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Crystallization and crystal manipulation of the *Pterocarpus angolensis* seed lectin

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The Man/Glc-specific legume lectin from the seeds of the African bloodwood tree (*Pterocarpus angolensis*) was crystallized in the presence of the disaccharide ligand Man(α1-3)ManMe. Small crystals initially appeared from a preliminary screen, but proved difficult to reproduce. The initial crystals were used to prepare microseeds, leading to a reproducible crystallization protocol. All attempts to obtain crystals directly of the ligand-free protein or of other carbohydrate complexes failed. However, the Man(α1-3)ManMe co-crystals withstand soaking with ten other carbohydrates known to bind to the lectin. Soaking for 15 min in 100 m*M* carbohydrate typically resulted in complete replacement of Man(α1-3)ManMe by the desired carbohydrate despite the involvement of lattice contacts at the binding site. Transferring the crystals for two weeks in carbohydrate-free artificial mother liquor resulted in the complete removal of the sugar from one of the two monomers in the asymmetric unit. Additional treatment of these crystals with 100 m*M* EDTA for two weeks resulted in removal of the structural calcium and manganese ions, which is accompanied by significant structural rearrangements of the loops that constitute the carbohydrate-binding site.

1. Introduction

Lectins represent a specific class of carbohydrate binding proteins distinct from enzymes and antibodies. Different lectin families are found in a wide range of organisms including viruses, bacteria, plants and animals. Their biological activities are diverse and include roles in innate immunity, bacterial and viral infection, sorting and trafficking of glycoproteins, development and differentiation in animals and defence mechanisms in plants (Taylor & Drickamer, 2003). The lectins from legume plants belong to one of the best studied lectin families (Sharon & Lis, 1990). Members of this family are found mainly in the seeds and sometimes in the vegetative parts of legume plants. Recently, family members were discovered in non-legume plants and in animals (Wang *et al.*, 2003; Velloso *et al.*, 2002). They show strong similarities on the level of their amino-acid sequences and tertiary structures, but encompass a wide range of carbohydrate specificities and quaternary structures (Loris *et al.*, 1998).

The carbohydrate binding activity of the legume lectins crucially depends on the presence of bound calcium and transition metal ions. These are necessary for a correct conformation of the carbohydrate binding site consisting of five loops that are variable in length, sequence and structure (Loris *et al.*, 1998; Sharma & Surolia, 1997). Monosaccharides are bound with rather low affinity (association constants of around 1 m*M*). Often, but not always, they show an enhanced affinity for oligosaccharides. The legume lectins have a highly conserved monomeric fold, but a variable quaternary structure (Bouckaert *et al.*, 1999; Loris, 2002). This quaternary structure variation allows for the selection of different arrangements of monovalent epitopes on a multivalent substrate, introducing a higher level of specificity and high avidity.

The seeds of the African bloodwood tree (*Pterocarpus angolensis*) contain a Man/Glc-specific member of the legume lectins (*P. angolensis* lectin; PAL), which we are studying with a combination of crystallographic and calorimetric techniques (Loris *et al.*, 2003; Loris *et al.*, 2004). Here we report the crystallization of this lectin, and

Table 1
Data collection parameters.

Crystal type	Resolution (Å)	R_{sym}	$\langle I/\sigma(I)\rangle$	Completeness (%)	Redundancy	Beamline
Man(α1-3)ManMe co-crystal	1.75	0.177†	13.1	98.6	6.9	BW7B
Manα Me soak	1.7	0.051	19.2	97.6	3.3	BW7A
Man(α1-2)ManMe soak	1.8	0.068	8.9	99.6	6.8	ID14-1
Man(α1-4)ManMe soak	2.05	0.119	11.7	99.9	5.7	X31
Man(α1-6)ManMe soak	2.05	0.111	9.8	97.3	4.3	X11
Man(α1-3) [Man(α1-6)]ManMe soak	1.85	0.081	17.0	99.7	4.6	BW7A
GlcNAc(β1-2)Man	1.8	0.078	13.2	98.4	3.9	X11
GlcNAc(β1-2)[Man(α1-3)]ManMe soak	1.85	0.079	14.6	97.6	7.9	X11
Glcα Me co-crystal	2.0	0.090	12.4	97.4	4.4	BW7B
Glc(α1-2)Fruβ soak	2.1	0.084	11.0	99.5	3.3	X11
Glc(α1-3)Fruβ soak	2.2	0.149	11.4	95.6	3.7	X11
Ligand removed	1.8	0.080	22.4	99.7	12.4	X11
EDTA-treated	2.3	0.067	12.6	99.5	4.3	X11

† The high R_{sym} value for the Man(α1-3)ManMe co-crystal is due to the inclusion of a low resolution pass that contains mainly weak data and does not indicate poor quality of the data.

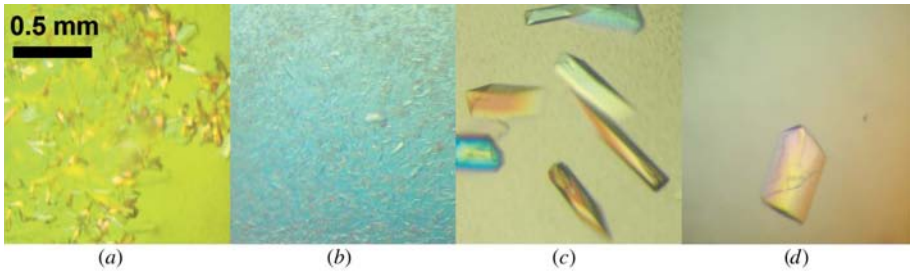

Figure 1
Crystals of PAL. (*a*) Original crystals of PAL–Man(α1-3)ManMe grown directly from the screen. (*b*) Usual outcome of a crystallization experiment of the PAL–Man(α1-3)ManMe complex in absence of seeding. (*c*) Large single crystals of PAL–Man(α1-3)ManMe obtained by microseeding. (*d*) EDTA-treated PAL crystal showing surface cracks. All four panels are shown on the same scale which is indicated in panel (*a*).

crystal manipulation to obtain different carbohydrate complexes as well as the metal-free state of the protein.

2. Material and methods

2.1. Protein purification

P. angolensis seeds were obtained from the Forestry Commission of Zimbabwe. The purification and properties of the *P. angolensis* seed lectin (PAL) will be described elsewhere. Briefly, extracts from uncoated grinded and defatted *P. angolensis* seeds were fractionated with ammonium sulfate. The 30–60% ammonium sulfate fraction was suspended in 100 m*M* NaCl and 10 m*M* phosphate pH 7.4 and then dialysed extensively against the same buffer. After removal of all remaining insoluble material by centrifugation (30 min at 24000*g*), the resulting solution was applied to a fetuin Sepharose column. After extensive washing to remove all unbound protein, the lectin was eluted using 0.3 *M* mannose in 100 m*M* NaCl and 10 m*M* phosphate pH 7.4.

2.2. Crystallization and crystal manipulation

The purified lectin was dialysed against 20 m*M* Tris pH 7.45 and concentrated to approximately 10 mg ml^{-1}. Crystallization conditions were screened using the hanging drop method with the Hampton Research Crystal Screen and Crystal Screen II kits. Both the uncomplexed lectin and complexes with Manα Me, Man(α1-2)ManMe, Man(α1-3)ManMe, Man(α1-6)ManMe, Man(α1-3)[Man(α1-6)]ManMe were tried in the crystallization trial. Crystals were obtained only in the presence of 10 m*M* Man(α1-3)ManMe.

For seeding experiments, 5–15 crystals were transferred to 15 µl precipitant solution (100 m*M* cacodylate pH 6.6, 200 m*M* $CaCl_2$, 18% PEG 8000) and mechanically crushed by pipetting and vortexing. This freshly prepared seed solution was then diluted 10,000–1,000,000-fold and used immediately. In a typical experiment, a drop containing 2 µl protein solution (10 mg ml^{-1} in 20 m*M* Tris pH 7.45) was mixed with 2 µl precipitant solution (100 m*M* cacolylate pH 6.6, 200 m*M* $CaCl_2$, 18% PEG 8000) and 1 µl seeds and equilibrated against 1 ml of precipitant solution in a hanging drop set-up.

Ligand replacement soaks were performed by transferring a PAL–Man(α1-3)ManMe co-crystal to 5 µl of artificial mother liquor [200 m*M* Ca-acetate, 100 m*M* Na-cacodylate pH 6.5 and 18%(*w*/*v*) PEG 8000] enriched with 100 m*M* of the desired carbohydrate ligand for at least 15 min in a hanging drop set-up. The transferred crystals were washed at least once in the ligand-enriched artificial mother liquor to ensure that as little as possible of the original Man(α1-3)ManMe would remain present. Crystals of the ligand-free and metal-free protein were obtained in a similar way, but by using artificial mother liquor without any carbohydrate and a slightly higher PEG concentration (23%) and longer soaking times (at least 2 weeks). Crystals of the metal-free protein were obtained by soaking ligand-free crystals in 100 m*M* cacodylate pH 6.6, 100 m*M* EDTA, 23% PEG 8000 for at least 2 weeks.

2.3. Data collection and structure determination

Crystals were mounted in thin-walled glass capillaries and all X-ray data were collected at room temperature on the EMBL beamlines of the DESY (Hamburg, Germany) and ESRF (Grenoble, France) synchrotrons. The data were processed with *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). Data were integrated with *DENZO*, merged with *SCALEPACK* and converted to structure factor amplitudes using the *CCP*4 program *TRUNCATE* (Collaborative Computational Project, Number 4, 1994). The statistics of the data collections are given in Table 1. Crystal structures were solved by the molecular replacement method using the program *AMoRe* (Navaza, 1994) and refined against a maximum likelihood target using the program *CNS* (Brünger *et al.*, 1998). For display and manual inspection, the program *TURBO* (Roussel & Cambillau, 1989) was used.

3. Results and discussion

3.1. Crystals of the *P. angolensis* lectin-Man(α1-3)ManMe complex

Crystallization conditions for PAL were screened using the Hampton Crystal Screen and Hampton Research Crystal Screen II kits. Crystallization of both the free protein and a number of

carbohydrate complexes were attempted, but crystals were obtained only for the complex with Man(α1-3)ManMe (Fig. 1*a*). These crystals diffracted up to 2.8 Å at EMBL beamline X11 of the DESY synchrotron. They belong to space group $P2_12_12_1$, have unit cell constants a = 56.9, b = 83.0 and c = 122.9 Å and contain a single PAL dimer in their asymmetric unit. Nevertheless, these crystals turned out to be difficult to reproduce.

Most repeat experiments resulted in showers of microcrystals (Fig. 1*b*), and only occasionally did macroscopic crystals appear. Therefore a microseeding protocol was developed (see *Material and methods*), which now leads to the reproducible growth of large single crystals (Fig. 1*c*). Again, this seeding technique worked only for the Man(α1-3)ManMe but generally failed to produce crystals of different ligand complexes or of the ligand-free protein. On a single occasion, however, a few isomorphous crystals of a PAL–GlcαMe were obtained in a single drop, but later on this could not be reproduced.

The crystal structure of the PAL–Man(α1-3)ManMe complex has been described elsewhere (Loris *et al.*, 2004). The asymmetric unit of these crystals contains a PAL dimer. The binding site of one of the two identical subunits (subunit B) is not involved in lattice contacts. However, the Man(α1-3)ManMe molecule present in the binding site of subunit A also interacts with a symmetry mate (Fig. 2). A single hydrogen bond is formed between the ring oxygen O5 of the reducing mannose and the amine group of the side chain of Asn68 of a neighbouring PAL molecule (Fig. 2). Several other hydrogen bonds, water bridges and van der Waals contacts can be observed for two residues that are part of the binding site (Asp136 and Glu221). Removal of the Man(O5)$\cdots$Asn68(NH_2) hydrogen bond probably is sufficient to reduce the strength of this lattice contact as to prevent nucleation and/or growth.

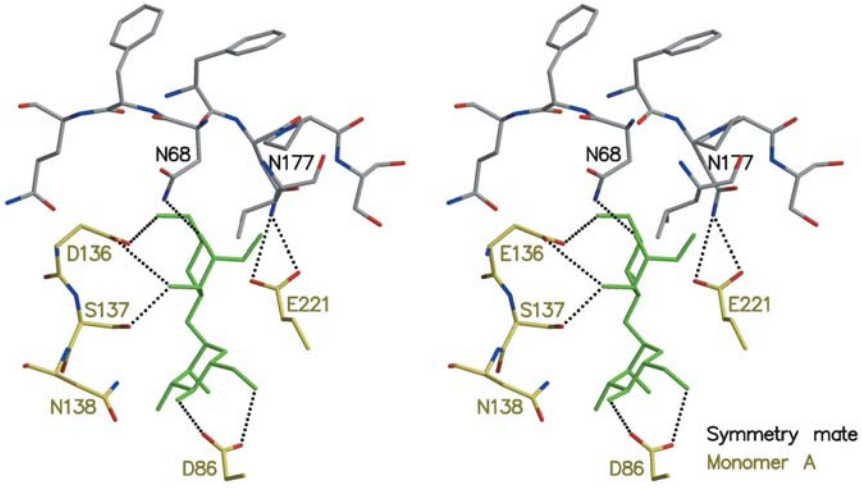

Figure 2
Stereoview of the lattice contact involving Man(α1-3)ManMe bound in binding site A. The disaccharide is shown interacting with some of the key residues of the binding site (Asp86, Glu136, Ser137, Asn138 and Glu221). Lattice contacts are present with two polypeptide stretches around Asn68 and Asn177. Hydrogen bonds are shown as dotted lines.

3.2. Preparation of different carbohydrate complexes by competition soaks

The problems of obtaining crystals of the uncomplexed protein as well as of complexes with other sugars than Man(α1-3)ManMe were frustrating, but could be explained from examination of the crystal structure of the Man(α1-3)ManMe complex. As one of the two binding sites is not involved in lattice contacts, it was rationa-

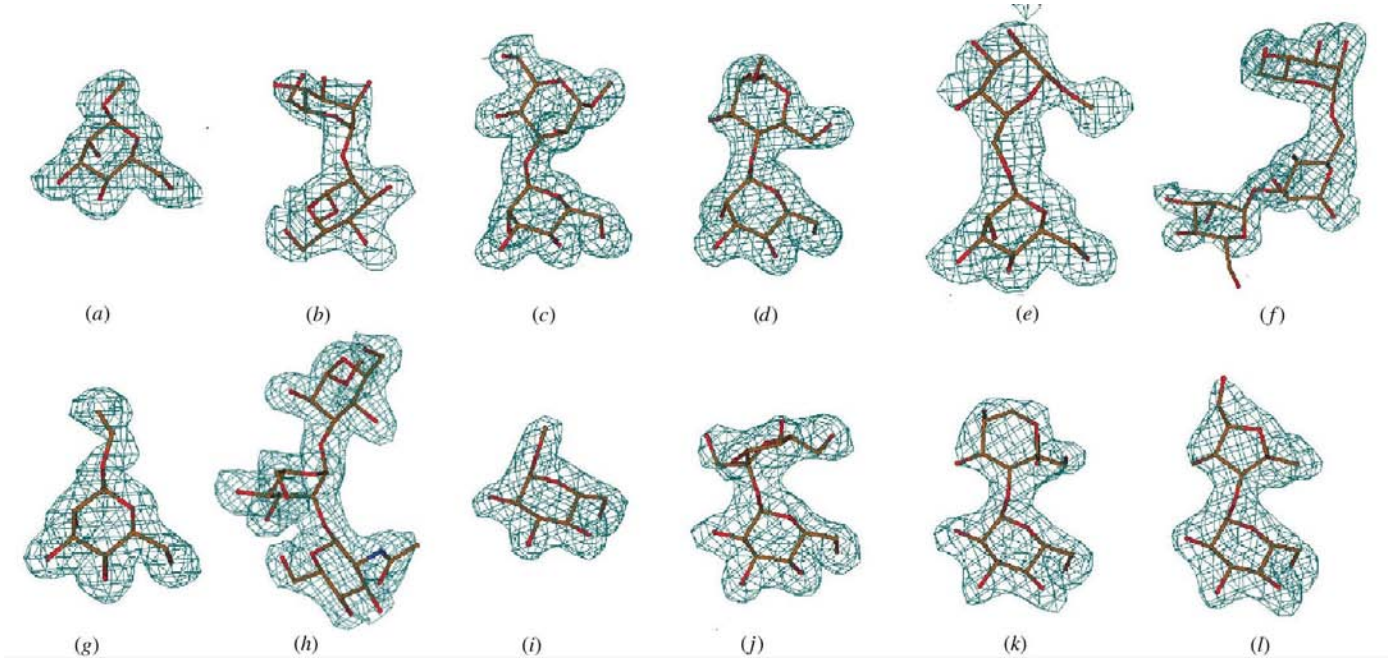

Figure 3
Electron densities of all sugars in the carbohydrate binding sites of the different PAL complexes. (*a*) ManαMe, (*b*) Man(α1-2)ManMe, (*c*) Man(α1-3)ManMe, (*d*) Man(α1-4)ManMe, (*e*) Man(α1-6)ManMe, (*f*)–(*g*) Man(α1-3) [Man(α1-6)]ManMe, (*h*) GlcNAc(β1-2)Man(α1-3)ManMe, (*i*) GlcαMe, (*j*) Glc(α1-2)Fruβ, (*k*) Glc(α1-3)Fru$p\beta$ and (*l*) Glc(α1-3)Fru*f*. In each case, the electron density seen in the binding site of monomer A is shown, except for (*g*) and (*l*) which correspond to monomer B.

lized that a short transfer of the crystals to a solution of artificial mother liquor containing a high concentration of a competing ligand (typically 100 m*M* was used) would replace the sugar bound in site B, while leaving the less accessible sugar in site A undisturbed. In this manner it was possible to produce crystals containing complexes with nine additional carbohydrate ligands: ManαMe, Man(α1-2)ManMe, Man(α1-4)ManMe, Man(α1-6)ManMe, Man(α1-3) [Man(α1-6)]ManMe, GlcNAc(β1-2)ManMe, GlcNAc(β1-2)Man(α1-3)ManMe, sucrose [Glc(α1-2)Fru$p\beta$] and turanose [Glc(α1-3)Fru$p\beta$/Glc(α1-3)Fru$f$]. Each of these crystals still diffracted to high resolution (at least 2.2 Å). Surprisingly, full substitution was obtained in each case in both binding sites (Fig. 3). In all cases but two, the conformations of the sugars are identical in both binding sites. The only two exceptions are the Man(α1-3) [Man(α1-6)]ManMe complex and the turanose complex. In the Man(α1-3) [Man(α1-6)]ManMe complex, only a single mannose residue is visible in binding site A and the rest of the sugar is disordered. The fully ordered conformation adopted by this trisaccharide in binding site B is not accessible in binding site A because of severe steric conflicts with a symmetry-related protein molecule (Loris *et al.*, 2004). In the case of the turanose complex, the fructose in binding site A is in its furanose configuration [Glc(α1-3)Fru$f$] while the one in binding site B has a pyranose configuration [Glc(α1-3)Fru$p\beta$]. In this case the difference between both binding sites is more difficult to explain (Loris *et al.*, 2003).

The soaking procedure apparently does not perturb the crystal lattice. Not only remain the unit cell constants quite identical, so does the lattice interaction involving binding site A. The proteins make identical hydrogen bonds and van der Waals interactions in each case. Interactions between the sugar and the symmetry-related protein vary. In the case of ManαMe, Man(α1-2)ManMe, and GlcαMe there is no direct lattice interaction involving the sugar molecule. For the other sugars, there are ligand-specific van der Waals contacts and/or hydrogen bonds.

3.3. Structure of the lectin with an empty carbohydrate binding site

A crystal lattice is a large co-operative unit, and an interaction that is too weak to support growth may still be strong enough to support the integrity of the crystal once it is formed. With this in mind, we were curious too see if it would also be possible to expel Man(α1-3)ManMe from the binding site rather than replacing it by another sugar. In order to do this, we decided to transfer PAL–Man(α1-3) ManMe co-crystals to mother liquor devoid of sugar. It was rationalized that in order to remove the carbohydrate completely, the de-soaking time probably needs to be longer than the times used in the competition soak experiments and that the de-soaking solution should be refreshed several times. Our crystals did not withstand multiple transfers to fresh mother liquor. They did, however, survive multiple transfers into mother liquor that was slightly enriched in PEG (23% PEG8000 instead of 18%) suggesting that crystal degradation was due to the absence of free protein rather than absence of the sugar.

The crystal structure of the 'ligand-free' crystals was determined by molecular replacement. Preliminary analysis of the electron density maps shows that (i) the overall structure nor the crystal packing have been disturbed by the treatment of the crystals, (ii) the binding site of molecule A still contains a bound Man(α1-3)ManMe molecule at full or almost full occupancy (Fig. 4*a*) and (ii) the binding site of molecule B is apparently free of carbohydrate and contains a number of ordered water molecules (Fig. 4*b*). A full analysis of this structure will be presented elsewhere when the refinement is finished.

3.4. De-metallization of the lectin in the crystal

The above described results prompted us to investigate whether PAL could also be de-metallized in the crystal. De- and re-metallization is expected to introduce significant conformational changes in and around the carbohydrate binding site and therefore are expected to be effected by crystal packing. Such manipulations were nevertheless shown to be possible in crystals of concanavalin A, where the process induces a change in space group (Bouckaert *et al.*, 1996; 2000). As the presence of a bound carbohydrate may stabilize the

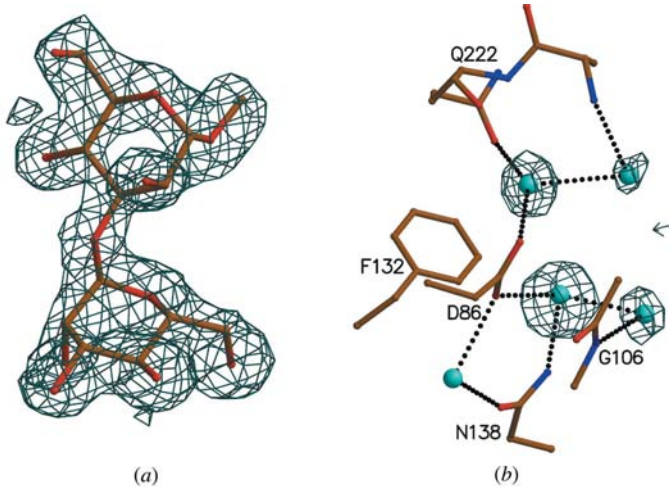

Figure 4
Electron densities observed in the carbohydrate binding sites of the 'ligand-free' structure of PAL. (*a*) Clear electron density is visible for the disaccharide Man(α1-3)ManMe in the binding site of monomer A. (*b*) Ordered water molecules are seen in the carbohydrate binding site of monomer B.

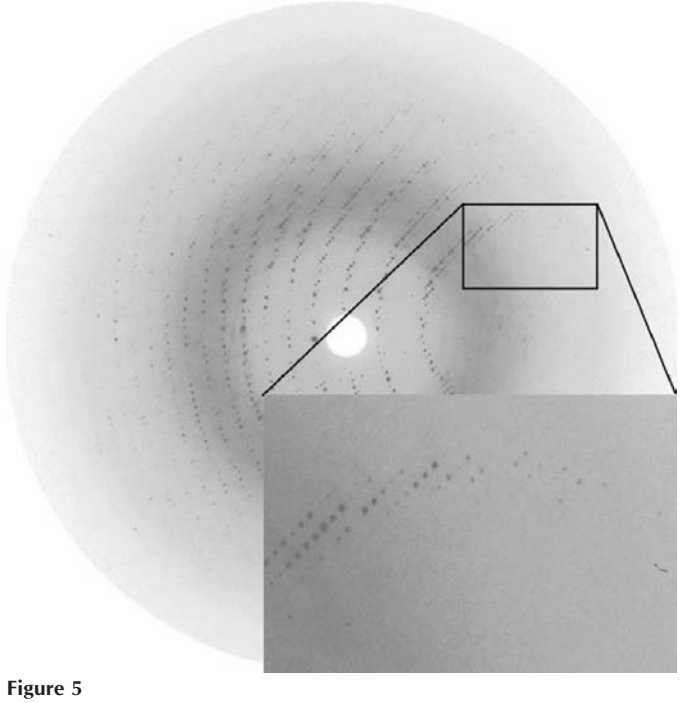

Figure 5
Diffraction pattern of a PAL crystal treated with EDTA for two weeks (see text for experimental details). Despite visual cracks on the surface of the crystal, the diffraction pattern is still clean and diffraction extends to 2.3 Å resolution. The crystal was exposed at room temperature for 8 s on EMBL beamline X11 of the DESY synchrotron (Hamburg, Germany). The rotation angle was 1.0°, the crystal-to-detector distance 150 mm and the wavelength 0.8115 Å.

active metal-bound conformation and prevent the metals from being removed, it was decided to perform these experiments on crystals that previously had undergone the desoaking procedure to remove the bound Man(α1-3)ManMe.

The carbohydrate-depleted crystals were transferred to a 5 µl drop of artificial mother liquor consisting of 23% PEG 8000, 0.1 *M* cacodylate pH 5.6 and 100 m*M* EDTA. Crystals were incubated in a hanging drop set-up for two weeks, after which they showed significant cracks on their surface (Fig. 1*d*). They also had become much more fragile and had to be handled with extreme care as they would easily break into many small pieces. Despite this damage, the crystals produced a high quality diffraction pattern extending to 2.3 Å resolution (Fig. 5).

The structure of the EDTA-treated crystal was determined by molecular replacement. Both calcium and manganese ions were shown to be absent in the two lectin subunits, and conformational changes can be seen in several loops that constitute the metal- and carbohydrate-binding sites. Most surprising about this structure is the observation of electron density for a carbohydrate in the binding site of subunit B. A detailed analysis of this structure will be presented elsewhere when the refinement is finished.

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