Importance of Hydrophobic and Polar Residues in Ligand Binding in the Family 15 Carbohydrate-Binding Module from *Cellvibrio japonicus* Xyn10C

Gavin Pell,[‡] Michael P. Williamson,[§] Christopher Walters,[§] Haomao Du,[‡] Harry J. Gilbert,*[,‡] and David N. Bolam[‡]

School of Cell and Molecular Biosciences, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU, United Kingdom, and Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2UH, United Kingdom

Received May 8, 2003; Revised Manuscript Received June 18, 2003

ABSTRACT: Modular glycoside hydrolases that degrade the plant cell wall often contain noncatalytic carbohydrate-binding modules (CBMs) that interact with specific polysaccharides within this complex macromolecule. CBMs, by bringing the appended catalytic module into intimate and prolonged association with the substrate, increase the rate at which these enzymes are able to hydrolyze glycosidic bonds. Recently, the crystal structure of the family 15 CBM (CBM15) from *Cellvibrio japonicus* (formerly *Pseudomonas cellulosa*) Xyn10C was determined in complex with the ligand xylopentaose. In this report we have used a rational design approach, informed by the crystal structure of the CBM15−ligand complex, to probe the importance of hydrophobic stacking interactions and both direct and water-mediated hydrogen bonds in the binding of this protein to xylan and xylohexaose. The data show that replacing either Trp 171 or Trp 186, which stack against xylose residues *n* and *n* + *2* in xylopentaose, with alanine abolished ligand binding. Similarly, replacing Asn 106, Gln 171, and Gln 217, which make direct hydrogen bonds with xylopentaose, with alanine greatly reduced the affinity of the protein for its saccharide ligands. By contrast, disrupting water-mediated hydrogen bonds between CBM15 and xylopentaose by introducing the mutations S108A, Q167A, Q221A, and K223A had little effect on the affinity of the protein for xylan or xylohexaose. These data indicate that CBM15 binds xylan and xylooligosaccharides via the same interactions and provide clear evidence that direct hydrogen bonds are a key determinant of affinity in a type B CBM. The generic importance of these data is discussed.

Carbohydrate−protein recognition plays a central role in many key biological processes, and thus understanding the nature of the interactions that determine the specificity and affinity of these macromolecular associations is of considerable importance. Carbohydrate-binding modules (CBMs), which are noncatalytic components of microbial plant cell wall hydrolases, represent an excellent model system for studying protein−carbohydrate recognition. Unlike other carbohydrate-binding proteins CBMs contain extended binding sites that can accommodate up to six sugar units in linear saccharide chains. Furthermore, the target polysaccharide may be decorated with a variety of different side chains, and thus these protein modules, which bind to the sugar backbone, are able to accommodate side chains that are highly variable in their nature and extent. The target ligands for these proteins are plant structural polysaccharides, which are the most abundant organic molecules in the biosphere. The interaction of CBMs with their ligands brings the appended catalytic module of the enzyme into intimate and prolonged association with the plant cell wall, resulting in a potentiation of catalytic activity (*1*, *2*).

On the basis of primary structure similarity, CBMs have been grouped into 31 protein families (http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html), some of which have been further divided into subfamilies, designated alphabetically, an example of which is CBM2a and CBM2b (*3*, *4*). The three-dimensional structures of representatives from over half the CBM families have been determined using X-ray crystallography or NMR spectroscopy (http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html). The data have revealed that the topology of CBM binding sites is complementary to the conformation adopted by their target saccharide ligands. Thus, the binding site of CBMs that interact only with crystalline polysaccharides, known as type A CBMs (*5*), is a planar hydrophobic surface that is composed of a linear strip of aromatic amino acids, mainly tryptophans and tyrosines (*6*). Similarly, CBMs which recognize individual polysaccharide chains, known as type B CBMs (*5*), accommodate their target ligands within clefts of variable depth, lined with polar and hydrophobic amino acids. There is substantial evidence that the stacking of aromatic residues against the sugar rings of polysaccharides and oligosaccharides is a key determinant of overall affinity and specificity in both type A and type B CBMs and, indeed, in protein−carbohydrate interactions in general (*4*, *7*−*14*). However, the relative importance of direct hydrogen bonds in ligand binding between type A and type B CBMs appears to be less conserved. Several studies have shown that in type A CBMs putative hydrogen-bonding residues generally do not play an important role in determining affinity for crystalline

* Corresponding author. Tel: +44-(0)191-2226962. Fax: +44-(0)-191-2228684. E-mail: H.J.Gilbert@Newcastle.ac.uk.
[‡] University of Newcastle upon Tyne.
[§] University of Sheffield.

10.1021/bi0347510 CCC: $25.00 © 2003 American Chemical Society
Published on Web 07/18/2003

Table 1: CBM15 Mutagenic Primers[a]

mutant	primer
N106A-F	5′-GGCTGGAGAGGCGCCGCATCAGGCAG-3′
N106A-R	5′-CTGCCTGATGCGGCGCCTCTCCAGCC-3′
S108A-F	5′-GGAGAGGCAACGCAGCGGGCAGTACCAGC-3′
S108A-R	5′-GCTGGTACTGCCCGCTGCGTTGCCTCTCC-3′
Q167A-F	5′-CCAGTGAAGCCAACTTGGCGATATTTGCCCAG-3′
Q167A-R	5′-CTGGGCAAATATCGCCAAGTTGGCTTCACTGG-3′
Q171A-F	5′-GCAGATATTTGCCGCGTTAAAAGAAGACTGG-3′
Q171A-R	5′-CCAGTCTTCTTTTAACGCGGCAAATATCTGC-3′
W176A-F	5′-GCCCAGTTAAAAGAAGACGCGTCAAAGGGCGAATG-3′
W176A-R	5′-CATTCGCCCTTTGACGCGTCTTCTTTTAACTGGGC-3′
W181A-F	5′-GTCAAAGGGCGAAGCGGATTGTCTGGC-3′
W181A-R	5′-GCCAGACAATCCGCTTCGCCCTTTGAC-3′
Q217A-F	5′-GCGCGCGATGTAGCAGTCGGTATCCAG-3′
Q217A-R	5′-CTGGATACCGACTGCTACATCGCGCGC-3′
Q221A-F	5′-CAAGTCGGTATCGCGGCCAAGGGAACACCCG-3′
Q221A-R	5′-CGGGTGTTCCCTTGGCCGCGATACCGACTTG-3′
K223A-F	5′-GGTATCCAGGCCGCGGGAACACCCGCC-3′
K223A-R	5′-GGCGGGTGTTCCCGCGGCCTGGATACC-3′

[a] Mutations that generated an alanine codon are underlined. Primers denoted -F and -R comprised the sense and antisense strands, respectively.

cellulose (*9*, *15*, *16*). By contrast, the mutation of residues in type B CBMs that are likely to make direct hydrogen bonds with oligosaccharides significantly reduces the affinity of these proteins for their ligands (*12*, *17*−*19*). The importance of water-mediated hydrogen bonds in CBM−ligand binding is, however, currently unknown, although there is some evidence which suggests that these indirect interactions may play a role in the binding of lectins to their ligands (*20*, *21*).

Recently, the crystal structure of the family 15 CBM (CBM15) from *Cellvibrio japonicus* (formerly *Pseudomonas cellulosa*) Xyn10C was determined in complex with the ligand xylopentaose (*22*). In this report we have used a rational design approach, informed by the crystal structure of the CBM15−ligand complex, to probe the importance of hydrophobic stacking interactions and hydrogen bonds in the binding of this type B CBM to both polysaccharide and oligosaccharide ligands. The data show that both hydrophobic stacking interactions and direct hydrogen bonds play a pivotal role in ligand binding, while most water-mediated hydrogen bonds appear to make less of a contribution to the overall affinity of CBM15 for xylan or xylohexaose.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions. The *Escherichia coli* strain used in this study was BL21(DE3): pLysS (Novagen) that harbored a recombinant of the expression vector pET16b (Novagen), designated pLS1, which encoded CBM15 fused to an N-terminal His_{10} tag (*22*). The cells were cultured in Luria broth (1 L in 2 L conical baffle flasks) supplemented with 50 μg/mL ampicillin at 37 °C and 180 rpm until the A_{600} reached 0.6, at which point expression of CBM15 was induced by addition of isopropyl β-thiogalactopyranoside to a final concentration of 1 mM and incubation for a further 4 h at 37 °C.

Generation of Mutants of CBM15. Derivatives of CBM15 were generated by the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions, using pLS1 as the template DNA. The primers that were employed in the mutagenesis PCR reactions are shown in Table 1. The complete sequences of the DNA encoding the CBM15 mutants were determined using an ABI 377 DNA sequenator and T7 forward and reverse primers to confirm that only the desired mutations had been introduced.

Protein Purification. Wild type and mutants of CBM15 were purified by immobilized metal affinity chromatography as described previously (*22*) using Talon resin (ClonTech). The purity of the protein was evaluated by SDS−PAGE. Protein concentration was determined from the calculated molar extinction coefficient of CBM15 at 280 nm (18400 M^{-1} cm^{-1}), with a reduced coefficient used for the tryptophan mutants of 12780 M^{-1} cm^{-1}.

Ligand Binding Studies. Ligand binding was determined by both affinity gel electrophoresis (AGE) and isothermal titration calorimetry (ITC). Affinity gel electrophoresis was performed as described previously (*23*) using oat spelt xylan (Ara:Xyl, 10:90; Sigma) as the ligand, which was included in the gels at concentrations ranging from 0.05 to 4 mg/mL. K_d values were calculated according to the method of ref *23* by determining the relative mobility of CBM15 in the presence and absence of ligand versus BSA (Sigma), the control nonbinding protein. ITC measurements were made at 25 °C following standard procedures (*24*) using a Microcal Omega titration calorimeter. The proteins were dialyzed extensively against 50 mM sodium phosphate buffer, pH 7.0, and the ligand was dissolved in the same buffer to minimize heats of dilution. During a titration experiment, the protein sample (100−300 μM), stirred at 300 rpm in a 1.4331 mL reaction cell maintained at 25 °C, was injected with 25 successive 10 μL aliquots of ligand comprising oat spelt xylan (20 mg/mL) or xylohexaose (10 mM; Megazyme International) at 200 s intervals. The molar concentration of CBM15 binding sites present in the xylan was determined as described previously (*22*). Integrated heat effects, after correction for heats of dilution, were analyzed by nonlinear regression using a single site-binding model (Microcal Origin, version 2.9). The fitted data yield the association constant (K_a) and the enthalpy of binding (ΔH). Other thermodynamic parameters were calculated using the standard thermodynamic equation: $-RT \ln K_a = \Delta G = \Delta H - T\Delta S$. To measure the change in heat capacity (ΔC_p), wild-type CBM15 was subjected to ITC at 25 and 35 °C, and

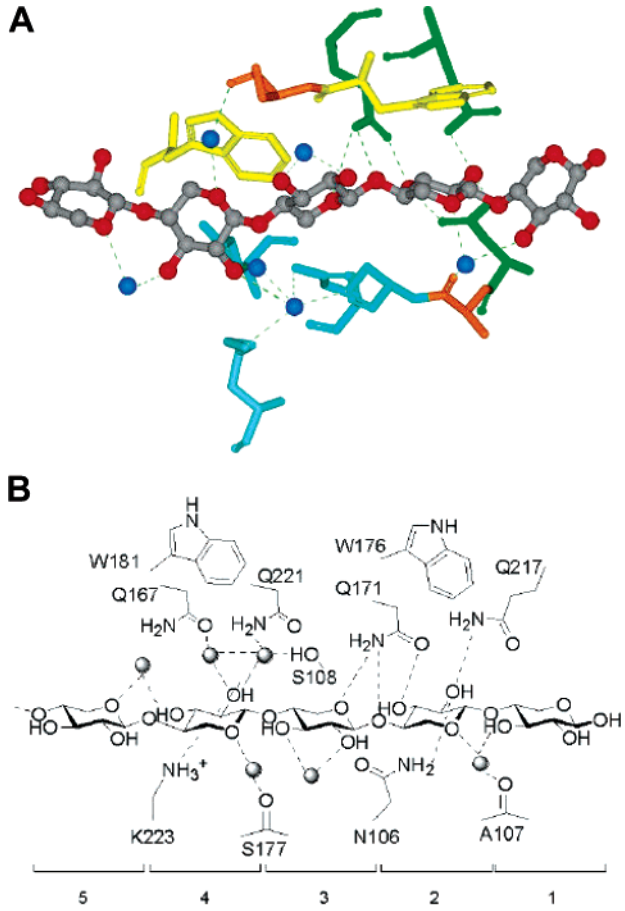

FIGURE 1: CBM15 residues identified as potentially involved in binding the ligand xylopentaose. Panel A: Crystal structure of CBM15 in complex with the ligand xylopentaose showing only the amino acids proposed to interact with the carbohydrate. Residues involved in hydrophobic stacking interactions with the ligand are colored yellow (W176, W181). Side chains forming direct hydrogen bonds with the sugar are shown in green (N106, Q171, Q217). The side chains of residues forming water-mediated hydrogen bonds with the ligand are displayed in blue (S108, Q167, Q221, and K223), while amino acids colored orange form water-mediated hydrogen bonds with xylopentaose via their backbone carbonyls (A107 and S177). Panel B shows the above residues in schematic form. In both panels hydrogen bonds are shown as dashed lines.

ΔC_p was determined using the equation:

$$\Delta C_p = \frac{\Delta H_2 - \Delta H_1}{T_2 - T_1}$$

where T_2 and ΔH_2 are the values from the higher temperature titration.

NMR Spectroscopy. Protein samples were approximately 200 μM in 10 mM sodium phosphate buffer (pH 6.5) containing 10% D_2O. NMR spectra were recorded at 30 °C on a Bruker DRX-500 spectrometer, and ^1H chemical shifts were referenced to an internal standard of 3-(trimethylsilyl)-propionate-2,2,3,3-d_4 at 0.00 ppm. Data were processed by FELIX (Accelrys Inc., San Diego, CA).

RESULTS AND DISCUSSION

Construction of CBM Mutants. In a previous study we reported the three-dimensional structure of CBM15 from *C. japonicus* Xyn10C in complex with xylopentaose (*22*). The data (Figure 1) revealed the amino acids that were in close proximity with the ligand and therefore likely to be the molecular determinants of specificity and affinity in CBM15. To investigate the relative importance of these residues in the binding of CBM15 to xylose polymers, they were each substituted for alanine, and the capacity of the resultant proteins to bind to xylan and xylohexaose was evaluated. The nine mutants of CBM15 generated were purified to electrophoretic homogeneity (data not shown), and the structural integrity of the mutant proteins was determined by NMR (Figure 2). The upfield ends of the different spectra (below 0.6 ppm) are derived from ring current-shifted protons which are in close contact with the π-orbitals of aromatic residues. Very minor changes in the positions or orientations of the aromatic rings cause significant changes to the chemical shifts of these ring current-shifted protons. The similarity of the spectra of wild type and mutants therefore suggests that no such changes in ring positions have occurred, and thus the packing in the vicinity of the rings is unaffected by the mutations. Thus the spectra demonstrate that the hydrophobic core of the proteins is essentially intact and that the CBM15 mutants have all folded into a correct three-dimensional structure. This view is supported by the fact that the upfield protons originate from several widely spaced regions of the protein, and thus we can safely conclude that none of the CBM15 mutations significantly perturbed the gross fold of the protein. This is consistent with the solvent-exposed location of these amino acids, which are therefore unlikely to play a key role in stabilizing the overall protein fold.

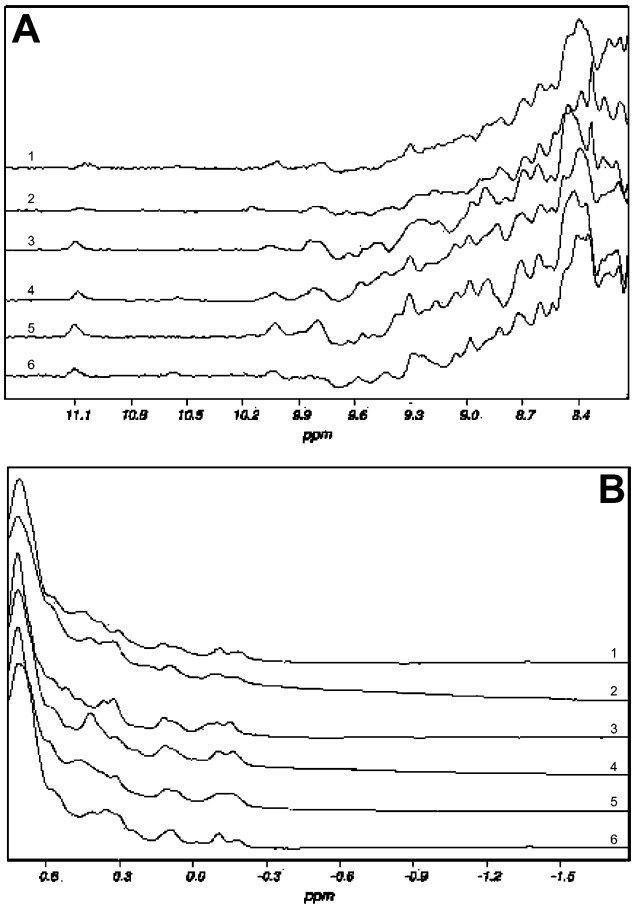

FIGURE 2: 1D NMR spectra of CBM15 mutants: panel A, downfield end of spectra; panel B, upfield end of spectra. The individual spectra are as follows: 1, Q171A; 2, Q217A; 3, W176A; 4, W181A; 5, N106A; 6, wild type.

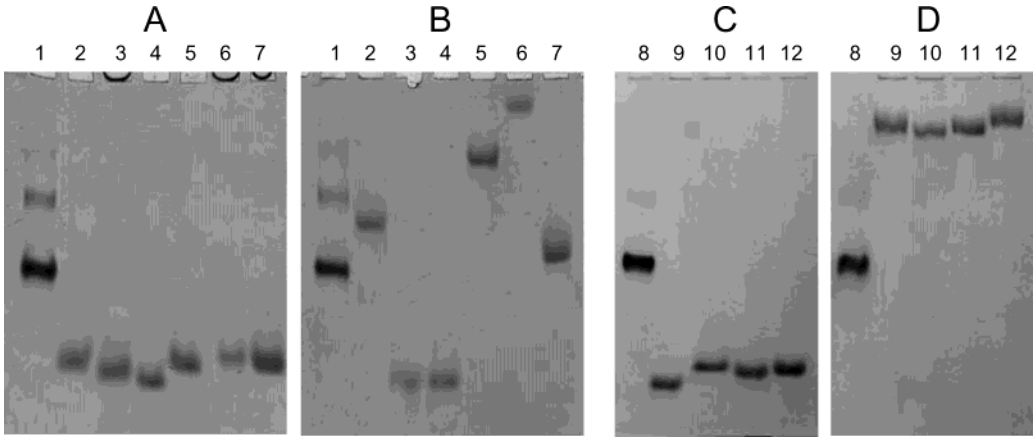

FIGURE 3: Affinity gel electrophoresis of CBM15 mutants binding to oat spelt xylan. Nondenaturing polyacrylamide gels containing no ligand are displayed in panels A and C, while gels containing 2 mg/mL oat spelt xylan are shown in panels B and D. Lanes: 1 and 8, BSA nonbinding control; 2, Q171A; 3, W176A; 4, W181A; 5, Q217A; 6, wild type; 7, N106A; 9, K223A; 10, Q221A; 11, Q167A; 12, S108A.

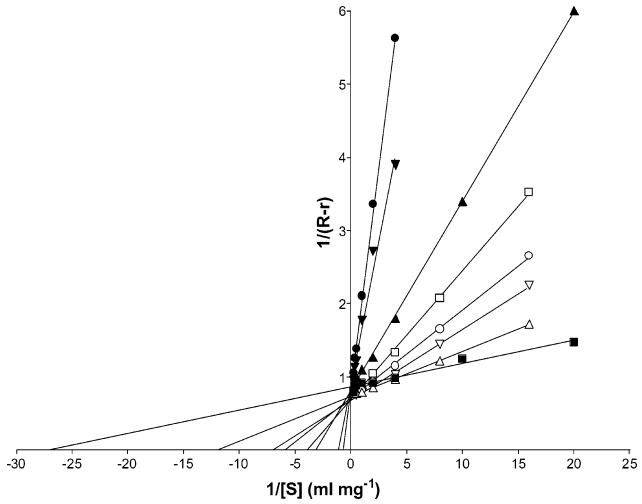

FIGURE 4: Plots used to determine the affinities of CBM15 mutants relative to wild type from affinity gel data. The data points are as follows: wild type (■), N106A (●), S108A (△), Q167A (○), Q171A (▼), Q217A (▲), Q221A (□), and K223A (▽). [S] = substrate concentration (mg mL^{-1}); R = relative mobility of the protein compared to BSA in the absence of the ligand; r = relative mobility of the protein compared to BSA in the presence of the ligand.

Biochemical Properties of CBM15 Mutants. The biochemical properties of the CBM15 mutants were initially assessed by AGE. Examples of typical AGE data and the plots used to determine relative affinity are shown in Figures 3 and 4, respectively. The full data set is shown in Table 2.

W176A and W181A. Both W176A and W181A displayed no affinity for xylan as judged by AGE (Table 2). Inspection of the crystal structure of CBM15 in complex with xylopentaose shows that Trp 176 and Trp 181 form hydrophobic stacking interactions with xylose residues n and $n + 2$, respectively, in the helical oligosaccharide. Thus, these two stacking interactions play a pivotal role in the binding of CBM15 to xylose polymers. This is entirely consistent with the literature, which is replete with reports demonstrating that the interaction of aromatic residues with sugar rings is a key element in the binding of CBMs to their target ligands and is a common theme in protein−carbohydrate interactions (*8*). For example, mutating or chemically modifying tryptophan or tyrosine residues in the binding site of CBM2a (*9*, *10*, *25*), CBM2b (*13*), CBM4 (*26*), CBM5 (*16*), CBM6 (*18*), CBM10 (*11*), and CBM22 (*12*) either abolished or greatly reduced the affinity of these proteins for their respective ligands. It should be noted that removal of tryptophan residues generally has a larger effect on ligand binding than loss of tyrosines (*9*). This may reflect the greater hydrophobicity of indole compared to phenolic rings (*14*).

Table 2: Affinity of Wild-Type and Mutant CBM15 Derivatives for Oat Spelt Xylan Determined by AGE

protein	K_D (mg/mL)	relative affinity (%)	protein	K_D (mg/mL)	relative affinity (%)
wild type	0.037	100.0	Q217A[c]	0.32	11.6
W176A[a]	ND[b]	0.0	S108A[d]	0.08	46.8
W181A[a]	ND	0.0	Q167A[d]	0.16	23.6
N106A[c]	1.46	2.5	Q221A[d]	0.23	16.1
Q171A[c]	0.89	4.1	K223A[d]	0.14	27.3

[a] Residues involved in hydrophobic stacking interactions with ligand. [b] ND: no binding detected. [c] Residues involved in direct hydrogen bonds with ligand. [d] Residues involved in water-mediated hydrogen bonds with ligand.

AGE of Mutants of Asn 106, Gln 171, and Gln 217. Each of these residues is within direct hydrogen-bonding distance of the xylopentaose ligand. Replacing any of these amino acids with alanine caused a significant decrease in affinity for both xylan and xylohexaose. The N106A mutation resulted in the largest loss in affinity (40-fold), while the Q217A mutant had a K_a value that was only 9-fold lower than that of the wild-type protein. These data indicate that each of the polar residues Asn 106, Gln 171, and Gln 217 forms direct hydrogen bonds with the ligand and that these interactions make a significant contribution to the overall affinity of the protein for xylan. It is interesting to note that although both Asn 106 and Gln 217 form a hydrogen bond with the C2OH of xylose-2 (Figure 1), the asparagine residue appears to be more important in ligand binding than the glutamine. This may reflect the lengths of the hydrogen bonds that Asn 106 and Gln 217 make with the C2OH of xylose-2, which are 2.62 and 3.05 Å, respectively.

There have been only two previous studies which have utilized three-dimensional structural data of CBM−ligand complexes to dissect the role of polar residues in ligand binding; these are CBM4 and CBM17 in complex with cellopentaose and cellotetraose, respectively. The crystal

structure of the *Cellulomonas fimi* CBM4-1−cellopentaose complex (*17*) in combination with a previous study (*26*) showed that the polar residues Gln 128, Gln 124, Arg 75, and Asn 50 made hydrogen bonds with the ligand β-glucan, and removal of any of these interactions resulted in a significant reduction in overall affinity (6−35-fold). In addition, mutation to alanine of direct H-bonding residues in the *Clostridium cellulovorans* CBM17−cellotetraose complex (*19*) resulted in a significant loss (8−25-fold) in affinity of the protein for cellohexaose. The crystal structure of unliganded CBM22-2 from *Clostridium thermocellum* Xyn10B also revealed several polar residues located within the ligand binding site that are conserved in this family of proteins (*27*). Removal of one of these residues, Glu 138, abolished xylan and xylotetraose binding without perturbing the three-dimensional structure of the protein (*12*). Although there is no structural data on CBM22-2 in complex with its ligands, the properties of the E138A mutant strongly indicate that Glu 138 makes one or more pivotal hydrogen bonds with the xylose polymers. Similarly, removal of Asn 120 from the ligand binding site of CBM6 from *C. thermocellum* Xyn11A resulted in a 145-fold reduction in affinity for xylan, pointing to the importance of hydrogen bonds in the binding of this protein module to its target ligands (*18*), although again there are no direct structural data to confirm this hypothesis. Interestingly, in both CBM6 and CBM22 the key H-bonding residue is relatively buried at the bottom of the binding cleft and would therefore be excluded from solvent on ligand binding. This would prevent the H-bond from exchanging between ligand and solvent as would occur with many of the more exposed H-bonding residues observed in type B CBMs, and this solvent exclusion may provide an explanation for the fundamental role played by these two residues in ligand binding in their respective CBMs.

Mutagenesis studies on CBMs that bind to crystalline polysaccharides, classified as type A modules, have also explored the role of polar residues in ligand binding. Despite being in an appropriate position to form a hydrogen bond(s) with cellulose, substitution of Glu 27 with alanine in the family 5 CBM from *Erwinia chrysanthemi* Cel5A did not diminish the affinity of the protein module for its ligand (*16*). Similarly, substituting most of the amino acids that are within hydrogen-bonding distance of cellulose in the CBM2a from *C. fimi* Xyn10A (*9*) or CBM1 from *Trichoderma reesei* Cel7A (*15*) did not cause a significant reduction in affinity for the polysaccharide. It has also been proposed that in CBM2b-1 from *C. fimi* Xyn11A Glu 257, Asn 292, and Gln 288 form hydrogen bonds with the ligand xylohexaose; however, the affinity of the treble mutant E257A/N292A/Q288A for xylooligosaccharides and xylan was only ~2-fold lower than that of the wild-type protein (*13*). Although CBM2bs bind to single polysaccharide chains, they cannot be classified as typical type B modules as they do not contain a clearly identifiable cleft. It is possible, therefore, that the relative importance of H-bonds in ligand binding in these proteins is similar to that observed in type A CBMs. It should be noted, however, that H-bonds are important in the binding of CBM17 to its ligand even though the protein contains only a shallow cleft (*19*), and thus the role of protein−sugar H-bonds at the interface of type A and type B CBMs appears to be somewhat variable.

The collective data dissecting the role of polar residues in CBM−ligand interactions indicate that, in proteins belonging to families 4, 6, 15, 17 and 22, hydrogen bonds play a key role in binding oligosaccharides. In contrast, in CBMs located in families 1, 2a, 2b, and 5, ligand binding is dominated by hydrophobic stacking interactions; there is no substantial evidence implicating polar residues as a major contributor to the affinity of these modules for polysaccharides. This may reflect the topology of the binding sites of CBMs. In the type A modules, such as CBM2a, CBM2b, and CBM5, the binding sites are very exposed, comprising planar binding surfaces. In contrast, the binding sites of type B CBMs in which polar residues play an important role in ligand binding consist of clefts of various depths. It is possible that the nature of the interactions with their ligands may influence the mobility of type A and type B CBMs once bound to their target polysaccharides. The capacity of type A modules to slide across the crystalline surface of their target ligands (*28*) may indicate that hydrophobic interactions are more amenable to this protein movement as they lack the directionality conferred by formation of energetically favorable hydrogen bonds. This movement is likely to maximize substrate accessibility to the catalytic module. In support of this conclusion, we note that if hydrophobic interactions were the sole determinant of carbohydrate recognition, then one would predict that such modules would bind a wide variety of ligands with the same conformation. This view is supported by the observation that type A CBMs are able to bind to the planar polysaccharides chitin and crystalline mannan in addition to crystalline cellulose (*16*, *29*). By this rationale, in type B CBMs the formation of strong H-bonds would prevent movement along their polysaccharide ligands. This movement, however, is precluded by both the twisted conformation of the ligand and the common occurrence of side chains in plant cell wall polysaccharides (*30*−*32*). It is possible, therefore, that the presence of favorable H-bonds in type B CBMs compensates, to some extent, for the reduced contribution to overall affinity of the hydrophobic stacking interactions compared to type A modules where the ligand presents a larger hydrophobic surface.

AGE of Mutants S108A, Q167A, Q221A, and K223A. The crystal structure of CBM15 in complex with xylopentaose indicates that Ser 108, Gln 167, Gln 221, and Lys 223 interact with the oligosaccharide via single ordered water molecules (Figure 1). AGE revealed that mutants of CBM15 in which Ser108, Gln 167, Gln 221, and Lys 223 have been substituted with alanine, displayed a modest decrease (2−6-fold) in affinity. These data indicate that amino acids in the binding site of CBM15 that interact with xylopentaose via water molecules generally make a less significant contribution to overall affinity than residues that form direct hydrogen bonds with the ligand.

ITC of the Binding of Wild-Type CBM15 and the Mutants N106A, Q171A, and Q217A to Their Ligands. The interaction of wild-type CBM15 and the three direct H-bonding mutants with xylan and xylohexaose was also investigated by ITC. Examples of the calorimetric data are shown in Figure 5, and the full data set is displayed in Table 3. The affinity of wild-type CBM15 for oat spelt xylan and xylohexaose was broadly similar, as were the changes in enthalpy and entropy associated with these binding events. The affinities of the

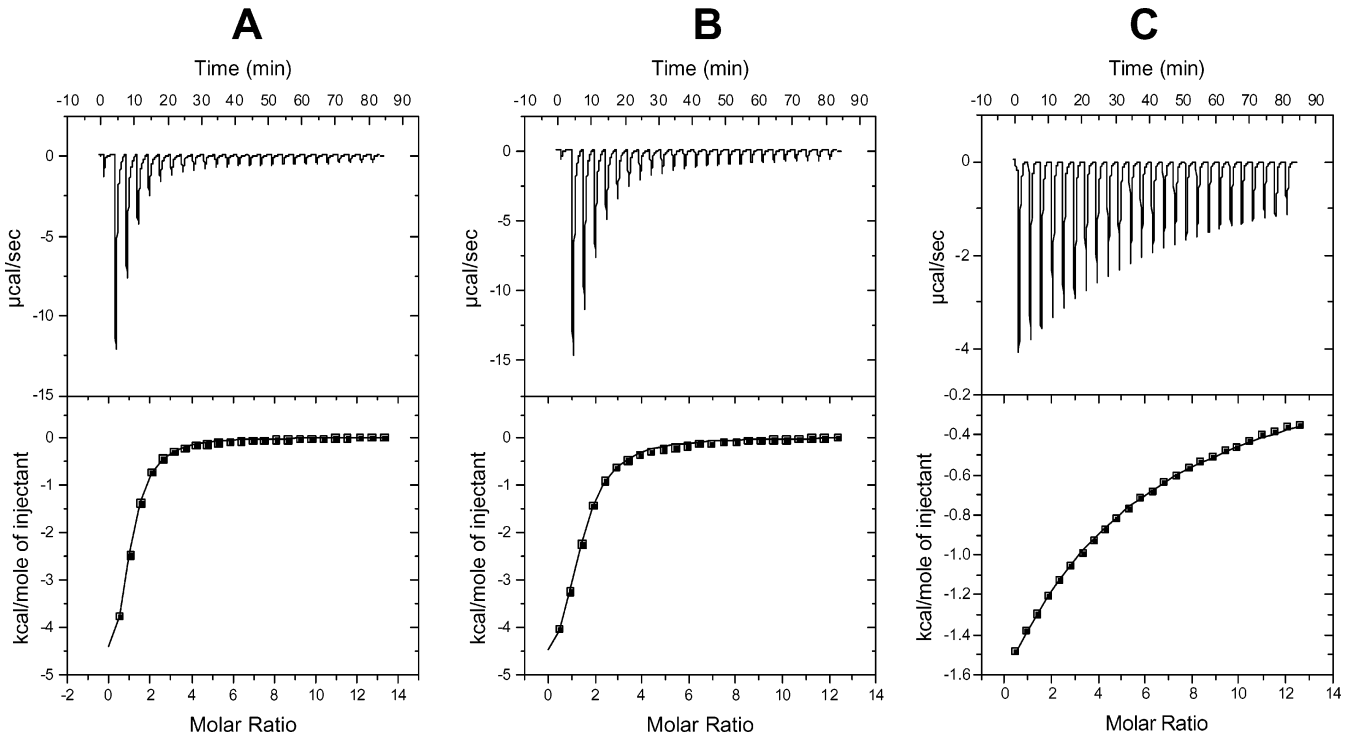

FIGURE 5: Typical ITC data for wild type, a water-mediated hydrogen bond mutant, and a direct hydrogen bond mutant of CBM15 titrated with 20 mg/mL oat spelt xylan. Panels: A, wild-type CBM15; B, S108A; C, N106A.

Table 3: Affinity of CBM15 Mutants Binding to Oat Spelt Xylan and Xylohexaose Determined by ITC

mutant	ligand	$K_a \times 10^{-3}$ (M^{-1})	ΔG (kcal mol^{-1})	ΔH (kcal mol^{-1})	$T\Delta S$ (kcal mol^{-1})	n
wild type	oat spelt xylan	20.3 ± 1.7	-5.9 ± 0.1	-8.8 ± 0.3	-2.9 ± 0.4	1.0 ± 0.0
N106A[a,b]	oat spelt xylan	0.5 ± 0.0				
Q171A[a,b]	oat spelt xylan	0.3 ± 0.1				
Q217A[a,b]	oat spelt xylan	0.7 ± 0.1				
S108A[c]	oat spelt xylan	15.7 ± 0.8	-5.7 ± 0.0	-9.4 ± 0.6	-3.7 ± 0.6	1.0 ± 0.0
Q167A[c]	oat spelt xylan	7.8 ± 0.0	-5.3 ± 0.0	-7.4 ± 0.1	-2.1 ± 0.1	1.1 ± 0.0
Q221A[c]	oat spelt xylan	4.7 ± 0.7	-5.0 ± 0.1	-8.7 ± 0.9	-3.7 ± 0.9	1.0 ± 0.0
K223A[c]	oat spelt xylan	7.3 ± 0.1	-5.3 ± 0.0	-9.6 ± 0.1	-4.3 ± 0.1	1.1 ± 0.1
wild type	xylohexaose	22.9 ± 1.0	-6.0 ± 0.0	-9.6 ± 0.2	-3.6 ± 0.2	1.0 ± 0.0
N106A[a,b]	xylohexaose	0.3 ± 0.1				
Q171A[a,b]	xylohexaose	0.2 ± 0.1				
Q217A[a,b]	xylohexaose	1.3 ± 0.1				
S108A[c]	xylohexaose	24.2 ± 2.0	-6.0 ± 0.1	-11.6 ± 0.4	-5.6 ± 0.5	0.9 ± 0.0
Q167A[c]	xylohexaose	6.6 ± 0.6	-5.2 ± 0.1	-8.6 ± 1.3	-3.3 ± 1.4	1.0 ± 0.1
Q221A[c]	xylohexaose	4.4 ± 0.5	-5.0 ± 0.1	-7.8 ± 1.1	-2.8 ± 1.2	1.0 ± 0.2
K223A[c]	xylohexaose	6.7 ± 0.8	-5.2 ± 0.1	-9.7 ± 0.9	-4.5 ± 1.0	1.1 ± 0.2

[a] Binding constants are an estimate of affinity. [b] Residues involved in direct hydrogen bonds with ligand. [c] Residues involved in water-mediated hydrogen bonds with ligand.

mutants N106A, Q171A, and Q217A were too low to provide accurate thermodynamic parameters ($K_a < 500$ M^{-1}) using either the polysaccharide or oligosaccharide as the ligand. Thus the ITC data are consistent with the AGE results, confirming that the three polar amino acids involved in direct H-bonding interactions with the carbohydrate, Asn 106, Gln 171, and Gln 217, all play a key role in ligand binding.

Previous studies on glycoside hydrolases have shown that several mutations in these enzymes cause a much larger decrease in activity against oligosaccharides compared to polysaccharides (*33*, *34*) (*29*), indicating that there may be significant differences in the way these biocatalysts interact with the two classes of substrate. Data presented in this paper indicate that the interactions between CBM15 and either xylan or xylooligosaccharides are very similar, implying that xylan, in addition to xylooligosaccharides, adopts a 3-fold helical structure in the binding site of the protein. This is entirely consistent with the known 3-fold helical conformation of xylan, which was previously determined by X-ray fiber diffraction (*30*), and it is likely, therefore, that xylooligosaccharides adopt the same minimum energy conformation in solution. This view, however, must be treated with some caution as cellooligosaccharides adopt a loose helical structure in solution with ϕ and ψ angles of $-77°$ and 82°, respectively (*35*), while crystalline cellulooligosaccharides display a 2-fold screw axis of symmetry with the glucose molecules twisted 180° relative to their neighbors (*36*). Studies investigating the effect of mutations on the binding of CBMs to a range of ligands are limited. Kormos et al. (*26*) showed that removal of polar residues from CBM4-1 from *C. fimi* Cel9B reduced binding to β-glucan and acid swollen cellulose to a similar extent, and substitution of the solvent-exposed aromatic residues with alanine virtually abolished the capacity of the protein to interact with

polysaccharides or oligosaccharides. Furthermore, the crystal structure of the CBM4-1 in complex with cellopentaose shows that the residues which form hydrogen bonds with the oligosaccharide are important in binding the polysaccharide (*17*). It is highly likely, therefore, that the interactions between CBM4-1 and both polysaccharides and oligosaccharides are very similar. Thus, we tentatively propose that the interactions by which specific CBMs bind to polysaccharides and oligosaccharides are conserved.

The ΔC_p of wild-type CBM15 was -299.6 cal mol^{-1} K^{-1}. This small negative value is typical of protein−carbohydrate interactions (*8*, *37*) and is thought to reflect a combination of polar and apolar interactions. Binding is not dominated by burial of apolar residues. This is consistent with both the hydrophobic stacking of the xylose rings *n* and *n* + 2 against tryptophan residues 176 and 181 observed in the CBM15−xylopentaose complex and also the important role of the direct hydrogen bonds between the protein and its ligand.

ITC of the Binding of the CBM15 Mutants S108A, Q167A, Q221A, and K223A to Their Ligands. Investigating the binding of derivatives of CBM15 that lack the side chain of amino acids Lys 223, Gln 221, Gln 167, or Ser 108 to polysaccharide and oligosaccharide ligands by ITC showed that the wild-type and mutant forms of this protein displayed similar affinity for the xylose polymers. Indeed, the K_a of S108A for both xylan and xylohexaose was indistinguishable from that of native CBM15. While these data are consistent with the AGE analysis, the cumulative ITC results display a more marked difference in affinity between residues involved in water-mediated and direct H-bonds with xylan. Differences in CBM affinities determined using these two techniques have been reported previously and may reflect the colloidal nature of the ligand in AGE (*32*, *38*). The ITC data further confirm that the water-mediated interactions between the four amino acids and either xylan or xylohexaose are not important determinants of the overall affinity of CBM15 for its ligands. The similarity in the affinity of these mutants for xylan and xylohexaose provides additional support for the view that the interactions between CBM15 and polysaccharide and oligosaccharide ligands are very similar. While the properties of K223A, Q221A, S108A, and Q167A suggest that water-mediated hydrogen bonds between CBM15 and its ligands are not important in binding, clearly mutagenesis strategies can only study water-mediated interactions between saccharide ligands and the amino acid side chains of CBM15. It is possible, therefore, that the interaction of the main chain carbonyl groups of Ser 177 and Ala 107 (Figure 1) with xylan and xylohexaose via water molecules could play an important role in ligand binding.

The observation that in complexes of model lectins with different saccharides the positions of ordered water molecules are often conserved has led to the assumption that they are important in ligand binding (*39*, *40*). In addition, in the complexes of peanut lectin with lactose and T-antigen the only apparent difference in ligand binding that explains the 20-fold increase in affinity of the latter sugar is the presence of two extra ordered water molecules which H-bond with the *N*-acetyl group of the T-antigen (*7*, *20*). In this report we show by site-directed mutagenesis that substitution of residues that appear to interact with ligand via ordered water molecules has little effect on overall affinity. Although these data imply that water-mediated hydrogen bonds are not important in the binding of CBM15 to its ligands, it should be emphasized that the distances of some of these indirect hydrogen bonds (3.2−3.4 Å) are rather long and thus these interactions are likely to be weak. Furthermore, as Gln 221, Ser 108, and Lys 223 all interact with xylopentaose via the same water molecule, removing only one of these three residues may not significantly disrupt this water-mediated hydrogen bond between the protein and the oligosacchairde. In addition, alternative solvent-mediated hydrogen bonds may occur between ligand and the mutant proteins, although a similar argument could be invoked for the introduction of mutations that remove direct H-bonding residues where any such events do not compensate for the loss in affinity in these mutants.

The thermodynamics of the interaction of CBM15 with xylose polymers is typical of the binding of proteins to soluble saccharides, which is invariably enthalpically driven, with an unfavorable entropic contribution (*4*, *8*, *17*, *18*, *24*, *27*). The molecular basis for the thermodynamic forces that drive these binding events, however, is somewhat controversial. It has been proposed that the favorable enthalpic component is the result of direct interactions between the protein and the ligand, while the unfavorable entropy is associated with a restriction in the mobility of the ligand on binding (*13*, *41*). Other authors stress the importance of solvent reorganization in determining the energetics of the interaction and suggest that the increased enthalpy is derived from water molecules on the surface of the macromolecules returning to bulk solvent (*42*−*44*). It is likely that both contribute at least partly to the energetics of each specific system.

Conclusions. In this report we have utilized the three-dimensional structure of a CBM in complex with an oligosaccharide ligand to dissect the importance of hydrophobic stacking interactions and both direct and indirect hydrogen bonds (via ordered water molecules) in the binding of the protein to its target carbohydrates. The data presented demonstrate that hydrophobic stacking interactions and direct hydrogen bonds between CBM15 and xylose-containing polymeric ligands are both important determinants of overall affinity, whereas water-mediated hydrogen bonds do not seem to play a critical role in the binding event. These results are consistent with other studies that have shown the importance of direct H-bonds in ligand binding in type B CBMs that accommodate their target saccharides within a cleft. In sharp contrast, in type A CBMs, where the binding site comprises a planar surface rich in aromatic amino acids, hydrophobic stacking interactions are the major determinants of both specificity and affinity. While we demonstrate that water-mediated hydrogen bonds between CBM15 and its ligands do not appear to play a key role in defining the overall affinity, until similar studies are carried out on other carbohydrate-binding proteins a generic inference on the role of ordered water molecules in protein−carbohydrate recognition cannot be drawn.

REFERENCES

1. Bolam, D. N., Ciruela, A., 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.
2. 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.

3. 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.
4. Simpson, P. J., Xie, H., Bolam, D. N., Gilbert, H. J., and Williamson, M. P. (2000) *J. Biol. Chem. 275*, 41137−41142.
5. Boraston, A. B., McLean, B. W., Kormos, J., Alam, M. M., Gilkes, N. R., Haynes, C. A., Tomme, P., Kilburn, D. G., and Warren, R. A. (1999) in *Recent Advances in Carbohydrate Bioengineering* (Gilbert, H. J., Davies, G. J., Henrissat, B., and Svensson, B., Eds.) pp 202−211, The Royal Society of Chemistry, Cambridge, U.K.
6. Tormo, J., Lamed, R., Chirino, A. J., Morag, E., Bayer, E. A., Shoham, Y., and Steitz, T. A. (1996) *EMBO J. 15*, 5739−5751.
7. Loris,R., Hamelryck, T., Bouckaert, J., and Wyns, L. (1998) *Biochim. Biophys. Acta 1383*, 9−36.
8. Toone, E. J. (1994) *Curr. Opin. Struct. Biol. 4*, 719−728.
9. McLean, B. W., Bray, M. R., Boraston, A. B., Gilkes, N. R., Haynes, C. A., and Kilburn, D. G. (2000) *Protein Eng. 13*, 801−809.
10. Nagy, T., Simpson, P., Williamson, M. P., Hazlewood, G. P., Gilbert, H. J., and Orosz, L. (1998) *FEBS Lett. 429*, 312−316.
11. Ponyi, T., Szabo, L., Nagy, T., Orosz, L., Simpson, P. J., Williamson, M. P., and Gilbert, H. J. (2000) *Biochemistry 39*, 985−991.
12. Xie, H., Gilbert, H. J., Charnock, S. J., Davies, G. J., Williamson, M. P., Simpson, P. J., Raghothama, S., Fontes, C. M., Dias, F. M., Ferreira, L. M., and Bolam, D. N. (2001) *Biochemistry 40*, 9167−9176.
13. 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.
14. Linder, M., Lindeberg, G., Reinikainen, T., Teeri, T. T., and Pettersson, G. (1995) *FEBS Lett. 372*, 96−98.
15. Linder, M., Mattinen, M.-L., Kontteli, M., Lindeberg, G., Stahlberg, J., Drakenberg, T., Reinikainen, T., and Pettersson, G. (1995) *Protein Sci. 4*, 1056−1064.
16. Simpson, H. D., and Barras, F. (1999) *J. Bacteriol. 181*, 4611−4616.
17. Boraston, A. B., Nurizzo, D., Notenboom, V., Ducros, V., Rose, D. R., Kilburn, D. G., and Davies, G. J. (2002) *J. Mol. Biol. 319*, 1143−1156.
18. Czjzek, M., Bolam, D. N., Mosbah, A., Allouch, J., Fontes, C. M., Ferreira, L. M., Bornet, O., Zamboni, V., Darbon, H., Smith, N. L., Black, G. W., Henrissat, B., and Gilbert, H. J. (2001) *J. Biol. Chem. 276*, 48580−48587.
19. Notenboom, V., Boraston, A. B., Chiu, P., Freelove, A. C., Kilburn, D. G., and Rose, D. R. (2001) *J. Mol. Biol. 314*, 797−806.
20. Ravishankar, R., Ravindran, M., Suguna, K., Surolia, A., and Vijayan, M. (1997) *Curr. Sci.*, 855−861.
21. Clarke, C., Woods, R. J., Gluska, J., Cooper, A., Nutley, M. A., and Boons, G. J. (2001) *J. Am. Chem. Soc. 123*, 12238−12247.
22. 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.
23. Takeo, K. (1984) *Electrophoresis 5*, 187−195.
24. 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.
25. Bray, M. R., Johnson, P. E., Gilkes, N. R., McIntosh, L. P., Kilburn, D. G., and Warren, R. A. (1996) *Protein Sci. 5*, 2311−2318.
26. Kormos, J., Johnson, P. E., Brun, E., Tomme, P., McIntosh, L. P., Haynes, C. A., and Kilburn, D. G. (2000) *Biochemistry 39*, 8844−8852.
27. Charnock, S. J., Bolam, D. N., Turkenburg, J. P., Gilbert, H. J., Ferreira, L. M., Davies, G. J., and Fontes, C. M. (2000) *Biochemistry 39*, 5013−5021.
28. Jervis, E., Haynes, C. A., and Kilburn, D. G. (1997) *J. Biol. Chem. 272*, 24016−24023.
29. Hogg, D., Pell, G., Dupree, P., Goubet, F., Martin-Orue, S. M., Armand, S., and Gilbert, H. J. (2003) *Biochem. J. 371*, 1027−1043.
30. Atkins, E. D. T. (1992) in *Xylan and Xylanases: Progress in Biotechnology* (Visser, J., Beldman, G., van Kusters, S., and Voragen, A. G. L., Eds.) pp 39−50, Elsevier Science Publishers B.V., Amsterdam.
31. Brett, C. T., and Waldren, K. (1996) *Physiology and Biochemistry of Plant Cell Walls*, Vol. 1, Chapman and Hall, London.
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. Charnock, S. J., Lakey, J. H., Virden, R., Hughes, N., Sinnott, M. L., Hazlewood, G. P., Pickersgill, R., and Gilbert, H. J. (1997) *J. Biol. Chem. 272*, 2942−2851.
34. Charnock, S. J., Spurway, T. D., Xie, H., Beylot, M. H., Virden, R., Warren, R. A., Hazlewood, G. P., and Gilbert, H. J. (1998) *J. Biol. Chem. 273*, 32187−32199.
35. Sugiyama, H., Hisamichi, K., Usui, T., Ishiyama, Sakai, K., and Ishiyama, J.-I. (2000) *J. Mol. Struct. 556*, 173−177.
36. Gessler, K., Krauss, N., Steiner, T., Betzel, C., Sandmann, C., and Saenger, W. (1994) *Science 266*, 1027−1029.
37. Tomme, P., Creagh, A. L., Kilburn, D. G., and Haynes, C. A. (1996) *Biochemistry 35*, 13885−13894.
38. Freelove, A. C., Bolam, D. N., White, P., Hazlewood, G. P., and Gilbert, H. J. (2001) *J. Biol. Chem. 276*, 43010−43017.
39. Elgavish, S., and Shaanan, B. (1998) *J. Mol. Biol. 277*, 917−932.
40. Ravishankar, R., Suguna, K., Surolia, A., and Vijayan, M. (1999) *Acta Crystallogr. D55*, 1375−1382.
41. Creagh, A. L., Ong, E., Jervis, E., Kilburn, D. G., and Haynes, C. A. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 12229−12234.
42. Lemieux, R. U. (1996) *Acc. Chem. Res. 29*, 373−380.
43. Williams, B. A., Chervenak, M. C., and Toone, E. J. (1992) *J. Biol. Chem. 267*, 22907−22911.
44. Ladbury, J. E. (1996) *Chem. Biol. 3*, 973−980.

BI0347510