The Family 6 Carbohydrate Binding Module CmCBM6-2 Contains Two Ligand-binding Sites with Distinct Specificities*§

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The microbial degradation of the plant cell wall is an important biological process, representing a major component of the carbon cycle. Enzymes that mediate the hydrolysis of this composite structure are modular proteins that contain non-catalytic carbohydrate binding modules (CBMs) that enhance catalytic activity. CBMs are grouped into sequence-based families, and in a previous study we showed that a family 6 CBM (CBM6) that interacts with xylan contains two potential ligand binding clefts, designated cleft A and cleft B. Mutagenesis and NMR studies showed that only cleft A in this protein binds to xylan. Family 6 CBMs bind to a range of polysaccharides, and it was proposed that the variation in ligand specificity observed in these proteins reflects the specific cleft that interacts with the target carbohydrate. Here the biochemical properties of the C-terminal cellulose binding CBM6 (CmCBM6-2) from Cellulibrio mixtus endoglucanase 5A were investigated. The CBM binds to the β1,4-β1,3-mixed linked glucans lichenan and barley β-glucan, cello-oligosaccharides, insoluble forms of cellulose, the β1,3-glucan laminarin, and xylooligosaccharides. Mutagenesis studies, informed by the crystal structure of the protein (presented in the accompanying paper, Pires, V. M. R., Henshaw, J. L., Prates, J. A. M., Bolam, D., Ferreira, L. M. A. Fontes, C. M. G. A., Henrissat, B., Planas, A., Gilbert, H. J., Czjzek, M. (2004) J. Biol. Chem. 279, 21560–21568), show that both cleft A and B can accommodate cello-oligosaccharides and laminarin displays a preference for cleft A, whereas xylooligosaccharides exhibit absolute specificity for this site, and the β1,4-β1,3-mixed linked glucans interact only with cleft B. The binding of CmCBM6-2 to insoluble cellulose involves synergetic interactions between cleft A and cleft B. These data show that CmCBM6-2 contains two binding sites that display differences in ligand specificity, supporting the view that distinct binding clefts with different specificities can contribute to the variation in ligand recognition displayed by family 6 CBMs. This is in sharp contrast to other CBM families, where variation in ligand binding is a result of changes in the topology of a single carbohydrate-binding site.

Microbial degradation of the plant cell wall is the primary mechanism by which organic carbon is recycled in the biosphere. The plant cell wall is a complex insoluble structure that is highly recalcitrant to biological attack (1, 2). To increase the efficiency of this degradative process microorganisms synthesize modular plant cell wall hydrolases in which the catalytic modules are attached via linker peptides to non-catalytic carbohydrate binding modules (CBMs) (3). CBMs potentiate catalytic activity by mediating prolonged and intimate association between the enzyme and its target substrate (4, 5), whereas some of these modules also increase substrate access by disrupting the crystalline structure of cellulose (6, 7), the major plant structural polysaccharide. The ligand specificity of CBMs generally reflects the substrate specificity of the catalytic module with xylan, mannan, laminarin, and cellulose binding CBMs located in xylanases, mannanases, laminarases, and cellulases, respectively (8, 9). It should be noted, however, that many plant cell wall hydrolases that do not hydrolyze cellulose contain CBMs that interact specifically with the crystalline form of this polysaccharide (2, 10–12).

Based on primary structural similarities CBMs have been grouped into 34 families (afmb.cnrs-mrs.fr/CAZY (13)). Although the ligand specificity of families 1, 5, and 10, which bind to crystalline polysaccharides, is invariant, there are considerable differences in carbohydrate recognition in other CBM families. For example, the CBM2 family contains proteins that bind to xylan (14) and crystalline cellulose (12), whereas different members of CBM4 recognize laminarin, individual cellulose chains, and xylan (8, 9) (15). The CBMs described to date are β-stranded proteins in which the topology of the carbohydrate-binding site reflects the nature of the target ligand. For example, CBMs that bind to crystalline cellulose (known as type A modules) contain a planar hydrophobic ligand binding surface (16–19). By contrast, CBMs that interact with individual polysaccharide chains (type B modules) accommodate their target ligands in a cleft of varying depth (9, 20, 21). In general both type A and type B CBMs interact with five or six saccharide units via hydrophobic stacking interactions between alternate sugar rings and aromatic amino acids, although hydrogen bonds also play an important role in carbohydrate recognition by type B modules (22, 23).

The three-dimensional structure of almost all type B CBMs determined to date conform to a classic lectin-like β-jelly roll in
which a single ligand-binding site comprises a shallow cleft on the concave surface of the protein. In CBM4 and CBM2, variation in cleft A and B was observed within the CBM6 family.

EXPERIMENTAL PROCEDURES

Source of Carbohydrates Used—All oligosaccharides and polysaccharides were purchased from Megazyme International (Bray County Wicklow, Ireland), except oat spelt xylan and hydroxyethylcellulose, which were obtained from Sigma, and cello-oligosaccharides, which were from Seikagaku Corp. (Japan). Acid-swollen cellulose was prepared from Avicel (PH101; Serva) as described previously (27).

Protein Expression and Purification—The regions of the CmCel5A gene (cel5A; Ref. 26) encoding CmCBM6-2 were amplified by the PCR using the primers 5′-CCCATATGGTAATCCGGACTATTC-3′ and 5′-CAAGATCTAATTGTGCTATCTGC-3′ (amplifies nucleotides 1475–1869 of cel5A). The reverse primer contains the cel5A stop codon. The amplified DNA was digested with NdeI and XhoI (restriction sites, shown in bold, were incorporated into the two primers) and cloned into the similarly restricted expression vector pET28a to generate pCf1. CmCBM6-2, encoded by pCf1, contains a N-terminal His$_6$ tag. To express CmCBM6-2 the Escherichia coli strain Tuner (Novagen), harboring pCf1, was cultured in Luria Bertani broth containing 10 µg/ml ampicillin at 37 °C to an optical density of 0.9. The temperature was then reduced to 16 °C, and when the absorbance had reached 0.7 isopropyl-$\beta$-D-thiogalactopyranoside was added to a final concentration of 0.1% (w/v), and bovine serum albumin was used as the non-binding negative control.

$^a$ Binding was evaluated by affinity gel electrophoresis as described under “Experimental Procedures.” Poly saccharide was added to the ligand gel at a final concentration of 0.1% (w/v), and bovine serum albumin was used as the non-binding negative control.

$^b$ +, binding; –, no binding.

$^c$ Mutation in cleft A.

$^d$ Mutation in cleft B.
to generate these mutants are listed in Table I, and each of the mutated CBM6 genes was sequenced by MWG Biotech (Germany) to ensure that only the appropriate mutations had been incorporated.

**Affinity Gel Electrophoresis**—Qualitative assessment of the capacity of CmCBM6-2 to bind to soluble polysaccharides was determined by affinity gel electrophoresis. The method was essentially as described by Freelove et al. (28) using the polysaccharide ligands at a concentration of 0.1% (w/v), and electrophoresis was carried out for 4 h. The non-binding protein, which was used as a negative control, was bovine serum albumin. When CmCBM6-2 bound to a polysaccharide ligand it could be visualized as a tight, but very slow migrating band, whereas in the gels lacking ligand (or ligand that the protein did not interact with) the CBM ran as a smear making it difficult to visualize.

**Isothermal Titration Calorimetry (ITC)**—ITC measurements were made following standard procedures (20) using a Microcal Omega titration calorimeter. The proteins were dialyzed extensively against 50 mM Na-HEPES buffer, pH 7.0, 5 mM CaCl₂, and the ligand was dissolved in the same buffer to minimize heats of dilution. During a titration experiment the protein sample stirred at 300 rpm in a 1.4331-ml reaction cell was injected with 25–50 successive 10-µl aliquots of polysaccharide (5–20 mg ml⁻¹) or oligosaccharide (7.5–20 mM) ligand at 200-s intervals. The molar concentration of CmCBM6-2-binding sites present in the polysaccharide ligands was determined as described previously (31). Integrated heat effects, after correction for heats of dilution, were analyzed by nonlinear regression using a single-site binding model (Micrcal Origin, Version 5.0). The fitted data yield the association constant (Kₐ), number of binding sites on the protein (n), and the enthalpy of binding (ΔH). Other thermodynamic parameters were calculated using the standard thermodynamic equation −RTlnKₐ = ΔG = ΔH − TΔS. Titrations were carried out in triplicate for most ligands, and the errors are the S.D. of the mean of these replicates.

**Binding to Insoluble Polysaccharides**—Qualitative assessment of binding to insoluble cellulose was carried out as follows: 200 µl of a 250 µg ml⁻¹ solution of CmCBM6-2 in 20 mM Tris-HCl, pH 8.0, containing 300 mM NaCl and 5% (v/v) Tween 20 (Buffer A) was mixed with 2 mg of insoluble polysaccharide. The reaction mixture was incubated on ice for 1 h with occasional mixing, after which the insoluble cellulose was pelleted by centrifugation at 13,000 × g for 2 min. The supernatant, comprising the unbound fraction, was removed, and all samples were analyzed by SDS-PAGE using a 12.5% gel. Controls with protein but no cellulose were included to ensure that no precipitation occurred during the assay period. Depletion isotherms to quantify the binding of wild type and mutants of CmCBM6-2 to both acid-swollen cellulose and Avicel were carried out as follows: protein (250 µl in Buffer A) at concentrations between 1 and 100 µM was added to 0.25 mg of cellulose and incubated on ice for 1 h with gentle mixing. The polysaccharide was then centrifuged at 13,000 × g for 1 min, and the A_{280} of the supernatant was measured to quantify the amount of free protein remaining after binding. Bound protein was calculated from the total minus the free. Controls with protein but no cellulose were included to ensure that no precipitation occurred during the assay period. The data were analyzed by nonlinear regression using a standard one-site binding model (GraphPad Prism, v2.01), and the Bₘₐₓ (amount of CBM bound at saturation) and Kₐ values were obtained from the regressed isotherm data. At least three separate binding isotherms were carried out for each protein.
**RESULTS**

**Ligand Specificity of CmCBM6-2—Cel5A contains an N-terminal GH5 catalytic domain and two family 6 CBMs, with the C-terminal module designated CmCBM6-2 (26). To evaluate the ligand binding specificity of CmCBM6-2, the protein module was expressed in *E. coli* and purified to electrophoretic homogeneity (Fig. 1). Affinity gel electrophoresis, Table II, shows that CmCBM6-2 binds to the β1,4-β1,3-mixed-linked glucans lichenan and barley β-glucan and the β1,3-glucan laminarin but does not display significant affinity for poorly substituted or highly decorated xylans, pectins such as polygalacturonic acid or rhamnogalacturonan I, potato or pectic β1,4-galactan, sugar beet arabinan, locust bean or carob galactomannan, and konjac glucomannan.**

ITC was used to quantify the interaction of CmCBM6-2 with its target ligands. Representative titrations that can be deconvoluted to give thermodynamic and binding parameters are displayed in Fig. 2, and the full data set is presented in Table III. ITC data that could only provide qualitative information on the binding of some ligands because the affinities were too low to obtain sigmoidal titration curves, are displayed in Table IV, and examples of these titrations are provided in theSupplemental Fig. 1. CmCBM6-2 binds to β1,3-β1,4 and mixed-linked β1,4-β1,3-glucans, and to xylooligosaccharides (Table III and Fig. 2) with affinities that are similar to most Type B CBMs from mesophilic microorganisms (29–32). The stoichiometry for the binding of CmCBM6-2 to cellulose is close to 1:2, indicating that the protein module contains two distinct ligand-binding sites that can each accommodate this oligosaccharide. The isotherms, however, display only one obvious binding event, and the data fit a single-site model well, suggesting that the two sites are non-interacting and have similar affinities for cellulose.

The isotherms, however, display only one obvious binding event, and the data fit a single-site model well, suggesting that the two sites are non-interacting and have similar affinities for cellulose. It was not possible, therefore, to deconvolute the ITC data (for the wild type protein) to determine the individual affinity for the oligosaccharide ligand at each discrete binding site. This issue was resolved using a mutagenesis approach described below.

Table III shows that the binding of CmCBM6-2 to its ligands is associated with negative enthalpy and entropy. The thermodynamics of the interaction of CmCBM6-2 with glucose- and xylose-based ligands is typical of the binding of proteins to soluble saccharides, which is invariably enthalpically driven, with an unfavorable entropic contribution (10, 20, 32).

**Identifying the Binding Sites in CmCBM6-2—Cleft A-Xylan binding CMB6 modules contain two distinct clefts that could comprise the ligand-binding site (21, 24). Mutagenesis, structural, and NMR data showed that only one of these sites, designated cleft A, could accommodate xylooligosaccharides or xylan (21, 24), whereas the other cleft, which was unable to interact with the target ligands, was defined as cleft B. Structural characterization of CmCBM6-2 (accompanying paper, Ref. 25) revealed that this protein also contains the same two putative binding clefts as the previously characterized CBM6s. To identify the location of the ligand-binding site(s) in CmCBM6-2, a mutagenesis strategy was employed. In the two xylan-binding CBM6 proteins cleft A contains two solvent-
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exposed aromatic residues that play a key role in ligand binding (21, 24), and Fig. 3 reveals that the equivalent amino acids in *C. mixtus* CBM6–2 are Trp-92 and Tyr-33 (25). Thus, the ligand specificity of mutants of CmCBM6–2 in which Trp-92 and Tyr-33 were substituted either individually (data not shown) or together (Table III) was evaluated. All three mutants bind lichenan and β-glucan with an affinity that resembles that of the wild type protein (Table III), indicating that the mutations introduced into cleft A do not disrupt binding to the β1,4-β1,3-mixed linked glucans. Although the mutants W92A, Y33A (data not shown), and W92A/Y33A bind to cellohexaose (Table III) with similar affinity to the wild type protein, the stoichiometry for cellohexaose was 1:1 (compared with 1:2 in the wild type protein), suggesting that the oligosaccharide binds to only a single site in the mutated CBMs. All three mutants of CmCBM6–2 bound to laminohexaose and laminarin weakly, but they did not interact with xylohexaose (Table IV and Supplemental Fig. 1). These data indicate that although the β1,3-linked ligands bind preferentially to cleft A, xylo-oligosaccharides display an absolute specificity for this site.

Cleft B—The crystal structure of CmCBM6–2 reveals an aromatic residue (Trp-39) and several polar amino acids (Gln-13, Ser-41, Arg-60, Glu-73, Lys-114 and Asn-116) within cleft B that are solvent-exposed and could thus play a role in ligand binding (Fig. 3). These residues were substituted with alanine, and the biochemical properties of the mutants were analyzed. The mutants S41A, R60A, and N116A could not be expressed in soluble form and, thus, were not studied further. Small amounts of soluble W39A were produced, and affinity gel electrophoresis revealed that the mutant binds to laminarin but not lichenan or barley β-glucan (Table IV). ITC revealed that Q13A and K114A bind to all the glucan ligands with affinities that are similar to the wild type protein (Table III), indicating that neither residue by itself plays a role in carbohydrate recognition in CmCBM6–2. This is rather surprising as data in the accompanying paper (25) indicate that these amino acids are in close proximity with the ligand at the distal subsites 1 and 4; however, it has been shown that not all residues in CBMs that make direct hydrogen bonds with the target carbohydrates contribute to overall affinity (29). In contrast, E73A does not bind to lichenan or β-glucan (Tables IV) but interacts with laminohexaose, lamarin, and cellohexaose (Table III). Although the affinity of cellohexaose for E73A is similar to W92A/Y33A, laminarin and laminohexaose bind more tightly to the cleft B mutant than the three cleft A mutants. These data indicate that both cleft A and cleft B bind to cellohexaose but only cleft B can accommodate the β1,4-β1,3-mixed linked glucans, lichenan and barley β-glucan, whereas laminohexaose and lamarin bind preferably to cleft A. The results also point to Glu-73 and Trp-39 playing a pivotal role in ligand binding in cleft B, a view supported by crystallographic data presented in the accompanying paper (25).

Size of the Ligand-binding Sites at Cleft A and Cleft B—To determine the extent of the ligand-binding sites in cleft A and cleft B, the affinity of E73A and W92A/Y33A for a range of cello-oligosaccharides was determined by ITC. E73A has a similar affinity for cello-oligosaccharides with a degree of polymerization (d.p.) between 2 and 6 (Table III and Fig. 2). Glucose also binds to E73A (Table IV and Supplemental Fig. 1) but with an affinity that was too low to accurately quantify. W92A/Y33A binds to cellobiose and cellohexaose with very similar affinities (Table III) and interacts weakly with cellobiose (Table IV) but does not accommodate glucose. These data are entirely consistent with the crystal structure of CmCBM6–2 presented in the accompanying paper (25), which shows that cleft A only forms interactions with two sugar rings, whereas cleft B comprises three binding sites for β1,4-configured glucans. These results are in sharp contrast to all other type B CBMs described to date, including the *C. thermocellum* CBM6, which contain binding sites that can accommodate between 4 and 6 sugar units of an oligo- or polysaccharide chain (21, 20, 31, 32).

To further probe the importance of cleft A and cleft B in ligand binding, key residues in both sites were substituted with alanine, generating the mutant W92A/Y33A/E73A. The binding data (Table IV, Fig. 2, and Supplemental Fig. 1) show that the triple mutant does not interact with any of the ligands tested. The observation that binding to β1,3- or β1,4-linked sugars is abolished only when both clefts A and B are disrupted, and that inactivation of either cleft individually does not abrogate binding to these saccharides, supports the view that these ligands are able to interact with the CBM at both cleft A and cleft B.

**Fig. 3.** Amino acids located in the binding site of CmCBM6–2 cleft A and cleft B. The amino acids on the surface of the ligand-binding sites of cleft A and cleft B are displayed in panels a and b, respectively. The terminal glucose in cleft A is derived from cello-oligosaccharides and laminarin. A molecule of cellobiose is shown bound in both clefts.
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**DISCUSSION**

The primary objective of this study is to determine whether the diversity in ligand specificity displayed by CBM6 modules reflects binding of these sugar polymers to distinct sites in different members of this protein family. *Cm*CBM6-2 interacts with the β1,4-β1,3-linked glucans, lichenan, and barley β-glucan in a cleft that is distinct from the ligand-binding site of the two xylan binding CBM6 modules described previously (21, 24). This observation is in direct contrast to all other Type B CBMs analyzed to date, where differences in ligand specificity within a family reflect subtle changes to a common binding site. For example, CBM4 contains proteins that bind to xylan, single cellulose chains, and laminarin, respectively (8, 9). Structural data show that in *Tm*CBM4, which interacts specifically with laminarin, the topology of the binding site is U-shaped, mirroring the solution conformation of the target ligand and, thus, explaining why the protein is unable to bind the linear polysaccharide cellulose (9). In family 2 CBMs differences in ligand specificity are mediated by the orientation of a single tryptophan in the binding site; in CBM2a this residue is planar with the other surface tryptophan residues, and thus, this protein can interact with the flat surface of crystalline cellulose (18). In CBM2bs the two tryptophans in the binding site are in a perpendicular orientation with respect to each other and, thus, are able to interact with xylose moieties n and n + 2 in xylan, which has a twisted 3-fold helical conformation (33, 34). Whether the diversity in ligand specificity observed in other CBM families reflects distinct binding sites, as occurs in CBM6, or variation of a common binding site remains to be elucidated.

The capacity of clefts A and B in *Cm*CBM6-2 to bind β1,4- and β1,3-linked glucans (although binding to the β1,3-linked glucans in cleft B is weak) is an unusual feature of this protein; the β-glucan binding CBMs characterized to date bind exclusively to either laminarin or cellulose but not to both ligands (9). The structural basis for the relaxed specificity of *Cm*CBM6-2 is, thus, an interesting issue. In both cleft A and B carbohydrate recognition is mediated by very few residues (see the accompanying paper, Ref. 25) that interact with two sugar residues in cleft A and three or four saccharide units in cleft B. The broad specificity of cleft A reflects the mechanism of ligand recognition; sugar binding is dominated by the stacking of two aromatic residues with a single sugar, and thus, a range of small gluco- or xylo-configured ligands can be accommodated. The occlusion of the distal region of cleft A (see the accompanying paper, Ref. 25) prevents recognition of the internal regions of polysaccharides, such as xylangs or β-glucans, and thus binding only occurs with the terminal sugars of these ligands. Because these polysaccharides have large d.p. values (>400),
the concentration of available binding sites is very low, and thus binding, is only evident to oligosaccharides derived from these polymers. The capacity of cleft A to bind to laminarin may reflect the extended helical conformation of the polysaccharide and, thus, the resultant internal U-shaped structure of this ligand (9), which may thus be able to interact with Trp-92 and Tyr-33 without forming steric clashes with the distal region of the cleft. An alternative, and more likely explanation is that laminarin has a d.p. of only ~25 (35), and thus, the concentration of terminal sugar residues on an equi-weight basis with the other polysaccharides evaluated is very high. The capacity of cleft B to bind a range of β-glucans reflects the topology of the subsites (see the accompanying paper, Ref. 25). Thus, subsites 4 and 3 can bind a β1,3-linked disaccharide and subsites 3 and 2 can accommodate only a β1,4-linked disaccharide, whereas subsites 2 and 1 can interact with either β1,4- or β1,3-linked disaccharides. It should be emphasized that because laminohexaose displays low affinity for cleft B, the interactions of β1,3-linked disaccharides with subsites 4 and 3 and subsites 1 and 2 are weak.

In the two xylan binding CBMs described previously (21, 24), cleft B does not bind sugar polymers because it is occluded by a proline-containing loop. In the accompanying paper (25) the structure of CmCBM6-2 shows that this loop is much smaller and lacks the rigidity conferred by proline, thus enabling cleft B to bind to saccharide chains. Sequence analysis reveals that several other CBM6 modules lack the proline, which occludes cleft B, as well as one of the two key aromatic residues that mediate ligand binding in cleft A (21, 24). It is likely, therefore, that in these proteins ligand binding occurs in cleft B but not cleft A. Thus, CmCBM6-2 may be a highly unusual family 6 protein in which both cleft A and cleft B are able to interact with carbohydrate ligands.

The vast majority of CBMs contain a single ligand-binding site, and thus, the presence of two independent glucan recognition sites with different specificities is a unique feature of CmCBM6-2. The other only CBMs reported to contain multiple sugar-binding sites are those located in families 13 and 20. CBM13s are members of the “ricin-superfamily” of lectins that have a β-trefoil fold (36–38). These proteins have three distinct sugar-binding sites that are all able to bind to the same mono- and oligosaccharide ligands, although the actual affinities for these molecules can vary between the different sites (39). CBM20s contain two discrete starch-binding sites that display very similar ligand specificities (40). The presence of multiple binding sites in protein modules has also been observed in the catalytic modules of starch modifying enzymes. Thus, the family 13 catalytic modules of amylases, cyclodextrinases, and other starch-modifying enzymes contain multiple domains designated A, B, and C, respectively. In barley α-amylase isozyme 1 domain C contains a maltooligosaccharide-binding site that is discrete from the active site cleft (41), as does the B-domain of amylosucrase from Neisseria polysaccharea (42). A recent study on the catalytic module of a family 13 β-agarase has also revealed an agaro-oligosaccharide-binding site that is distinct from the active site of the enzyme (43).

The ligand specificity of CmCBM6-2 is broadly consistent with the biological role of the enzyme, which hydrolyzes β1,4-β1,3-mixed-linked glucans such as lichenan and barley β-glucan in addition to soluble forms of cellulose (26). Thus, the CBM binds to both these polysaccharides, and by bringing these substrates into close proximity with the catalytic module is likely to potentiate enzyme activity, as reported previously for several other CBMs (4, 5). The biological rationale for the interaction of CmCBM6-2 with the β1,3-linked glucan polymer laminarin is less clear because Cel5A has no detectable activity against this polysaccharide (26), and the terrestrial bacterium that expresses this endoglucanase is unlikely to encounter a marine polysaccharide. It may, therefore, simply be a standby effect and, thus, not related to the biological function of the enzyme.

In conclusion, this report shows that CmCBM6-2 binds to β-glucan ligands at two distinct binding clefts. Both sites are able to recognize β1,3- and β1,4-linked glucan chains (although β1,3-glucan binds weakly to cleft B), a feature that is not evident in other CBMs, which display absolute selectivity for either laminarin or cellulose (9). A further unusual feature of CmCBM6-2 is that although both binding sites interact with β1,3 and β1,4 homopolymers of glucose, only cleft B is able to bind β1,3:β1,4-mixed-linked glucans, whereas xylooligosaccharides interact exclusively with cleft A. In contrast, the variation in ligand specificity evident in other CBM families is through changes to a common binding site. The CBM6 scaffold, comprising two distinct binding clefts that in conjunction bind to insoluble ligands but individually display significant differences in specificity, confers the remarkable plasticity in carbohydrate recognition displayed by this family of proteins. The structural basis for the unique binding properties of CmCBM6-2 is reported in the accompanying paper (25).

REFERENCES
1. Hogg, D., Pell, G., Dupree, P., Goubet, F., Martin-Orue, S. M., Armand, S., and Gronenborn, A. M. (1989) Biochemistry 28, 7241–7257
2. Boraston, A. B., Notenboom, V., Warren, R. A., Kilburn, D. G., and Davies, G. J. (2002) Biochem. J. 369, 665–675
3. Abou Hachem, M., Nordberg Karlsson, E., Bartonek-Roxa, E., Raghothama, S., Fontes, C. M., Dias, F. M., Ferreira, L. M., and Gilbert, H. J. (2001) Biochemistry 40, 1099–1105
4. Black, G. W., Hazlewood, G. P., Millward-Sadler, S. J., Laurie, J. I., and Davies, G. J. (1991) Biochem. J. 276, 573–5751
5. CmCBM6 Contains Two Discrete Binding Sites
6. Boraston, A. B., Nurizzo, D., Notenboom, V., Ducrons, V., Rose, D. B., Kilburn, D. G., and Davies, G. J. (2002) J. Biol. Chem. 276, 9176–9184
7. Raghothama, S., Simpson, P. J., Russinovitch, Z., Doon, V., Rose, D. B., Davies, G. J. (2003) J. Biol. Chem. 278, 191–1156
8. Boraston, A. B., Revett, T. J., Asteria, C. N., Notenboom, V., Ferreira, L. M., and Davies, G. J. (2003) Structure 11, 665–675
9. Borgaonkar, S., Borthakur, J., Durrant, A. J., Kilburn, D. G., and Davies, G. J. (2001) Biochem. J. 369, 1027–1043
10. Boraston, A. B., Nurizzo, D., Notenboom, V., Ducrons, V., Rose, D. B., Davies, G. J. (2002) J. Biol. Chem. 276, 9176–9184
11. Hogg, D., Pell, G., Dupree, P., Goubet, F., Martin-Orue, S. M., Armand, S., and Gilbert, H. J. (2003) Biochem. J. 371, 1027–1043
12. References
13. Coutinho, P. M., and Henrissat, B. (1999) in Recent Advances in Carbohydrate Bioengineering (Gilbert, J. J., Davies, G., Henrissat, B., and Svensson, B., eds) pp. 3–12, The Royal Society of Chemistry, Cambridge, UK
14. Cm–glucan ligands at two distinct binding clefts. Both sites are
15. References
16. References
17. References
18. References
19. References
20. References
21. References
22. References
23. References
24. References
25. References
26. References
CmCBM6 Contains Two Discrete Binding Sites

25. Pires, V. M. R., Henshaw, J., Prates, J. A. M., Bolam, D., Ferreira, L. M. A., Fontes, C. M. G. A., Henrissat, B., Planas, A., Gilbert, H. J., Czjzek, M. (2004) J. Biol. Chem. 279, 21560–21568
26. Fontes, C. M., Clarke, J. H., Hazlewood, G. P., Fernandes, T. H., Gilbert, H. J., and Ferreira, L. M. (1998) Appl. Microbiol. Biotechnol. 48, 552–559
27. Wood, T. M. (1988) Methods Enzymol. 160, 18–25
28. Freelove, A. C., Bolam, D. N., White, P., Hazlewood, G. P., and Gilbert, H. J. (2001) J. Biol. Chem. 276, 43010–43017
29. Xie, H., Bolam, D. N., Nagy, T., Szabo, L., Cooper, A., Simpson, P. J., Lakey, J. H., Williamson, M. P., and Gilbert, H. J. (2001) Biochemistry 40, 5700–5707
30. Boraston, A. B., Tomme, P., Amandoron, E. A., and Kilburn, D. G. (2000) Biochem. J. 350, 933–941
31. Szabo, L. Jamal, S., Xie, H., Charnock, S. J., Bolam, D. N., Gilbert, H. J., and Davies, G. J. (2001) J. Biol. Chem. 276, 49061–49065
32. Charnock, S. J., Bolam, D. N., Nurizzo, D., Szabo, L., McKie, V. A., Gilbert, H. J., and Davies, G. J. (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 14077–14082
33. Simpson, P. J., Bolam, D. N., Cooper, A., Ciruela, A., Hazlewood, G. P., Gilbert, H. J., and Williamson, M. P. (1999) Structure 7, 853–864
34. Simpson, P. J., Xie, H., Bolam, D. N., Gilbert, H. J., and Williamson, M. P. (2000) J. Biol. Chem. 275, 41137–41142
35. Boraston, A. B., Warren, R. A., and Kilburn, D. G. (2001) Biochemistry 40, 14679–14685
36. Notenboom, V., Boraston, A. B., Williams, S. J., Kilburn, D. G., and Rose, D. R. (2002) Biochemistry 41, 4246–4254
37. Fujimoto, Z., Kuno, A., Kaneko, S., Yoshida, S., Kobayashi, H., Kusakabe, I., and Mizuno, H. (2000) J. Mol. Biol. 300, 573–583
38. Fujimoto, Z., Kuno, A., Kaneko, S., Kobayashi, H., Kusakabe, I., and Mizuno, H. (2002) J. Mol. Biol. 316, 65–78
39. Scharpf, M., Connelly, G. P., Lee, G. M., Boraston, A. B., Warren, R. A., and McIntosh, L. P. (2002) Biochemistry 41, 4255–4263
40. Williamson, M. P., Le Gal-Coeffet, M. F., Sorimachi, K., Furniss, C. S., Archer, D. B., and Williamson, G. (1997) Biochemistry 36, 7535–7539
41. Robert, X., Haser, R., Gottschalk, T. E., Ratajczak, F., Driguez, H., Svensson, B., and Aghajari, N. (2003) Structure 11, 973–984
42. Skov, L. K., Mirza, O., Sprogøe, D., Dar, I., Renaud-Simeen, M., Albenne, C., Monsan, P., and Gajhede, M. (2002) J. Biol. Chem. 277, 47741–47747
43. Allouch, J., Jam, M., Helbert, W., Barbye, T., Kloareg, B., Henrissat, B., and Czjzek, M. (2003) J. Biol. Chem. 278, 47171–47180
