Properties of a family 56 carbohydrate-binding module and its role in the recognition and hydrolysis of β-1,3-glucan

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BH0236 from Bacillus halodurans is a multimodular β-1,3-glucanase comprising an N-terminal family 81 glycoside hydrolase catalytic module, an internal family 6 carbohydrate-binding module (CBM) that binds the nonreducing end of β-1,3-glucan chains, and an uncharacterized C-terminal module classified into CBM family 56. Here, we determined that this latter CBM, BhCBM56, bound the soluble β-1,3-glucan laminarin with a dissociation constant (Kd) of ~26 μM and displayed higher affinity for insoluble β-1,3-glucans with Kd values of ~2–10 μM but lacked affinity for β-1,3-glucooligosaccharides. The X-ray crystal structure of BhCBM56 and NMR-derived chemical shift mapping of the binding site revealed a β-sandwich fold, with the face of one β-sheet possessing the β-1,3-glucan-binding surface. On the basis of the functional and structural properties of BhCBM56, we propose that it binds a quaternary polysaccharide structure, most likely the triple helix adopted by polymerized β-1,3-glucans. Consistent with the BhCBM56 and BhCBM6/56 binding profiles, deletion of the CBM56 from BH0236 decreased activity of the enzyme on the insoluble β-1,3-glucan curdlan but not on soluble laminarin; additional deletion of the CBM6 also did not affect laminarin degradation but further decreased curdlan hydrolysis. The pseudo-atomic solution structure of BH0236 determined by small-angle X-ray scattering revealed structural insights into the nature of avid binding by the BhCBM6/56 pair and how the orientation of the active site in the catalytic module factors into recognition and degradation of β-1,3-glucans. Our findings reinforce the notion that catalytic modules and their cognate CBMs have complementary specificities, including targeting of polysaccharide quaternary structure.

Polysaccharides are a class of macromolecules that, due to their ubiquity in terrestrial and aquatic plants, arthropods, and microbes, comprise the most abundant organic polymers on Earth. The turnover of these carbohydrate macromolecules is attributed primarily to the metabolic action of microbes. Largely by deploying members of the glycoside hydrolase (GH) superfamily of enzymes, microorganisms break down polysaccharides as the initial step in their metabolism. These enzymes break glycosidic bonds using a hydrolytic catalytic mechanism and are presently classified into over 130 amino acid sequence-based families. Remarkably, the specificities represented by the multitude of characterized GHs span the majority of known polysaccharides.

Although GHs are typically described by the activity of their catalytic modules, these enzymes are often multimodular and contain additional functionalities within their architectures. The most common type of ancillary module is the non-catalytic carbohydrate-binding modules (CBMs) (2, 3). Through their specific ability to bind carbohydrates, these modules target the parent enzyme to a particular carbohydrate and maintain its association with the substrate, thereby enhancing the catalytic activity of the enzyme by this proximity effect. Like GHs, CBMs are classified into families, which currently number >70 and are based on amino acid sequence identity. CBMs have also been classified into three types: A, B, and C (2, 3). The type A CBMs bind crystalline polysaccharides, such as cellulose and chitin, but display no significant binding to individual glycan chains. In contrast, the type B CBMs are described as “endo” binders and recognize internal regions of individual, usually soluble, glycan chains. Finally, type C CBMs are the “exo” binders that recognize the termini of individual glycan chains. Like the GHs, the specificities of characterized CBMs include a large variety of the polysaccharides that comprise Earth’s biomass. Among these are CBMs in families 4, 6, 13, 32, 39, 43, 52, 54, and 56 that recognize β-1,3-glucans (4–12). Where detailed characterization is available, the properties of these β-1,3-glucan–specific CBMs indicate binding most consistent with type B or C classification.

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This article contains supplemental Table S1.

The atomic coordinates and structure factors (code 5T7A) have been deposited in the Protein Data Bank (https://www.pdb.org/).

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3 The abbreviations used are: GH, glycoside hydrolase; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid disodium salt; CBM, carbohydrate-binding module; ITC, isothermal titration calorimetry; NSD, normalized spatial discrepancy; L7, β-1,3-glucocoehlase; SAXS, small-angle X-ray scattering; SSRL, Stanford Synchrotron Radiation Lightsource; RMSD, root mean square deviation; PDBe, Protein Data Bank; HSQC, heteronuclear single quantum coherence; Rg, radius of gyration; BisTris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; SEC, size exclusion column; Nbound, binding capacity (or density) of CBM-binding sites present on insoluble polysaccharide.
CBM56 structure and binding

![Figure 1. The modular schematics of BH0236 and the constructs used in this study. Numbers above the schematics indicate the amino acid numbering for the modular boundaries.]

Found in seaweed, terrestrial plants, fungi, and bacteria, β-1,3-glucans are common components of terrestrial and aquatic biomass, where this family of polysaccharide has a variety of biological roles. For example, in seaweed, specifically brown macroalgae, the β-1,3-glucan laminarin functions as a storage polysaccharide (13). This highly soluble polysaccharide has a mean degree of polymerization of 25 and can contain up to four β-1,6-linked branches per molecule (14). In species of higher plants, a linear, unbranched β-1,3-glucan called callose precedes the deposition of cellulose and plays an important role in the development and response to many biotic and abiotic stresses (15, 16). Pachyman is also a linear β-1,3-glucan isolated from Wolfiporia extensa (also called Poria cocos). This polymer has a degree of polymerization of ~255 and has limited solubility (17). Scleroglucan is also a fungal β-1,3-glucan produced by Sclerotium sp. that contains frequent β-1,6-linked branch points, providing a tendency for this polysaccharide to gel, rather than be completely insoluble. β-1,3-glucans play a structural role in the fungal cell wall, along with β-1,6-glucans, mannoproteins, and chitin (7). Like pachyman, curdlan is an insoluble highly polymerized and unbranched β-1,3-glucan exopolysaccharide that is produced by some environmental bacteria, such as Agrobacterium sp. and Alcaligenes sp. (18). Thus, β-1,3-glucans are a biologically widespread family of glucans whose physicochemical properties, and therefore biological roles, are influenced by their degrees of polymerization and β-1,6-branching.

A common feature of β-1,3-glucans, regardless of their source and degree of β-1,6-branching, is their tendency to form helices, which in turn have a propensity to associate into a quaternary structure comprising a triple helix of parallel chains. Within these structures, imperfections in the association of individual chains can lead to disordered loop regions or the formation of duplexes (18). The highly polymerized nature often observed in the β-1,3-glucan family of polysaccharides necessitates that their breakdown is initiated by endo-β-1,3-glucoanases (EC 3.2.1.39), which typically fall into GH families 16, 17, 55, 64, 81, and 128. Notably, these endo-β-1,3-glucanases often possess one or more β-1,3-glucan-binding CBMs. An emerging theme in the structural analyses of endo-β-1,3-glucanases and β-1,3-glucan-specific CBMs is the recognition of the helical tertiary structure adopted by β-1,3-glucan chains (19). What remains unclear, however, is whether the aggregated state of β-1,3-glucan chains, which we consider either the formation of specific quaternary structure or the association of glucan chains into insoluble aggregates, influences the recognition of β-1,3-glucans by CBMs.

In this study, we examine the ability of BH0236, a β-1,3-glucan–specific glycoside hydrolase from Bacillus halodurans, to bind β-1,3-glucans. This enzyme is multimodular comprising a GH81 catalytic module (BhGH81) (20), an internal family 6 CBM (BhCBM6) (5), and a putative C-terminal family 56 CBM (Fig. 1). Our recent structural and functional studies of BhGH81 indicate that this catalytic module can recognize the duplex and/or triplex quaternary structure of β-1,3-glucans as part of its catalytic abilities (20). BhCBM6 is also well-characterized and known to bind the non-reducing end of β-1,3-glucan chains and β-1,3-glucooligosaccharides, therefore classifying it as a type C CBM (5). To illuminate the details of a relatively understudied CBM family, here we determine the structure of the C-terminal CBM56 (BhCBM56), examine its β-1,3-glucan–binding properties, outline its contribution to β-1,3-glucan degradation by its parent enzyme, and solve the overall solution structure of BH0236 to provide complete structural context for β-1,3-glucan recognition by the CBMs and catalytic module.

Results

The interaction of BhCBM56 with β-1,3-glucans

The structure and binding properties of BhCBM6 were determined previously (5); thus, we focused primarily on examining the isolated CBM56 module (BhCBM56) and the CBM tandem (BhCBM6/56) (Fig. 1). The gene fragments encoding these modules were recombinantly expressed in Escherichia coli, and the resulting proteins were purified to enable their characterization. Quantitative depletion isotherm analysis using insoluble pachyman and curdlan revealed that both BhCBM56 and BhCBM6/56 bind to these polysaccharides (Fig. 2 and Table 1). BhCBM56 and BhCBM6/56 displayed dissociation constants ($K_d$) for pachyman in the micromolar range that were an order of magnitude different and with BhCBM56 having the weaker affinity of the two proteins. To examine the possibility of avid binding of the CBM tandem, we also determined the $K_d$ of BhCBM6 for pachyman and found it to be 2-fold weaker than that of BhCBM56. We interpret these results as consistent with the high affinity of the BhCBM6/56 tandem for this polysaccharide resulting from avidity, whereby the individual binding sites in the tandem simultaneously interact with adjacent binding motifs for the respective CBMs on the polysaccharide, thus increasing the apparent affinity of the tandem. The density of BhCBM6/56-binding sites on pachyman,
as determined by $N_o$, was roughly 50% of that for BhCBM6 and 5% of that for BhCBM6/56, suggesting that proximal binding motifs on the polysaccharide to allow avid binding of the BhCBM6/56 protein occur with a relatively low frequency.

Both BhCBM56 and BhCBM6/56 bound to curdlan with $K_d$ and $N_o$ values generally similar to those observed for pachyman. We could not detect significant binding of BhCBM6 to curdlan. Given the less profound differences in $K_d$ for BhCBM56 and BhCBM6/56 when compared with the pachyman results and the lack of clear binding of BhCBM6 to curdlan, it is difficult to conclude that there is avid binding to this polysaccharide. However, the general pattern of higher affinity and lower binding capacity for the BhCBM6/56 tandem is similar to that observed for pachyman and consistent with a degree of avid binding. Toward an explanation for the less profound degree of avid binding on curdlan, we note that the chains of this polysaccharide are estimated to have a high degree of polymerization up to 12,000 glucose units (21). In contrast, the average degree of polymerization for pachyman is 255. As BhCBM6 binds the non-reducing ends of $\beta$-1,3-glucan chains, the binding sites present per mass on curdlan would be significantly less frequent than on pachyman. Our inability to detect substantial binding of BhCBM6 probably reflects a low density of BhCBM6-binding sites on curdlan and does not necessarily indicate a complete lack of binding. A low density of BhCBM6-binding sites on curdlan would probably manifest as a lower degree of avidity in BhCBM6/56 binding.

Figure 2. Binding isotherm analysis of insoluble $\beta$-1,3-glucan binding for BhCBM56 with curdlan (A), BhCBM6/56 with curdlan (B), BhCBM6 with pachyman (C), BhCBM56 with pachyman (D), and BhCBM6/56 with pachyman (E). Error bars, S.D. of triplicate measurements. Where error bars are not visible, the error was smaller than the size of the data marker. Solid lines, best fits to a bimolecular interaction model.

| Table 1 | Affinity of CBM constructs for insoluble $\beta$-1,3-glucans |
|---------|------------------------------------------------------------|
|         | Polysaccharide | CBM construct | $N_o$ | $K_d$ |
| Curdlan | BhCBM56       | $1.2 \pm 0.1$ | 3.7 ± 0.6 |
|         | BhCBM6/56     | $0.5 \pm 0.1$ | 2.2 ± 0.5 |
| Pachyman| BhCBM6        | $2.3 \pm 0.2$ | 21.6 ± 3.4 |
|         | BhCBM56       | $18.1 \pm 0.9$ | 10.0 ± 1.4 |
|         | BhCBM6/56     | $1.0 \pm 0.1$ | 1.2 ± 0.2 |

$^a$ The binding capacity (or density) of CBM-binding sites present on the insoluble polysaccharide.
We also evaluated the ability of BhCBM56 and BhCBM6/56 to bind β-1,3-glucooligosaccharides and laminarin by isothermal titration calorimetry (ITC). BhCBM56 was unable to bind β-1,3-glucooligosaccharides up to seven sugar units long (L7). BhCBM6/56 bound to L7 with a 1:1 stoichiometry and binding parameters the same as those previously determined for BhCBM6 on its own (5), suggesting that L7 is binding only to the CBM6 module and not to the CBM56 present in this construct (data not shown). BhCBM56 did bind to laminarin and yielded an isotherm consistent with a simple bimolecular binding event (Fig. 3A). Fitting the data with a bimolecular binding model yielded a $K_d$ of 25.9 ± 1.0 μM and a stoichiometry of 0.11 ± 0.0 molecules of L10/CBM-binding site, which equates to 1 CBM-binding site/92.9 ± 1.0 glucose subunits in the polysaccharide. The change in enthalpy of binding ($\Delta H$), change in entropy ($\Delta S$), and change in Gibbs free energy ($\Delta G$) were determined to be $-27.5 \pm 0.4$ kcal/mol, $-71.1 \pm 1.3$ cal/mol/K, and $-6.3 \pm 0.1$ kcal/mol, respectively. These values are somewhat typical of CBM–polysaccharide interactions (2, 3), whereas the affinity is roughly 2–6-fold lower than the affinities determined for binding to insoluble β-1,3-glucans.

In contrast, laminarin-binding experiments with BhCBM6/56 yielded more complex isotherms with multiple binding phases (Fig. 3B). Fitting these data with a model that accounts for two separate classes of binding sites gave a high-affinity site with a $K_d$ of 120.1 ± 9.7 μM and a stoichiometry of 0.04 ± 0.00 molecules of L10/CBM-binding site, which equates to 1 CBM-binding site/255.2 ± 7.3 glucose subunits and a $\Delta H$, $\Delta S$, and $\Delta G$ of $-53.4 \pm 0.7$ kcal/mol, $-147.7 \pm 2.1$ cal/mol/K, and $-9.4 \pm 0.5$ kcal/mol, respectively. The very high affinity (nanomolar) of this binding site combined with its infrequency in laminarin (1/∼255 glucose units) suggests that this represents simultaneous binding of the CBMs in the tandem to CBM6 and CBM56 sites that are infrequently close in space, resulting in avid binding. The low-affinity site had a $K_d$ of 3.7 ± 0.2 μM and a stoichiometry of 0.32 ± 0.01 molecules of L10/CBM-binding site, which equates to 1 CBM-binding site/31.7 ± 0.4 glucose subunits in the polysaccharide. The $\Delta H$, $\Delta S$, and $\Delta G$ were $-14.2 \pm 0.3$ kcal/mol, $-22.6 \pm 1.1$ cal/mol/K, and $-7.4 \pm 0.1$ kcal/mol, respectively. These values are very similar to those previously estimated for the binding of isolated BhCBM6 to laminarin (5). Furthermore, as the average degree of polymerization for the glucan chains comprising laminarin is 25, and each chain possesses a single non-reducing end ligand for the CBM6, we expect a stoichiometry close to 1 binding site/∼25 glucose residues, which was indeed the case here and was also observed previously for isolated BhCBM6 (5). We acknowledge in this scenario that a third type of interaction is probably also occurring: independent interaction of the CBM56 module with its binding site on laminarin. However, the affinity of this third interaction is expected to be only ~6-fold different from the CBM6–laminarin interaction, making it difficult to discriminate between the two independent CBM interactions. Therefore, it is likely that the fitting of the second and low affinity class of interaction in part also represents a component of the independent CBM56–laminarin equilibrium.

The structure of BhCBM56 and identification of the binding site

There are presently no structures of a CBM56 module; thus, to illuminate the molecular details of β-1,3-glucan recognition by BhCBM56, we solved the X-ray crystal structure of this ~95-

![Figure 3. Representative isothermal titration calorimetric analysis of laminarin binding to BhCBM56 (A) and BhCBM6/56 (B). The top panels show the baseline-corrected thermograms for the titration of laminarin at a concentration of 22.5 or 13.5 mM (calculated based on equivalents of β-1,3-glucodecaose) into BhCBM56 (at 120 μM) or BhCBM6/56 (at 78 μM), respectively. In the bottom panels, the integrated heats of dilution resulting from titration of laminarin into matched buffer lacking protein are shown as solid circles. The integrated experimental heats corrected for the heat of dilution are shown as solid squares. Solid lines, best-fit lines for a simple bimolecular interaction model in A and a two-site binding model in B.](image-url)
CBM56 structure and binding

Table 2
Data collection and structure refinement statistics for BhCBM56

| Data Collection | Beaml ine | Space Group | Cell Dimensions a, b, c (Å) | Wavelength (Å) | Resolution (Å) | Rmerge | Rfree |
|----------------|----------|-------------|-----------------------------|----------------|----------------|--------|-------|
| SSRL BL 11-1   | C2       |             |                             | 0.89/1.6       | 36.1-1.60      | 0.059  | 0.073 |
|                 |          |             |                             |                | 39.52-1.60     | 0.045  | 0.052 |
|                 |          |             |                             |                | 39.40-1.60     | 0.052  | 0.052 |

To assist in confirming this surface of BhCBM56 as the laminarin-binding site, we generated alanine substitutions at four positions: Tyr-961, Asp-963, His-965, and Trp-1015 (the positions of Tyr-961 and Trp-1015 are also indicated in Fig. 4B). The relative affinities of these mutant CBMs were compared with the unmodified CBM by affinity electrophoresis using laminarin as the ligand (Fig. 5 and Table 3). The D963A and Y961A mutants bound to laminarin but with affinities below a quantifiable range, whereas the H965A mutant had a roughly 5-fold lower affinity than the unmutated CBM, indicating the importance of these residues in the interaction. The W1015A mutant had an affinity that, when considering the error, was not substantially lower than that of the unmodified CBM.

The β-1,3-glucanase activity of BH0236

With the identification of the C-terminal module of BH0236 as a CBM56 with β-1,3-glucan-binding activity, we sought to probe the role of the CBMs in β-1,3-glucan degradation by this enzyme. We generated three engineered versions of BH0236, all with N-terminal hexahistidine tags. The first construct, which we refer to as BH0236, comprised the entire sequence with the exception of the N-terminal 27-amino acid-residue secretion signal peptide (Fig. 1). The remaining two constructs were based on the BH0236 construct but with the CBM56 deleted (called BH0236Δ56) and with both CBMs deleted (called BhGH81) (Fig. 1). Our previous activity studies of BhGH81 using fluorophore-assisted carbohydrate electrophoresis revealed activity on laminarin and curdlan after 1 h, but significant activity on pachyman was only observed after an extensive overnight incubation; no activity on scleroglucan was detected, even with extended digestion (20). Using a reducing sugar assay to detect the release of new reducing sugars after enzyme treatment using these same four substrates, we tested the activity of full-length BH0236. After a 1-h digestion, there was clear activity on laminarin and curdlan (Fig. 6A). Consistent with the previous results using BhGH81, BH0236 displayed no activity on scleroglucan, and the enzyme appeared to have low activity on pachyman, but variability in the results made this latter observation inconclusive. On the basis of these results, using the reducing sugar assay, we examined the activity of the all three enzyme constructs on laminarin and curdlan (Fig. 6B). There was no statistically significant difference observed between the three proteins on soluble laminarin. In contrast, BH0236Δ56 had a significantly lower activity on curdlan than BH0236 (~3-fold lower), whereas the activity of the catalytic module alone, BhGH81, was lower than both BH0236Δ56 (~2-fold lower) and BH0236 (~6-fold lower). These results indicate that the CBMs play an insignificant role in the degradation of a soluble β-1,3-glucan but have an important role in the efficient degradation of an insoluble β-1,3-glucan.
CBM56 structure and binding

SAXS analysis of BH0236

Overall, BH0236 is a quite large enzyme, whose N-terminal catalytic region comprises four distinct domains, whereas its C-terminal polysaccharide-binding region contains two functionally distinct CBMs that are important to the full enzyme’s catalytic activity on insoluble β-1,3-glucan. To understand the functional contributions of the CBMs in the context of the complete enzyme structure, we performed solution small-angle X-ray scattering analysis on our BH0236 construct, which is a protein that we were unable to crystallize for high-resolution structural analyses. Values for the radius of gyration ($R_g$) measured by the Guinier method and with Gnom were similar, at 40.4 ± 0.1 and 42.2 ± 0.1 Å, respectively. The maximum dimension ($D_{max}$) was 129.3 Å, and the Porod volume was 155,361 Å³. The average particle density calculated using the Porod volume and the molecular weight of BH0236 (theoretical 114,518.8 Da) was 1.22 g cm⁻³, which is consistent with the density expected for a monodisperse solution of a monomeric protein (23). Kratky and Porod-Debye plots of the scattering data revealed profiles expected for a protein lacking significant flexibility (Fig. 7, A and B) (23).

To generate an ab initio shape for BH0236, we ran DAMMIF 10 times and averaged the resulting models. This yielded an average χ-value of 1.34 ± 0.01 and an average normalized spatial discrepancy (NSD) of 0.68 ± 0.06, indicating good agree-
were not significantly different from one another. The model independently run 10 times and consistently resulted in models that regions were modeled as dummy atoms. BUNCH was independently with the ab initio shape derived with DAMMIF (Fig. 7D).

Discussion

Isolated BhCBM56 bound insoluble β-1,3-glucans (pachyanman and curdlan) with affinities in the range of typical CBM–polysaccharide binding (2) and bound to soluble laminarin, although with a \( K_d \sim 3–5 \) fold greater than that for the insoluble polysaccharides. Given that other characterized β-1,3-glucon–binding CBMs, which are of type B or C, bound tightly to both laminarin and β-1,3-glucooligosaccharides, we found it unusual that BhCBM56 displayed no detectable affinity for β-1,3-glucooligosaccharides. This suggests that the CBM selects for a shared property between the soluble and insoluble β-1,3-glucans that is absent in β-1,3-glucooligosaccharides. We postulate that this property is most likely the ability of highly polymerized β-1,3-glucans to form higher-order structures. All three of the β-1,3-glucans identified as ligands for BhCBM56 are known to contain triple-helical quaternary structures. Insoluble β-1,3-glucans, like pachyanman and curdlan, have an extra degree of complexity, as their triple-helical β-1,3-glucan domains are thought to be cross-linked by strand sharing, thus giving the polysaccharides additional internal structure and insolubility (18). In comparison with β-1,3-glucooligosaccharides, such as L7, to which BhCBM56 does not bind, the higher degree of polymerization of laminarin, pachyanman, and curdlan may contribute to their recognition by BhCBM56. However, we note that the maximum dimension of the identified laminarin-binding face on BhCBM6 is \( \sim 22–24 \) Å, whereas the dimension of L7 in its single-helical conformation is \( \sim 18–20 \) Å, making it seem unlikely that a degree of polymerization beyond seven would result in the difference between binding and no binding. Furthermore, if BhCBM56 were able to bind more extended regions of single β-1,3-glucan chains, we would still expect a relatively high density of binding sites on laminarin. For example, BhCBM6 binds once per laminarin chain (average degree of polymerization of 25), as does the β-1,3-glucan–binding type B CBM4 from Thermotoga maritima (4, 5, 24). In contrast, BhCBM56 binds once per ~100 glucose units, or approximately once every four laminarin chains. Based on the physical dimensions of BhCBM56, if one conservatively assumes a binding footprint of 5–10 glucose units, then the CBM binds to only ~5–10% of the glucose units present in laminarin. It is notable that only ~5% of laminarin isolated from Laminaria digitata, the source of laminarin used here, forms triple helices (25). The consistency between our measured binding capacity and the estimated triple-helical content of laminarin implies that this quaternary structure may constitute the structure to which BhCBM56 binds.

Additional support for the argument that BhCBM56 specifically recognizes the quaternary structure of β-1,3-glucans comes from its comparison with PiCBM39. PiCBM39 specifically binds β-1,3-glucans, and its X-ray crystal structure determined when bound to laminarihexaose elegantly revealed features consistent with binding the triple-helical quaternary structure of β-1,3-glucopolysaccharides, such as laminarin (12). Although the residues of the laminarin-binding surface on PiCBM39 are not well-conserved with BhCBM56, the general location and sizes of the binding surfaces, revealed by the complex of PiCBM39 and the NMR mapping of BhCBM56, match very closely (Fig. 8), suggesting not only fold conservation between the two modules but similar mechanisms of recognizing the quaternary structure of β-1,3-glucans.

Overall, therefore, we presently favor the hypothesis that the quaternary structure of polymerized β-1,3-glucans contributes to their recognition by BhCBM56. Although the mechanism by which this module may bind higher-order structures in β-1,3-glucans remains a matter of speculation, our interpretation of the data suggests that it may be the triple-helical regions that are bound by the protein, in which case the binding site on the polysaccharide may comprise patches of spatially adjacent glucose residues contributed by separate glucan chains that are brought in proximity by the intertwining of the polysaccharide.
CBM56 structure and binding

Figure 6. The activity of BH0236 from B. halodurans. A, a screen of BH0236 activity against four different \( \beta \)-1,3-glucans. Error bars, S.D. of triplicate samples. B, activity comparison of BH0236 and truncated variants on laminarin and curdlan. Error bars, S.D. of sextuplicate samples. **, \( p < 0.01 \); ***, \( p < 0.001 \). All experiments used 0.2% (w/v) \( \beta \)-1,3-glucan, 50 nM enzyme, and the HABH reducing sugar assay to detect the generation of new reducing sugar (expressed as absorbance units).

The role of the CBMs in the ability of BH0236 to degrade an insoluble substrate is therefore not unusual. However, this enzyme provides a unique model in that the catalytic module can recognize the quaternary structure of the polysaccharide substrate, the CBM6 module specifically recognizes the non-reducing end of \( \beta \)-1,3-glucan chains, and the terminal CBM56 prefers to bind aggregated \( \beta \)-1,3-glucan chains, probably of a specific tertiary or quaternary structure. To place these observations in the context of the structure of the complete enzyme, we performed a SAXS analysis to generate a pseudo-atomic model representing the solution structure of the full enzyme. Overall, the SAXS-based model showed the organization of the modules in three dimensions to approximate the order in which the modules appear in the primary structure; the N-terminal catalytic module separated from the distal C-terminal CBM56 by the central CBM6 (Fig. 7D). The CBMs were in close association; however, their relative orientations and resulting presentation of glucan-binding sites were such that with CBM6 binding the non-reducing end of a \( \beta \)-1,3-glucan chain, it would not be possible for the CBM56 to bind the same glucan chain without severe bending of the polysaccharide chain (Fig. 7E). This implies that the most likely contribution to the avid binding of the BhCBM6/56 tandem is the simultaneous binding of each CBM to separate but proximal sugar chains present in the \( \beta \)-1,3-glucan quaternary structure, either a triple helix or aggre-
Similarly, the active site of the catalytic module is also oriented away from the binding sites of the CBMs, again suggesting recognition and hydrolysis of a glucan chain distinct from that bound by either of the CBMs. Overall, this implies a mode of cooperation between the catalytic module and CBMs in recognizing substrate that is somewhat random in three-dimensional space and without specific directional coordination, a scenario that has been suggested for other multimodular glycoside hydrolases (28).

The β-1,3-glucanase BH0236 from *Bacillus halodurans* has served as one of the models for the structural and functional examination of family 81 glycoside hydrolases. The catalytic module of this enzyme, BhGH81, has displayed unexpected architectural and functional features that are consistent with an
ability to recognize the quaternary structure of its β-1,3-glucan substrate (20). This capability appears to be mirrored by the CBM56 module in this enzyme, which selectively binds insoluble β-1,3-glucans, probably by binding domains in the polysaccharide with triple-helical quaternary structure. This study reinforces the notion that catalytic modules and their cognate CBMs typically have complementary specificities, including targeting polysaccharide quaternary structure, but extends this concept beyond crystalline β-1,4-glucan polysaccharides to include other polysaccharides that adopt defined quaternary structures.

Experimental procedures

Materials

β-1,3-glucan oligosaccharides, curdlan and pachyman were obtained from Megazyme International Ireland Ltd. (Bray, Ireland). Scleroglucan was obtained from V-labs (Covington, LA). All reagents, chemicals, and other carbohydrates were purchased from Sigma unless otherwise specified.

Cloning

The BhGH81 catalytic module fragment (residues 29–790) was cloned previously, as was the BhCBM6 construct (residues 791–926) (5, 20). Gene fragments encoding the BhCBM56 (amino acids 927–1021) and the tandem of CBMs, referred to as BhCBM56/56 (residues 791–1021), were amplified by PCR from *B. halodurans* genomic DNA (ATCC BAA-125D), and their products were cloned into pET28a via the engineered restriction sites using a standard molecular biology procedure as was used previously to clone the BhCBM6 gene fragment (5) (see supplemental Table S1 for primers). Gene fragments encoding BH0236Δ56 (residues 29–926) and BH0236 (residues 29–1021) were amplified by PCR and inserted in pET28a vector using primers designed for the “In-Fusion HD Cloning” method (Clontech) (see supplemental Table S1 for primers). Site-directed mutations were introduced using the “megaprimmer” PCR method (29) into BhCBM56 (W1015A, H965A, D963A, and Y961A). The resulting gene fusions encoded an N-terminal six-histidine tag fused to the protein of interest by an intervening thrombin protease cleavage site. Bidirectional DNA sequencing was used to verify the fidelity of each construct.

Protein expression and purification

All recombinant expression vectors were transformed into *E. coli* BL21 Star (DE3) cells (Invitrogen), and proteins were produced using 2 × YT medium supplemented with kanamycin (50 μg/ml). Briefly, bacterial cells transformed with the appropriate expression plasmid were grown at 37 °C until the culture reached an optical density of 0.9 at 600 nm. Gene expression and protein production were then induced by the addition of isopropyl β-D-1-thiogalactopyranoside to a final concentration of 0.5 mM, followed by overnight incubation at 16 °C with shaking. Cells were harvested by centrifugation and disrupted by chemical lysis (30). Proteins were purified from the cleared cell lysate by Ni²⁺-immobilized metal affinity chromatography followed by size-exclusion chromatography using a Sephacryl S-100 or S-300 column (GE Healthcare). Purified protein was concentrated using a stirred-cell ultrafiltration device with a 1000 or 10,000 molecular weight cut-off membrane (Millipore).

Protein concentration was determined by measuring absorbance at 280 nm and using calculated molar extinction coefficients of 36,440 cm⁻¹ M⁻¹ for BhCBM6, 21,430 cm⁻¹ M⁻¹ for BhCBM56, 189,540 cm⁻¹ M⁻¹ for BhGH81, 225,980 cm⁻¹ M⁻¹ for BH0236Δ56, 57,870 cm⁻¹ M⁻¹ for BhCBM56/56, and 247,410 cm⁻¹ M⁻¹ for BH0236 (31).

Enzyme activity assays

Reducing sugar assays used 50 nm enzyme with 0.2% β-1,3-glucan (laminarin, curdlan, scleroglucan, or pachyman) in 20 mM Tris-HCl, pH 7.0, at 37 °C with shaking. The initial screen for activity was performed using 1-h incubation of the polysaccharide with enzyme; samples were performed in triplicate. Upon identifying laminarin and curdlan as suitable substrates, pilot time courses were performed to determine an incubation time that resulted in less than 50% of the maximum conversion of substrate, which was chosen to be 30 min. The activities of the BhGH81, BH0236Δ56, and BH0236 constructs on lami-
narin and curdlan were then compared using a 30-min incubation; experiments were performed in sextuplicate. Samples were analyzed by removing 4.2 μl of the reaction mix and adding it to 250 μl of working reagent, containing 10% of reagent A (p-hydroxybenzoic acid hydrazide at 50 g/liter in 5% hydrochloric acid) and 90% of reagent B (12.5 and 1.1 g/liter trisodium citrate and calcium chloride dihydrate, respectively, in 2% sodium hydroxide). The samples were then incubated at 100 °C for 10 min before reading the absorbance at 410 nm. Negative control reactions were obtained using no enzyme or heat-killed enzymes.

Reaction products were analyzed by fluorophore-assisted carbohydrate electrophoresis in a protocol adapted from Robbins et al. (32). Briefly, 0.5 μM enzyme was incubated overnight in the presence of 5 mg ml<sup>−1</sup> of β-1,3-glucan or β-1,3-glucoligosaccharides in a 10-μl reaction volume in 20 mM Tris-HCl, pH 8.0. The reactions were stopped by the addition of 1 ml of ice-cold 100% ethanol, and the samples were dried using a vacuum concentrator at ~50 °C for 2 h. Overnight labeling of the sugar products was carried out at 37 °C by adding 5 μl of a solution of 0.2 M 8-aminonaphthalene-1,3,6-trisulfonic acid disodium salt (ANTS) in 15% acetic acid and 5 μl of 1 M sodium cyanoborohydride in dimethyl sulfoxide to the dried samples. The ANTS-labeled products were then dried and resuspended in 25 μl of loading dye (0.015% bromphenol blue plus 10% glycerol in 62 mM Tris-HCl, pH 6.8). Approximately 0.5–1 μg of ANTS-labeled product was loaded onto a 35% polyacrylamide gel with a 10% stacking gel and electrophoresed at a constant 100 V for 30 min, followed by 1 h at 300 V at 4 °C in native running buffer (25 mM Tris-HCl, 0.2 M glycine). Gels were immediately visualized and imaged under UV light.

**CBM binding**

ITC was performed as described previously (5) using a VP-ITC (MicroCal, Northampton, MA) in 50 mM potassium phosphate buffer (pH 7.0) at 25 °C. BhCBM56 was used at a concentration of 120 μM, and laminarin at a concentration of 22.5 mM (calculated based on equivalents of β-1,3-glucododecaose, L10) was titrated into protein. BhCBM56/56 was used at a concentration of 78 μM and laminarin at 13.5 mM. Laminarin solutions were prepared by mass using buffer saved from the last step of extensive dialysis of the protein solutions. All solutions were filtered and degassed immediately before use. An ideal alternative would have been to titrate CBM into polysaccharide, so-called reverse titrations, which were tried. However, the proteins suffered aggregation problems at high concentrations, thus precluding this experimental setup. Therefore, titrations were performed in the standard manner but were analyzed using a model-fitting mode whereby the CBM present in the sample cell was treated as the ligand and the polysaccharide as the macromolecule. Analysis of the BhCBM56 data by fitting an one-site binding model gave suitable fits as judged by a runs test of the residuals from the fitting process using a two-site binding model revealed this model to give an acceptable fit. All data show the average and S.D. of three independent titrations.

For depletion binding isotherms, pachyman and curdlan were individually suspended in 50 mM potassium phosphate (pH 7.0) for 1 h, followed by vacuum filtration; this procedure was repeated with 1 M potassium phosphate (pH 7.0) and three times with water. The resulting washed polysaccharides were then resuspended in a small volume of water and lyophilized. Polysaccharide suspensions were created by mass in the desired buffer immediately before use. Depletion assays for both BhCBM6/56 and BhCBM56 were performed in 50 mM potassium phosphate (pH 7.0) using procedures described previously (33, 34). Briefly, triplicate samples were incubated at room temperature under continuous tumbling for 1 h in 1.5-ml microcentrifuge tubes containing either 2.6 mg (pachyman) or 6.5 mg (curdlan) of polysaccharide and a defined amount of CBM construct in a total reaction volume of 650 μl. The final polypeptide concentrations for BhCBM6/56 and BhCBM56 ranged from 0.25 to 40 μM and from 0.5 to 80 μM, respectively. After equilibration, the samples were centrifuged at 12,000 rpm for 10 min, and the resulting supernatant was analyzed by UV absorbance at 280 nm to determine the concentration of unbound CBM. Control samples containing CBM but no polysaccharide were performed in parallel to represent the total amount of CBM. Data were analyzed as described previously, using a binding model accounting for a single class of binding site on the β-1,3-glucan polysaccharide (33).

Affinity electrophoresis was performed and analyzed as described previously (34, 35) using 10% (w/v) polyacrylamide gels polymerized with and without the inclusion of laminarin ranging in concentration from 0 to 1.8 mM, (based on equivalents of β-1,3-glucododecaose). A 10-μg sample of each protein was electrophoresed in the gel, followed by staining of the gels Coomassie Blue R250 in 25% (v/v) methanol and 10% (v/v) acetic acid and destained with 25% (v/v) methanol and 10% (v/v) acetic acid. Binding to polysaccharide was visualized as reduced mobility of the protein on these gels relative to the nondenaturing gel lacking polysaccharide. Bovine serum albumin was used as a non-interacting reference protein.

**General crystallography procedures**

Crystals were obtained using sitting-drop vapor diffusion for screening and hanging-drop vapor diffusion for optimization, all at 18 °C. For data collection, single crystals were flash-cooled with liquid nitrogen in crystallization solution supplemented with a cryoprotectant optimized for each crystal form as given below. Diffraction data were collected on the beamline 11-2 at the Stanford Synchrotron Radiation Source (SSRL) as indicated in Table 2. All diffraction data were processed using MOSFLM and SCALA (36). All data collection and processing statistics are shown in Table 3. For all structures, manual model building was performed with COOT (37), and refinement of atomic coordinates was performed with REFMAC (38). The addition of water molecules was performed in COOT with FINDWATERS and manually checked after refinement. In all data sets, refinement procedures were monitored by flagging 5% of all observation as “free” (39). Model validation was performed with MOLPROBITY (40). All model statistics are shown in Table 3.
Coordinates and structure factors have been deposited with the PDB codes indicated below.

**BhCBM56 structure determination**

Crystals of BhCBM56 (20 mg/ml) were obtained in 21% PEG 3350 and 0.1 M BisTris/HCl, pH 5.5. A bromide derivative was obtained by soaking a crystal in crystallization solution containing 25% (v/v) ethylene glycol and 1 M potassium bromide for 10 min before data collection. A heavy atom substructure comprising seven bromide atoms was determined using autoSHARP (41). An initial model was then built using ARP/wARP (42). This built model was used to solve the structure of CBM56 by molecular replacement using PHASER.

### NMR spectroscopy

Uniformly $^{13}$C/$^{15}$N-labeled BhCBM56 was expressed and purified in a similar manner to that described above, with the following exceptions. Bacterial cells transformed with CBM56-encoding plasmid were grown in M9 minimal medium supplemented with 1 g/liter $^{15}$NH$_4$Cl, 2 g/liter $[13C]$glucose as the sole nitrogen and carbon sources, respectively. At induction, 5 ml/liter $^{13}$C/$^{15}$N-BioExpress-1000 medium (Cambridge Isotope Laboratories) was added and left at 16 °C with shaking overnight.

Uniformly 2 mM $^{13}$C/$^{15}$N-labeled BhCBM56 was dialyzed into a solution containing 25 mM Tris-HCl, pH 7.5, 50 mM NaCl, in 90% H$_2$O, 10% D$_2$O for NMR data collection. All NMR data were collected at 30 °C on a 600-MHz Varian INOVA spectrometer equipped with a room temperature triple resonance probe. Backbone resonance assignments of BhCBM56 were determined by collecting and analyzing $^1$H-$^1$H HSQC, HNCACb, CBCAcoNH, HNCO, and HNcaCO experiments, using PHASER.

The low-resolution shapes of the protein construct were determined *ab initio* from the scattering curve using the program DAMMIF (48). These solutions were subsequently compared and averaged with the program DAMAVER (49), which also computes the NSD values for the groups of *ab initio* models. In all cases, calculations led to highly similar forms with NSD values ranging between 0.643 and 0.705. The combined *ab initio* and rigid-body modeling program BUNCH (50) was used to generate models from the scattering data using the available atomic coordinates over a series of scattering data sets. The atomic coordinates of BhCBM56 determined by X-ray crystallography in this study, BhGH81 (PDB codes 5T49 and 5T4G), and BhCBM6 (PDB code 1W9S (5)) were used as three independent rigid bodies. Superimposition of the BUNCH-generated models was performed using the program SUPCOMB (51). The goodness of fit of the models was assessed with the program CRYOSOL (47).

### Small-angle X-ray scattering

SAXS data were collected at the Cornell High Energy Synchrotron Source (Cornell University, Ithaca, NY) on the G1 beamline, which is combined with an in-line size exclusion column (SEC). BH0236 at 34.2 mg ml$^{-1}$ was loaded onto a Superdex 200 SEC before measuring the scattering pattern using an exposure time of 1–5 s at room temperature. The wavelength was 1.245 Å with a sample-to-detector distance set at 1500 mm, leading to scattering vectors $q$ (where $q = 4 \sin \theta / \lambda$, where $2 \theta$ is the scattering angle) ranging from 0.007 to 0.7 Å$^{-1}$. Background scattering was measured for the sample using the void volume of the SEC and subsequently subtracted from the protein scattering pattern after proper normalization and correction for detector response.

The $R_g$ values were derived from the Guinier approximation: $I(q) = I(0) \exp(-q^2R_g^2/3)$, where $I(q)$ is the scattered intensity and $I(0)$ is the forward scattered intensity (46). The radius of gyration and $I(0)$ are inferred from the slope and the intercept, respectively, of the linear fit of $\ln[I(q)]$ versus $q^2$ in the $q$ range $q \times R_g < 1.3$. The distance distribution function $P(r)$ was calculated on the merged curve by the Fourier inversion of the scattering intensity $I(q)$ using GNOM (47). The $P(r)$ function was also used to calculate the $R_g$ taking into account the whole data collected.

The low-resolution shapes of the protein construct were determined *ab initio* from the scattering curve using the program DAMMIF (48). These solutions were subsequently compared and averaged with the program DAMAVER (49), which also computes the NSD values for the groups of *ab initio* models. In all cases, calculations led to highly similar forms with NSD values ranging between 0.643 and 0.705. The combined *ab initio* and rigid-body modeling program BUNCH (50) was used to generate models from the scattering data using the available atomic coordinates over a series of scattering data sets. The atomic coordinates of BhCBM56 determined by X-ray crystallography in this study, BhGH81 (PDB codes 5T49 and 5T4G), and BhCBM6 (PDB code 1W9S (5)) were used as three independent rigid bodies. Superimposition of the BUNCH-generated models was performed using the program SUPCOMB (51). The goodness of fit of the models was assessed with the program CRYOSOL (47).

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