X4 Modules Represent a New Family of Carbohydrate-binding Modules That Display Novel Properties*

Received for publication, December 5, 2003, and in revised form, March 4, 2004
Published, JBC Papers in Press, March 5, 2004, DOI 10.1074/jbc.M313317200

David N. Bolam†, Hefang Xie‡, Gavin Pell‡, Deborah Hogg‡, Greta Galbraith‖, Bernard Henriassat‖, and Harry J. Gilbert‡‡

From the †School of Cell and Molecular Biosciences, University of Newcastle upon Tyne, The Agriculture Building, Newcastle upon Tyne NE1 7RU, United Kingdom and §Architecture et Fonction des Macromolécules Biologiques, UMR 6098, CNRS & Universités Aix-Marseille I & II, 31 Chemin J. Aiguier, F-13402 Marseille Cedex 20, France

The hydrolisis of the plant cell wall by microbial glycoside hydrolases and esterases is the primary mechanism by which stored organic carbon is utilized in the biosphere, and thus these enzymes are of considerable biological and industrial importance. Plant cell wall-degrading enzymes in general display a modular architecture comprising catalytic and non-catalytic modules. The X4 modules in glycoside hydrolases represent a large family of non-catalytic modules whose function is unknown. Here we show that the X4 modules from a Cellulibrio japonicus mannanase (Man5C) and arabino-furanosidase (Abf62A) bind to polysaccharides, and thus these proteins comprise a new family of carbohydrate-binding modules (CBMs), designated CBM35. The Man5C-CBM35 binds to galactomannan, insoluble amorphous mannan, glucomannan, and manno-oligosaccharides but does not interact with crystalline mannan. Abf62A-CBM35 interacts with unsubstituted oat-spelt xylan but not substituted forms of the hemicellulose or xylo-oligosaccharides, and requires calcium for binding. This is in sharp contrast to other xylan-binding CBMs, which interact in a calcium-independent manner with both xylo-oligosaccharides and decorated xylans.

The plant cell wall comprises the most abundant source of renewable carbon on the planet. This extensive resource is made available to the biosphere through the action of microbial glycoside hydrolases, which are thus of considerable biological and industrial importance. Plant cell walls are composed of a complex network of polysaccharides that are highly inaccessible to enzyme attack. Glycoside hydrolases that degrade the plant cell wall are generally modular enzymes comprising catalytic and non-catalytic modules that are joined via flexible linker sequences. Many of these non-catalytic modules bind to specific oligo- and polysaccharides derived from the plant cell wall and are thus defined as carbohydrate-binding modules (CBMs, Ref. 2). By localizing the appended catalytic module onto the surface of the (mainly) insoluble polysaccharide substrates, CBMs potentiate the activity of glycoside hydrolases against these composite structures (3, 4). Thus, CBMs play a pivotal role in the capacity of glycoside hydrolases to degrade the plant cell wall.

Based on sequence similarities, CBMs have been grouped into families (afmb.cnrs-mrs.fr/CAZY, Ref. 5). Currently there are 34 CBM families, 32 of which contain modules from prokaryotic enzymes whereas only families 1 and 29 contain fungal proteins (afmb.cnrs-mrs.fr/CAZY, Ref. 5). Three-dimensional structures of representatives of over half of the CBM families demonstrate that these proteins generally adopt a β-jelly roll fold (2). Structural data has also shown that the topology of the ligand-binding site of CBMs varies. In Type A CBMs, which interact with the flat surfaces crystalline polysaccharides such as cellulose, the binding site comprises a hydrophobic planar surface that contains a linear strip of exposed aromatic amino acids (6–9), while in Type B CBMs, which interact with individual polysaccharide chains, the ligand is accommodated within a cleft of varying depth that extends the length of the protein (10–13). In contrast to lectins, there is a low density of hydrogen bonds between CBMs and their target saccharides (2). While lectins contain multiple binding sites that interact with mono- or disaccharides of complex carbohydrates (14), CBMs generally contain a single binding site that accommodates 5–6 saccharide units (13, 15). Although all Type A or B CBMs characterized to date are monovalent (contain only one binding site), co-operativity between multiple CBMs in a single enzyme can lead to significant increases in affinity compared with the individual modules (16–18). Ligand specificity in Type A CBMs is generally invariant, while in the Type B modules polysaccharide recognition is variable within a family and normally reflects the catalytic activity of the enzyme from which it is derived (10–13).

In addition to known CBMs, plant cell wall hydrolases often contain non-catalytic modules of unknown function. This is exemplified by three plant cell wall-degrading enzymes from Cellulibrio japonicus, Xyn10B (xylanase), Abf62A (arabino-furanosidase), and Est1A (acetyl xylan esterase) that contain an identical ~150-amino acid module, termed X4, whose role in enzyme function is unclear (19, 20). This X4 module is also present in a mannanase (Man5C) from the same organism (21). To understand the mechanism by which glycoside hydrolases attack the complex composite structure that comprises the...
**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Culture Conditions, and Plasmids—**The *Escherichia coli* strains Tuner (Novagen), C41 (DE3), a gift from Prof. A. R. Fersht at the Medical Research Council, Cambridge, UK), JM83 (DE3), and JM83 were used in this study. The plasmid vectors used were pGEX-2T (Amersham Biosciences), pRSET (Invitrogen), pET16b, and pET22b (Novagen), while the recombinant plasmids and the proteins they encode are shown in Fig. 1. To generate pDB1, encoding Abf62A-CBM35, the region of the cognate gene, abf62A comprising nucleotides 457–945 was amplified by PCR using the primers: 5′-GCGGATC-CTTCTTCGACCTGTTGGCC-3′ and 5′-GCGGATTCATTTACTG-TATGAGGAGGATGAC-3′, which contain BamHI and EcoRI restriction sites, respectively, and the resultant DNA fragment was cloned into BamHI/EcoRI-restricted pRS3. The plasmid pDB2, encoding GST-Abf62A-CBM35, was produced by cloning the same region of *albyen* 62A present in pDB1 into BamHI/EcoRI-digested pGEX-2T. The plasmid pGP1, encoding Man5C-CBM35, was generated by amplifying nucleotides 589–981 of *man5C* using the primers 5′-GCGCATATGATGGCCGCGAGCGAGCGAAAG-3′ and 5′-GCGCTCAAGGCGGCTGCGACGATGGTGCTGCTGCTGC-3′, which contain Ndel and XhoI restriction sites, respectively, and the resultant DNA was cloned into similarly restricted and pET22b. Construction of pDH25 encoding the glycoside hydrolase family 5 (GH5) catalytic module of Man5C was described previously (21). The recombinant plasmid pDH26, which encodes Man5C-CBM35-GH5, was generated by amplifying the region of *man5C* comprising nucleotides 589–2495 using the primers 5′-GCGCATATGATGCCGCGAGCGAGCGAAAG-3′ and 5′-GCGCTCAAGGCGGCTGCGACGATGGTGCTGCTGCTGC-3′, which contain Ndel and BamHI restriction sites, respectively. The amplified DNA was digested with Ndel and BamHI and cloned into similarly restricted pET16b. In pDB1, pDH25, and pDH26 the encoded protein module is appended to an N-terminal His tag, while in pGP1 the encoded CBM35 module has a C-terminal His tag. In pDB2 the encoded GST-Abf62A-CBM35 was produced by cloning the same region of *albyen* 62A present in pDB1 into BamHI/EcoRI-digested pGEX-2T.

**Expression and Purification of C. japonicus Proteins in E. coli—**To generate the proteins encoded by recombinants of pET16b and pRSET, the *E. coli* strains Tuner or C41 harboring pDH26 and pDB1, respectively, were cultured in LB supplemented with 50 μg/ml ampicillin (1000 ml in 2-liter conical baffle flasks) at 37 °C and 180 rpm to an OD<sub>600</sub> nm of 1.5 (OD<sub>600</sub> nm ≈ 0.6). The culture was then cooled to 16 °C, before expression of CBM35 was induced by addition of isopropyl β-D-thiogalactoside (IPTG) to a final concentration of either 0.1 mM for C41 cells or 0.2 mM for Tuner cells and incubation at 16 °C for a further 15 h. The cells were harvested by centrifugation at 4500 × g for 10 min at 4 °C and resuspended in one-fifth volume 20 mM Tris/HCl buffer, pH 8.0, containing 300 mM NaCl before lysis by sonication and centrifugation (25,000 × g) for 15 min at 4 °C to produce cell-free extract. The CBM35 proteins were purified from cell-free extract by immobilized metal affinity chromatography (IMAC) as described previously (22). Using Talo<sub>N</sub>- resin (Clontech). To generate Man5C-CBM35, the recombinant plasmid pDB1 was transformed into *E. coli* strain JM83 harboring pGP1, and recombinant protein was purified as described above except that the expression of the recombinant protein was induced at 37 °C for 3 h using a final concentration of 1 mM IPTG. To produce GST-Abf62A-CBM35, E. coli JM83 containing pDB2 was cultured as described above except that expression of the recombinant protein was induced at 30 °C for 4 h using a final IPTG concentration of 0.5 mM. The GST fusion protein was purified from cell-free extract by glutathione-Sepharose (Amersham Biosciences) affinity chromatography as described previously (22). Protein concentration was determined from the calculated molar extinction coefficients at 280 nm, which were 80,700 M<sup>−1</sup> cm<sup>−1</sup> for Abf62A-CBM35, 19,700 M<sup>−1</sup> cm<sup>−1</sup> for Man5C-CBM35, 30,440 M<sup>−1</sup> cm<sup>−1</sup> for GST-Abf62A-CBM35, 120,700 M<sup>−1</sup> cm<sup>−1</sup> and 96,230 M<sup>−1</sup> cm<sup>−1</sup> for GST-Abf62A-CBM35, Man5C-CBM35, Man5C-CBM35-GH5, and GH5 alone, respectively.

**Sources of Sugars Used—**All oligosaccharides and polysaccharides were purchased from Megazyme International, except glucuronoxylan, birchwood xylan, oat-spelt xylan, and hydroxethyl cellulose (HEC), which were obtained from Sigma. Bacterial microcrystalline cellulose, acid swollen cellulose, and the soluble and insoluble fractions of oat-spelt xylan were prepared as described previously (24). To generate insoluble degalactosylated mannan (DGM, 0.5 g of carob galactomannan (high viscosity, galactose/mannose = 1:4) was treated with 10 units of *C. japonicus α-galactosidase CalZa 29* in 10 ml of 50 mM sodium phosphate buffer, pH 7.0, at 37 °C for 18 h. Removal of the galactose side chains caused the mannann chains to aggregate and become insoluble. The precipitated mannan was washed extensively in distilled water. The monosaccharide content of the insoluble DGM polysaccharide, determined by acid hydrolysis followed by HPLC, revealed that the ratio of galactose/mannose was 1:50.

**Affinity Gel Electrophoresis (AGE)—**AGE was performed as described previously (26) using oat-spelt xylan (arabinose/xylose = 1:10), rye arabinoxylan (high viscosity; arabinoxylan/xylose = 1:1), 4-O-methylglucuronoxylan, birchwood xylan (glucuronic acid/xylose = 1:10; Ref. 27), barley β-glucan (medium viscosity), hydroxyethylcellulose, konjac glucomannan (low viscosity; glucose/mannose = 2:3), carob galactomannan (low viscosity; galactose/mannose = 1:4), debranched α,1,5-arabinoxylan (sugar beet), β,1,4-galactan (potato), and rhamnogalacturonan (soybean) as ligands. The ratio of sugars in each polysaccharide was provided by the manufacturer. Briefly, the continuous gels contained 7.5% w/v admixture in 25 mM Tris, 250 mM glycine buffer, pH 8.3. For ligand-containing gels, glycine was added to the separating gel mixtures to 0.01-5 mg/ml prior to polymerization. Native polyacrylamide gels, with and without ligand, were polymerized at the same time and were electrophoresed in the same gel tank. The proteins (5 μg) were electrophoresed at 25 °C and 10 mA/gel in gels with and without ligand for 2 h. GST was used as a negative, non-interacting control. Proteins were visualized by Coomassie Blue staining. The migration distances of the CBMs and the reference protein were measured from the bottom of the gel. The protein bands evident on the gels, and these data were used to determine the dissociation constants (K<sub>D</sub>) from plots of 1/(R<sub>c</sub> - r) versus 1/C according to the affinity equation shown in Equation 1,

\[
1/(R_c - r) = 1/(R_c - R_0) + 1/(K_D/C)
\]

where r is the relative migration distance of the CBM in the presence of ligand in the gel, R<sub>c</sub> is the relative migration distance of the free CBM in the absence of ligand, R<sub>0</sub> is the relative migration distance of the complex at high excess of ligand where all CBM molecules are fully complexed, C is the concentration of the ligand in the gel, and K<sub>D</sub> is the dissociation constant of CBM for the macromolecular ligand. K<sub>D</sub> values were determined as the inverse of the absolute value of the intercept on the abscissa of data plotted according to the affinity equation. All migration distances of the CBMs were measured relative to the migration of the reference protein GST.

**Creation of the Apo Form of Abf62A-CBM35—**Purified Abf62A-CBM35 at a concentration of 400 μM in 50 mM sodium HEPES buffer, pH 8.0, was passed through a 20 × 1.5-cm column containing a 10-ml bed volume of Chelex-100 (Sigma) under the flow of gravity. The buffer used for ITc was treated in the same way to remove any traces of calcium.

**Gel Filtration Chromatography—**Gel filtration was performed using a HiLoad 16/60 Superdex 75 column (Amersham Biosciences) attached...
X4 Modules Represent a New Family of CBMs

22955

to a Bio-Rad Biologic HR FPLC system. The column was calibrated using Sigma gel filtration molecular weight markers ranging from 12.4 to 66 kDa. Blue dextran (2 MDa) was used to determine the column void volume. Protein (1 ml) was loaded on the column and run at 1 ml/min in 50 mM sodium HEPES buffer, pH 8.0, for 120 min. Where appropriate, the reducing agent Tris(2-carboxyethyl)phosphine (TCEP) was added to the sample and the running buffer at a final concentration of 10 mM.

Isothermal Titration Calorimetry (ITC)—ITC measurements were made at 25 °C using a Microcal Omega titration calorimeter. The Man5C-CBM35 and Abf62A-CBM35 modules were diazoyl extensively against 50 mM sodium HEPES buffer, pH 8.0, and the ligands were dissolved in the same buffers to minimize heats of dilution. Where appropriate, TCEP, calcium, or EDTA at final concentrations of 1, 5, and 10 mM, respectively, were added to Abf62A-CBM35 and carbohydrate ligand prior to ITC. During a titration experiment, the protein sample (150–600 μM), stirred at 300 rpm in a 1.4331-ml reaction cell maintained at 25 °C, was injected with 25–50 successive 10-μl aliquots of ligand comprising polysaccharide (25–250 mg/ml) or oligosaccharide (5–15 mM), at 200-s intervals. The apo form of Abf62A-CBM35 (150 μM) was titrated against 3 mM calcium chloride to determine whether calcium bound independently to the protein. Prior to the titration the cell was washed out with 5 mM EDTA, followed by an extensive Chelex-treated buffer wash to remove any traces of the metal ion from the machine. Integrated heat effects, after correction for heats of dilution, were analyzed by non-linear regression using a single site-binding model (Microcal Origin, version 5.0). Fitted data yield the association constant (K_a) and the enthalpy of binding (ΔH). Other thermodynamic parameters were calculated using the standard thermodynamic equation: -RTlnK_a = ΔG = ΔH - TΔS. The c values (product of the association constant × the concentration of the acceptor) were 3–30. At least two independent titrations were performed for each ligand tested. The molar concentration of Man5C-CBM35 binding sites present in galactomannan and glucomannan was determined by altering the concentration of polysaccharide used for regression of the isotherm until the fit yielded a value of 1 for n (number of binding sites on each molecule of CBM). The assumption that n = 1 was based on the elugosaccharide titrations, which all displayed a stoichiometry of 1:1. For analysis of the xylan binding data with Abf62A-CBM35, this approach could not be used because the CBM did not interact with xylo-oligosaccharides. Instead, the binding data was fitted by treating the CBM in the sample cell as the ligand and the polysaccharide as the acceptor. This gives accurate values for K_a and ΔH, but not n (in this case the number of binding sites on each molecule of polysaccharide).

Binding to Insoluble Polysaccharides—The binding of the CBM35 proteins to insoluble polysaccharides (acid-swollen cellulose, bacterial microcrystalline cellulose, DGM, ivory nut mannan, and insoluble oast-selt xylan) was determined qualitatively using SDS-PAGE. Pure protein sample and Tris(2-carboxyethyl) buffer, pH 8.0, was mixed with 2 mM polysaccharide in a final volume of 100 μl. Tubes were incubated on ice for 1 h, with regular gentle mixing before being centrifuged at 13,000 × g for 1 min and the supernatant, containing unbound protein, carefully removed. The polysaccharide pellet was then washed in 100 μl of the same buffer, before being resuspended in 50 μl of 10% (w/v) SDS and boiled for 10 min to dissociate any bound protein. Controls with protein but no polysaccharide were included to insure that no precipitation occurred during the assay period. Bound and unbound fractions were analyzed by SDS-PAGE using a 12.5% (w/v) polyacrylamide gel.

Enzyme Assays—The mannanase activity of the Man5C derivatives Man5C-CBM35-GH5 and GH3 and GH5 was evaluated by HPLC with mannanose linked glucose polymers, rhamnogalacturonan, or the polysaccharide from xylan as substrates using equal concentrations of protein. The Man5C derivatives (50 nM) were incubated with 5 mg/ml ivory nut mannan or insoluble DGM in 50 mM sodium phosphate/12 mM citrate (PC) buffer, pH 6.5 at 37 °C for up to 5 h in a total volume of 0.5 ml. At regular time intervals, a 40-μl aliquot was removed, the enzyme was inactivated by boiling for 10 min, and mannobiose, the primary reaction product, was quantified by HPLC following the method of Hogg et al. (21). To determine the rate of mannanhydrolysis, the hexasaccharide (0.6 mM) was incubated with 40 nM enzyme in PC buffer, pH 6.5 at 37 °C for up to 30 min in a total volume of 0.4 ml, and the release of mannotetraose was quantified as described above.

RESULTS AND DISCUSSION

Identification of the CBM35 Family of Protein Modules—Previous studies identified a protein module, originally termed X4, comprising ~150 amino acids in three C. japonicus enzymes, Xyn10B (formerly XynB), Abf62A (formerly XynC), and Est1A (formerly XynD) that are involved in the hydrolysis of xylan. All three enzymes contain an identical N-terminal region that comprises a typical family 2a CBM joined via a serine-rich linker to the X4 module (19, 20). As the X4 modules in these Cellulbio oss enzymes are shown to bind polysaccharides (see below), henceforth these sequences will be designated as a new family of CBMs (family 35, CBM35). When the primary structure of Abf62A-CBM35 was used to query databases using BLAST, a number of sequences that display similarity to this sequence were identified in enzymes that modify carbohydrates, including glycoside hydrolases and lyases that attack the hemimellulose and pectic polysaccharides, respectively, within the plant cell wall. Based on sequence similarities the CBM35 modules can be grouped into three clads containing modules derived from xylan/pectin-modifying enzymes, mannanases, and isomalto-oligosaccharide-modifying enzymes, respectively. The alignment of the CBM35s and phylogenoms of this family are displayed in Fig. 2. It should be noted that while CBM35 constitutes a discrete protein family, it displays a distant relationship with family 6 CBMs. The observation that CBM35s lack the three aromatic residues (e.g. Trp-92, Tyr-33, and Trp-39 in CBM6–2 from Cellulbio oss mixtu Cell5A), which play a pivotal role in ligand recognition by CBM6 proteins (28), provides further support for the view that CBM35 and CBM6 comprise discrete families of CBMs.

Expression of the CBM35 Modules—To determine the function of CBM35s in glycoside hydrolases we have focused on two enzymes from C. japonicus, Man5C and Abf62A (20, 21). The mannanase comprises a family 5 and family 10 CBM, which are joined by typical serine-rich linker sequences, and a CBM35 module abutted to the GH5 catalytic module. The region of the arabinoarufuramosidase and mannanase genes encoding the respective CBM35a were cloned into E. coli expression vectors, and the encoded proteins, fused to a His tag supplied by the vector, were produced in soluble form in the host bacterium. The two proteins were purified by IMAC. Purified Abf62A-CBM35 contained both monomeric and dimeric species as judged by non-reducing SDS-PAGE and gel filtration; however, the addition of reducing agent converted the protein to its monomeric form (Fig. 3), suggesting that dimerization was the result of an interchain disulfide bond. The subsequent biochemical characterization of Abf62-CBM35 was carried out in the presence of reducing agent (unless stated otherwise) to ensure that it was maintained in its monomeric form.

Family 35 CBMs Bind to Polysaccharides—The biochemical properties of the two CBM35 modules were initially evaluated using AGE. As a result of its high pl, Abf62A-CBM35 was fused to glutathione S-transferase (GST-Abf62A-CBM35) to ensure migration into non-denaturing gels. Examples of the affinity gels and subsequent plots used to quantify binding are displayed in Figs. 4 and 5, respectively, and the full data set is presented in Table I. The CBM35 module from Man5C (Man5C-CBM35) binds to galactomannan and glucomannan but does not interact with substituted or unsubstituted xylans, soluble β-linked glucose polymers, rhamnogalacturonan, or the pectin side chains arabinan and galactan. The capacity of Man5-C-CBM35 to bind insoluble polysaccharides was evaluated using SDS-PAGE to monitor bound and unbound protein. Example data are shown in Fig. 6 and the full data set presented in Table I. Man5C-CBM35 does not bind to Avicel, acid-swollen cellulose, bacterial crystalline cellulose, or ivory nut mannan, but does associate with DGM, an insoluble form of mannan generated by the enzymic removal of galactose side chains from galactomannan. The polysaccharide chains in

Downloaded from http://www.jbc.org/ by guest on July 24, 2018
A. caviae-Xyl3
A. caviae-XylD
A. orientalis-ORF.CZA382.11
S. coelicolor-ORF.SCF34.24
S. globispora-CtsZ-1
L. innocua-ORF.Lin2538-1
B. circulans-CmgtA-1
B. circulans-CmgtB-1
L. innocua-ORF.Lin2540
L. monocytogenes-ORF.Lmo2446
S. globispora-CtsY
Bacillus.P-358-pelA-1
C. japonicus-Abf62A
C. japonicus-Est1A
C. japonicus-Xyn10B
C. thermocellum-Man26B
C. thermocellum-ManA
C. saccharolyticus-ManA
C. japonicus-PelA
Pircymes.ep-Mann
Pircymes.ep-Manc
D. thermophilum-ManA
C. japonicus-ManG5

Consensus motifs

FIG. 2. Alignment of CBM35 modules (panel A) and phylograms (panels B and C). The CBM35 sequences used in the alignment (panel A) and phylogram are from the following genes, with their EMBL/GenBank accession numbers in brackets.

A. caviae-Xyl3, xylanase 3 from Aeromonas caviae (EC 3.2.1.8) [D88553]; A. caviae-XylD, xylanase D from A. caviae (EC 3.2.1.8) [U86340]; A. orientalis-ORF.CZA382.11 from Amycolatopsis orientalis (EC not determined (n.d.)) [AL078635]; S. coelicolor-ORF.SCF34.24, ORF SCF34.24 from Streptomyces coelicolor A3 (EC n.d.) [AL109974]; S. globispora-CtsZ-1, 6-glucosyltransferase (CtsZ) from Sporosarcina globispora C11 (EC 2.4.1.19) [AB073929]; L. innocua-ORF.Lin2538-1, ORF Lin2538 from Listeria innocua (EC n.d.) [NC_003212]; L. monocytogenes-ORF.Lmo2446, ORF Lmo2446 from L. monocytogenes EGD-e (EC n.d.) [NC_003210]; S. globispora-CtsZ-2, 6-glucosyltransferase (CtsZ) from S. globispora C11 (EC 2.4.1.19) [AL078635]; B. circulans-CmgtA-1, cyclosomalto-oligosaccharide glucanotransferase from B. circulans T-3040 (EC 2.4.1.19) [D61382]; B. circulans-CmgtB-1, cyclosomalto-oligosaccharide glucanotransferase from B. circulans T-3040 (EC 2.4.1.19) [D61382]; B. circulans-CmgtB-2, cyclosomalto-oligosaccharide glucanotransferase from B. circulans T-3040 (EC 2.4.1.19) [D61382]; L. innocua-ORF.Lin2540, ORF Lin2540 from L. innocua (EC n.d.) [NC_003212]; L. monocytogenes-ORF.Lmo2446, ORF Lmo2446 from L. monocytogenes EGD-e (EC n.d.) [NC_003210]; S. globispora-CtsY, isomaltsyltransferase (CtsY) from S. globispora C11 (EC 2.4.1.19) [AB073929]; Bacillus.P-358-pelA-1, pectate lyase P358 from Bacillus sp. P-358 (EC n.d.) [AB062880]; Bacillus.P-358-pelA-2, pectate lyase P358 from Bacillus sp. P-358 (EC n.d.) [AB062880]; C. japonicus-Abf62A, arabinofuranosidase 62A from C. japonicus (EC 3.2.1.55) [X54523]; C. japonicus-Est1A, esterase 1A from C. japonicus (EC n.d.) [X58956]; C. japonicus-Xyn10B, xylanase 10B from C. japonicus (EC 3.2.1.8) [X54523]; C. japonicus-PelA, pectate lyase A from C. japonicus (EC 4.2.2.2) [AF279264]; S. coelicolor-ORF.SCF34.27c, ORF SCF34.27c from S. coelicolor (EC n.d.) [AL359215]; A. globiformis-Dex, isomaltodextranase from Arthrobacter globiformis (EC 3.2.1.94) [D30761]; A. orientalis-ORF.CZA382.11 from Amycolatopsis orientalis (EC not determined (n.d.)) [AL078635]; S. coelicolor-ORF.SCF34.24, ORF SCF34.24 from Streptomyces coelicolor A3 (EC n.d.) [AL109974]; S. globispora-CtsZ-1, 6-glucosyltransferase (CtsZ) from Sporosarcina globispora C11 (EC 2.4.1.19) [AB073929]; L. innocua-ORF.Lin2538-1, ORF Lin2538 from Listeria innocua (EC n.d.) [NC_003212]; L. monocytogenes-ORF.Lmo2446, ORF Lmo2446 from L. monocytogenes EGD-e (EC n.d.) [NC_003210]; S. globispora-CtsZ-2, 6-glucosyltransferase (CtsZ) from S. globispora C11 (EC 2.4.1.19) [AL078635]; B. circulans-CmgtA-1, cyclosomalto-oligosaccharide glucanotransferase from B. circulans T-3040 (EC 2.4.1.19) [D61382]; B. circulans-CmgtB-1, cyclosomalto-oligosaccharide glucanotransferase from B. circulans T-3040 (EC 2.4.1.19) [D61382]; B. circulans-CmgtB-2, cyclosomalto-oligosaccharide glucanotransferase from B. circulans T-3040 (EC 2.4.1.19) [D61382]; L. innocua-ORF.Lin2540, ORF Lin2540 from L. innocua (EC n.d.) [NC_003212]; L. monocytogenes-ORF.Lmo2446, ORF Lmo2446 from L. monocytogenes EGD-e (EC n.d.) [NC_003210]; S. globispora-CtsY, isomaltsyltransferase (CtsY) from S. globispora C11 (EC 2.4.1.19) [AB073929]; Bacillus.P-358-pelA-1, pectate lyase P358 from Bacillus sp. P-358 (EC n.d.) [AB062880]; Bacillus.P-358-pelA-2, pectate lyase P358 from Bacillus sp. P-358 (EC n.d.) [AB062880]; R. marinus-ManA, /H9252-mannanase A from Rhodothermus marinus (EC 3.2.1.78) [X90947]; C. saccharolyticus-MaA, /H9252-mannanase from Caldicellulosiruptor saccharolyticus (EC 3.2.1.78) [U39812]; R. marinus-ManA, /H9252-mannanase A from Rhodothermus marinus (EC 3.2.1.78) [X90947]; C. thermocellum-Man26B, /H9252-mannanase 26B from Clostridium thermocellum (EC 3.2.1.78) [AB044406]; C. thermocellum YS-ManA, /H9252-mannanase from Clostridium thermocellum (EC 3.2.1.78) [AB044406].
A from *C. thermocellum* YS (EC 3.2.1.78) [AJ242666]; *Pyromyces* sp.-ManA, β-mannanase A from *Pyromyces* sp. (EC 3.2.1.78) [X91857]; *Pyromyces* sp.-ManB, β-mannanase B from *Pyromyces* sp. (EC 3.2.1.78) [X97408]; *Pyromyces* sp.-ManC, β-mannanase C from *Pyromyces* sp. (EC 3.2.1.78) [X97520]; *D. thermophilum*-ManA, β-mannanase from *Dictyoglomus thermophilum* (EC 3.2.1.78) [AF013989]; *C. japonicus*-Man5C, endo-β-1,4-mannanase 5C from *C. japonicus* (EC n.d.) [AY187033]. Residues that are conserved are highlighted. The three clades within the CBM35 family are displayed in the phylogram in panel B. These are CBM35 modules from: (i) xylan/pectin-modifying enzymes, (ii) mannanases, (iii) isomaltooligosaccharide-modifying enzymes. In panel C a phylogenetic tree comprising CBM35 and CBM6 is displayed. The bar shows the genetic distance of 0.1 substitutions per nucleotide.
DGM are likely to associate in a less ordered fashion than those found in crystalline mannans and are therefore analogous to amorphous cellulose where discrete polysaccharide chains are able to interact with CBMs that contain a ligand binding cleft (18). Thus, Man5C-CBM35 appears to exhibit features typical of Type B CBMs, which accommodate individual polysaccharide chains in a binding site that displays a cleft topology (10, 15), but are unable to interact with the flat surfaces of highly crystalline ligands such as ivory nut mannan.

Analysis of the polysaccharide binding properties of the Abf62A-CBM35 module were carried out using GST-CBM35 for soluble ligands and the His-tagged CBM35 module for insoluble polysaccharides. The data show that the protein binds to both soluble (Figs. 4 and 5) and insoluble oat-spelt xylan (Fig. 6) but displays very weak affinity for soluble arabinoxylan from rye (Fig. 5 and Table I). AGE analysis also revealed slight retardation of Abf62A-CBM35 by glucuronoxylan, although binding was too weak to quantify (Fig. 4 and Table I). The protein does not interact with birchwood xylan, soluble, or insoluble forms of cellulose, β-glucan, galactomannan, glucomannan, or pectins (Table I). These data demonstrate that Abf62A-CBM35 is a xylan-specific CBM that interacts preferentially with unsubstituted forms of the polysaccharide. The results presented above reveal that both Abf62A-CBM35 and Man5C-CBM35 display polysaccharide binding properties and thus justify the reclassification of X4 modules as family 35 CBMs (CBM35).

The Use of ITC to Measure Ligand Binding of Man5C-CBM35—ITC was used to measure binding of Man5C-CBM35 to both polysaccharide and oligosaccharide ligands. Examples of these titrations are presented in Fig. 7, and the full data set
is displayed in Table II. The thermodynamics of the interaction of Man5C-CBM35 with polysaccharides and oligosaccharides is enthalpy-driven with the change in entropy making an unfavorable contribution to ligand binding. This pattern of energetics is typical of the binding of proteins to soluble saccharides (12, 14, 18, 29). The CBM binds to manno-oligosaccharides with a stoichiometry of 1:1 displaying maximal affinity for mannopentaose and mannohexaose. Binding of Man5C-CBM35 to mannopentaose and mannohexaose was GST-Abf62A-CBM35, while Abf62A-CBM35 was used to evaluate binding to insoluble polysaccharide (see Fig. 1). + indicates binding; − indicates no binding. Values in parentheses are $K_a$ (mg/ml) determined by quantitative AGE.

| Soluble polysaccharide                  | Man5C-CBM35 $^a$ | Abf62A-CBM35 $^a$ |
|----------------------------------------|------------------|-------------------|
| Carob galactomannan                    | +$^+$            | −                 |
| Konjac glucomannan                     | +$^+$            | −                 |
| Hydroxyethylcellulose                  | −                | −                 |
| Barley β-glucan                        | −                | −                 |
| Oat-splet xylan                         | +$^+$            | −                 |
| Rye arabinoxylan                       | +$^+$            | −                 |
| Glucuronoxylan                         | +$^+$            | −                 |
| Birchwood xylan                        | −                | −                 |
| Debranched arabinoxylan                 | −                | −                 |
| Rhamnogalacturonan                     | −                | −                 |
| Galactan                               | −                | −                 |
| Insoluble polysaccharide               | −                | −                 |
| Acid-swollen cellulose                  | −                | −                 |
| Bacterial microcrystalline              | −                | −                 |
| cellulose                              | −                | −                 |
| Ivory nut mannan                       | −                | ND$^b$            |
| Degalactosylated mannan                | +$^+$            | ND$^b$            |
| Oat-splet xylan                         | −                | +$^+$            |

$^a$ Abf62A-CBM35 construct used in assessing binding to soluble polysaccharides was GST-Abf62A-CBM35, while Abf62A-CBM35 was used to evaluate binding to insoluble polysaccharide (see Fig. 1). $^+$ indicates binding; − indicates no binding. $^b$ Values in parentheses are $K_a$ (mg/ml) determined by quantitative AGE.

$^c$ +$^+$ indicates very weak binding. $^d$ Not determined.

is displayed in Table II. The thermodynamics of the interaction of Man5C-CBM35 with polysaccharides and oligosaccharides is enthalpy-driven with the change in entropy making an unfavorable contribution to ligand binding. This pattern of energetics is typical of the binding of proteins to soluble saccharides (12, 14, 18, 29). The CBM binds to manno-oligosaccharides with a stoichiometry of 1:1 displaying maximal affinity for mannopentaose and mannohexaose. Binding of Man5C-CBM35 to mannopentaose and mannohexaose was GST-Abf62A-CBM35, while Abf62A-CBM35 was used to evaluate binding to insoluble polysaccharide (see Fig. 1). + indicates binding; − indicates no binding. Values in parentheses are $K_a$ (mg/ml) determined by quantitative AGE.

| Soluble polysaccharide                  | Man5C-CBM35 $^a$ | Abf62A-CBM35 $^a$ |
|----------------------------------------|------------------|-------------------|
| Carob galactomannan                    | +$^+$            | −                 |
| Konjac glucomannan                     | +$^+$            | −                 |
| Hydroxyethylcellulose                  | −                | −                 |
| Barley β-glucan                        | −                | −                 |
| Oat-splet xylan                         | +$^+$            | −                 |
| Rye arabinoxylan                       | +$^+$            | −                 |
| Glucuronoxylan                         | +$^+$            | −                 |
| Birchwood xylan                        | −                | −                 |
| Debranched arabinoxylan                 | −                | −                 |
| Rhamnogalacturonan                     | −                | −                 |
| Galactan                               | −                | −                 |
| Insoluble polysaccharide               | −                | −                 |
| Acid-swollen cellulose                  | −                | −                 |
| Bacterial microcrystalline              | −                | −                 |
| cellulose                              | −                | −                 |
| Ivory nut mannan                       | −                | ND$^b$            |
| Degalactosylated mannan                | +$^+$            | ND$^b$            |
| Oat-splet xylan                         | −                | +$^+$            |

$^a$ Abf62A-CBM35 construct used in assessing binding to soluble polysaccharides was GST-Abf62A-CBM35, while Abf62A-CBM35 was used to evaluate binding to insoluble polysaccharide (see Fig. 1). $^+$ indicates binding; − indicates no binding. $^b$ Values in parentheses are $K_a$ (mg/ml) determined by quantitative AGE.

$^c$ +$^+$ indicates very weak binding. $^d$ Not determined.

is displayed in Table II. The thermodynamics of the interaction of Man5C-CBM35 with polysaccharides and oligosaccharides is enthalpy-driven with the change in entropy making an unfavorable contribution to ligand binding. This pattern of energetics is typical of the binding of proteins to soluble saccharides (12, 14, 18, 29). The CBM binds to manno-oligosaccharides with a stoichiometry of 1:1 displaying maximal affinity for mannopentaose and mannohexaose. Binding of Man5C-CBM35 to mannopentaose and mannohexaose was GST-Abf62A-CBM35, while Abf62A-CBM35 was used to evaluate binding to insoluble polysaccharide (see Fig. 1). + indicates binding; − indicates no binding. Values in parentheses are $K_a$ (mg/ml) determined by quantitative AGE.

| Soluble polysaccharide                  | Man5C-CBM35 $^a$ | Abf62A-CBM35 $^a$ |
|----------------------------------------|------------------|-------------------|
| Carob galactomannan                    | +$^+$            | −                 |
| Konjac glucomannan                     | +$^+$            | −                 |
| Hydroxyethylcellulose                  | −                | −                 |
| Barley β-glucan                        | −                | −                 |
| Oat-splet xylan                         | +$^+$            | −                 |
| Rye arabinoxylan                       | +$^+$            | −                 |
| Glucuronoxylan                         | +$^+$            | −                 |
| Birchwood xylan                        | −                | −                 |
| Debranched arabinoxylan                 | −                | −                 |
| Rhamnogalacturonan                     | −                | −                 |
| Galactan                               | −                | −                 |
| Insoluble polysaccharide               | −                | −                 |
| Acid-swollen cellulose                  | −                | −                 |
| Bacterial microcrystalline              | −                | −                 |
| cellulose                              | −                | −                 |
| Ivory nut mannan                       | −                | ND$^b$            |
| Degalactosylated mannan                | +$^+$            | ND$^b$            |
| Oat-splet xylan                         | −                | +$^+$            |

$^a$ Abf62A-CBM35 construct used in assessing binding to soluble polysaccharides was GST-Abf62A-CBM35, while Abf62A-CBM35 was used to evaluate binding to insoluble polysaccharide (see Fig. 1). $^+$ indicates binding; − indicates no binding. $^b$ Values in parentheses are $K_a$ (mg/ml) determined by quantitative AGE.

$^c$ +$^+$ indicates very weak binding. $^d$ Not determined.

is displayed in Table II. The thermodynamics of the interaction of Man5C-CBM35 with polysaccharides and oligosaccharides is enthalpy-driven with the change in entropy making an unfavorable contribution to ligand binding. This pattern of energetics is typical of the binding of proteins to soluble saccharides (12, 14, 18, 29). The CBM binds to manno-oligosaccharides with a stoichiometry of 1:1 displaying maximal affinity for mannopentaose and mannohexaose. Binding of Man5C-CBM35 to mannopentaose and mannohexaose was GST-Abf62A-CBM35, while Abf62A-CBM35 was used to evaluate binding to insoluble polysaccharide (see Fig. 1). + indicates binding; − indicates no binding. Values in parentheses are $K_a$ (mg/ml) determined by quantitative AGE.

| Soluble polysaccharide                  | Man5C-CBM35 $^a$ | Abf62A-CBM35 $^a$ |
|----------------------------------------|------------------|-------------------|
| Carob galactomannan                    | +$^+$            | −                 |
| Konjac glucomannan                     | +$^+$            | −                 |
| Hydroxyethylcellulose                  | −                | −                 |
| Barley β-glucan                        | −                | −                 |
| Oat-splet xylan                         | +$^+$            | −                 |
| Rye arabinoxylan                       | +$^+$            | −                 |
| Glucuronoxylan                         | +$^+$            | −                 |
| Birchwood xylan                        | −                | −                 |
| Debranched arabinoxylan                 | −                | −                 |
| Rhamnogalacturonan                     | −                | −                 |
| Galactan                               | −                | −                 |
| Insoluble polysaccharide               | −                | −                 |
| Acid-swollen cellulose                  | −                | −                 |
| Bacterial microcrystalline              | −                | −                 |
| cellulose                              | −                | −                 |
| Ivory nut mannan                       | −                | ND$^b$            |
| Degalactosylated mannan                | +$^+$            | ND$^b$            |
| Oat-splet xylan                         | −                | +$^+$            |

$^a$ Abf62A-CBM35 construct used in assessing binding to soluble polysaccharides was GST-Abf62A-CBM35, while Abf62A-CBM35 was used to evaluate binding to insoluble polysaccharide (see Fig. 1). $^+$ indicates binding; − indicates no binding. $^b$ Values in parentheses are $K_a$ (mg/ml) determined by quantitative AGE.

$^c$ +$^+$ indicates very weak binding. $^d$ Not determined.
FIG. 7. Representative ITC data of Man5C-CBM35 binding to oligo- and polysaccharides. The upper parts of each panel show the raw binding heats, the lower parts show the integrated binding heats minus the dilution control heats fitted to a single-site binding model. Ligands were at the following concentrations: mannohexaose (5 mM), mannopentaose (5 mM), carob galactomannan (5 mg/ml), konjac glucomannan (5 mg/ml). Man5C-CBM35 was at 600 μM.
Man5C-CBM35. It would appear, therefore, that several subsites in Man5C-CBM35 are able to bind to glucose or mannosyl residues, and the protein is able to interact with either an axial (mannose) or equatorial (glucose) O2 at these locations. This promiscuity in ligand recognition is similar to CBM29-2, which is able to bind gluco- or manno-configured sugars at each of its six subsites, and in two of these subsites the same amino acid is able to interact with an equatorial or axial O2 (15). In contrast to CBM29, the inability of Man5C-CBM35 to bind to a homopolymer of glucose indicates that either the interaction with an axial O2 is a critical element of sugar binding in at least one subsite, and/or an equatorial O2, by making a steric clash with the protein at one or more subsites, prevents the CBM35 from binding to cellobiose or cellulose. CBM27 is also able to bind glucosamannan but is unable to interact with cellobiose or insoluble regenerated cellulose (11), again suggesting that glucose can bind at selected subsites but is precluded from others. The crystal structures of CBM27 in complex with mannose-based ligands reveal that the protein makes hydrogen bonds with the axial O2 of mannose at subsites 2, 3, and 4. While the equatorial O2 of glucose can be tolerated in subsites 1, 2, and 5, steric clashes prevent the sugar from binding at subsites 3 and 4 explaining why the protein does not bind to cellulose. A similar selectivity for mannose at specific subsites can be invoked to explain why Man5C-CBM35 binds to glucosamannan but not cellulose or cellobiose-polysaccharides.

**Man5C-CBM35 Potentiates Mannanase Activity Against Insoluble Amorphous Mannan**—To evaluate whether Man5C-CBM35 potentiates mannanase activity, derivatives of Man5C comprising the GH5 catalytic module (GH5) and GH5 fused to CBM35 (Man5C-CBM35-GH5) were expressed in *E. coli*, and the catalytic activity of these proteins was evaluated. Both derivatives of Man5C display similar activities against mannhexaoose and insoluble ivory nut mannan; however, Man5C-CBM35-GH5 hydrolyzes insoluble DGM five times faster than GH5 alone (data not shown). Addition of the Man5C-CBM35 to GH5 in *trans* (as discrete proteins) in various ratios ranging from 1:1 to 10:1 (CBM35/GH5) did not increase the activity of the mannanase against any of the substrates evaluated (data not shown). These results suggest that the activity of the mannanase is compromised by restricted access to insoluble DGM, but that the enzyme is able to rapidly access soluble substrates. CBM35, by bringing the catalytic module in the Man5C derivative CBM35-GH5 into intimate and prolonged association with DGM, increases enzyme access to the substrate leading to more efficient catalysis. The CBM does not potentiate mannanase activity in *trans*, indicating that the module does not mediate its affect by disrupting the interchain interactions in mannan, which is in contrast to some CBM2a proteins that enhance cellulase action by disrupting the surface of crystalline cellulose, leading to an increase in substrate access (30, 31). The inability of CBM35 to improve the activity of the catalytic module of Man5C against ivory nut mannan is consistent with the observation that the CBM does not bind to the crystalline polysaccharide. Overall, these data demonstrate that Man5C-CBM35 displays properties similar to several cellulose (crystalline and non-crystalline) and xylan-binding CBMs, which have also been shown to enhance the catalytic activity of appended glycoside hydrolases against insoluble polysaccharides by increasing enzyme-substrate proximity (3, 4, 32).

**Calcium Mediates Binding of Abh62A-CBM35 to Xylan**—For ITC studies, the CBM35 module linked to a His tag was titrated with oat-spelt xylan in HEPS buffer. In the presence of 5 mM calcium the protein bound to xylan; however, when the divalent ion was replaced with 10 mM EDTA no interaction between Abh62A-CBM35 and the polysaccharide was evident. (Fig. 8). It should be noted that when Abh62A-CBM35, which had not been treated with reducing agent (exists as a dimer/monomer), was titrated with oat-spelt xylan the affinity was similar to the monomeric form of the CBM. However, aggregation of the polysaccharide occurred implying that the two xylan-binding sites in the disulfide-mediated dimer cross-link individual xylan chains (data not shown). Xylan aggregation by CBMs has also been demonstrated by the three linked CBM6 modules in *Clostridium stercorarium* xylanase II 11A (18). This phenomenon is also well established in the lectin field where multiple binding sites on the proteins mediate cross-linking of complex multivalent carbohydrates (14).

To investigate the role of the calcium in ligand-binding in more detail, an apo form of Abh62A-CBM35 was produced by treating the protein with Chelex (see “Experimental Procedures”). ITC shows that the apo form of the CBM does not interact with oat-spelt xylan in HEPES buffer; however, the protein binds tightly to the polysaccharide in the presence of 5 mM CaCl2 (Fig. 8 and Table III). The ΔH and TΔS values for the binding of the CBM35 to oat-spelt xylan are negative, similar to Man5C-CBM35 and other CBMs that interact with soluble polysaccharides (10, 11, 15). Titration of the apo form of Abh62A-CBM35 with calcium demonstrates that the protein binds tightly to the divalent metal ion (Fig. 8 and Table III). These data indicate that Abh62A-CBM35 displays an absolute requirement for calcium when binding to xylan, and furthermore, the protein is able to interact with the metal ion in the absence of the polysaccharide.

Previous studies have shown that CBMs from families 4, 6, 9, and 22 contain one or more calcium ions located at sites remote from the ligand binding cleft, suggesting a structural role for the metal (10, 29, 33–35). In support of this view, removal of calcium from both cellulose and xylan-binding CBM4s reduced the temperature at which the proteins unfolded by 8 °C (34) and 23 °C (35), respectively. However, the loss of this metal in both family 4 and 22 CBMs did not influence ligand binding. This report therefore provides one of the first examples of calcium playing a direct role in the binding of a CBM to its target ligand. Indeed, the demonstration that the metal ion also mediates the binding of a family X9 CBM to xylan2 suggests that the involvement of calcium in the association of CBMs with their target ligands may be a common phenomenon. Although the role of calcium in the interaction of CBMs

| Ligand                  | $K_a \times 10^{-3}$ | Δ$G$ kcal mol$^{-1}$ | Δ$H$ kcal mol$^{-1}$ | TΔS kcal mol$^{-1}$ |
|------------------------|----------------------|----------------------|----------------------|---------------------|
| Manohexaoose           | 8.4 (±0.1)$^b$       | -5.3 (±0.0)          | -12.2 (±0.0)         | -6.9 (±0.0)         |
| Mannopentaose          | 6.7 (±0.1)           | -5.2 (±0.0)          | -12.3 (±0.0)         | -7.1 (±0.1)         |
| Carob galactomannan    | 5.8 (±0.1)           | -5.1 (±0.0)          | -13.0 (±0.1)         | -7.9 (±0.1)         |
| Konjac glucosamannan   | 5.6 (±0.2)           | -5.1 (±0.0)          | -13.1 (±0.2)         | -8.0 (±0.2)         |

$^a$ Values in parentheses are standard deviations from the fit.

$^b$ $n$ is the number of binding sites on the protein.

A. B. Boraston, personal communication.
with polysaccharides and oligosaccharides has not been extensively studied, the importance of this metal in the binding of lectins to carbohydrates is well established (14, 18).

**Abf62A-CBM35 Only Binds to Poorly Substituted Xylans**

To further investigate the interaction of Abf62A-CBM35 with carbohydrates, ITC was performed using 4-O-methylglucuronomethylglucuronoxylan and xylohexaose as ligands (data not shown). No significant binding was observed with either of the sugars tested, confirming that the protein targets extended unsubstituted regions of xylan.

The ligand specificity of Abf62A-CBM35 is in sharp contrast to the other xylan-binding CBMs described to date, which are all able to interact with substituted and unsubstituted forms of the hemicellulose with similar affinities (10, 12, 18). Substituted xylans contain arabinose and/or 4-O-methylglucuronic acid groups attached to O2 and O3s of the xylose backbone. Structural studies on CBM15 from *C. japonicus* Xyn10C complexed with xylopentaose have revealed how xylan side chains can be accommodated. Six of the ten C2-OH and C3-OH groups in the pentasaccharide are solvent-exposed and therefore

![TABLE III](0x0000)

**Affinity of Abf62A-CBM35 for oat-spelt xylan and calcium as determined by ITC**

| Protein              | Ligand         | $K_A \times 10^{-4}$ | $\Delta G$  | $\Delta H$  | $T \Delta S$ |
|----------------------|----------------|----------------------|--------------|--------------|--------------|
| Monomer              | Oat-spelt xylan| 4.1 ($\pm 0.1$)     | -6.3 ($\pm 0.0$) | -11.0 ($\pm 0.5$) | -4.7 ($\pm 0.5$) |
| Monomer + 10 mM EDTA | Oat-spelt xylan| NB                   | NB           | NB           | NB           |
| Monomer (apo form)   | Oat-spelt xylan| NB                   | NB           | NB           | NB           |
| Monomer (apo form)   | Calcium        | 53.5 ($\pm 0.4$)    | -7.8 ($\pm 0.0$) | -17.8 ($\pm 0.1$) | -10.0 ($\pm 0.1$) |

* Titrations were carried out in the presence of 5 mM CaCl2 and reducing agent (1 mM TCEP).
* Values in parentheses are standard deviations from at least two separate titrations.
* NB, no binding.
* The apo form of the monomer was prepared by treatment with Chelex-100 to remove bound divalent metal ions.
References

1. Brett, C. T., and Waldren, K. (1996) in Physiology and Biochemistry of Plant Cell Walls. Topics in Plant Biology (Black, M., and Charlewood, B., eds) Chapman and Hall, London

2. Boraston, A. B., McLean, B. W., Kormos, J., Alam, M. M., Gilkes, N. R., Hines, C. A., Tomme, P., Kilburn, D. G., and Warren, R. A. (1999) in Recent Advances in Carbohydrate Bioengineering. (Gilbert, H. J., Davies, G. J., Henriusass, B., and Svensson, B., eds) pp. 202–211, The Royal Society of Chemistry, Cambridge, UK

3. Bolam, D. N., Czjzek, M., McQueen-Mason, S., Simpson, P., Williamson, M. P., Rixon, J. E., Boraston, A., Hazlewood, G. P., and Gilbert, H. J. (1998) Biochem. J. 331, 775–781

4. Gill, J., Rixon, J. E., Bolam, D. N., McQueen-Mason, S., Simpson, P. J., Williamson, M. P., Hazlewood, G. P., and Gilbert, H. J. (1999) Biochem. J. 342, 473–480

5. Coutinho, P. M., and Henriusass, B. (1999) in Recent Advances in Carbohydrate Bioengineering (Gilbert, J. J., Davies, G., Henriusass, B., and Svensson, B., eds) pp. 3–12, The Royal Society of Chemistry, Cambridge

6. Kraulis, J., Clare, G. M., Nilges, M., Jones, T. A., Pettersson, G., Knowles, J., and Groenenboorn, A. M. (1989) Bioinformatics 5, 743–757

7. Raghotama, S., Simpson, P. J., Szabo, L., Nagy, T., Gilbert, H. J., and Williamson, M. P. (2000) Biochemistry 39, 978–984

8. Tervo, J., Lainem, R., Charnock, A. J., Morag, E., Bayer, A. E., Shoham, Y., and Stelt, T. A. (1996) EMBO J. 15, 5739–5751

9. Xu, G. Y., Ong, E., Gilkes, N. R., Kilburn, D. G., Muhendaraj, D. R., Harris-Brandts, M., Carver, J. P., Kay, L. E., and Harvey, T. S. (1995) Biochemistry 34, 6993–7009

10. Charnock, S. J., Bolam, D. N., Turkenburg, J. P., Gilbert, H. J., Ferreira, L. M., and Davies, G. J. (2000) Biochemistry 39, 5013–5021

11. Boraston, A. B., Revett, T., Bolam, D. N., Czjzek, M., Nurizzo, D., and Davies, G. J. (2003) Structure 11, 665–675

12. Szabo, L., Jamal, S., Xie, H., Charnock, S. J., Bolam, D. N., Gilbert, H. J., and Davies, G. J. (2001) J. Biol. Chem. 276, 49661–49665

13. Boraston, A. B., Nurizzo, D., Notenboom, V., Deros, D., Rose, D. B., Kilburn, D. G., and Davies, G. J. (2002) J. Mol. Biol. 319, 1143–1156

14. Lis, H., and Sharon, N. (1998) Chem. Rev. 98, 637–674

15. Charnock, S. J., Bolam, D. N., Nurizzo, D., Szabo, L., McKin, V. A., Gilbert, H. J., and Davies, G. J. (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 14077–14082

16. Linder, M., Salovouri, I., Roushoned, L., and Teeri, T. T. (1996) J. Biol. Chem. 271, 21368–21372

17. Bolam, D. N., Xie, H., White, P., Simpson, P. J., Hancock, S. M., Williamson, M. P., and Gilbert, H. J. (2001) Biochemistry 40, 2468–2477

18. Boraston, A. B., McLean, B. W., Chen, G., Li, A., Warren, R. A., and Kilburn, D. G. (2002) Mol. Microbiol. 43, 187–194

19. Ferreira, L. M., Wood, T. M., Williamson, G., Faulds, C., Hazlewood, G. P., Black, G. W., and Gilbert, H. J. (1993) Biochem. J. 294, 349–355

20. Kellett, L. E., Poole, D. M., Ferreira, L. M., Durrant, A. J., Hazlewood, G. P., and Gilbert, H. J. (1999) Biochem. J. 342, 5707–5716

21. Kilburn, D. G. (1991) Bio/Technology 9, 187–195

22. Glaudemans, C. P. J., and Timell, T. E. (1958) Biochem. J. 77, 1043–1046

23. Xie, H., Gilbert, H. J., Charnock, S. J., Davies, G. J., Williamson, M. P., Simpson, P. J., Raghotama, S., Fontes, C. M., Dias, F. M., Ferreira, L. M., and Bolam, D. N. (2001) Biochemistry 40, 9167–9176

24. Xie, H., Bolam, D. N., Nagi, S., Szabo, L., Cooper, S., Simpson, P. J., Lakey, J. H., Williamson, M. P., and Gilbert, H. J. (2001) Biochemistry 40, 5700–5707

25. Simpson, P. J., Xie, H., Bolam, D. N., Gilbert, H. J., and Williamson, M. P. (2000) J. Biol. Chem. 275, 41137–41142

26. Halstead, J. R., Fransen, M. P., Eberhart, R. Y., Park, A. J., Gilbert, H. J., and Hazlewood, G. P. (2000) FEBS Microbiol. Lett. 192, 197–203

27. Takei, K. (1984) Electrophoresis 5, 187–195

28. Glaudemans, C. P. J., and Timell, T. E. (1958) J. Am. Chem. Soc. 80, 941–941

29. Henzaw, J., Bolam, D. N., Pires, V. M., Czajek, M., Henriusass, B., Ferreira, L. M., Fontes, C. M., and Gilbert, H. J. (2004) J. Biol. Chem. 279, 21552–21559

30. Czajek, M., Bolam, D. N., Mosiah, A., Allouch, J., Fontes, C. M., Ferreira, L. M., Bernet, O., Zamboni, V., Darbon, H., Smith, N. L., Black, G. W., Henriusass, B., and Gilbert, H. J. (2001) J. Biol. Chem. 276, 48580–48587

31. Din, N., Damade, H. G., Gilkes, N. R., Miller, R. C., Jr., Warren, R. A., and Kilburn, D. G. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 11380–11385

32. Din, N., Gilkes, N. R., Tekkant, B., Miller, R. C., Warren, R. A., and Kilburn, D. G. (1991) Bio/Technology 9, 1096–1099

33. Black, G. W., Hazlewood, G. P., Millward-Sadler, S. J., Laurie, J. L., and Gilbert, H. J. (1995) Biochem. J. 307, 191–196

34. Notenboom, V., Boraston, A. B., Kilburn, D. G., and Rose, D. R. (2001) Biochemistry 40, 6248–6256

35. Johnson, P. E., Creagh, A. L., Brun, E., Joe, K., Tomme, P., Haynes, C. A., and Henrissat, B. (1999) in Recent Advances in Carbohydrate Bioengineering (Gilbert, J. J., Davies, G. J., Henriusass, B., and Svensson, B., eds) pp. 3–12, The Royal Society of Chemistry, Cambridge

36. Jouleau, J. P., Comtat, J., and Ruel, K. (1992) in Xylan and Xylanases: Progress in Biotechnology (Voragen, A. G. L., ed) Vol. 7, pp. 1–15, Elsevier Science Publishers B. V., Amsterdam

Downloaded from http://www.jbc.org/ by guest on July 24, 2018
X4 Modules Represent a New Family of Carbohydrate-binding Modules That Display Novel Properties
David N. Bolam, Hefang Xie, Gavin Pell, Deborah Hogg, Greta Galbraith, Bernard Henrissat and Harry J. Gilbert

J. Biol. Chem. 2004, 279:22953-22963.
doi: 10.1074/jbc.M313317200 originally published online March 5, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M313317200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 33 references, 12 of which can be accessed free at http://www.jbc.org/content/279/22/22953.full.html#ref-list-1