with a three-dimensional set of strong terms gave the approximate coordinates for the main minimum at x, y, z of 0.25, 0.25 and 0.04. A three-dimensional search with 1562 strongest reflections and a fine grid of 0.3 Å located the position at x, y, z = 33.7, 33.8 and 4.2 Å with an *R*-factor of 0.36.

Least-squares refinement

A complete tetramer, not including the metal ions, was positioned in the $P2_12_12_1$ unit cell according to the molecular replacement results. The conventional crystallographic *R*-factor at this stage was 0.53.

Least-squares refinement of the model was carried out in two distinct stages. First, the rotational and translational parameters of the individual subunits were refined according to a protocol suggested by Derewenda (1989), as follows. After five cycles of restrained, least-squares refinement (Hendrickson and Konnert, 1979) the model structure, consisting of four independent subunits of concanavalin A, was superimposed, by a least-squares method of best molecular fit (CCP4 Program suite, Daresbury Laboratory), onto the resulting structure. As a result, the *R*-factor dropped from the initial value of 0.53 to 0.38. The new structure was subjected iteratively to the same procedure until convergence with an *R*-factor value of 0.21.

In the second stage of refinement, a difference Fourier map with amplitudes equal to $F_{obs} - F_{calc}$ was calculated and displayed on an Evans and Sutherland computer graphics system (PS300) using the FRODO program (Jones, 1978). The highest maxima corresponded to the expected positions of the metal ions. Four additional, large peaks were interpreted as corresponding to the bound mannoside molecules. A Fourier map of the electron density calculated with amplitudes equal to $\div F_{obs} - F_{calc} \div$ was also displayed. It showed that, during the refinement, the side-chain of Leu99 had been incorrectly shifted into the density corresponding to the saccharide. The position of this side-chain was corrected and the metal ions were introduced in their appropriate positions. Least-squares refinement then converged with an *R*-factor of 0.19.

At this stage the model was reviewed extensively and many minor corrections were made. Particular attention was given to the vicinity of the bound saccharide. Careful manual intervention followed by refinement against the X-ray data led to a substantial improvement in the quality of the model and in the clarity of the resultant electron density maps although no significant reduction in the *R*-factor was observed. Several water molecules were identified and were included in the model. At no stage was the saccharide molecule included in the refinement.

An atomic model of methyl α-D-mannopyranoside from the FRODO structure library was fitted into the final difference-electron density map using the positions of the axial and equatorial ring substituents as major criteria. Contacts between the saccharide and the protein were not considered at this stage. It was found that the mannoside could be positioned unambiguously in the experimentally determined electron density. Simple geometric optimization of hydrogen-bond lengths did not affect the fit of the model to the electron density.

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