Structural Role of Calcium for the Organization of the Cellulosome of *Clostridium thermocellum*†

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ABSTRACT: The cellulosome of *Clostridium thermocellum* is a multipolypeptide complex of structural and catalytic subunits. Several of the catalytic subunits have at the carboxyl end a conserved duplicated region (CDR) which interacts with internally repeated elements (IREs) of scaffolding subunits such as CipA. This interaction requires calcium. The two parts of the CDR region here designated CDR1 and CDR2 (closest to the carboxyl end) each consist of about 20 amino acid residues. As shown in our previous paper [Choi, S. K., & Ljungdahl, L. G. (1996) *Biochemistry* 35, 4897−4905], treatment of the cellulosome with ethylenediaminetetraacetic acid (EDTA) under aerobic conditions disintegrates the cellulosome with formation of truncated catalytic subunits. The cleavage is at a specific asparagine residue located within CDR1 and occurs with complete loss of CDR2. Two branched peptides containing the amino acid sequences of CDR1 and CDR2 (designated bCDR1 and bCDR2) were synthesized, and specific antibodies were raised against them. These antibodies did not cross react with bCDR1 or bCDR2, respectively. After sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and Western blotting, it was observed that about 15 subunits of the cellulosome reacted with anti-bCDR1 and anti-bCDR2. In a similar experiment with EDTA-treated cellulosomes, these subunits reacted with anti-bCDR1 but not with anti-bCDR2, showing that they lost the bCDR2 epitope and were truncated. The peptide bCDR1 binds calcium, whereas bCDR2 does not. Furthermore, bCDR1 but not bCDR2 binds to CipA, presumably at IRE regions. This binding requires calcium. A model is proposed for the binding of the catalytic subunits to CipA which involves CDR1, an IRE, and calcium.

Clostridium thermocellum is a thermophilic anaerobic bacterium that when grown on cellulose produces a cellulolytic protein complex with high activity toward crystalline cellulose (Lamed et al., 1985; Hon-nami et al., 1986). The complex is composed of perhaps as many as 26 different subunits and is termed cellulosome (Lamed et al., 1983; Kohring et al., 1990). The cellulosome has a highly ordered structure and consists of structural and catalytically active subunits (Béguin et al., 1992; Felix & Ljungdahl, 1993; Bayer et al., 1994).

The gene of the structural subunit *cipA* (cellulosome-integrating protein) has been sequenced (Gerngross et al., 1993). It encodes a protein of 196 800 Da containing nine internally repeated elements (IREs)[1] or cohesins (Bayer et al., 1994) and a cellulose-binding domain (CBD). The following 10 genes encoding catalytic subunits of the cellulosome have been sequenced: *celA* (Béguin et al., 1985), *celB* (Grepinet & Béguin, 1986), *celD* (Joliff et al., 1986), *celE* (Hall et al., 1988), *celF* (Navarro et al., 1991), *celG* (Lemaire & Béguin, 1993), *celH* (Yagüe et al., 1990), *celS* (Wang et al., 1993), *celX* (Hall et al., 1988), and *xynZ* (Grepinet et al., 1988). The gene products expressed in *Escherichia coli* have been characterized and by immunological analyses found to be polypeptides of the cellulosome. All of them contain a conserved duplicated region (CDR) or dockerin, composed of CDR1 and CDR2, which each contain 22−24 amino acid residues. CDR1 and CDR2 are linked together by 8−15 amino acid residues, and they are in most subunits found close to the C-terminal (Aubert et al., 1992). The CDRs are not essential for catalytic activity (Hall et al., 1988), but they are involved in the binding of the catalytic subunits to IREs of CipA (Tokatlidis et al., 1991; Fujino et al., 1992; Salamitou et al., 1994; Yaron et al., 1995).

In our previous paper (Choi & Ljungdahl, 1996), we reported that Ca is the main metal of the cellulosome and that it is tightly bound to it. Treatment of the cellulosome with EDTA decreased its ability to hydrolyze crystalline cellulose but had little effect on the endoglucanase activity. The treatment with EDTA released the Ca from the cellulosome, which disintegrated. This occurred with truncation of catalytic subunits. It was demonstrated with CelS that the cleavage occurred after asparagine residue 681 and involved the removal from the C-terminal end of 60 amino acid residues containing most of the CDR1 domain and the complete CDR2 region. Asparagine 681 is part of the CDR1 domain and of CelS, and it is conserved and found in CDRs

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[1] Abbreviations: CBD, cellulose-binding domain; CDR, conserved duplicated region (Dockerin); bCDR, branched CDR; EDTA, ethylenediaminetetraacetic acid; IRE, internally repeated element (Cohesion); SDS, sodium dodecyl sulfate.

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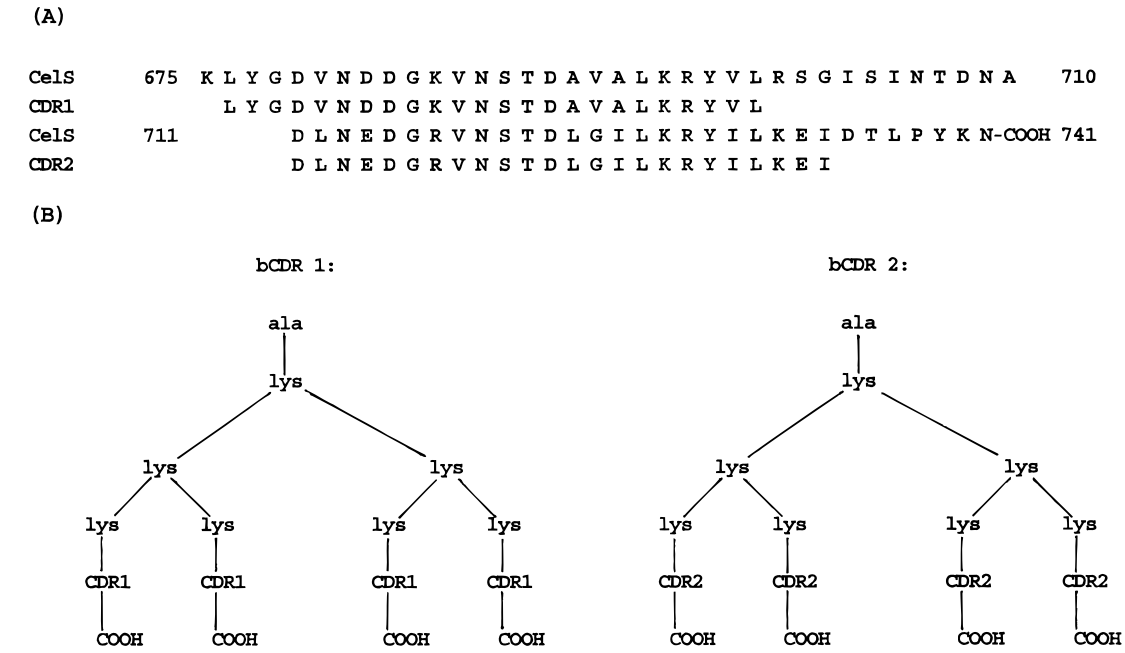

FIGURE 1: Amino acid sequences used for synthesis of branched peptides. (A) The C-terminal sequence of subunit CelS of the cellulosome contains conserved reiterated regions (CDR1 and CDR2). (B) Peptides bCDR1 and bCDR2 were chemically synthesized using a branched lysine core and an Advanced ChemTech MPS 250 Peptide Synthesizer. The numbers are residues counted from the N-terminal end.

of several of the catalytic subunits. Our results indicated that several of them were truncated by the EDTA treatment in a way similar to that of CelS. This is confirmed in this paper, in which we show that CDR1, but not CDR2, binds Ca and that the binding of CDR1 to the IREs of CipA requires Ca.

MATERIALS AND METHODS

Bacterial Growth and Isolation of the Cellulosome. C. thermocellum JW20 was grown anaerobically at 60 °C with 1% (w/v) Avicel PH-101 (FMC Corp.) as the carbon source, and the cellulosome was purified as described in the preceding paper (Choi & Ljungdahl, 1996).

Disintegration of the Cellulosome and Purification of the 46 and 71 kDa Truncated Subunits. The cellulosome was disintegrated aerobically by incubation in a solution containing 50 mM Tris (pH 7.5), 0.1 M NaCl, and 5 mM EDTA at 37 ° for 24 h. The 46 and 71 kDa polypeptides which are truncated forms of CelA and CelS (Choi & Ljungdahl, 1996) were purified from the EDTA-treated cellulosomal preparation using a 1.25 × 20 cm Q-Sepharose column (Pharmacia) equilibrated with 30 mM Tris (pH 7.5) and an eluting gradient from 0 to 0.45 M NaCl. The 46 kDa polypeptide was eluted at 0.03 M NaCl and the 71 kDa polypeptide at 0.2 M NaCl. The 46 kDa polypeptide was applied to a Phenyl Superose HR 5/5 column (0.5 × 5 cm, Pharmarcia) equilibrated with 40% $(NH_4)_2SO_4$ in 30 mM Tris (pH 7.5). It eluted as one peak with a linear gradient of 40% $(NH_4)_2SO_4$ to 0% $(NH_4)_2SO_4$ in 30 mM Tris (pH 7.5). The 71 kDa polypeptide was further purified using a Phenyl Sepharose column (1.25 × 20 cm, Pharmacia) equilibrated with 40% $(NH_4)_2SO_4$ and 30 mM Tris (pH 7.5) and eluted with an $(NH_4)_2SO_4$ gradient from 40 to 0%. The main fraction consisted of almost pure 71 kDa protein as revealed by SDS−PAGE. It contained 7.83 μmol of Ca per gram of protein. To eliminate the Ca, the protein was incubated in 50 mM Tris (pH 7.5) and 0.1 mM EDTA for 3 h at 37 °C. It was then applied to Superdex 75WR 10/30 column (1.0 × 30 cm, Pharmacia), followed by a Phenyl Superose column eluted with an $(NH_4)_2SO_4$ gradient from 20 to 0%.

Synthesis of Multiple Antigenic Peptides (MAPs). On the basis of sequences of CDR1 and CDR2 of CelS, two branched peptides in tetrameric forms were synthesized onto branched lysine backbones (Posnett et al., 1988). The peptide bCDR1 corresponding to CDR1 contained the residues 676−699, and peptide bCDR2 corresponding to CDR2 consisted of residues 711−734 of CelS (Figure 1). The homogeneity of the peptides was checked both with high-performance liquid chromatography (HPLC) and by analysis of total amino acid compositions on a 120A amino acid analyzer (Applied Biosystems Inc., Forster City, CA).

Polyclonal Antibody Production toward bCDR1 and bCDR2. The synthetic peptides (0.5 mg in 0.5 mL) were mixed with 0.5 mL of Complete Freund's Adjuvant (Sigma), emulsified, and injected intramuscularly into adult New Zealand white rabbits. A booster injection was done in a manner identical to that of the primary injection after 3 weeks except that Incomplete Freund's Adjuvant was used. Blood (25−30 mL) was drawn 3 weeks after the second injection. The antibody titer was determined by enzyme-linked immunosorbent assay.

Electrophoresis and ^{45}Ca Binding to Immobilized Peptides. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS−PAGE) was performed according to Laemmli (1970). Calcium binding to bCDR1 and bCDR2 was assayed using the method described by Maruyama et al. (1984). Peptides in the SDS gel were transferred by electrophoresis to a PVDF membrane using a miniprotein transblotting apparatus (Bio-Rad) (Towbin et al., 1979). The membrane was washed with 10 mM imidazole (pH 6.8), 3 mM $MgCl_2$, and 100 mM KCl for 1 h and then incubated with ^{45}Ca (1 μCi/mL) for 10 min in the same buffer. The membrane was washed with deionized water three times for 5 min, dried at room temperature for 3 h, and exposed to X-ray film.

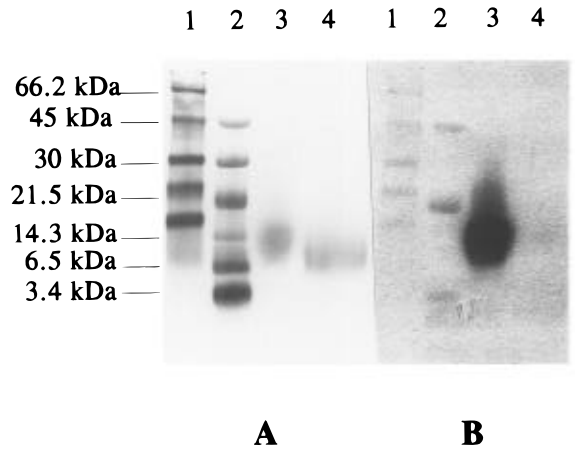

FIGURE 2: Calcium binding of bCDR1 and bCDR2. Proteins on SDS−polyacrylamide gel (3−20%) were transblotted to a PVDF membrane. A calcium binding test was done as described in Materials and Methods. (A) Ponceau S staining of the membrane. (B) Autoradiography of a ^{45}Ca-overlaid membrane. Lanes 1 and 2 are large and small molecular mass markers. Lane 3 is bCDR1, and lane 4 is bCDR2.

Modified Western Blotting for Interactions of Cellulosomal Polypeptides with bCDR1 and bCDR2. Cellulosomal subunits from SDS−polyacrylamide gels were transblotted to PVDF membranes. Detection of cellulosomal proteins which have epitopes of CDR1 or CDR2 was done with Western blotting with anti-bCDR1 and anti-bCDR2 using an alkaline phosphatase immunoblot kit (Bio-Rad). To detect interactions between cellulosomal proteins and calcium, modified Western blotting was used. PVDF membranes transblotted from SDS-gels were blocked by immersion in a 3% gelatin solution for 1 h and then overlaid with 0.1 mg/mL bCDR1 or bCDR2 in the presence or in the absence of 21 mM $CaCl_2$ for 2 h. The membranes were then washed with 20 mM Tris (pH 7.5), 500 mM NaCl, and 0.05% Tween 20 two times for 10 min. They were finally overlaid with $^1/_{2000}$-fold anti-bCDR1 or anti-bCDR2 for 2 h and washed with above washing solution before being reacted with $^1/_{3000}$-fold goat anti-rabbit IgG conjugated with alkaline phosphatase for 1 h.

RESULTS

Characterization of Synthetic Multiple Antigenic Peptides and Their Calcium Binding and Immunologic Properties. Two multiple antigenic peptides, bCDR1 and bCDR2, were synthesized on the basis of CelS amino acid sequences (Figure 1). The bCDR1 and bCDR2 migrated as broad bands on SDS−PAGE, assumed due to their branched forms. Only bCDR1 bound calcium (Figure 2, lane 3). It should be noted that some of the proteins used as molecular mass markers showed some capacity to bind Ca. The two polyclonal antibody preparations raised against bCDR1 and bCDR2 reacted specifically with respective peptides, and no cross reactivity was observed.

Reactions of Antibodies with 71 and 46 kDa Cellulolytic Subunits and Other Cellulosomal Polypeptides. In the preceding paper (Choi & Ljungdahl, 1996), we showed that the 71 kDa polypeptide corresponded to a truncated form of CelS missing the CDR2 domain and part of the CDR1 domain. The results showed that the 46 kDa polypeptide corresponded to CelA and suggested that it also was

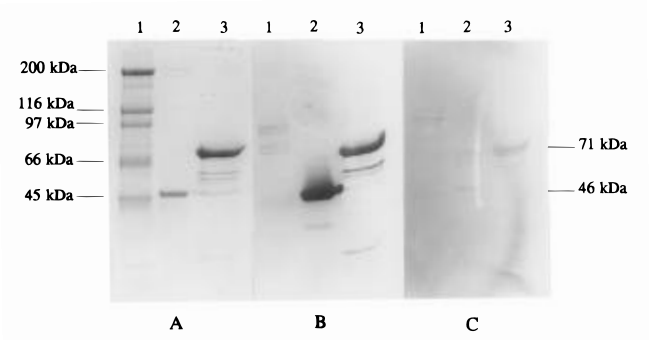

FIGURE 3: Reactions of the 46 and 71 kDa cellulosomal subunits with anti-bCDR1 and anti-bCDR2. (A) Coomassie blue staining, (B) Western blotting with anti-bCDR1, and (C) Western blotting with anti-bCDR2. Lane 1 is molecular mass markers. Lane 2 is 46 kDa protein, and lane 3 is 71 kDa protein.

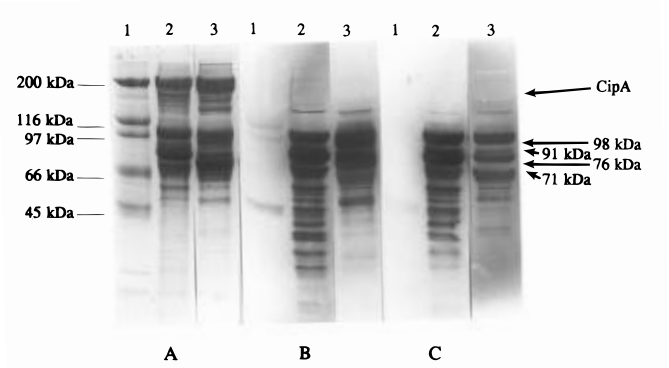

FIGURE 4: Reactions of cellulosomal subunits before and after EDTA treatment with anti-bCDR1 and anti-bCDR2; (A) Coomassie blue staining, (B) Western blot using anti-bCDR1, (C) Western blot with anti-bCDR2. Lane 1 is molecular mass markers. Lane 2 is cellulosomal subunits before EDTA treatment, and lane 3 is cellulosomal subunits after EDTA treatment for 24 h at 37 °C.

truncated. The purified polypeptides when examined with SDS−PAGE appeared close to homogeneity as shown in Figure 3 (panel A, lanes 2 and 3). Anti-bCDR1 recognized both the 46 and 71 kDa polypeptides, but there was no reaction with anti-bCDR2 (Figure 3B,C). The 71 kDa polypeptide has six amino acid residues (L-Y-G-D-V-N) at the C-terminal common with bCDR1 but is missing the complete bCDR2 sequence (Choi & Ljungdahl, 1996). The results are in complete agreement with previous observations that CelS is cleaved after the asparagine residue 681 to yield the 71 kDa truncated form. The results are identical for the 46 kDa subunit which demonstrates that this subunit is missing the CDR2 domain but still has, at least, part of the CDR1 domain. We conclude that CelA is cleaved in the same manner as CelS by the EDTA treatment to yield the 46 kDa truncated subunit.

Previous results indicated that several of the catalytically active subunits of the cellulosome were truncated by the EDTA treatment (Choi & Ljungdahl, 1996). A comparison with Western blotting was therefore made between samples of the cellulosome before and after EDTA treatment (Figure 4). The untreated cellulosome reacted in a similar manner with anti-bCDR1 and anti-bCDR2, indicating that the subunits contained both domains. Both antisera reacted with at least 17 different subunits of the cellulosome (panels B and C, lane 2). Unexpected was that a number (at least seven) of polypeptides with masses below 45 kDa reacted with both antisera. This indicates that the cellulosome contains several smaller polypeptides having the CDR domains and which apparently do not stain well with

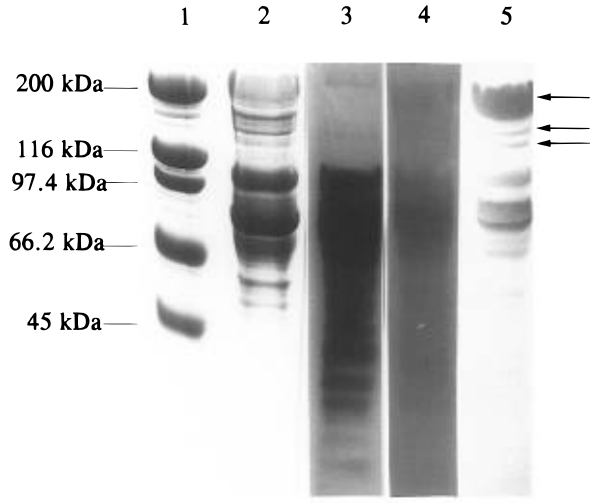

FIGURE 5: Interactions of cellulosomal subunits with bCDR1 in the presence and absence of calcium. SDS−PAGE (7.5%) of cellulosomal subunits was analyzed for interaction with bCDR1 using modified Western blotting as described in Materials and Methods. Lane 1 is molecular mass markers stained with Coomassie blue. Lane 2 is cellulosomal subunits stained with Coomassie blue. Lane 3 is Western blotting of cellulosomal subunits with anti-bCDR1 without treatment with bCDR1. Lane 4 is Western blotting as for lane 3 after application of bCDR1. Lane 5 is Western blotting as for lane 4 in the presence of calcium.

Coomassie blue. These peptides have not previously been recognized (Lamed et al., 1983; Kohring et al., 1990). It should be noted that CipA and several other polypeptides with masses over 120 kDa did not react with the antibodies, indicating that these do not have the CDR sequences.

After the EDTA treatment, several polypeptides reacted with anti-bCDR1 but fewer with anti-bCDR2. This confirmed that many of the subunits were truncated in much the same way as CelS and CelA. A number of the smaller subunits (less than 45 kDa) were not found in the EDTA-treated cellulosome which indicates that they also were truncated. By comparison in Figure 4 of lane 3 of panel B and lane 3 of panel C, it can be concluded that the 91, 71, and 45 kDa polypeptides react with anti-bCDR1 but not with anti-bCDR2. But, it is also clear that some polypeptides after the EDTA treatment still react with anti-bCDR2. This may be explained by the fact that the truncation reaction was not complete or that not all polypeptides undergo truncation during the EDTA treatment.

Interaction between bCDR1 and CipA Occurs in the Presence of Calcium. It has been shown that the CDR anchors the catalytic subunits to the scaffolding subunits CipA of the cellulosome (Tokatlidis et al., 1991). Thus, CelD and XynZ carrying the CDR1 and CDR2 bind to CipA, whereas the same truncated subunits lacking CDR do not bind. Calcium may be involved in the binding. Our observation that CDR1, but not CDR2, binds Ca prompted us to test if Ca is needed for the binding of CDR1 to CipA and to other cellulosomal subunits. This was done with the modified Western blotting method. The results are shown in Figure 5. The cellulosomes used had not been EDTA-treated. Lane 3 (Figure 5) is a regular Western blotting (control) with anti-bCDR1 or anti-bCDR2. It confirms the results of Figure 4. In lanes 4 and 5, the membrane after being blocked with 0.3% gelatin was treated with a solution containing 0.1 mg/mL bCDR1 without (lane 4) and in the presence of 21 mM $CaCl_2$ (lane 5) prior to the application of anti-bCDR1. The treatment with bCDR1 without Ca resulted in a rather diffused pattern with weak reactions of the antibody with the cellulosomal polypeptides. This may be due to the extensive washing needed to remove unbound bCDR1. No binding of anti-bCDR1 was observed to CipA or to peptides larger than 120 kDa. In the presence of Ca, anti-bCDR1 reacted with several polypeptides and especially with CipA, clearly demonstrating the binding of bCDR1 to CipA. A similar experiment with bCDR2 replacing bCDR1 showed that bCDR2 does not bind to CipA either in the presence or the in the absence of Ca. As noted with arrows (Figure 5), two bands below the CipA band showed Ca-dependent binding of bCDR1, which indicates that polypeptides other than CipA may also function as scaffolding proteins.

DISCUSSION

The cellulosome of *C. thermocellum* consists of perhaps as many as 26 polypeptides with masses over 45 kDa (Kohring et al., 1990). Most of these have cellulolytic activities corresponding to endoglucanases, cellobiohydrolases, and xylanases. Other polypeptides, notably CipA, have structural functions serving as scaffolds. As shown in this paper (Figure 3), the cellulosome may also contain a number of subunits smaller than 45 kDa. These were identified using antisera (anti-bCDR1 and anti-bCDR2) produced against the two domains of the conserved duplicated region found in many of the larger catalytically active subunits.

The cellulosome has a very tight structure not easily disrupted by denaturing agents. It has been shown that the

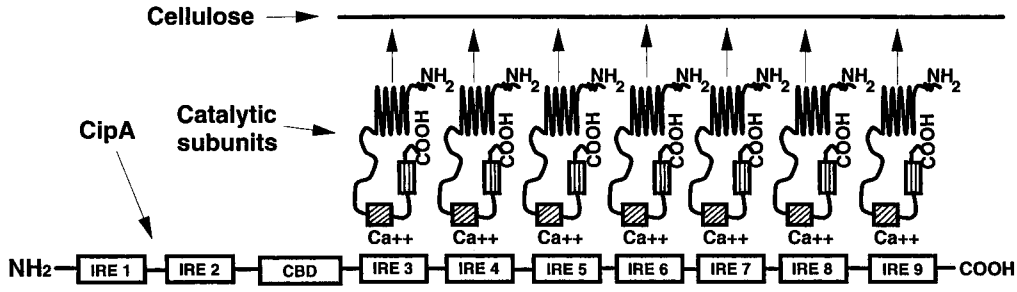

FIGURE 6: Model proposed for the structure of the cellulosome of *C. thermocellum*. CipA is the scaffolding subunit which contains nine IREs and a CBD (cellulose binding site) (Gerngross et al., 1993). The catalytic subunit exemplified by CelD (Juy et al., 1992; Joliff et al., 1986) has an α-barrel catalytic site and also contains the conserved duplicated sequence, of which the diagonally striped boxes represent CDR1 and the horizontally striped boxes CDR2. Calcium is required for the binding of CDR1 of catalytic subunits to the IREs of the scaffolding subunit. Arrows indicate the multienzymatic attack on the cellulose chain. The distance between arrows may be four cellobiose units (Mayer et al., 1987).

CDRs of the catalytic subunits interact with the internally repeating elements of CipA (Tokatlidis et al., 1991; Fujino et al., 1992; Yaron et al., 1995). Calcium enhances this interaction. It has been proposed that CDR1 may have calcium binding ability. This was based on the amino acid sequence of CDR1 of CelD, which has features similar to the EF-hand motif of Ca^{2+}-binding sites of some calcium-binding proteins (Chauvaux et al., 1990). However, these authors could not demonstrate that Ca binds to CDR1 of CelD; instead, they showed that CelD has three calcium binding sites involving aspartic acid residues 246, 361, and 523, none of which is within the CDR1 sequence (584−605) (Chauvaux et al., 1995).

The demonstrations that removal of Ca with EDTA were accompanied by formation of truncated polypeptides lacking most of the CDR1 and the complete CDR2 and that these polypeptides bind less Ca (Choi & Ljungdahl, 1996) indicated that Ca binds to the CDRs. Here we show that, of the synthetic peptides bCDR1 and bCDR2, only bCDR1 binds Ca. In addition, we demonstrated that Ca is required for the binding of bCDR1 to CipA and also to some other cellulosomal subunit. We did not find an interaction between bCDR2 and CipA.

On the basis of these observations, we have modified previous models for the organization of the cellulosome (Fujino et al., 1993; Bayer et al., 1994) (Figure 6). In this model, we consider the sequence for CipA (Gerngross et al., 1993) and the three-dimensional structure of CelD (Juy et al., 1992). CipA has nine internally repeating elements, two of which are located between the cellulose binding domain and the N-terminal and differ from the other seven, which are highly homologous. IRE3 and IRE7 interact with CelD through the CDR (Salamitou et al., 1994; Fujino et al., 1992). The model takes this into account by showing that IREs 3−9 bind enzymatic subunits. This does not mean that IRE1 and IRE2 are unable to bind enzymatic subunits; it only shows that they are different from the other IREs. We assume that the IREs have calcium binding sites and that calcium is needed for a very tight binding of CDR1 to the IRE (Figure 6). This calcium-mediated protein interaction may explain the durable structure of the cellulosome. CelD, as found by X-ray crystallography, has an N-terminal β-barrel packed against a larger, mostly α-helical domain shaped like an α-barrel with 12 helices connected by loops forming the active site (Juy et al., 1992). The C-terminal includes the CDR region. It appeared disordered and was not included in the crystal structure model by Juy et al. (1992). This allows some flexibility in the attachment of the catalytic subunits to the CipA. Since we did not find any interaction between bCDR2 with CipA, it is not possible at the present time to assign a function for this domain.

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