Apo- and Cellopentaose-bound Structures of the Bacterial Cellulose Synthase Subunit BcsZ*

Received for publication, February 2, 2011, and in revised form, March 4, 2011 Published, JBC Papers in Press, March 25, 2011, DOI 10.1074/jbc.M111.227660

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Cellulose, a very abundant extracellular polysaccharide, is synthesized in a finely tuned process that involves the activity of glycosyl-transferases and hydrolases. The cellulose microfibril consists of bundles of linear β-1,4-glucan chains that are synthesized inside the cell; however, the mechanism by which these polymers traverse the cell membrane is currently unknown. In Gram-negative bacteria, the cellulose synthase complex forms a trans-envelope complex consisting of at least four subunits. Although three of these subunits account for the synthesis and translocation of the polysaccharide, the fourth subunit, BcsZ, is a periplasmic protein with endo-β-1,4-glucanase activity. BcsZ belongs to family eight of glycoside hydrolases, and its activity is required for optimal synthesis and membrane translocation of cellulose. In this study we report two crystal structures of BcsZ from Escherichia coli. One structure shows the wild-type enzyme in its apo form, and the second structure is for a catalytically inactive mutant of BcsZ in complex with the substrate cellopentaose. The structures demonstrate that BcsZ adopts an (α/α)_6-barrel fold and that it binds four glucan moieties of cellopentaose via highly conserved residues exclusively on the nonreducing side of its catalytic center. Thus, the BcsZ-cellopentaose structure most likely represents a posthydrolysis state in which the newly formed nonreducing end has already left the substrate binding pocket while the enzyme remains attached to the truncated polysaccharide chain. We further show that BcsZ efficiently degrades β-1,4-glucans in in vitro cellulase assays with carboxymethyl-cellulose as substrate.

Cellulose, one of the most abundant biopolymers in nature, is produced by most vascular plants and a large number of algae, but is also found in some bacteria and even tunicates (1, 2). The cellulose microfibril consists of linear chains of glucose molecules that are linked via β-1,4-glycosidic bonds (β-1,4-glucan) and are bundled together to form cable-like structures outside the cell (3, 4). A key enzyme in the biosynthesis of cellulose is the membrane-embedded cellulose synthase, which catalyzes the polymerization of UDP-activated glucose molecules (5, 6) and, presumably, also the translocation of the growing polysaccharide across the cell membrane (7). The bacterial cellulose synthase (Bcs) complex is predicted to form a trans-envelope secretion system comprising at least four subunits (6). Although the BcsA, -B, and -C subunits are involved in synthesizing and translocating the β-1,4-glucan across the inner and the outer membrane, the fourth subunit, BcsZ, is a periplasmic protein with endo-β-1,4-glucanase activity (8).

BcsZ belongs to family 8 of glycoside hydrolases (GH-8) that adopts an (α/α)_6-barrel architecture, consisting of two rings of six parallel α-helices with opposing orientation in both rings (9). GH-8 enzymes hydrolyze glycosyl bonds with a pair of acidic residues in a reaction that inverts the anomeric configuration at the new reducing end (inverting hydrolases) (10). Studies with the Acetobacter ximon and Agrobacterium tumefaciens homologs of BcsZ (CMCax and CelC, respectively) demonstrate their profound effect on cellulose production in vivo (11–13). In general, depleting the glucanase activity leads to a reduced rate of cellulose secretion, whereas increasing the concentration of CMCax in the growth medium of A. ximon stimulated the formation of cellulose (11, 12, 14). Similar effects were observed in plants. In Arabidopsis thaliana the absence of the membrane bound endo-β-1,4-glucanase KORRIGAN led to architectural alterations in the primary cell wall (15), suggesting that cellulose production and deposition require the orchestrated activities of glycosyl-transferases and hydrolases. Although the biological purpose of the hydrolytic activity during cellulose synthesis is unknown, it is possible that glucanases are required for the controlled alignment of individual β-1,4-glucan strands to form the cellulose microfibril. Alternatively, it has been suggested that glucanases might cleave lipid-linked intermediates during the polymerization process (13); the presence of such lipid-linked precursors during cellulose synthesis, however, has not been confirmed unambiguously for either plant or bacterial cellulose synthases.

To understand the biological role of BcsZ during cellulose synthesis, we determined two crystal structures of the enzyme from Escherichia coli. One structure shows the wild-type BcsZ in its apo form, whereas the second represents a catalytically inactive mutant of BcsZ (E55Q) bound to cellopentaose. Fur-

* The abbreviations used are: Bcs, bacterial cellulose synthase; CMC, carboxymethyl-cellulose; GH, glycoside hydrolase; r.m.s.d., root mean square deviation; Se-Met, selenomethionine.
thermore, we demonstrate that BcsZ hydrolyzes high molecular weight β-1,4-glucans in vitro by using carboxymethyl-cellulose (CMC) as substrate.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification of BcsZ—The mature region of the BcsZ gene (residues 22–368) was amplified from E. coli K12 genomic DNA and cloned with a C-terminal His₆ tag into the pET20b vector using the NcoI and XhoI restriction sites. The pET20b vector encodes an N-terminal PelB signal sequence for secretion into the periplasm. BcsZ expression was induced with 0.5 mM isopropyl β-D-thiogalactopyranoside in E. coli C43 cells at an A₆₀₀ of 0.4 for 3 h at 30 °C. The cells were harvested by centrifugation and resuspended in 20 mM Tris, pH 7.5, and 0.3 M NaCl (RS buffer). Cells were lysed using a microfluidizer, and the whole cell extract was centrifuged in a Beckman Optima ultracentrifuge for 30 min at 42,000 rpm. The supernatant was incubated with 1 ml nickel-nitrilotriacetic acid-agarose (Qiagen) for 45 min at 4 °C, and the resin was packed in a gravity flow chromatography column and washed with 100 ml of RS buffer containing 20 mM imidazole. BcsZ was eluted in 10 ml of RS buffer containing 200 mM imidazole. The eluted protein was concentrated to a 500-μl final volume and loaded onto a Superdex S200 gel filtration column (GE Healthcare) eluted in 10 ml of RS buffer containing 200 mM imidazole. The eluted protein was concentrated to 100 μl (determined by UV absorbance with a calculated extinction coefficient of 106,005 M⁻¹ cm⁻¹ at 280 nm), spun at 100,000 g for 15 min, and set up for crystallization. The E55Q mutant was introduced into the wild-type BcsZ sequence using the Stratagene QuikChange mutagenesis kit, and the mutated gene was sequenced to confirm the presence of the desired mutation. The BcsZ mutant was purified as described for wild-type BcsZ. Selenomethionine (Se-Met)-derivatized BcsZ was produced in C43 cells grown in M9 minimal medium supplemented with 1 mg/ml Lys, Thr, and Phe, 0.5 mg/ml Leu, Ile, and Val, as well as 0.6 mg/ml L-Se-Met. The purification of Se-Met-derivatized BcsZ was as described for wild-type BcsZ.

Crystallography Data Collection—Wild-type and Se-Met-derivatized BcsZ were crystallized at 17 °C by vapor diffusion after mixing 2 μl of 100 μM BcsZ with an equal volume of well solution containing 30–40% PEG300 and 0.1 M sodium citrate/phosphate buffer, pH 4.2. Crystals appeared within 5 h and continued to grow over a period of 7 days after which they were directly frozen in liquid nitrogen. The E55Q mutant was crystallized in the presence of 10 mM cellopentaose, 12–18% PEG3350, and 50 mM sodium citrate, pH 7.0. The crystals were cryoprotected by a stepwise transfer into crystallization solution containing 25% glycerol and flash frozen in liquid nitrogen. Diffraction data for all crystals were collected at the Argonne National Laboratory, SER-CAT beam lines 22-ID and 22-BM.

Diffraction data were indexed, integrated, and scaled with Denzo and Scalepack as part of the HKL2000 software package (16). For experimental phasing, the positions of all 40 expected selenium sites were determined from a single-wavelength anomalous dispersion data set using the SHELXD&E software as part of the CCP4 software package (17, 18). The obtained phases from SHELXE were of sufficient quality to allow automatic model building for most of the structure in ARP/wARP (19), which built approximately 90% of the model. The electron density in some loop regions was significantly improved after 4-fold noncrystallographic symmetry averaging in PIRATE (20, 21) and a second round of model building in ARP/wARP using the density-modified phases. The dataset obtained from Se-Met-derivatized BcsZ crystals was significantly better than that of wild-type BcsZ crystals (see Table 1); hence, the Se-Met dataset was used for model building and refinement of the apo-BcsZ structure. Both structures were refined in Refmac5 (18) including TLS groups (Translation/Libration/Screw), as determined by the TLSMD web server (22, 23). Water molecules and structure factors for the apo-BcsZ and the BcsZ-cellopentaose structures were deposited at the Protein Data Bank under ID codes 3QXF and 3QXQ, respectively. All figures were prepared in PyMOL (25).

Cellulase Assay—CMC-containing agar plates were prepared by dissolving 2% CMC and 1.5% agar in LB medium. The solution was autoclaved, cooled, and supplemented with 0.5 mM isopropyl β-D-thiogalactopyranoside and 0.1 mg/ml ampicillin prior to pouring plates. 20 μg of purified protein was spotted onto the plates, followed by incubation at 37 °C for 48 h. The agar plates were stained with 2% Congo Red solution (26) for 1 h at 25 °C and destained in 1 M NaCl for 2 h.

RESULTS

Architecture of BcsZ—The E. coli BcsZ protein was expressed with an N-terminal PelB signal sequence for secretion into the periplasm and a C-terminal His₆ tag to facilitate purification. The wild-type BcsZ protein as well as its E55Q mutant was purified via nickel affinity and size exclusion chromatography as described under “Experimental Procedures” and concentrated to 100 μM prior to crystallization. We solved the structure of apo-BcsZ by the single-wavelength anomalous dispersion method with Se-Met-derivatized BcsZ, and this structure served as the search model for solving the BcsZ-cellopentaose complex by molecular replacement. Of the total 368 residues, the apo- and cellopentaose-bound structures of BcsZ include residues 24–360 and 23–360, respectively.

Apo-BcsZ crystallized in space group P1 with four copies per crystallographic asymmetric unit and diffracted x-rays to 1.75 Å resolution (Table 1). Its structure adopts a classical (α/α)₆-barrel fold, in which six pairs of antiparallel α-helices form an inner and outer ring (Fig. 1A). This architecture forms a deep, sickle-shaped groove on one side of the molecule, which, by comparison with other GH-8 hydrolases, serves as the substrate binding pocket (27). Glu336 and Asp243, the putative pair of catalytic residues, are localized at the center of this groove where the curvature is highest (Figs. 1A and 2B). Above the (α/α)₆-barrel, three β-sheets flank the substrate binding groove, creating a funnel on top of the hexameric rings (Fig. 1B). Sheet 1 is formed by loops connecting helices 1/2 and 3/4, sheet 2 by the connections of helices 5/6 and 7/8, and sheet 3 by loops between helices 7/8 and 9/10, respectively (Fig. 1, A and B). Despite only sharing 26% sequence identity
with CMCax from *A. xylinum* (28), the Cα backbones of both structures align with 1.9 Å r.m.s.d. The main differences between both structures lie in the pronounced organization of the β-sheets surrounding the BcsZ substrate binding groove and the formation of a short, two-stranded β-sheet near the C terminus of BcsZ (Fig. 1).

**TABLE 1**

| Crystallographic statistics | Apo-wild type | Apo-Se-Met | Cellopentaose complex |
|-----------------------------|--------------|------------|-----------------------|
| **Data collection**         |              |            |                       |
| Resolution limit (Å)        | 50–1.75 (1.78–1.75) | 50–1.85 (1.88–1.85) | 35–2.2 (2.24–2.2) |
| Space group                 | P1           | P1         | P21                   |
| Cell constants (Å<sup>3</sup>) | 54.9, 88.0, 92.1/69.97, 74.10, 78.03 | 54.8, 87.9, 91.7/69.96, 74.36, 78.22 | 90.6, 99.5, 93.3/90.0, 103.0, 90.0 |
| Unique reflections          | 248,650      | 131,388    | 108,218               |
| Mean I/σ (I)               | 28 (2.2)     | 18.5 (6.2) | 13.8 (2.2)            |
| R<sub>sym</sub><sup>b</sup> | 0.13 (0.65)  | 0.07 (0.23) | 0.09 (0.57)          |
| Completeness (%)            | 95.9 (94.3)  | 97.8 (96.4) | 98.4 (96.8)          |
| Redundancy                  | 3.8 (2.8)    | 4.7 (4.5)  | 3.4 (3.2)             |
| **Refinement**              |              |            |                       |
| Resolution range (Å)        | 50–1.85      | 35–2.2     |                       |
| NCS<sup>c</sup>             | 4            | 4          |                       |
| R<sub>work</sub><sup>d</sup> (%) | 13.6       | 17.7       |                       |
| R<sub>free</sub><sup>e</sup> (%) | 17.8       | 22.1       |                       |
| No. of reflections          |              |            |                       |
| Total                       | 121,857      | 76,666     |                       |
| Model geometry              |              |            |                       |
| r.m.s.d. bond length (Å)    | 0.008        | 0.005      |                       |
| r.m.s.d. bond angle (°)     | 0.92         | 0.87       |                       |
| Average B-factor (Å<sup>2</sup>) | 10.0 | 12.9 |                       |
| Main chain                  | 15.5         | 14.1       |                       |
| Side chain                  | Cellopentaose| 35.4       |                       |
| Water                       | 23.5         | 15.0       |                       |
| No. of atoms                |              |            |                       |
| Total                       | 12,365       | 12,261     |                       |
| Protein                     | 10,960       | 11,196     |                       |
| Cellopentaose               | 224          |            |                       |
| Water                       | 1,405        | 841        |                       |
| Ramachandran analysis<sup>f</sup> | In preferred regions | 96.7 | 96.6 |                       |
| In allowed regions          | 3.3          | 3.4        |                       |
| Outliers                    | 0.0          | 0.0        |                       |

<sup>a</sup> Values in parentheses refer to the highest resolution shell.

<sup>b</sup> R<sub>sym</sub> = ΣhklsI(hkls) − I(hkls)/ΣhklsI(hkls), where I(hkls) is the integrated intensity of the reflection.

<sup>c</sup> No. of BcsZ protomers/crystallographic asymmetric unit.

<sup>d</sup> R<sub>work</sub> = Σ|Fo| − |Fc|)/Σ|Fo|, where Fo and Fc are observed and calculated structure factors.

<sup>e</sup> R<sub>free</sub>: As for R<sub>work</sub> but for 5% of randomly selected reflections that were omitted from the refinement.

<sup>f</sup> Performed in COOT.

**FIGURE 1. BcsZ adopts an (α/α)₆-barrel fold.** BcsZ is shown as a cartoon from a top (A) and side (B) view. The inner and outer helices are shown in dark and pale green, respectively, and the three β-sheets flanking the substrate binding cleft are shown in blue. The catalytic residues Glu<sup>55</sup> and Asp<sup>243</sup> are shown as red sticks. Helices are labeled H1–H12 from the N to the C terminus. The C-terminal residues 349–357 of BcsZ form a two-stranded β-sheet (colored orange) on the opposite side of the substrate binding pocket.
Substrate Binding to BcsZ—We stabilized the BcsZ-cellopentaose complex by mutating Glu55, the putative proton donor during catalysis (27, 29), to a Gln (E55Q mutant). Crystallization experiments were carried out in the presence and the absence of cellopentaose, and those conditions producing crystals only in the presence of cellopentaose were further optimized. The BcsZ-cellopentaose complex crystallized in space-group P21 with four copies in the crystallographic asymmetric unit and diffracted x-rays to 2.2 Å resolution (Table 1). The presence of cellopentaose was clearly visible in a difference Fourier ($F_o - F_c$) electron density map after rigid body refinement of the four protomers in the asymmetric unit (Fig. 2A).

Fig. 2 shows that BcsZ binds cellopentaose in a large groove that is primarily formed between the loops connecting helices 3/4 and 5/6. In contrast to the interaction of cellopentaose with the cellulose-degrading GH-8 hydrolase CelA from Clostridium thermocellum (27), BcsZ interacts with cellopentaose exclusively via residues on the nonreducing side of the catalytic center at positions $-1$ to $-4$ (30) (Fig. 2B). The glucosyl residue at the reducing end of cellopentaose forms H-bonds via its OH-1, OH-2, and OH-3 hydroxyl groups to side chains of Gln$^{55}$, Asp$^{110}$, Tyr$^{182}$, and Asp$^{116}$, respectively. At position $-2$, the glucosyl molecule interacts with Trp$^{113}$ and forms an H-bond to Ser$^{112}$ via its OH-6 hydroxyl. Side chains of Asp$^{110}$, Asn$^{112}$, and Phe$^{170}$ interact with the glucosyl moiety at the $-3$ position. The carbonyl of Gly$^{169}$ forms the only contact to the moiety at position $-4$ via an H-bond to its OH-2 hydroxyl group. All interactions between cellopentaose and BcsZ occur via highly conserved residues. BcsZ is shown as a surface with residues colored according to their conservation (blue, highest; green, lowest). The figure was prepared from an alignment of 50 BcsZ sequences using the CONSURF web server (39).
a single contact with BcsZ; its OH-2 hydroxyl forms an H-bond with the backbone carbonyl of the highly conserved Gly\textsuperscript{169} (Fig. 3). The glucosyl residue at the nonreducing end of cellopentaose does not interact with BcsZ. All interactions between cellopentaose and BcsZ observed in our structure are mediated by highly conserved amino acids (Fig. 3B) and are thus likely to represent a biologically important mode of interaction.

Close inspection of our electron density maps shows additional weak density adjacent to Tyr\textsuperscript{242} and Tyr\textsuperscript{332} in the substrate binding groove, which cannot be accounted for by water or other molecules in the crystallization buffer. This density likely represents two weakly bound glucoside moieties at positions −1 and +1. Cellopentaose is represented as a yellow stick model.

**Comparison of Apo- and Cellopentaose-bound Structure of BcsZ**—The structures of BcsZ in its apo form and in complex with cellopentaose align neatly with a r.m.s.d. between C\textalpha{} atoms of only 0.3 Å. In the apo conformation, the side chain of Glu\textsuperscript{55} is part of an H-bond network involving Asp\textsuperscript{116} and Tyr\textsuperscript{182}. In the cellopentaose-bound structure, however, the position of the γ-carboxylate of Glu\textsuperscript{55} (mutated to Gln in this structure) is occupied by the OH-3 hydroxyl of the reducing sugar of cellopentaose. Hence, the side chain of Gln\textsuperscript{55} rotates by 180 degrees to a position where it would be in close proximity to the oxygen of the scissile glucosidic bond (27) (Fig. 4).

**Hydrolytic Activity of BcsZ**—We tested the BcsZ hydrolitic activity utilizing CMC, SigmaCell Type 20, or methyl-cellulose as substrates. These substrates did not yield detectable hydrolytic activity when measured in solution using either the 3,5-dinitrosalicylic acid or p-hydroxybenzoic acid hydrazide methods (31–33), which detect the increasing concentration of reducing sugars as a result of the enzymatic activity (data not shown). However, when BcsZ-overexpressing *E. coli* cells were grown on agar plates containing 2% CMC, a clear halo appeared around the colonies after staining with Congo Red (26), a marker for β-1,4- and β-1,3-glycosidic bonds (31) (data not shown). To further verify that the observed effects were dependent on BcsZ, we spotted an equal amount of purified wild-type BcsZ as well as its E55Q mutant onto the CMC-agar plates and incubated at 37 °C for 48 h, followed by Congo Red staining. Fig. 5 shows that the purified BcsZ protein efficiently degrades CMC when embedded in agar plates. Purified cellulase from *Aspergillus niger* (Tokyo Chemical Industry, GenBank entry CAA03658.1) and bovine serum albumin (BSA) were used as controls. The clear halos indicate areas with degraded CMC against a dark background of nondegraded substrate.

**DISCUSSION**

Cellulose biosynthesis, both in plants and bacteria, requires the coordinated actions of multiple proteins that lead to the synthesis and secretion of a β-1,4 glucan chain. In Gram-negative bacteria, the β-1,4-glucan chain is synthesized from UDP-activated glucose by an integral inner membrane enzyme (6, 12, 34). To reach its final destination, the glucan chain must be transported across the inner membrane, the periplasmic space, and the outer membrane. Outside the cell, multiple glucan chains can align to form higher order aggregates (microfibrils), a process that at least in part might be driven by the low water solubility of β-1,4-glucans (35).

The role BcsZ maintains throughout this process is unclear. *In vitro* cellulose digestion assays demonstrate that BcsZ has very low activity toward cellulose substrates, including CMC or...
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Sigmacell. These observations may be explained by the recalcitrant nature of the cellulose microfibril. Unlike many cellulases involved in cellulose degradation, BcsZ lacks a carbohydrate binding domain to anchor it to the cellulose microfibril and increase its effectiveness (10). Hence, it is conceivable that BcsZ performs its biological function prior to the formation of the inert cellulose microfibril. The observation that it efficiently degrades agar-embedded CMC (Fig. 5) supports this idea because the glucan chains might have been partially dissociated due to the heating process required for preparing the plates.

In our structure, cellopentaose is bound to the highly conserved −1 through −4 sites of BcsZ (Fig. 2B). This can be interpreted as a posthydrolysis state in which the newly formed oligosaccharide, located at the reducing side of the catalytic center, has already dissociated from the BcsZ-product complex. The apparent preference of BcsZ to occupy its −1 to −4 substrate binding sites suggests that a minimal substrate would consist of at least six glucose units. A similar preference for cellohexaose or longer glucan chains has been observed for CMCax from A. xylinum (36).

The interaction of cellopentaose with BcsZ observed in our structure is in contrast to its interaction with CelA, a GH-8 hydrolase that is involved in cellulose degradation in C. thermocellum. Bound to a catalytically inactive mutant of CelA, cellopentaose occupies sites +2 through −3 in an intermediate state of catalysis (27). Interestingly, site −4, formed by a highly conserved Gly residue in BcsZ, is absent in CelA. A sequence comparison as well as the superimposition of the BcsZ and CelA structures identify a Gly-Phe-Ala insertion in BcsZ within the loop connecting helices 5 and 6 (Figs. 1 and 3B). This insertion is highly conserved in BcsZ and contains Gly169, which makes the only contact with cellopentaose at position −4. The presence of this binding site might explain why BcsZ binds cellopentaose in a posthydrolysis state, and CelA in an intermediate state of hydrolysis.

Our data support the idea that BcsZ is involved in the translocation process of individual β-1,4-glucan chains across the periplasm and the outer membrane. The observation that the outer membrane component of the cellulose synthase complex (BscC) is not essential for cellulose synthesis (37, 38) raises the possibility that partially assembled cellulose synthase complexes lacking BscC might lead to the accumulation of glucan chains in the periplasm. BcsZ might then degrade these polymers into water-soluble oligosaccharides. Another, or additional, function of BcsZ could be the cleavage of the translocating polysaccharide chains, thereby influencing the formation of cellulose microfibrils outside the cell. Further insights into the biological functions of BcsZ will require the reconstitution of functional bacterial cellulose synthase complexes in vitro.

Acknowledgments—We thank Owen Pornillos, Lukas Tamm, and Michael Wiener for critical comments on the manuscript and are grateful to Caitlin Hubbard and Peter Horanyi for discussions.

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