Crystallization, neutron data collection, initial structure refinement and analysis of a xyloglucan heptamer bound to an engineered carbohydrate-binding module from xylanase

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Carbohydrate-binding modules (CBMs) are discrete parts of carbohydrate-hydrolyzing enzymes that bind specific types of carbohydrates. Ultra high-resolution X-ray crystallographic studies of CBMs have helped to decipher the basis for specificity in carbohydrate–protein interactions. However, additional studies are needed to better understand which structural determinants confer which carbohydrate-binding properties. To address these issues, neutron crystallographic studies were initiated on one experimentally engineered CBM derived from a xylanase, X-2 L110F, a protein that is able to bind several different plant carbohydrates such as xylan, β -glucan and xyloglucan. This protein evolved from a CBM present in xylanase Xyn10A of *Rhodothermus marinus*. The protein was complexed with a branched xyloglucan hepta-saccharide. Large single crystals of hydrogenous protein ($\sim 1.6\text{ mm}^3$) were grown at room temperature and subjected to H/D exchange. Both neutron and X-ray diffraction data sets were collected to 1.6 Å resolution. Joint neutron and X-ray refinement using *phenix.refine* showed significant density for residues involved in carbohydrate binding and revealed the details of a hydrogen-bonded water network around the binding site. This is the first report of a neutron structure of a CBM and will add to the understanding of protein–carbohydrate binding interactions.

1. Introduction

Bacterial carbohydrate-degrading enzymes have potential biotechnological uses, for example in biorefinery processes. In addition to their catalytic domains, these enzymes often have one or more carbohydrate-binding modules (CBMs). Many CBMs aid the hydrolytic capacity of the catalytic domain through several proposed mechanisms, including immobilizing and concentrating the catalytic domain on the surface of its substrate (Hervé *et al.*, 2010; Tomme *et al.*, 1988). Such CBMs may have importance in industrial enzymes, such as in the production of biofuels and highly refined carbohydrate products. CBMs have also become important as probes for use in the analysis of complex carbohydrates as, similarly to antibodies (Hervé *et al.*, 2011; Pattathil *et al.*, 2010), they can bind to and detect the presence of specific carbohydrate structures directly in biological tissues and processed biomaterials (Filonova *et al.*, 2007; Hervé *et al.*, 2011; Knox, 2012;

Luis *et al.*, 2013; Sandquist *et al.*, 2010; von Schantz, Schagerlöf *et al.*, 2014).

To develop specific probes to solve challenges in research or biotechnological processes, we can not only rely on naturally existing CBMs but can also engineer CBMs to specifically fit particular applications, for instance in terms of ligand selectivity, binding kinetics or temperature optimum. For instance, the first probe that specifically detected only nonfucosylated xyloglucans was developed through genetic engineering of a CBM that originally showed recognition of a broad range of carbohydrates (Gunnarsson *et al.*, 2006). In the future, one can envisage the rational design of carbohydrate-specific probes *in silico* for a particular application. Given the vast complexity of carbohydrates, such efforts are likely to represent daunting tasks without a more detailed knowledge of their structures and binding interactions. Understanding the fundamental interactions of carbohydrates and carbohydrate-binding proteins such as CBMs represents a first step in this direction. Numerous protein–carbohydrate crystal structures have been determined to date, including those involving CBMs (Gilbert *et al.*, 2013) and antibodies (Dingjan *et al.*, 2015). Many of these interactions involve H atoms, and some are mediated by the stacking of carbohydrate rings against hydrophobic or aromatic surfaces, CH– π interactions, hydrogen bonding and other polar interactions. Water molecules are often found to mediate interactions between the protein and its carbohydrate ligand. To learn more about these interactions, we recently determined a number of X-ray crystal structures of an apo and ligand-bound CBM, X-2, and its mutant X-2 L110F (Fig. 1).

X-2 was originally engineered from the carbohydrate-cross-reactive CBM4-2 found in xylanase Xyn10A of the thermophilic bacterium *Rhodothermus marinus* (Nordberg-Karlsson *et al.*, 1997). This protein belongs to CBM family 4 (one of 71 currently recognized CBM families; <http://www.cazy.org>). The development of X-2 was achieved through the use of combinatorial library technology and phage-display selection based on ligand binding (Cicortas Gunnarsson *et al.*, 2007). X-2 is specific for xylan (and its synthetic mimic oligoxylose). Further mutagenesis identified the single mutant X-2 L110F (von Schantz *et al.*, 2012) that, similar to the wild-type module, is able to bind multiple carbohydrates including not only xylan but also β -glucan and xyloglucan (von Schantz, Håkansson *et al.*, 2014; von Schantz *et al.*, 2012). In both CBM variants residue 110 stacks against the ligand in the cavity of the CBM ligand-binding site (Fig. 1). Importantly, X-2 L110F (and its immediate predecessor X-2), in contrast to other similar modules (including CBM4-2), crystallizes sufficiently well to allow the development of CBM–ligand complex crystals (Gullfot *et al.*, 2010; von Schantz *et al.*, 2012; von Schantz, Håkansson *et al.*, 2014).

H atoms are often critical for protein–carbohydrate interactions. Unfortunately, these atoms are often not readily visible in structures determined by X-ray crystallography, even at ultra high resolution. Owing to this, their positions and their involvement in binding have to be indirectly proposed or inferred. At 1.0 Å resolution we were able to observe unassigned $F_o - F_c$ density corresponding to some H atoms in

the protein, in the ligand and on a few well ordered water molecules. However, the data were not strong enough to support the modelling of explicit positions for H atoms (von Schantz, Håkansson *et al.*, 2014; von Schantz *et al.*, 2012). From these previous ultra high-resolution X-ray crystallographic studies, many details of carbohydrate binding were observed. To address the lack of detailed knowledge of H-atom positions, water involvement, hydrogen bonding and polar and nonpolar interactions between carbohydrates and X-2, neutron studies were pursued.

Neutron crystallography is a unique and useful tool for detailed studies of enzyme mechanisms, ligand/drug-binding interactions and the role of solvent in protein structure and function. Neutrons scatter approximately equally well from C, N, O and H atoms, compared with X-rays, which scatter from clouds of electrons around the nucleus with a magnitude that depends on the number of electrons. Neutrons scatter from atomic nuclei and, with a few exceptions, most elements scatter neutrons with similar magnitude. Hydrogen has a negative neutron scattering length and contributes to a large, incoherent background in neutron diffraction experiments (Langan & Chen, 2013). As such, it is common practice to replace hydrogen with its isotope ^2H (or deuterium; D), as it has a small incoherent cross-section and a large positive scattering length comparable to those of C, N and O atoms. The two most common approaches to H/D exchange are either to express the protein of interest in cells under fully deuterated conditions or to exchange labile H in a protein with D

Figure 1

Ligand-binding cleft of X-2 L110F in complex with XXXG as determined by ultra high-resolution X-ray crystallography. The stick diagram of XXXG is labelled and letters define each X and G as defined by the xyloglucan nomenclature (Fry *et al.*, 1993). Monomer numbers are labelled. The X-ray coordinates shown are from PDB entry 4bj0 (von Schantz, Håkansson *et al.*, 2014).

Table 1

Data collection and processing.

Values in parentheses are for the outer shell.

Method	X-ray	Neutron
Diffraction source	MAX IV lab synchrotron	FRM-II nuclear research reactor
Wavelength (Å)	1.04	2.68
Temperature (K)	293	293
Detector	MAR 165 CCD	Cylindrical neutron image-plate detector
Rotation range per image (°)	1	0.4
No. of images	135	290
Exposure time per image	30 s	30–45 min
Space group	$P2_12_12$	$P2_12_12$
a, b, c (Å)	44.9, 49.7, 72.7	44.9, 49.7, 72.7
α, β, γ (°)	90, 90, 90	90, 90, 90
Resolution range (Å)	29.3–1.60 (1.70–1.60)	45.0–1.60 (1.65–1.60)
$R_{\text{merge}}^{\dagger}$ (%)	4.5 (25.5)	12.7 (50.2)
No. of unique reflections	21610 (3389)	20520 (1485)
Completeness (%)	97.7 (97.2)	91.1 (80.3)
Mean $I/\sigma(I)$	28.6 (7.8)	6.6 (1.8)
$R_{\text{work}}/R_{\text{free}}^{\ddagger}$ (%)	16.3/20.3	22.7/25.6

$\dagger R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$. $\ddagger R_{\text{work}} = \frac{\sum_{hkl} ||F_{\text{obs}}| - |F_{\text{calc}}||}{\sum_{hkl} |F_{\text{obs}}|} \times 100$. R_{free} is calculated in the same way as R_{work} but using 5% of the reflections that were excluded from refinement.

(Cuypers *et al.*, 2013; Chen *et al.*, 2012). In contrast to X-rays, neutron diffraction of deuterated protein crystals can definitively reveal the positions of H/D atoms, even at medium resolution. The need for very large crystals ($>1 \text{ mm}^3$) and lengthy data-collection processes deters many researchers from attempting to obtain neutron crystallographic data (Blakeley *et al.*, 2008; Langan & Chen, 2013).

Here, we describe the details of the preparation of large X-2 L110F crystals, H/D exchange, room-temperature X-ray and neutron data collection and joint X-ray/neutron refinement using *phenix.refine*. Model refinement and detailed analysis is not complete, but we report some of the interesting preliminary details that are apparent in the structure. Our work also shows that it is possible to obtain large H/D-exchanged crystals in a few months from only 7 mg of starting protein material and that neutron diffraction data collection to high resolution (1.6 Å) is possible in ~ 10 d using a monochromatic reactor-based neutron source. This is also the first neutron structure of a CBM and as such adds to our growing knowledge of sugar-binding interactions as observed using neutrons.

2. Materials and methods

2.1. Macromolecule production

His-tagged X-2 L110F (molecular weight 19 kDa), genetically engineered from CBM4-2 of the Xyn10A xylanase from *R. marinus*, was produced using *Escherichia coli* T7 Express (New England Biolabs, Ipswich, England). The folded protein was purified from the soluble fraction obtained after sonication of bacterial cells by metal-ion affinity chromatography using Ni-NTA (Qiagen, Hilden, Germany) as described previously (von Schantz *et al.*, 2012).

2.2. Crystallization

The protein solution (13 mg ml^{-1}) was mixed with a branched xyloglucan heptasaccharide [XXXG, made up of a linear $\beta(1 \rightarrow 4)$ -linked glucan tetrasaccharide backbone that is decorated with α -linked xylopyranosyl residues on its first three saccharide units at C-6; Fig. 1; CAS Registry No. 121591-98-8; Megazymes, Bray, Ireland; Fry *et al.*, 1993; 50 mM final concentration] and the crystallization additive spermine tetrachloride (20 mM final concentration). The mixture was incubated at room temperature for 1 h and then spun down at $13\,000 \text{ rev min}^{-1}$ for 30 min. The supernatant was carefully removed and used to set up crystallization trays. Initially, drop volumes were scaled up to 30 and 50 μl in 24-well Linbro plates using microbridges and glass rod supports from Hampton Research. Large single crystals grew in several of these and these conditions were replicated in even larger volumes.

Large drops of 110 μl total volume were made by mixing the protein:ligand solution with precipitant solution (25% PEG 1500, 0.1 M MES pH 5.5) and additive in a 50:50:10 μl ratio. These were set up in nine-well glass plates in a Hampton Research Sandwich Box setup. The reservoir solution contained a higher PEG concentration to facilitate vapour diffusion in the large trays (the reservoir consisted of 25 ml 28% PEG 1500, 0.1 M MES pH 5.5). Crystals appeared in ~ 4 weeks and grew to maximum size after eight weeks (Fig. 2). For all crystallization screening trials, scaling up and setting up of the final large drops that produced neutron-sized crystals, only 7 mg of protein in total was used. This is significant as we typically use 100–300 mg of protein to screen and ultimately produce crystals sufficiently large for neutron data collection.

Single crystals were mounted and subjected to H/D exchange by placing them in a quartz capillary, wicking them well and introducing a perdeuterated liquid plug on either side of the crystal prior to sealing the ends with beeswax (Fig. 2). The crystals were H/D-exchanged for ~ 2 months prior to both room-temperature X-ray and neutron data collection.

2.3. Data collection and processing

Large, single H/D-exchanged crystals of X-2 L100F were selected for both room-temperature X-ray and neutron data

Figure 2
Photograph of a large, single crystal of X-2 L110F in complex with XXXG mounted in a quartz capillary undergoing H/D exchange (dimensions $\sim 1.3 \times 1.2 \times 1.0 \text{ mm}$; 1.6 mm^3) and a representative monochromatic neutron diffraction image collected using BIODIFF at FRM-II.

collection (Fig. 2). Neutron diffraction data were collected using the BIODIFF instrument at the FRM-II reactor source (TUM, Germany) and the crystals were shown to diffract very well to at least 1.6 Å resolution (Table 1).

BIODIFF is a monochromatic diffractometer equipped with a cylindrical neutron image-plate detector (Niimura *et al.*, 1994; Maatel, Voreppe, France). The cylindrical area detector of BIODIFF provides a large coverage of reciprocal space, thereby allowing a large number of Bragg reflections to be recorded simultaneously (Fig. 2). The readout resolution of the image-plate scanner was set to 250 µm. The data set was collected at room temperature using a pyrolytic graphite monochromator (PG002) set to a wavelength of 2.68 Å. A total of 290 images were recorded with a rotation range of 0.4° per frame and an exposure time of between 30 and 45 min per image. The intensities of the reflections were integrated and scaled with *DENZO* (v.1.96.2) and *SCALEPACK* (v.1.98.2) (Otwinowski & Minor, 1997).

X-ray data were collected from a smaller H/D-exchanged crystal at room temperature on the I911-2 Cassiopeia beam-

line at MAX IV lab, Lund, Sweden. I911-2 is a fixed-wavelength station (1.04 Å) equipped with a 165 mm MAR Mosaic CCD detector. Diffraction data were collected to 1.60 Å resolution as 135 images with a 1° oscillation step and 30 s exposure per image. Data processing and scaling were performed using *XDS* and *XSCALE* (Kabsch, 2010). The data-set statistics are shown in Table 1. The refined room-temperature X-ray model, including ligand, Ca²⁺ and solvent, was used for the initial joint refinement. *phenix.refine* alone and joint mode were used for the X-ray alone and the joint neutron and X-ray refinement, respectively (Adams *et al.*, 2002, 2009). The starting model for the X-ray refinement alone was PDB entry 4bj0 (von Schantz, Håkansson *et al.*, 2014). The joint refinement is in progress and R_{work} and R_{free} are reported in Table 1. Iterative refinement involved several rounds of maximum-likelihood-based refinement of individual coordinates, individual B factors and occupancies against both neutron and X-ray data. After every round of refinement, the model was manually checked against $F_o - F_c$ and $2F_o - F_c$ positive nuclear density maps in *Coot*. This facilitates the proper placement of waters and certain D atoms.

3. Results and discussion

3.1. Nuclear density for the protein and ligand

The ligand appears to have undergone very good H/D exchange, with most exchanged D atoms clearly visible in $2F_o - F_c$ nuclear density maps. Several of the sugars in XXXG are well ordered and visible, specifically residue numbers BGC1169, BGC1170 and half of BGC1171, and the branched xylose molecules YYS1173 and half of YYS1175. Two of the saccharide units were totally disordered, with very little interpretable density visible at the 1.5σ contour level:

Figure 3
Representative nuclear density maps of several key residues after several rounds of *PHENIX* joint refinement and manual checking in *Coot*. The H/D atoms are shown in white; $2F_o - F_c$ nuclear density is shown as a blue mesh and is contoured at 1.5σ. (a) His146 and the hydrogen bond to YYS1173, (b) Arg115 as a hydrogen-bond donor to YYS1173, (c) hydrogen-bonded water network adjacent to the ligand-binding cleft.

BGC1172 and XYS1174. This is in contrast to the high-resolution X-ray crystal structure, in which there is excellent density for the entire ligand. This may be owing to several factors, the most pertinent being that the current data were collected at room temperature with possibly lower occupancy of the ligand, and most likely the significantly lower resolution (1.6 *versus* 1.0 Å). However, for the well ordered parts of the ligand it is possible to observe the atomic details of hydrogen-bonding interactions between XXXG and the ligand-binding cleft of X-2 L110F. Here, we report the initial structural analysis. Further refinement and detailed analysis is ongoing and will be reported at a later date.

3.2. Ligand-binding interactions

There are several hydrogen-bonding interactions between the protein and ligand, and the details are visible in the $2F_o - F_c$ nuclear density maps (Figs. 3*a*, 3*b* and 3*c*). The neutron data confirm our understanding of the Phe110–BGC1171–Phe69 stacking interaction that was first observed in the X-ray crystal structure. However, there are extensive hydrogen-bonding and water-mediated interactions in XXXG ligand binding that can only be seen by neutron crystallography. A few of these are shown in Figs. 3(*a*), 3(*b*) and 3(*c*).

His146 was observed as neutral, with the imidazole ring engaged in a hydrogen bond as a donor to the O4 of XYS1175 and an acceptor from DO2 of XYS1173 (Fig. 3*a*). Arg115 is very well exchanged and shows strong scattering from the large number of D atoms in the side chain. Arg115 is a hydrogen-bond donor to two sugars in XXXG: O2 of XYS1173 and O6 of BGC1170 (Fig. 3*b*).

Adjacent to the bound ligand there is a series of well ordered, hydrogen-bonded water molecules that form a tight network between residues Phe110, Asn107, Thr104 and Glu112 (Fig. 3*c*). The waters S25, S26 and S29 are connected to the protein and to each other through strong hydrogen bonds (distances as indicated in Fig. 3*c*). Even though this network is located next to the ligand-binding pocket, there is only one weak hydrogen bond to the ligand between S25 and DB on O2 of BGC1171 (distance of ~ 3.5 Å). The water network coordinates a series of hydrogen bonds to the above-mentioned amino-acid side chains. These interactions culminate in these residues being appropriately positioned for direct hydrogen bonds to the sugar.

There are several other interactions that are still preliminary and are not shown here. The nuclear density for the Tyr149 side chain indicates that the OD group acts as a hydrogen-bond donor to O3 of XYS1173 and a hydrogen-bond acceptor from Ser102. The Thr74 side-chain DG1 is a hydrogen-bond donor to Gln72 and a hydrogen-bond acceptor from the XYS1175 DO3 atom.

It is noteworthy that the nuclear density clearly shows positive $2F_o - F_c$ peaks corresponding to H/D atoms in both X-2 L110F and the XXXG ligand. In contrast to the readily visible density in nuclear maps, the corresponding maps for the ultra high-resolution X-ray data show no discernable peaks. Of particular interest are residues Tyr149 and His146.

For neither of these is there any corresponding $F_o - F_c$ electron density at 1.0σ for H atoms in key positions, even at 1.0 Å resolution.

4. Conclusions

Our initial analysis of the neutron structure of X-2 L110F in complex with the branched polysaccharide XXXG reveals numerous details of hydrogen-bonding interactions and shows the interactions of a well ordered solvent network with the protein. A common criticism of neutron protein crystallography is that a large amount of protein is required to produce very large crystals and that it takes too long. In the current study, we show that it is possible to grow large crystals (~ 1.6 mm³) from only 7 mg of starting material. This can be achieved by scaling up the volume of well defined conditions, with minor adjustments, and can be performed in a reasonable amount of time (~ 3 –4 months). In addition, data collection at BIODIFF yielded a $\sim 91\%$ complete data set to 1.6 Å resolution in ~ 10 d. This demonstrates that it is feasible to pursue high-resolution neutron structures in a reasonable amount of time using relatively low amounts of protein. Based on these initial results, we envisage that neutron crystallographic studies of other members of the large, diverse set of CBMs will add substantial value to our understanding of the diverse interactions between these modules and their target carbohydrates. It is expected that insight into these interactions extending beyond that already achieved by analysis of the large number of existing structures of CBM–ligand complexes established using X-ray crystallography will allow us to further develop CBMs as tools in bioscience and biorefinery practice.

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