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Role of Scaffolding Protein CipC of *Clostridium cellulolyticum* in Cellulose Degradation

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The role of a miniscaffolding protein, miniCipC$_1$, forming part of *Clostridium cellulolyticum* scaffolding protein CipC in insoluble cellulose degradation was investigated. The parameters of the binding of miniCipC$_1$, which contains a family III cellulose-binding domain (CBD), a hydrophilic domain, and a cohesin domain, to four insoluble celluloses were determined. At saturating concentrations, about 8.2 μmol of protein was bound per g of bacterial microcrystalline cellulose, while Avicel, colloidal Avicel, and phosphoric acid-swollen cellulose bound 0.28, 0.38, and 0.55 μmol of miniCipC$_1$ per g, respectively. The dissociation constants measured varied between 1.3×10^{-7} and 1.5×10^{-8} M. These results are discussed with regard to the properties of the various substrates. The synergistic action of miniCipC$_1$ and two forms of endoglucanase CelA (with and without the dockerin domain [CelA$_2$ and CelA$_3$, respectively]) in cellulose degradation was also studied. Although only CelA$_2$ interacted with miniCipC$_1$ (K_d, 7×10^{-9} M), nonhydrolytic miniCipC$_1$ enhanced the activities of endoglucanases CelA$_2$ and CelA$_3$ with all of the insoluble substrates tested. This finding shows that miniCipC$_1$ plays two roles: it increases the enzyme concentration on the cellulose surface and enhances the accessibility of the enzyme to the substrate by modifying the structure of the cellulose, leading to an increased available cellulose surface area. In addition, the data obtained with a hybrid protein, CelA$_3$-CBD$_{CipC}$, which was more active towards all of the insoluble substrates tested confirm that the CBD of the scaffolding protein plays an essential role in cellulose degradation.

Cellulolytic microorganisms have evolved a whole range of cellulases with various specificities which synergistically degrade natural cellulose. These enzymes have a modular structure, and several of them consist of one or two catalytic domains and a cellulose-binding domain (CBD) separated by linker sequences rich in proline and hydroxyamino acids. The CBDs, like the catalytic domains of cellulases (or xylanases), which have been classified into 12 distinct families of glycosyl hydrolase on the basis of sequence similarities, have been assigned to nine different families based on primary-structure similarities (44). The structure of three of them (belonging to families I, II, and V) has been resolved by using nuclear magnetic resonance techniques (4, 5, 24, 47). The CBD of the scaffolding protein of *Clostridium thermocellum* has been crystallized, and its three-dimensional structure has been recently resolved (28, 45).

It has frequently been demonstrated (6, 7, 19, 21, 43) that enzyme activity on various cellulosic substrates is affected by removal of the CBD. For example, removal of the CBDs from *Trichoderma reesei* CBHI and CBHII reduced the enzyme activity on Avicel but had no effect with soluble substrates (43). A truncated form of CenA from *Cellulomonas fimi*, lacking its CBD, has been found to be less active towards Avicel and bacterial cellulose but more active on acid-swollen cellulose and carboxymethyl cellulose (19). Recent experiments in which the CBD from *Clostridium stercorarium* xylanase was joined to endoglucanase IV of *Ruminococcus albus* showed that grafting of this CBD enhanced the enzyme activity on insoluble cellulose (23). All of these results suggest that CBDs, by limiting the diffusion of the enzymes on the substrate surface, might favor the hydrolysis of cellulose.

The function of the CBDs does not seem to be limited to attaching enzymes to the substrate surface; they might play a direct role in cellulose hydrolysis. In 1950, Reese et al. (39) proposed a model according to which two components may act consecutively in the degradation of cellulose: a noncatalytic component, called the S factor (for swelling factor), or C1, which makes the substrate more accessible to the catalytic component, called Cx. It was subsequently suggested (7, 8, 42) that CBDs might correspond to the C1 factor described by Reese et al., which exposes new cellulose regions to the enzymes by means of a mechanism which has not been elucidated. Indeed, Din et al. (7, 8) have shown that the CBD (family II) of endoglucanase A (CenA) from *C. fimi* disrupts the structure of cotton fibers, making the substrate more accessible to enzyme attack. This CBD acts synergistically with the catalytic domain of the enzyme and might play a role similar to that of the C1 factor of Reese et al. It cannot be assumed, however, that all CBDs have the same function, and one must keep in mind that all of these studies (7, 8, 42) concern only the role of cellulolytic enzymes CBDs produced in nonaggregated cellulolytic systems, where the various enzymes were released separately into the culture medium.

Clostridium cellulolyticum (11, 15), like the majority of cellulolytic clostridia, such as *C. thermocellum* (1–3, 12, 26, 27), *C. cellulovorans* (9), and *C. papyrosolvens* (16, 36), produces a large cellulolytic complex (about 600 kDa), called a cellulosome, in which several cellulases are tightly bound to a scaffolding protein called CipC. This scaffolding protein possesses a family III CBD which, as is known to occur in the case of *C. thermocellum* and *C. cellulovorans* (20, 32), might facilitate the anchoring of the cellulosome to the surface of the cellulose.

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Although the role of the cohesin domain as a receptor for the catalytic subunit has been established (25, 40, 41, 48), the role of other CipC domains, hydrophilic domains, and CBDs has not been clearly elucidated. The purpose of this study was to investigate the involvement of the CBD of scaffolding protein CipC in cellulose degradation. We used the cellulosomal endoglucanase CelA, a two-domain enzyme which possesses a catalytic domain belonging to glycosyl hydrolase family 5 and a C-terminal dockerin domain (10, 13). CelA does not contain any CBD, and we recently demonstrated that formation of a complex between CelA and a truncated form of CipC, called miniCipC$_1$ (which contains the CBD, hydrophilic domain 1 [HD$_1$], and cohesin domain 1 [C$_1$]), occurs between the dockerin domain of CelA and C$_1$ of miniCipC$_1$ (34). In the present paper, we report on the synergistic action between endoglucanase CelA and truncated forms of CipC with which CelA either does or does not interact.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *Escherichia coli* BL21 (DE3) was used as the host for pET-22b(+) (Novagen) derivative expression vectors (pETCip1, pETCip2, and pETCip4) (34), and *E. coli* TG1 was used as the host for pJF118EH (14) derivative vectors (pA2, pA3, and pA25) (13). *E. coli* was grown at 37°C in Luria-Bertani medium supplemented with ampicillin (100 μg/ml) when required. *C. cellulolyticum* ATCC 35319, which was used as the source of genomic DNA, was grown anaerobically at 32°C on basal medium supplemented with cellobiose as the carbon and energy source (17).

DNA manipulation. Chromosomal DNA was obtained from *C. cellulolyticum* as described by Quiviger et al. (38). Large-scale and small-scale plasmid purifications were performed by using the alkaline lysis method (30) (Qiagen Kit [Qiagen]). Digestion was performed as instructed by the manufacturer.

Expression and purification of recombinant proteins. The expression and purification of recombinant proteins miniCipC$_1$ (CBD-HD$_1$-C$_1$) and miniCipC$_0$ (CBD-HD$_1$) were monitored as previously described (34).

The DNA fragment encoding CBD$_{CipC}$ was amplified by PCR from total chromosomal DNA of *C. cellulolyticum* by using *Pwo* DNA polymerase (Boehringer Mannheim). For this amplification, two synthetic primers, *cip157* (5′-ttcccgggcatatgTTTGCAGCAGGTACTG-3′) and *cip162* (5′-acccaagctttctagattaAGGAGTTGAACCGTAAGCAAGAGT-3′), having partial homology with DNA regions of the DNA fragment encoding CBD$_{CipC}$ (in uppercase letters) were used. An *Sma*I-*Nde*I polylinker (including the ATG codon) and one *Hin*dIII site were created in this way upstream and downstream, respectively, of the coding sequence. A stop codon was also created downstream of the sequence. This amplified fragment was digested with *Nde*I and *Hin*dIII and ligated into *Nde*I-*Hin*dIII-linearized plasmid pET-22b(+). The resulting plasmid was called pETCip4. Two different inserts from two independent PCR events were sequenced (Genome Express Society) by using a Perkin-Elmer 373 fluorescent sequencing apparatus (Applied Biosystems dye terminator method).

The gene encoding chimeric protein CelA$_3$-CBD$_{CipC}$ was constructed as described below. Plasmid pA2 (13), encoding the entire form of CelA, was digested with *Asu*II and *Hin*dIII, generating two fragments. The smaller (about 300 bp), encoding the dockerin domain, was deleted and replaced with a synthetic linker (5′-cgaagccaagcccgggacaagta-3′/5′-agcttacttgtcccgggcttggctt-3′) designed to introduce an *Sma*I site between the *Asu*II and *Hin*dIII sites. The resulting plasmid, pA21, contained the DNA fragment coding for the catalytic domain of endoglucanase A (devoid of the region encoding the dockerin domain) and the linker region. The DNA fragment encoding the CBD of CipC was amplified as previously described, by using synthetic primers *cip182* (5′-tccccgggatcgaaggtagggatatcTTTGCAGCAGGTACTTGG-3′) and *cip162*. Upstream of the CBD coding sequence, synthetic primer *cip182* created restriction sites for *Sma*I and *Eco*RV. The other primer, *cip162*, as previously described, created a stop codon and an *Hin*dIII site downstream of the fragment coding for the CBD. This amplified fragment was digested with *Sma*I and *Hin*dIII and inserted into pA21. The resulting plasmid was called pA25, and as described above, two different inserts from two independent PCR events were sequenced.

The production and purification of CBD$_{CipC}$, miniCipC$_0$ (CBD-HD$_1$), miniCipC$_1$ (CBD-HD$_1$-C$_1$), and chimeric protein CelA$_3$-CBD$_{CipC}$ were performed as described by Pagès et al. (34). Proteins CelA$_2$ and CelA$_3$ were produced and purified as previously described (13).

Substrates. Carboxymethyl cellulose (CMC, medium viscosity) was purchased from Sigma, and Avicel PH101 was purchased from Fluka. Phosphoric acid-swollen cellulose (PASC) was obtained by using the method developed by Walseth (46), with Avicel PH101. Avicel is a heterogeneous suspension in which cellulose microfibrils are aggregated. A more homogeneous suspension (called colloidal Avicel) was therefore prepared. Colloidal Avicel was obtained by milling Avicel PH101 (5 g/liter) for 5 h at 4°C in distilled water with a shaker. After sedimentation (for 12 h at 4°C), two distinct phases were obtained: the pellet, containing the nondisaggregated cellulose particles, and the supernatant, corresponding to a homogeneous suspension in which the cellulose particles were mostly disaggregated. The supernatant (consisting of a colloidal suspension) was concentrated by ultrafiltration with an Amicon concentrator and a 30K Millipore membrane. The cellulose concentration was calculated from the dry weight of an aliquot fraction. Bacterial microcrystalline cellulose (BMCC) was a generous gift from C. Boisset and B. Henrissat (CERMAV, Grenoble, France).

Enzyme assays. A 1-ml volume of a diluted enzyme solution was mixed with 4 ml of either BMCC (10 g/liter), Avicel (10 g/liter), colloidal Avicel (10 g/liter), PASC (10 g/liter), or CMC (10 g/liter) in 25 mM potassium phosphate buffer (pH 7)–0.3% NaN_3. For the insoluble substrates (BMCC, Avicel, colloidal Avicel, and PASC), the reaction mixtures were incubated at 37°C with gentle shaking. At various incubation times, aliquot fractions were centrifuged (for 20 min at 10,000 × *g*) to sediment any residual polysaccharides. The supernatants were centrifuged again (for 20 min at 10,000 × *g*), and the sugars produced were measured by the Park and Johnson ferricyanide method (35). One international unit of activity corresponds to 1 μmol of D-glucose equivalent released per min. The same procedure (except for the centrifugation) was used to determine the CMCase activities of the different solutions. The protein concentration was determined by the method of Lowry et al. (29), with bovine serum albumin as the standard.

Determination of cellulose-binding capacity and dissociation constants. Samples of purified miniCipC$_1$ (between 10 and 150 μg) were added to 2-ml microcentrifuge tubes containing cellulose in 25 mM potassium phosphate buffer (pH 7). The final volume of the assay mixture was 1 ml. The capacity of miniCipC$_1$ to bind to BMCC, Avicel, colloidal Avicel, and PASC was determined by using various amounts of miniCipC$_1$ and a constant amount of cellulose (between 0.5 and 5 mg, depending on the type of cellulose). MiniCipC$_1$ was incubated with cellulose for 1 h at 4°C with gentle shaking. After centrifugation (4,000 × *g* for 10 min), the amount of residual protein in the supernatant fluid (free miniCipC$_1$) was determined colorimetrically by the method of Lowry et al. (29). The amount of miniCipC$_1$ bound to the cellulose was calculated by subtracting the amount of free miniCipC$_1$ from the total amount added. The data were analyzed by drawing double-reciprocal plots of 1/bound miniCipC$_1$ versus 1/free miniCipC$_1$ (20).

Pretreatment of cellulose with miniCipC$_1$. PASC was incubated with miniCipC$_1$ (20 μg/mg of cellulose) at 20°C for 30 min in 25 mM potassium phosphate buffer (pH 7)–0.3% NaN_3. The cellulose was washed with sterile water by vacuum filtering through a 1.6-μm-pore-size glass filter (glass microfiber filter GF/D [Whatman]). The miniCipC$_1$ was thus eluted from the cellulose with the sterile water. A control experiment was performed without adding any protein. After being washed, the cellulose was resuspended in 25 mM potassium phosphate buffer (pH 7)–0.3% NaN_3. The cellulose concentration was estimated by determining the dry weight of an aliquot. Treated PASC was used for the enzyme assays at 10 g/liter as previously described.

RESULTS

Expression and purification of three truncated forms of CipC and chimeric protein CelA$_3$-CBD$_{CipC}$. Three truncated forms of CipC, corresponding to the CBD alone (163 amino acid residues), the peptide miniCipC$_0$ (269 amino acid residues) containing the CBD and the first HD$_1$, and the peptide miniCipC$_1$ (414 amino acid residues) containing the CBD, HD$_1$, and C$_1$, were expressed in the cytoplasm of *E. coli* (Fig. 1B). A chimeric protein called CelA$_3$-CBD$_{CipC}$, obtained by fusing the CBD of CipC to the catalytic domain of CelA, was also produced (Fig. 1D). Each protein was purified by using a procedure based on the cellulose-binding capacity of the CBD of CipC as described previously (34). Proteins were eluted from cellulose by H_2O treatment. The molecular masses of the CBD, miniCipC$_0$, miniCipC$_1$, and CelA$_3$-CBD$_{CipC}$ determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (18,000, 29,000, 44,000, and 61,000 Da) are in close agreement with the predicted molecular masses (17,496, 28,564, 43,546, and 60,761 Da, respectively).

Analysis of cellulose-miniCipC$_1$ affinity and binding capacity. The ability of miniCipC$_1$ to bind to cellulose was investigated. The binding parameters, i.e., the dissociation constant (K_d) and the binding capacity (micromoles of miniCipC$_1$ bound per gram of cellulose), were determined with various cellulosic substrates. The results are given in Table 1. The substrates used in this study differed in terms of the index of crystallinity, available surface area, intermolecular hydrogen bonding organization, and cellulose chain orientation. BMCC, the most crystalline cellulose used, has a crystallinity index of

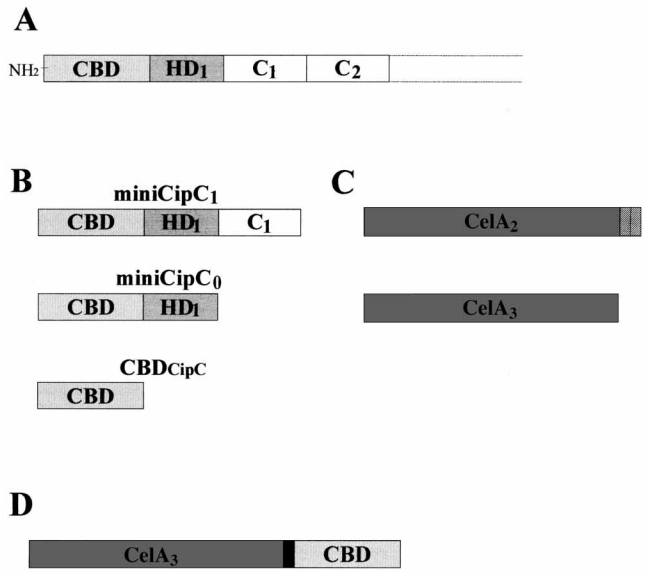

FIG. 1. Schematic diagrams of the recombinant proteins used in this study. (A) Known N-terminal part of CipC. (B) Truncated forms of CipC, i.e., the 414-amino-acid polypeptide called miniCipC$_1$ (which contains the CBD, HD$_1$, and C$_1$ of CipC), the 269-amino-acid polypeptide called miniCipC$_0$, and the 163-amino-acid polypeptide which contains only the CBD of CipC. (C) Two forms of endoglucanase A, with (CelA$_2$; predicted molecular mass, 50,802 Da) or without (CelA$_3$; predicted molecular mass, 43,194 Da) the C-terminal dockerin domain. Purification of the two forms was performed as described by Fierobe et al. (14). (D) Chimeric protein CelA$_3$-CBD$_{CipC}$. The 11 amino acid residues between CelA$_3$ and the CBD$_{CipC}$ are represented by the black box.

76%, whereas the crystallinity index of Avicel is 47% (22). Moreover, the available surface area of BMCC (which consists mainly of cellulose of the Iα type) is at least 100-fold that of Avicel (which consists mainly of cellulose of the Iβ type) (18, 31). Avicel is a heterogeneous cellulose preparation obtained from wood, in which microfibrils of cellulose are aggregated. Colloidal Avicel is a homogeneous suspension in which the microfibrils are disaggregated and the number of available binding regions increases without affecting the degree of crystallinity. PASC, on the other hand, is more amorphous. Moreover, undefined structural changes occur when type I cellulose is swollen in concentrated phosphoric acid. In PASC, a type II cellulose (6), the cellulose chains are arranged in antiparallel orientation. The present results show that miniCipC$_1$ interacts with high affinity with all of the insoluble celluloses tested. The linearity of the double-reciprocal plots (in the range of concentrations given in Materials and Methods) suggests that only one type of high CBD-cellulose interaction occurs. When very large concentrations of miniCipC$_1$ were used, a new class of sites having weak affinity was also detected (data not shown)

TABLE 1. Adsorption of miniCipC$_1$ to insoluble substrates

Substrate	K_d[a] (M)	Binding capacity (μmol/g of cellulose)
BMCC	3.5×10^{-8}	8.2
Avicel	1.3×10^{-7}	0.28
Colloidal Avicel	4.0×10^{-7}	0.38
PASC	1.5×10^{-8}	0.55

[a] The dissociation constant is defined as $1/B = (K_d/B_{max} \times 1/F) + 1/B_{max}$, where B is the bound miniCipC$_1$ concentration and F is the free miniCipC$_1$ concentration.

TABLE 2. Comparison of CelA$_2$ and CelA$_2$-miniCipC$_1$ complex activities

Substrate	Hydrolysis (IU/μM)			Degree of synergism[b]
	CelA$_2$	MiniCipC$_1$	Complex[a]	
BMCC	0.55	0	0.68	1.23
Avicel	1.7	0	2.1	1.24
Colloidal Avicel	2.2	0	3.4	1.54
PASC	30	0	47	1.55

[a] The stoichiometry of the CelA$_2$-miniCipC$_1$ complex is 1:1.
[b] The degree of synergism is defined as the ratio of complex activity plus miniCipC$_1$ activity to endoglucanase CelA$_2$ activity.

with Avicel and PASC. The binding parameters of miniCipC$_1$ could not, however, be clearly correlated with the degree of crystallinity of the cellulose used. The high binding value obtained with BMCC, compared to Avicel, with the same quantity of substrate may be due to the fact that a larger area was available for miniCipC$_1$. PASC (type II cellulose) seems, however, to be the preferential binding substrate for miniCipC$_1$.

To determine whether soluble carbohydrates might compete with Avicel for miniCipC$_1$ binding, CMC (1, 2, or 5 mg) was added in some assays. The lack of competition observed between Avicel and CMC suggests that miniCipC$_1$ has little or no affinity for CMC.

The cellulose-binding affinity and binding capacity of CBD alone towards Avicel were also measured: K_d, 1.4×10^{-7} M; binding capacity, 0.25 μmol/g of cellulose. These values are identical to those obtained with miniCipC$_1$ and suggest that the presence of HD$_1$ and C$_1$ (both of which were present in the miniCipC$_1$ polypeptide) do not affect the cellulose-binding properties of CBD$_{CipC}$.

Synergistic action between CelA$_2$ (with the dockerin domain) and miniCipC$_1$. We previously observed that endoglucanase CelA$_2$, but not CelA$_3$ (Fig. 1C), interacted with miniCipC$_1$ with an apparent equilibrium K_d of 7×10^{-9} M (34). The activity of CelA$_2$ on BMCC, Avicel, colloidal Avicel, PASC, and CMC was measured and compared with that of the CelA$_2$-miniCipC$_1$ complex. No increase in the activity of the CelA$_2$-miniCipC$_1$ complex compared to the activity of CelA$_2$ alone toward CMC was detected. With each insoluble substrate tested, however, the presence of miniCipC$_1$ induced an increase in CelA$_2$ activity, while miniCipC$_1$ alone did not release any soluble sugars from any of these substrates. In each case, a degree of synergism, defined as the ratio between complex activity and endoglucanase activity (both in international units/micromolar), was calculated. These data are given in Table 2. The degree of synergism observed when CelA$_2$ formed a complex with miniCipC$_1$ was about the same with all four insoluble substrates, although slightly higher values were obtained with colloidal Avicel and PASC.

The amount of reducing sugars released by endoglucanase CelA$_2$ and by reaction mixtures containing CelA$_2$ and various concentrations of miniCipC$_1$ was investigated. The results are given in Fig. 2. As described previously, miniCipC$_1$ alone did not release any soluble sugars from cellulose (Table 2) but enhanced the release of soluble carbohydrates by endoglucanase CelA$_2$ from BMCC (Fig. 2A), Avicel (Fig. 2B), colloidal Avicel (Fig. 2C), and PASC (Fig. 2D). The degree of synergism depended on the cellulosic substrate and on the amount of miniCipC$_1$. When miniCipC$_1$ was used at concentrations above 10 μM (with Avicel, colloidal Avicel, and PASC) or 20 μM (with BMCC), the synergism decreased. Table 3 gives the maximum degree of synergism obtained with each of the four

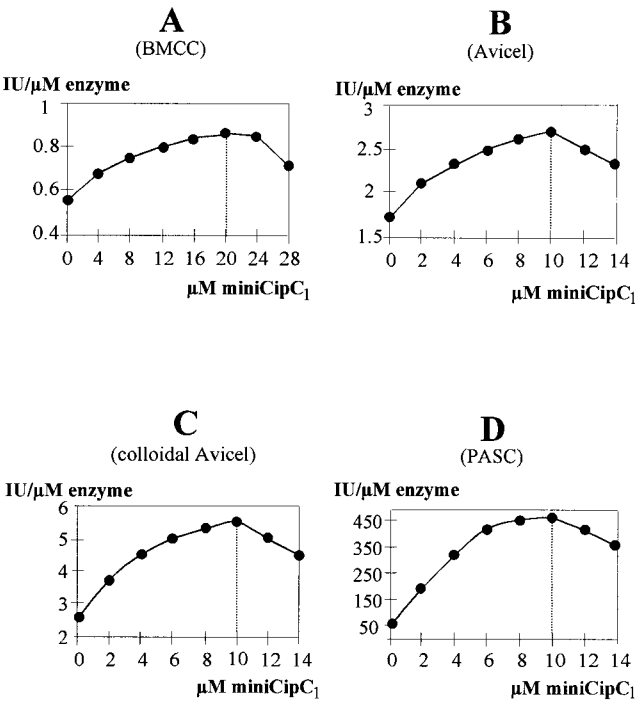

FIG. 2. Hydrolysis of insoluble substrates by $CelA_2$ and mixtures containing $CelA_2$ and various quantities of the nonhydrolytic polypeptide $miniCipC_1$. (A) Hydrolysis of BMCC. (B) Hydrolysis of Avicel. (C) Hydrolysis of colloidal Avicel. (D) Hydrolysis of PASC. The enzymatic reaction was monitored as described in Materials and Methods, by using 0.6 μM enzyme for BMCC degradation, 0.3 μM enzyme for Avicel and colloidal Avicel degradation, and 0.02 μM enzyme for PASC degradation. With each substrate tested, a control assay was performed by using $miniCipC_1$ and the substrate alone. In each enzymatic reaction, 40 mg of cellulose was used.

substrates. In each case, the amount of $miniCipC_1$ present was in great excess in comparison with the amount of endoglucanase A. This indicates that $miniCipC_1$ plays an essential role in $CelA_2$ cellulose degradation, regardless of its concentrating effects: it might expose additional hydrolyzable regions, perhaps by modifying the structure of the cellulose. This synergism seems to depend directly on the cellulose structure. With a type II substrate such as PASC, having the lowest degree of crystallinity and a cellulose chain arrangement different from that of Avicel or BMCC, the optimum synergism can be as high as 8. The amount of new sites exposed by $miniCipC_1$ therefore seems to be greater on PASC than on Avicel, colloidal Avicel, and BMCC. With BMCC, the disrupting effect of $miniCipC_1$ is relatively weak. This may have been due to the structural differences between this cellulose and the other substrates tested: BMCC consists mainly of Iα cellulose, whereas Avicel is composed mainly of Iβ cellulose, and these two celluloses display different patterns of intermolecular hydrogen bonding. The disrupting effect of $miniCipC_1$ might be relatively limited in the case of Iα cellulose.

Synergistic action between truncated forms of CipC and nonbound forms of endoglucanase A. If CipC plays a direct role in the degradation of cellulose, like factor C1 described by Reese et al., a synergistic action between two components which cannot interact (34), such as $miniCipC_0$ and $CelA_2$ or $miniCipC_1$, $miniCipC_0$ and $CelA_3$ (without its dockerin domain), is likely to occur. The possibility of a synergistic action occurring between these protein forms was investigated by using colloidal Avicel (Fig. 3A) and PASC (Fig. 3B). The experimental conditions were those that previously yielded maximum synergism between $CelA_2$ and $miniCipC_1$. The results showed that the presence of $miniCipC_0$ or $miniCipC_1$ enhanced endoglucanase cellulose hydrolysis. With all of the substrates tested, similar degrees of synergism were observed between the various unbound partners. Similar results were obtained with CBD_{CipC} and $CelA_2$ or $CelA_3$ (data not shown). The increase in the amount of reducing sugars released never resulted, in any of these cases, from enzyme concentration at the cellulosic substrate surface. The synergistic action observed here probably resulted only from the disrupting effect of CBD_{CipC}. Table 3 gives the $miniCipC_1$ concentrating and disrupting effects ($CelA_2$) and the disrupting effect alone ($CelA_3$).

Similar results were obtained when PASC was pretreated with $miniCipC_1$, which was removed prior to adding $CelA_2$ or $CelA_3$. $CelA_2$ was 2.7-fold more active toward this substrate, and $CelA_3$ was 3-fold more active. The endoglucanase activities of $CelA_2$ and $CelA_3$ toward PASC were 40 and 70 IU/μM, respectively, and those toward pretreated PASC were 108 and 207 IU/μm, respectively. These results confirm that $miniCipC_1$ was able to generate new hydrolyzable regions, increasing the number of available hydrolyzable sites.

Study of cellulose degradation by chimeric protein $CelA_3$-CBD_{CipC}. The activity of endoglucanase $CelA_3$ (without the dockerin domain) on crystalline cellulose is weak. Grafting of the CBD of CipC to the catalytic domain of endoglucanase A enhanced the activity of the enzyme toward insoluble substrates, especially PASC (Table 4), but not toward soluble substrates such as CMC (data not shown). PASC is the preferential insoluble substrate of $CelA_3$; however, the release of reducing sugars from PASC induced by $CelA_3$ stops after 1 h. In the case of the chimeric protein, the soluble sugars were released at a 2.5-fold higher rate than in the case of $CelA_3$, and this increased catalytic rate remained for at least 4 h (data not shown). This suggests that the activity of $CelA_3$ is rapidly stopped (after 1 h) due to the lack of hydrolyzable sites and that the presence of the CBD enhances the activity of $CelA_3$, probably by increasing the number of hydrolyzable sites. In this case, the number of hydrolyzable sites is not a limiting factor. Similar results were obtained with Avicel. On the other hand, the activity of a reaction mixture containing the chimeric protein and an excess of $miniCipC_1$ (10 μM) toward PASC was evaluated and compared with the activity of the chimeric protein. In this case, the chimeric protein activity was slightly inhibited (25%) (Table 4), probably due to competition between the two CBDs for the same sites.

TABLE 3. Maximum degrees of synergism observed between $CelA_2$ and $CelA_3$ and nonhydrolytic peptide $miniCipC_1$[a]

Substrate	Maximum degree of synergism	
	$CelA_2$/$miniCipC_1$[b]	$CelA_3$/$miniCipC_1$[c]
BMCC	1.4	ND
Avicel	1.6	ND
Colloidal Avicel	3.2	2.3
PASC	8	4.2

[a] The enzymatic reaction was monitored by using 0.6 μM $CelA_2$ or $CelA_3$ for BMCC degradation, 0.3 μM for Avicel and colloidal Avicel degradation, and 0.02 μM for PASC degradation. The maximum degree of synergism was obtained when the enzymes were incubated with 10 μM $miniCipC_1$ for Avicel, colloidal Avicel, and PASC degradation and with 20 μM $miniCipC_1$ for BMCC degradation.

[b] The synergism observed resulted from the $miniCipC_1$ concentrating action and $miniCipC_1$ disrupting action.

[c] The synergism observed resulted only from the $miniCipC_1$ disrupting action, since $CelA_3$ cannot interact with $miniCipC_1$. ND, not determined.

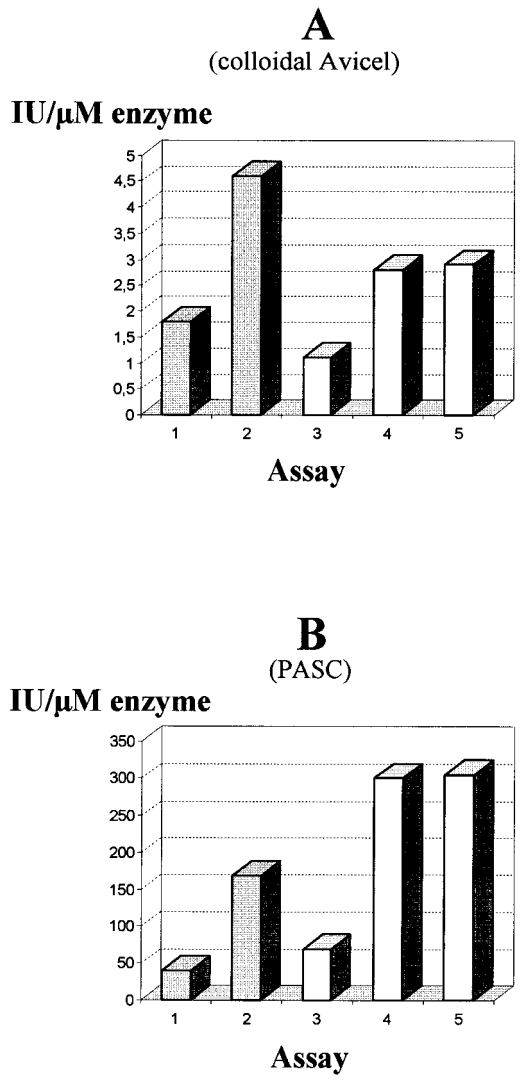

FIG. 3. Synergism between endoglucanase A and two truncated forms of CipC. The amount of reducing sugars released from colloidal Avicel (A) and PASC (B) (40 mg per enzymatic reaction) by endoglucanases $CelA_2$ and $CelA_3$ (assays 1 and 3) was compared with the amount of soluble sugars released by mixtures containing $CelA_2$ plus $miniCipC_0$ (assay 2), $CelA_3$ plus $miniCipC_1$ (assay 4), and $CelA_3$ plus $miniCipC_0$ (assay 5). The enzymatic reaction was monitored as described in Materials and Methods, by using 0.3 and 0.02 μM $CelA_2$ or $CelA_3$ for colloidal Avicel and PASC activities, respectively, and 10 μM truncated CipC forms.

DISCUSSION

The involvement of the CBD of *C. cellulolyticum* scaffolding protein CipC in the degradation of cellulose was investigated. First, the binding parameters of a truncated form of CipC called $miniCipC_1$ were determined with four insoluble substrates, and second, a synergistic action of $miniCipC_1$ and endoglucanase A was found to occur.

The parameters of the interaction between a truncated form of CipC called $miniCipC_1$ and four insoluble celluloses were investigated. The adsorption of $miniCipC_1$ was found to depend on the nature of the cellulose substrate. The K_d measured with all of the substrates tested varied roughly within a 10-fold range, while the apparent binding capacity (micromoles per gram of cellulose) on BMCC was 30-fold higher than on Avicel. The high binding capacity of $miniCipC_1$ might be attributable to the greater BMCC microfibril surface area in this case than with Avicel or the Avicel derivatives. The theoretical BMCC area varies between 100 and 300 m^2/g (depending on the homogeneity of the BMCC preparation), and the experimental Avicel surface was 1 or 10 m^2/g (depending on the homogeneity of the Avicel preparation) (18, 31). The $miniCipC_1$ binding parameters determined with Avicel were very similar to those of the CBD alone, indicating that the presence of the hydrophilic and cohesin domains does not affect the Avicel-binding properties of CBD. The binding parameters of the CBD of CipC are comparable to those of the CBD of CbpA from *C. cellulovorans* and the CBD of CipB from *C. thermocellum* (20, 37). These two CBDs, like the CBD of CipC, have been classified as belonging to family III (20, 28, 32, 37) based on the similarities between their primary structures, and it is worth noting that the CBDs of CbpA and CipB exhibit 44 and 40% identity with the CBD of CipC, respectively. All of the residues found, on the basis of the CipB-CBD three-dimensional structure, to interact with cellulose chains (45) are conserved, however, in CipC and CbpA. The affinities of the three CBDs for a given microcrystalline cellulose substrate such as Avicel were found to be in the same range. As suggested in the case of the CbpA CBD (20), the CBD of CipC has little or no affinity for CMC. This suggests that either the three-dimensional arrangement of cellulose chains is a prerequisite for the binding of the CBD to occur or CBD_{CipC} binding is inhibited by the presence of carboxymethyl substituents. It has been recently suggested that the CipB CBD may interact selectively with glucosyl moieties located on three adjacent chains of the cellulose structure (45).

The activity of $CelA_2$ associated with $miniCipC_1$ on insoluble substrates was investigated and compared with the activity of $CelA_2$ alone. On all of the substrates tested, the measured activities of the complex were higher than those of the enzyme alone. This enhancement was relatively small, however, and these data are in line with those obtained by Kruus et al. (25), who showed that the activity of the cellobiohydrolase CelS from *C. thermocellum* ATCC 27405 on Avicel increased by 29% when CelS was bound to a truncated form of CipA containing the CBD and the cohesin 3 domain of the protein. A more unexpected finding was the increase observed when an excess of $miniCipC_1$ was added, since in this particular case the maximum degree of synergism between $CelA_2$ and $miniCipC_1$ on PASC could be as high as 8. A decrease in the degree of synergism between $miniCipC_1$ and the complex was observed, however, when $miniCipC_1$ was in very large excess (10 or 20 μM). This is likely to have been due to competition for the same sites, preventing the complex from binding to the substrate under these experimental conditions. In addition, a lower but significant level of enhancement of endoglucanase activity was also observed when endoglucanase A was not as-

TABLE 4. Comparison of $CelA_3$ and chimeric protein $CelA_3$-CBD_{CipC} activities on various insoluble substrates

Substrate	Hydrolysis (IU/μM)		
	$CelA_3$	$CelA_3$-CBD_{CipC}	$CelA_3$-CBD_{CipC}/$miniCipC_1$[a]
BMCC	0.4	0.5	ND
Avicel	1.0	1.8	ND
Colloidal Avicel	1.2	1.8	ND
PASC	70	182	145

[a] The enzymatic reaction was monitored by using 0.02 μM chimeric protein for PASC degradation and 10 μM $miniCipC_1$. ND, not determined.

sociated with the truncated form of CipC or when the substrate had been pretreated with this nonhydrolytic polypeptide. All of these results suggest that the CBD of CipC may have two concomitant effects: (i) it may anchor the enzyme to the cellulose surface, thus increasing its concentration close to the substrate, and (ii) it may modify the structure of the cellulose. This action may lead to the exposure of new sites that can be hydrolyzed by the enzyme. These newly hydrolyzable regions are likely to result in an increase in the available surface area of the cellulose, as previously described in the case of the CBD of CenA from *C. fimi* (7, 8). In addition, it has been observed that the presence of the CBD of CipC prevents the flocculation of BMCC or PASC (data not shown). These experiments indicate that the presence of the CBD leads to macroscopic changes in cellulose. More extensive studies are required, however, to determine the exact role of the CBD, and we cannot conclude that this macroscopic effect is the only change in cellulose structure induced by the CBD.

Grafting of the CBD of CipC onto the catalytic domain of CelA enhanced the catalytic domain activity on all of the insoluble substrates tested, especially PASC. It has been frequently demonstrated that CBD plays an important role in enzyme activity (6, 7, 21, 23, 44). Likewise, grafting of the CBD of XynA from *C. stercorarium* to the catalytic domain of a heterologous endoglucanase IV from *R. albus* enhanced the activity of the enzyme toward insoluble cellulose (23). Karita et al. suggested that the increase in activity may have depended only on the concentration of the catalytic domain on the substrate surface and, hence, that all of the known CBDs may have different degrees of involvement in cellulose degradation. The CBD of scaffolding protein CipC obviously plays an essential role in cellulose degradation. The disrupting action of the CBD creates additional hydrolyzable regions which can then simultaneously undergo multicutting by the cellulosomal enzymes, resulting in efficient cellulose degradation.

Although the intimate mechanism whereby the clostridial cellulosome degrades native cellulose has not been elucidated, all of the experiments performed up to now have thrown some light on several aspects of this phenomenon, namely, (i) the cohesin-dockerin interaction resulting in cellulosome assembly, (ii) the cellulose-binding role of the CBD of the scaffolding protein, (iii) the disrupting effect of the cellulose and/or the creation of hydrolyzable sites newly exposed to the CBD of the scaffolding protein, (iv) the synergistic action of the various cellulosomal subunits with different catalytic properties, and (v) the changes in cellulosome conformation upon binding to cellulose (33). In the final analysis, elucidation of the mechanism whereby this specialized machinery degrades cellulose in its natural biotope should provide clues to the evolutionary meaning of this kind of complex, which has been detected only in anaerobic organisms.

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