STRUCTURAL ANALYSIS OF SEMI-SPECIFIC OLIGOSACCHARIDE RECOGNITION BY A CELLULOSE-BINDING PROTEIN OF THERMOTOGA MARITIMA REVEALS ADAPTATIONS FOR FUNCTIONAL DIVERSIFICATION OF THE OLIGOPEPTIDE PERIPLASMIC-BINDING PROTEIN FOLD

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Running head: T. maritima CBP Structure

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Periplasmic-binding proteins (PBPs) constitute a protein superfamily that binds a wide variety of ligands. In prokaryotes, PBPs function as receptors for ATP-binding cassette or tripartite ATP-independent transporters and chemotaxis systems. In many instances PBPs bind their cognate ligands with exquisite specificity, distinguishing, for example, between sugar epimers or structurally similar anions. By contrast, oligopeptide-binding proteins (OpBPs) bind their ligands through interactions with the peptide backbone, but do not distinguish between different side-chains. The extremophile Thermotoga maritima possesses a remarkable array of carbohydrate-processing metabolic systems, including the hydrolysis of cellulosic polymers. Here we present the crystal structure of a T. maritima cellobiose-binding protein (tm0031), tmCBP, which is homologous to OpBPs. tmCBP binds a variety of lengths of β(1→4) linked glucose oligomers, ranging from two rings (cellobiose), to five (cellopentaose). The structure reveals that binding is semi-specific. The disaccharide at the non-reducing end binds specifically; the other rings are located in a large, solvent-filled groove where the reducing end makes several contacts with the protein, thereby imposing an upper limit of the oligosaccharides that are recognized. Semi-specific recognition, in which a molecular class, rather than individual species, is selected provides an efficient solution for the uptake of complex mixtures.

Periplasmic binding proteins (PBPs) are soluble ligand-binding components of ATP-binding cassette (1) or tri-partite ATP-independent transporters (2), and chemotaxis systems (3). Members of the PBP superfamily mediate uptake of many primary metabolites in bacteria such as amino acids (4), carbohydrates (5), ions (6) and polyamines (7). Ligands are subsequently transported across the membrane by accessory proteins which couple transport to ATP-hydrolysis (1) or H+/M⁺ motive force (2).

PBPs form a structural superfamily which is classified into three groups according to the ordering of β-strands in the core of the two domains that characterize this protein fold (group I/ribose-binding protein fold (8), group II/maltose-binding protein fold (9), and group III/Vitamin B12-binding protein fold (10)) (11). Each of the domains forms a three-layered α/β/α fold, divided by a two or three β-strand hinge. The ligand-binding site is situated between the two domains. The interactions of these binding surfaces are contributed largely by amino acid side-chains located in the loops connecting the alternating β-strands and α-helices of each domain.

PBPs undergo a hinge-bending motion upon addition of ligand allowing the two interfaces, which are solvent-exposed in the absence of ligand, to completely envelop the ligand in the closed form, thus mimicking the...
desolvated environment of a protein core (8,12-14). The resulting binding sites typically form extensive specific interactions with their cognate ligands, enabling highly specific discrimination between anomic or epimeric carbohydrates (15), differently sized carbohydrates (15,16) or chemically similar anions (17). By contrast group II di/oligopeptide-binding proteins (OpBPs) which bind peptides that range from two to nine amino acids (18,19) are semi-specific and show little discrimination between side-chains of bound peptides (20). In these proteins recognition is mediated primarily through hydrogen bonds to the peptide main-chain atoms, whereas the side-chains are placed in non-specific pockets that accommodate both polar and non-polar amino acid side-chains through interactions with differentially ordered water molecules (19-21). Any of the twenty amino acids are bound by OpBPs with little sequence dependent variations in dissociation constants ($K_d$) (19).

The hyperthermophilic bacterium *Thermotoga maritima* MSB8 is an extensively studied model for extremophiles and a potential source of many enzymes and metabolic pathways of biotechnological interest (22). Its genomic sequence reveals a remarkable array of diverse carbohydrate metabolism pathways (23,24). Among these are systems for the uptake and processing of a variety of β-linked oligosaccharides. Metabolite transport is encoded by a large number of ABC-transporters and related proteins (23). It has been difficult to assign function reliably to the PBP components of these, because of the distant sequence relationships between *T. maritima* and biochemically characterized model organisms. A combination of *in vitro* binding studies (25) and transcriptional expression profiling of cultures grown in the presence of various carbohydrate substrates (26,27) has shown that five of twelve PBPs that are homologous to OpBPs, bind not oligopeptides, but various carbohydrates. The X-ray crystal structure of one of these, the β-1,4-mannobiose-binding protein (PDB code 1VR5), was recently solved in the open conformation in the absence of its cognate ligand (structure determined and deposited by the Joint Center for Structural Genomics (28), but otherwise undocumented). As expected, based on amino acid similarity, the overall structure of 1VR5 is similar to known OpBPs. In the absence of a ligand the mode of ligand binding in this protein remains unknown. These observations raise the question of how the OpBP fold has adapted to recognize sugars, and what, if any, specificity is encoded.

To answer these questions we undertook structural studies of tm0031, which was previously shown to bind the β-linked disaccharides cellobiose and laminaribiose (25). In additional ligand-binding studies, we establish that this cellobiose binding protein (tmCBP) binds not only disaccharides but also oligosaccharides up to five sugar monomers in length. X-ray crystallographic structural analysis reveals that only the first two sugar rings are recognized specifically though hydrogen bonds and van der Waals interactions with the protein. Additional rings are placed into a large solvent filled cavity in the interior of the binding pocket where there is little specific recognition of the ligand polar and non-polar groups, in a manner similar to the recognition of peptides in the OpBP fold (19). Comparison of superimposed apo-1VR5 with ligand-bound tmCBP suggests that the former possesses similar adaptations to bind its cognate ligand(s). Consequently, we postulate that this binding pocket imposes limited specificity between oligosaccharides other than through specific recognition of the first two sugar rings and the sterics of the cavity that imposes length constraints. This mode of sugar binding has not been observed in other carbohydrate-binding PBPs and represents a novel mechanism of carbohydrate recognition in this protein superfamily.

**EXPERIMENTAL PROCEDURES**

Over-expression and Purification- The tmCBP plasmid was a gift from the laboratory of K. Noll. Protein was expressed and purified as described (25).

Circular Dichroism- Circular dichroism (CD) measurements were carried out on a Jasco CD spectrophotometer. Thermal denaturations were determined by measuring the CD signal at 222 nm (1 cm path length) as a function of temperature using 1.0 µM protein (10 mM Tris-HCl pH 7.8,
150 mM NaCl). In the absence of the chemical denaturant guanidinium chloride (GdCl) tmCBP is too stable to exhibit temperature-induced denaturation. To determine the apparent thermal transition midpoint \( \text{app}T_m \) in the absence of GdCl a series of thermal melts in the presence of decreasing amounts of GdCl was used to extrapolate to 0 M GdCl (16). Protein samples were incubated for 15 minutes prior to collecting data. Each measurement includes a 3-second averaging time for data collection and a 60 second equilibration period at each temperature. Data were fit to a two-state model to determine the \( \text{app}T_m \) values (29).

**Crystallization and Data Collection** - Crystals of tmCBP were grown in the presence of 1 mM ligand. The cellopentaose complex was crystallized by hanging drop vapor diffusion in drops containing 2 µl of the protein solution mixed with 2 µl of 0.1 M NaCacodylate pH 6.3-6.5, 18-24 % (w/v) PEG 4000 and 0.2 M magnesium acetate. The cellobiose complex was crystallized by hanging drop vapor diffusion in drops containing 2 µl of the protein solution mixed with 2 µl of 0.1 M MES pH 6.5, 0.2 M ammonium sulfate, 30 % (w/v) PEG MME 5000. Diffraction-quality crystals typically grew within one week at 17 °C. Crystals grown in the presence of cellohexaose resulted in a cellopentaose complex (data not shown) due to the low purity of the oligosaccharides (~95% pure) and the presence of contaminating cellopentaose. The crystals of the cellobiose and cellopentaose complexes diffract to 1.50 Å resolution and belong to the P4(1)2(1)2 space group (\( a/b=107.3 \) Å, \( c=118.2 \) Å) and the P2(1) space group (\( a=62.1 \) Å, \( b=101.5 \) Å, \( c=108.3 \) Å, \( \beta=94.1 ° \)) respectively (Table 1). Crystals were transferred stepwise to the precipitant solution containing 10 % ethylene glycol for cryoprotection, mounted in a nylon loop, and flash frozen in liquid nitrogen. All data were collected at 100K at the SER-CAT 22ID beam line at the Advanced Photon Source. Diffraction data were scaled and integrated using XDS (30).

**Structure Determination Methods, Model Building and Refinement** - The structure of the cellobiose complex was determined to a resolution of 1.50 Å by molecular replacement using the N- and C-terminal domains of the β-1,4-mannobiose binding protein (PDB code 1VR5) separately as search models in the program PHASER (31); the individual domains rather than the entire molecule were used to allow for changes in the inter-domain hinge-bending angles. One protein molecule bound with cellobiose in the binding pocket was found in the asymmetric unit and was refined to \( R_{cryst} \) and \( R_{free} \) values of 19.2 % and 21.6% respectively (Table 1). The final model for the cellobiose complex includes two intact monomers, two cellobiose molecules, and 500 water molecules. The structure of cellopentaose complex was determined to a resolution of 1.5 Å by molecular replacement using the cellobiose-bound form as a search model in the program PHASER (31). Two molecules bound with cellopentaose in the binding pocket were found in the asymmetric unit and was refined to \( R_{cryst} \) and \( R_{free} \) values of 20.4 % and 22.5 % respectively (Table 1). The final model for the cellopentaose complex includes two intact monomers, two cellopentaose molecules, and 887 water molecules. The cellobiose and cellopentaose complexes are in nearly identical conformations, with RMSD of 0.21 Å for alignment of all atoms in both crystal forms (excluding waters and ligand). Manual model building was carried out in the programs O and COOT and refined using REFMAC5 (32-34). The models exhibits good stereochemistry as determined by PROCHECK and MolProbity; final data collection and refinement statistics are listed in Table 1 (35,36). No residues are Ramachandran outliers.

PDB coordinates and structure factors of cellobiose and cellopentaose complexes have been deposited in the RCSB Protein Data Bank under the accession codes 2O7I and 3I5O respectively.

**RESULTS**

**Thermal Stability and Ligand Binding** - The stability of the *T. maritima* cellobiose-binding protein (tmCBP) was determined by thermal denaturation using CD. In the absence of the chemical denaturant GdCl, no significant change in the CD signal (up to 100 °C) could be observed as a function of temperature (data not shown), and all subsequent measurements were carried out in the presence of GdCl (Figure 1). Melting curves were found to fit a two-state model (29,37); an
apparent thermal transition midpoint ($^{app}T_m$) of 108°C in the absence of GdCl was determined by linear extrapolation of a series of melting point determinations carried out at different concentrations of GdCl (15) (Figure 1).

Ligand-mediated shifts in $^{app}T_m$ in the presence of 2M GdCl, were used to assess the binding of various cellulose hydrolysates ranging from 2-sugar rings (celllobiose) to 6-sugar rings (cellohexaose). Protein stability increases non-linearly with oligosaccharide length (Figure 1), clearly delineating a minimal recognition unit and a maximal length, where no more contribution is made to protein stability. The disaccharide celllobiose binds relatively weakly, as evidenced by a small shift in $^{app}T_m$ and a change in cooperativity; a significant increase is observed with the trisaccharide, cellotriose; beyond five sugar units, there is no significant increase in stability. These results indicate that the first three sugar rings make the greatest contribution towards the free energy of ligand binding, although the binding pocket binds five optimally, but not necessarily maximally.

Overall Three-Dimensional Structure- The protein was co-crystallized with celllobiose and cellopentaose. In these two complexes, the tmCBP structure adopts the closed conformation and has the α/β fold that is characteristic of OpBPs and shares the highest structural homology with Salmonella typhimurium OpBP (stOpBP, PDB code 1JEV) ($C_\alpha$ RMSD of 2.8 Å) (38,39) even though the amino acid sequence similarity among them is low (18 % identity). Alignment of the individual domains of the 1VR5 search model reveals $C_\alpha$ RMSD values of 1.3 and 1.1 Å for the N- and C-terminal domains respectively. Like stOpBP, tmCBP is a three-domain protein with the carbohydrate-binding site located in a deep groove at the domain interface (Figure 2). The topology of β-strands in domains I and III belongs to the group II PBP sub-family.

Oligosaccharide Binding Pocket- The cellopentaose complex defines a ligand-binding site that spans the entire interface formed by domains I, II and III (Figure 2). The minimal celllobiose-binding site is found at one extreme of this groove, at the interface between domains I and III (Figure 2). As is observed in other, highly specific carbohydrate-binding PBPs, the two sugar rings are bound by an extensive network of hydrogen bonds that largely satisfy the dual potential of the hydroxyls (i.e. both the proton donor, and the oxygen acceptor). A total of 19 hydrogen bonds are made with the celllobiose. Of these, 15 hydrogen bonds are formed by a network of polar interactions with protein side-chain, main-chain, and specifically bound water molecules mediating recognition of the hydroxyls in the two sugars (Figure 3). Two polar amino acids (N216, D383) contribute four of the ten hydrogen bonds, three are made by the protein main-chain (A14O, G13O, F234N), three are made with aromatic binding pocket residues, through either polar or aromatic hydrogen bonds. The remaining hydrogen-bonding potential is satisfied by water molecules (Figure 3). The oxygen from the hemiacetal of the second sugar ring (B) forms a hydrogen bond with two water molecules, one of which also interacts with the protein; the hemiacetal oxygen from the first ring (A) forms an intramolecular hydrogen bond with the C3 hydroxyl of the second ring (B) (Figure 3). Three additional tryptophan residues (W381, W384 and W536) form extensive van der Waals contacts with both rings of the celllobiose.

Adjacent to this minimal disaccharide binding site is a large cavity that is shielded from bulk solvent, but completely filled with well-defined water molecules (Figures 3 and 4). This region lacks the aromatic and polar amino acids lining the disaccharide-binding site. In the cellopentaose-bound form the extra three rings are placed into this cavity (Figures 3 and 4). However, unlike the celllobiose-bound form there is little specific recognition and of the sugar rings (Figure 3). These additional rings have the potential to form a total of 26 hydrogen bonds through their hydroxyls and hemiacetal oxygens, but only four hydrogen bonds are made with the protein. One is formed with the C6 hydroxyl of the third ring (C) and the carboxylate of D262; three other hydrogen bonds are formed by the main chain of three glycine residues (G101, G103 and G104), at the tip of the cavity (the cap), with the hydroxyls of the fifth ring (E). Ordered water molecules satisfy the remaining hydrogen-bonding potential of the three sugar rings (Figure 3).
Ligand-binding Cavity Water Structure- 80 water molecules fill the cavity of the cellobiose complex, of which 66 form a primary solvation shell that directly contact the protein or carbohydrate polar groups (Figure 4). A further 13 form a secondary solvation shell that are bound only by water molecules in the primary solvation shell. The remaining one ordered water molecule is hydrogen-bonded to the secondary shell (tertiary solvation shell). 75 waters are retained in the cavity of cellopentaose complex, of which 67 are in the primary solvation shell and eight in secondary solvation shell (Figure 4). No ordered tertiary shell water molecules are found in the cellopentaose complex; the single tertiary shell water being displaced by the extra rings of the carbohydrate. Many of the water molecules in the primary solvation shell form an approximately tetrahedral hydrogen-bonding network, thereby constructing an ice-like structure within the cavity (Figure 4). The secondary solvation shell forms between one to four hydrogen bonds, with the remaining hydrogen-bonding potential being satisfied by interactions with bulk water.

The positions of the ordered water molecules change little upon ligand binding. Binding of the pentasaccharide displaces one of the 13 waters in the secondary shell and the single tertiary shell water being displaced by the extra rings of the carbohydrate. Many of the water molecules in the primary solvation shell form an approximately tetrahedral hydrogen-bonding network, thereby constructing an ice-like structure within the cavity (Figure 4). The secondary solvation shell forms between one to four hydrogen bonds, with the remaining hydrogen-bonding potential being satisfied by interactions with bulk water.

The remaining five amino acids differ in only two positions: L233 which forms van der Waals interactions with the O3 hydroxyl is replaced with a phenylalanine in 1VR5; G232 which forms van der Waals interactions with the O2 hydroxyl, is replaced with an asparagine in 1VR5. The latter change is accompanied by a backbone movement in 1VR5, which prevents a steric clash that would occur with the axial C2 hydroxyl of mannose.

The remainder of the ligand-binding cavity, which in tmCBP is relatively non-specific, differs significantly between the aligned structures: the loops and helices contain substitutions, insertions/deletions; the backbone is found in different conformations (Figure 5). No definitive conclusions can be drawn about 1VR5 in the absence of a cognate ligand, but it appears that this region also forms a cavity that is larger than the molecular envelope of an individual oligosaccharide chain, suggesting semi-specific recognition.

DISCUSSION

We have demonstrated that the Thermotoga maritima tm0031 ORF encodes for a periplasmic polymeric carbohydrate-binding protein that binds β(1→4) linked glucose molecules that range in size from two to five sugar rings. The mode of oligosaccharide recognition is unusual in that this binding protein is specific for only part of the ligand, whereas other sugar-binding proteins in the PBP superfamily typically are exquisitely specific. Instead tmCBP selects a molecular class, rather than a single species. Based on comparison with the apo structure of the β-1,4-mannobiose-binding protein, it appears that its ligands may bind in a similar manner, although the boundaries of the cavity, which determine sugar length and branch selectivity are not well-defined in the absence of a cognate ligand. Oligosaccharides are selected by specific recognition of the disaccharide at the non-reducing end (the “hook”), whereas the rest of the oligosaccharide with mixed linkages is placed into a solvent-filled cavity. The reducing end of cellopentaose forms a few specific hydrogen bonds. This “capping” interaction might impose an upper limit on the length of an oligosaccharide
that is recognized by tmCBP, and subsequently transported across the membrane. The nonselective cavity is filled with a remarkable arrangement of ordered water molecules, divided into a semi-permanent layer that forms a tetrahedral ice-like hydrogen-bonding network, and a more loosely bonded layer that is partially displaced by the bound oligosaccharide. It is therefore possible that different branches and linkages can be accommodated within the steric limitation of the cavity, enabling pre-concentration of wider array of plant- (40), marine algal- (41), and fungal-based (42) β-carbohydrates. For instance, the pentameric β(1→4) sugar does not occupy the entire volume of the cavity, occupying only a fraction of one region; other linkages of oligosaccharides can be positioned in the remaining cavity space (Figure 6).

*T. maritima* possesses an extensive sacchrolytic metabolic system based on an extracellular cellulas that hydrolyze polysaccharides to shorter chains which are then transported across the membrane for phosphorylation and further cleavage (22). In this scheme, tmCBP acts as a molecular sieve, with the “hook” selecting only a subset of β-oligosaccharides, and the “cap” imposing a size limit that can be transported and function as a substrate for the intracellular β-glucose hydrolases. Such semi-specific recognition systems present an efficient solution for the processing of complex mixtures down to common metabolic feedstocks.

**REFERENCES**


**FOOTNOTES**

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Source was supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract No. W-31-109-Eng-38.

FIGURE LEGENDS

FIGURE 1. Thermal denaturation of tmCBP determined by circular dichroism. A. Thermal denaturation of tmCBP in 2M GdCl in the absence (■) or presence of 1mM cellobiose (●), cellotriose (▲), cellotetraose (▼), cellopentaose (♦), cellohexaose (+). Solid lines in (A) were fit to a two-state model that accounts for the native and denatured baseline slopes (37). Inset, appTm values as a function of the number of sugar rings. B. The appTm value in the absence of denaturant were obtained by extrapolation of a series of thermal melting curves determined at different concentrations of GdCl. Solid lines represent linear fits to the observations.

FIGURE 2. Overall structure of tmCBP. A. The cellobiose complex. B. The cellopentaose complex. Domain I (blue), domain II (cyan) and domain III are indicated (yellow, β-strands; orange, β-strands in the hinge; bound sugars shown in ball and stick representation: red, oxygens; gray, carbons).

FIGURE 3. The sugar-binding pocket and cavity. A,B. The cellobiose complex. C,D. The cellopentaose complex. Hydrogen-bonding interactions are indicated with black dashed lines drawn between the ligand and amino acid side-chains and waters (red spheres) that do no interact with the protein. Hydrogen bonds between the ligand and water molecules that interact with the protein are indicated with orange dashed lines.

FIGURE 4. The solvent structure in the ligand-binding cavity. A. The disaccharide binds specifically at one end of the binding-site groove in a site defined by a cluster of aromatic residues (the “hook”). The adjacent cavity is filed with 80 well-defined water molecules. B. The non-reducing end of the cellopentaose also binds to the hook. The three other rings are placed into the adjacent cavity which contains 75 well-defined water molecules. C. Stereo diagram of water structure in the cellopentaose complex. Hydrogen-bonding interactions are indicated with black dashed lines. Water molecules are colored according to their solvation layer (water molecules in the primary solvation shell, red; secondary solvation shell, green; tertiary solvation shell, orange).

FIGURE 5. Comparison of the binding sites of tmCBP and the apo-protein of the β-1,4-mannobiose binding protein, 1VR5. The N- and C-terminal domains of 1VR5 (cyan with side-chains shown in ball and stick representation: red, oxygens; yellow, carbons) were superimposed individually onto the cellopentaose complex of tmCBP (blue with side-chains shown in ball and stick representation: red, oxygens; yellow, carbons).

FIGURE 6. The carbohydrate selectivity of the ligand binding cavity. Different linkages of carbohydrates (blue, β-(1,4/1,3/1,3); cyan, β-(1,3/1,3); gray, cellopentaose) beyond the hook can be modeled to occupy different regions of the cavity adjacent to the cellobiose binding site (gray surface representation).
Table 1. Data collection and refinement statistics

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**Non-hydrogen atoms in refinement**

| Protein       | 4812     | 9775   |
| Water         | 500      | 887    |
| Ligand        | 23       | 112    |
| R<sub>cry</sub>/R<sub>free</sub> (%) | 19.2/21.6 | 20.4/22.5 |

**r.m.s.d. from ideal**

| Bond lengths (Å) | 1.12 | 1.07 |
| Bond angles (°)  | 0.007| 0.005|
| B-factors (Å$^2$) |
| Main-Chain      | 15.8 | 21.2 |
| Side-Chain      | 16.8 | 24.3 |
| Ligand          | 12.5 | 20.8 |
| Water           | 26.1 | 30.7 |

**Ramachandran Plot**

| Allowed (%) | 100 | 100 |
| Favored (%) | 97.7 | 97.8 |

<sup>a</sup>Number in parentheses represent values in the highest resolution shell

<sup>b</sup>R<sub>free</sub> is the R-factor based on 5% of data excluded from refinement
Figure 1
Figure 3
Figure 5:
Figure 5:
Figure 6:
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