Interaction between *Clostridium thermocellum* endoglucanase CelD and polypeptides derived from the cellulosome-integrating protein CipA: stoichiometry and cellulolytic activity of the complexes

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Four mini-scaffoldins were constructed from modules derived from the *Clostridium thermocellum* cellulosome-integrating protein CipA. Cip7 and Cip6 contained one and two cohesin modules respectively. Cip14 and Cip16, also containing one and two cohesin modules respectively, were flanked by a cellulose-binding domain. Endoglucanase CelD formed stable complexes with all mini-scaffoldins. Analytical ultracentrifugation of the complexes showed that 1 mol of CelD bound per mol of Cip14, and 2 mol of CelD bound per mol of Cip16. Under the conditions used for assaying cellulase activity, 96 % of CelD alone bound to Avicel. Association with Cip14 or Cip16 increased the cellulose binding of CelD to 99 %, while association with Cip7 or Cip6

decreased binding to 79 and 75 % respectively. The hydrolytic activity of CelD against Avicel was increased 3-fold in complexes with Cip14 and Cip16, but remained substantially the same in complexes with Cip6 and Cip7. Addition of whole CipA also enhanced the efficiency of Avicel hydrolysis by CelD. However, even at an optimal ratio of the components, CelD–CipA complexes were somewhat less active than complexes of CelD with Cip14 or Cip16. These results suggest that the synergism observed between CelD and Cip14 or Cip16 is mostly due to the presence of the cellulose-binding domain, which promotes productive binding of the enzyme.

INTRODUCTION

Clostridium thermocellum produces a high-molecular-mass exocellular protein complex termed the cellulosome, which actively hydrolyses crystalline cellulose [1]. The cellulosome is composed of several hydrolytic enzymes attached to a large, enzymically inactive protein termed CipA. Attachment of each catalytic component is mediated by a conserved, non-catalytic domain termed the dockerin domain. Dockerin domains bind specifically to a set of highly conserved, complementary domains termed cohesin domains, which are repeated nine times within the sequence of CipA [2–5]. Binding of the cellulosome to cellulose is mediated by a cellulose-binding domain (CBD) located between the second and third cohesin domains of CipA [4,6].

A 1:1 stoichiometry has been assumed for the interaction between individual cohesin and dockerin domains [5,7], but this has never been formally established. In addition, in spite of many publications devoted to the cellulosome and its components (for reviews, see [5,8–10]), the mechanism of cellulose hydrolysis by the cellulosome is not yet clear. It is well known that the integrity of the cellulosome is critical for the hydrolysis of crystalline cellulose, and dissociated components have weak, if any, activity against this substrate [1,11]. Partial reconstitution experiments have demonstrated that combination of individual catalytic subunits with CipA enhances their activity against crystalline cellulose [12–14], although the activity of the reconstituted complexes is much lower than the activity of the native cellulosome. Two factors may account for the stimulation of cellulolytic activity by CipA. On the one hand, complex formation with CipA associates cellulases with a CBD. In cellulase systems

consisting of non-associated enzymes, most cellulases contain a CBD [15], which enhances their affinity for cellulose and potentiates their activity [16,17]. On the other hand, cellulases bound to CipA are clustered, and it may be that clustering of the enzymes at definite points on the substrate surface facilitates the degradation process, by allowing cellulases to act on regions that are already partly disrupted by previous hydrolysis events [18,19].

Previous data have shown that intact CipA greatly increased the activity of *C. thermocellum* endoglucanase CelD against Avicel [14]. In order to assess the relative contributions of the CBD and of the clustering effect provided by CipA, we compared the synergistic effects of four ‘mini-CipAs’ and of intact CipA on the hydrolysis of cellulose by CelD. Cip7 and Cip6 consist of one and two copies respectively of the seventh cohesin domain of CipA. Cip14 and Cip16 comprise the CBD of CipA plus one and two copies respectively of the seventh cohesin domain. The stoichiometry of the complexes between CelD and Cip14 or Cip16 was established by equilibrium sedimentation analysis.

EXPERIMENTAL

Bacterial strains, plasmids and media

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* TG1 was used for cloning and as a host for pBC SK[−]-derived plasmids. The pCT603 plasmid carrying the *celD* gene was harboured by *E. coli* JM101. Histidine-tagged proteins were produced in *E. coli* BL21*recA* harbouring the multicopy plasmid pREP4, which carries the *lacI* gene. The *recA*

Abbreviations used: CBD, cellulose-binding domain; *p*-NPC, *p*-nitrophenyl β -D-cellobioside.

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Table 1 Bacterial strains and plasmids used

Strains and plasmids	Relevant features	Source or reference
Strains		
JM101	<i>F'</i> <i>traD36 proA⁺ proB⁺ lacI^q lacZΔM15/supE thi Δ(lac-proAB)</i>	[42]
TG1	<i>Δ(lac-pro) thi supE hsdD5/F' tra-36 proA⁺ B⁺ lacI^q lacZΔM15</i>	[43]
BL21 recA ⁻	<i>ompT, recA: miniTn5 Tet^R</i>	The present study
Plasmids		
pREP4	Kan ^R , <i>lacI</i>	Qiaexpress [®] kit, Qiagen
pBC SK ⁻	Cam ^R , cloning vector	Stratagene [®]
pQE-31	Amp ^R , expression vector encoding six N-terminal His residues	Qiaexpress [®] kit, Qiagen
pCip1	pBC SK ⁻ derivative encoding the 7th cohesin domain of CipA, plus an adjacent stop codon, framed by <i>Bam</i> HI and <i>Xba</i> I sites	[25]
pCip3	pBC SK ⁻ derivative encoding the 7th cohesin domain of CipA, plus the adjacent linker segment, framed by <i>Bam</i> HI and <i>Bgl</i> II sites	The present study
pCip9	pBC SK ⁻ derivative encoding the CBD of CipA, plus the adjacent linker segment, framed by <i>Bam</i> HI and <i>Bgl</i> II sites	The present study
pCip7	pQE-31 derivative encoding the 7th cohesin domain of CipA, fused to six His residues, plus an adjacent stop codon	[25]
pCip6	pQE-31 derivative encoding a polypeptide with two copies of the 7th cohesin domain of CipA, fused to six His residues	The present study
pCip14	pQE-31 derivative encoding a polypeptide with the CBD and 7th cohesin domain of CipA, fused to six His residues	The present study
pCip16	pQE-31 derivative encoding a polypeptide with the CBD and two copies of the 7th cohesin domain of CipA, fused to six His residues	The present study
pCT603	pUC8 derivative encoding endoglucanase CelD	[26]

mutation was introduced into BL21 [20] by transduction using a P1 lysate of CSH126 [21] and selecting for tetracyclin resistance. Strains were grown at 37 °C in Luria–Bertani medium [22]. The following antibiotics were added, depending on the plasmids present in the host: ticarcillin (100 µg/ml), chloramphenicol (30 µg/ml), kanamycin (25 µg/ml).

Construction of plasmids encoding different combinations of CBDs and cohesin domains

DNA manipulations were performed as described in [23]. DNA segments encoding protein domains to be combined were amplified by PCR, using primers containing appropriate restriction sites (Figure 1). The segments encoding the seventh cohesin domain and the CBD of CipA were amplified from pCT1450 [7] and pCip102 [14] respectively. The segment encoding the cohesin domain to be inserted at the 3' end of the artificial gene was flanked in 5' by an *Eco*RI and a *Bgl*II site and in 3' by an *Xba*I site, which provided a stop codon. Other segments were flanked in 5' by an *Eco*RI and a *Bgl*II site, and in 3' by a *Bam*HI site. The amplified segments were cloned between the *Eco*RI and *Xba*I sites or the *Eco*RI and *Bam*HI sites respectively of pBC SK⁻. The absence of spurious mutations was verified by sequencing.

The inserts carried by the resulting plasmids, i.e. pCip9, pCip1 and pCip3, can be recombined and concatemerized in a controlled manner. For this, a segment to be inserted next to the 5' end of a second segment is excised with *Eco*RI and *Bam*HI and recloned between the *Eco*RI and *Bgl*II sites of the plasmid containing the 3' segment. The 5' end of the resulting insert is flanked again by an *Eco*RI site and a *Bgl*II site, but the *Bam*HI–*Bgl*II junction between the two segments cannot be re-cut by *Bam*HI or *Bgl*II. Concatemers containing defined segments in a determined order could thus be constructed by sequential cloning steps. After piecing together the desired sequence elements, the resulting DNA fragments were excised with *Bgl*II and *Sac*I and recloned between the *Bam*HI and *Sac*I sites of pQE-31. The resulting constructs were used to overproduce the encoded polypeptides, which contained an N-terminal His₆ tag for Ni²⁺-affinity purification [24].

Protein purification

Cultures of BL21(pREP4) harbouring pCip6, pCip7, pCip14 or pCip16 were grown at 37 °C to an *D*₆₆₀ of 0.6–0.7. Isopropyl β-D-thiogalactoside was added at a final concentration of 0.5 mM, and the cells were further cultivated for 5 h. Cip7 was purified by Ni²⁺-affinity chromatography from *E. coli* BL21(pREP4)(pCip7) as described previously [25]. Cip6 was purified from *E. coli* BL21(pREP4)(pCip6) using the same procedure, except that the preparation was purified further by gel filtration using LKB Ultrogel AcA44. In the case of Cip14 and Cip16, the proteins were adsorbed on to the Ni²⁺-nitriloacetate resin and the column was washed with 2 vol. of 40 mM Tris/HCl, pH 7.5 (buffer A), then with 3 vol. of buffer A containing 0.3 M NaCl, 0.1 % Tween 20 and 10 mM β-mercaptoethanol. Elution was performed with a gradient of imidazole (0–0.5 M) in buffer A. Fractions containing protein were collected and dialysed against buffer A. The dialysed sample was applied to a MonoQ (5/5R) column (Pharmacia) equilibrated with buffer A. After washing the column with buffer A, proteins were eluted with 20 ml of a 0.5–1 M NaCl gradient. Purified proteins were dialysed against buffer A, concentrated by ultrafiltration and kept as aliquots at –20 °C. Yields per litre of culture were 25 mg for Cip7, 5 mg for Cip6, 19 mg for Cip14 and 7 mg for Cip16. CipA was purified from *E. coli* BL21(pREP4)(pCip102) as described previously [14]. The intact 68 kDa form of CelD was purified from inclusion bodies produced in *E. coli* JM101(pCT603) [26], as described previously [27].

Protein determination

The concentrations of Cip6, Cip7, Cip14, Cip16 and CelD were determined on the basis of *A*₂₈₀ values. Molar absorption coefficients, calculated from aromatic amino acid content [28], were (M⁻¹·cm⁻¹): 6760 for Cip7 [25], 13 520 for Cip6, 40 600 for Cip14, 46 200 for Cip16, 112 100 for CipA and 123 200 for CelD.

Complex formation

Complexes between CelD and Cip6, Cip7, Cip14, Cip16 or CipA were generated by incubating the proteins together for 30 min at

Figure 1 Construction of clones encoding Cip7, Cip6, Cip14 and Cip16

Restriction enzymes: B, *BamHI*; Bg, *BglII*; E, *EcoRI*; Sc, *SacI*; X, *XbaI*. The numbering of amino acid residues begins at the initial methionine encoded by the *cipA* gene. See the text for details.

4 °C in 50 mM Tris/HCl buffer, pH 7.5, containing 10 mM CaCl₂ (buffer B). Complexes were analysed by native gel electrophoresis in 10% polyacrylamide, using the Laemmli buffer system [29] without SDS and β -mercaptoethanol.

Analytical ultracentrifugation

The molecular masses of CelD and of complexes formed between stoichiometric amounts of CelD and Cip14 or Cip16 were determined by sedimentation equilibrium in a Beckman XL-A analytical ultracentrifuge. Samples were dialysed extensively against 50 mM Tris/HCl buffer, pH 7.5, containing 10 mM CaCl₂ and 100 mM NaCl. The A_{280} of the samples was adjusted to 0.4 by dilution with dialysis buffer. Portions of 0.15 ml of

each sample and of the corresponding dialysis buffer were introduced into the sectors of standard double-sector cells (aluminium-filled Epon centrepieces) over 20 μ l of FC-43 mineral oil (3M). Centrifugation was performed at 11000 rev./min (9200 g_{av}) and 20 °C. After 22 h, equilibrium was reached and the protein distribution was analysed by continuous radial scanning of the A_{280} in each cell. For each sample, 10 successive scans were recorded and averaged to improve the signal-to-noise ratio. The rotor was then accelerated to 40000 rev./min (122000 g_{av}). After 5 h at high speed, the meniscus was depleted and the absorbance was recorded again to obtain the baseline value in each cell. The data were used to determine the molecular mass of each sample by means of the Sedimentation Equilibrium Single Data Set Analysis program of the Optima X-LA Data Analysis Software

(Beckman, Palo Alto, CA, U.S.A.). The 'Ideal I' model of this program gave the best fit. For calculations, the partial specific volume of the proteins was assumed to be 0.73 ml/g. The buffer density was 1.005 g/ml.

Adsorption of proteins on to cellulose

The adsorption of CelD and complexes was studied by incubating 190 pmol of CelD, complexed or not with an equimolar amount of Cip7, Cip6, Cip14 or Cip16, with 2 ml of 0.5 % (w/v) Avicel or amorphous cellulose in 50 mM Mes buffer, pH 6.0, containing 10 mM CaCl_2 (buffer C) for 15 min at 60 °C with occasional mixing. Subsequently, cellulose was allowed to settle under gravity for 5 min. An aliquot of the supernatant was collected carefully, centrifuged to remove any remaining cellulose particles and assayed for CelD activity using *p*-nitrophenyl β -D-cellobioside (*p*-NPC) as a substrate (unadsorbed activity). Adsorbed activity was calculated by subtracting unadsorbed activity from total activity (activity before adsorption).

Enzyme assays

Activity against Avicel or acid-swollen amorphous cellulose (prepared according to [30]) was determined by incubating CelD or complexes at 60 °C in the presence of a 0.5 % (w/v) substrate suspension in buffer C. After suitable time intervals, aliquots of the suspension were centrifuged and the supernatant was assayed for reducing sugars, using D-glucose as a standard [31].

The hydrolysis of *p*-NPC was assayed by monitoring the release of *p*-nitrophenol [32].

RESULTS

Complex formation

Non-denaturing gel electrophoresis showed that CelD formed stable complexes with all forms of mini-CipA. Upon titration of CelD with increasing amounts of Cip14 (Figure 2A) or Cip16 (Figure 2B), a new band corresponding to the complex appeared, whereas the intensity of the band corresponding to free CelD decreased. At the equivalence point, only the band representing the complex was observed, indicating that dissociation did not occur to a significant extent during electrophoresis. Similar results were obtained using Cip7 and Cip6 (P. Béguin, unpublished work). Only one form of complex appeared upon titrating CelD against Cip16 (Figure 2B) or Cip6 (P. Béguin, unpublished work). However, both Cip6 and Cip16 contain two cohesin domains, and should form two types of complexes, depending on whether one or both of the cohesin domains are occupied. The fact that only one complex was observed suggests either that steric hindrance prevents more than one cohesin domain being occupied or that binding to the first domain facilitates binding to the second one, leading to the preferential formation of complexes in which both domains are occupied. The equivalence point of the titration of CelD against Cip16 would suggest a stoichiometry of about 1.2 mol of CelD per mol of Cip16 (Figure 2). However, absolute quantities of proteins are difficult to determine accurately. In order to determine the stoichiometry of the complexes more reliably, the molecular masses of CelD, CelD–Cip14 and CelD–Cip16 were measured by sedimentation equilibrium analysis. In each case, the protein concentration profile at equilibrium was compatible with a monodisperse sample, even for the CelD–Cip16 complex, for which no acceptable fit was obtained with a two-component model ('Ideal 2' model of the program). The calculated molecular masses were 65 kDa for CelD alone, 107 kDa for the complex between CelD and Cip14, and 172 kDa for the complex between

Figure 2 Complex-formation between CelD and Cip14 or Cip16

CelD was mixed with increasing amounts of Cip14 or Cip16 and the mixture was analysed by non-denaturing PAGE. (A) Titration of 95 pmol of CelD with Cip14. Lane 1 contains 190 pmol of Cip14 alone. Molar ratios of CelD/Cip14 were: lane 2, 1:0.2; lane 3, 1:0.5; lane 4, 1:0.8; lane 5, 1:1; lane 6, 1:1.5; lane 7, 1:2. Lane 8 contains 95 pmol of CelD. (B) Titration of 95 pmol of CelD with Cip16. Lane 1 contains 52 pmol of Cip16 alone. Molar ratios of CelD/Cip16 were: lane 2, 1:0.4; lane 3, 1:0.55; lane 4, 1:0.8; lane 5, 1:0.9; lane 6, 1:1; lane 7, 1:1.1. Lane 8 contains 95 pmol of CelD.

CelD and Cip16. Given the actual masses of CelD (68 kDa), Cip14 (39 kDa) and Cip16 (56 kDa), these values are in good agreement with those expected for the CelD monomer and for complexes with stoichiometries Cip14–CelD and Cip16–(CelD)₂. Thus the absence of Cip16–CelD complexes, even in the presence of excess Cip16, suggests that the binding of CelD to Cip16 is co-operative. A possible explanation may be that cohesin domains within the Cip16 molecule interact with each other, partially masking both binding sites, until a dockerin domain is bound to one of the sites.

Adsorption of CelD and of CelD–mini-CipA complexes on to cellulose

Preliminary data indicated that Cip7 and Cip6 did not bind to Avicel, whereas Cip14 and Cip16 bound quantitatively (I. Kataeva, unpublished work). At protein/substrate ratios similar to those used in hydrolysis experiments, the binding of CelD alone to Avicel or to amorphous cellulose was nearly complete (96 % and 98 % respectively). The addition of equimolar amounts of Cip14 or Cip16 decreased the percentage of unbound CelD to about 1 % (99 % binding) in the case of Avicel, and to less than 0.5 % in the case of amorphous cellulose. Cip7 and Cip6, which contain no CBD, decreased to some extent the adsorption of CelD on to Avicel (to 79 % and 75 % respectively) or amorphous cellulose (91 % and 88 % respectively). This effect may be due to the masking of the dockerin domain of CelD on interaction with the cohesin domains of Cip7 and Cip6. Previous data indeed suggest that dockerin domains increase the hydrophobicity of proteins, and may enhance their affinity for cellulose [27,33,34].

Figure 3 Hydrolysis of Avicel (A and B) and amorphous cellulose (C and D) by CelD and by complexes of CelD with Cip14 (A and C) or Cip16 (B and D)

Hydrolysis was performed in 2 ml of 0.5% (w/v) Avicel, or in 1 ml of 0.5% (w/v) amorphous cellulose, both suspended in buffer C. (A) Hydrolysis of Avicel by CelD and Cip14. Curve 1, 350 pmol of Cip14; curves 2–5, 190 pmol of CelD plus 0 pmol (curve 2), 88 pmol (curve 3), 175 pmol (curve 4) or 350 pmol (curve 5) of Cip14. (B) Hydrolysis of Avicel by CelD and Cip16. Curve 1, 380 pmol of Cip16; curves 2–6, 190 pmol of CelD plus 0 pmol (curve 2), 48 pmol (curve 3), 96 pmol (curve 4), 190 pmol (curve 5) or 380 pmol (curve 6) of Cip16. (C) Hydrolysis of amorphous cellulose by CelD and Cip14. Curve 1, 1750 pmol of Cip14; curves 2–5, 95 pmol of CelD plus 0 pmol (curve 2), 88 pmol (curve 3), 175 pmol (curve 4) or 1750 pmol (curve 5) of Cip14. (D) Hydrolysis of amorphous cellulose by CelD and Cip16. Curve 1, 480 pmol of Cip16; curves 2–6, 95 pmol of CelD plus 0 pmol (curve 2), 24 pmol (curve 3), 48 pmol (curve 4), 480 pmol (curve 5) or 960 pmol (curve 6) of Cip16.

Cellulose hydrolysis by CelD alone and CelD complexed with Cip14, Cip16, Cip7, Cip6 or CipA

CelD, Cip14 and Cip16

Although Cip14 and Cip16 did not change the amount of substrate-bound CelD significantly, the addition of Cip14 or Cip16 increased by up to 3-fold the activity of CelD towards Avicel and amorphous cellulose (Figure 3). The effect was dependent on the mini-CipA/CelD ratio, with little increase past the equivalence point. No significant difference was observed between Cip14 and Cip16. Thus CelD complexed to Cip16 appeared to have the same average activity as when complexed to Cip14.

Avicel hydrolysis by CelD and CelD-containing complexes stopped after only a small fraction of the substrate had been degraded. This could be due to inactivation of the enzyme, to product inhibition or to exhaustion of the most easily degraded fraction of the substrate. Neither CelD nor any of the CelD–mini-CipA complexes lost significant activity against *p*-NPC when incubated for 5 days at 60 °C in the same buffer as used for cellulose hydrolysis. Thus inactivation is unlikely to account for the early termination of hydrolysis. The addition of 5 mM cellobiose was required to inhibit the hydrolysis of 1 mM *p*-NPC by 50% (I. Kataeva, unpublished work). In the case of Avicel hydrolysis, product accumulation was too low to cause significant inhibition. This effect may play some role in the case of amorphous cellulose, which is degraded more extensively. However, addition of a fresh batch of Avicel to CelD or CelD–Cip14 complexes resulted in a new burst of activity, indicating that early termination of Avicel hydrolysis was due to the limited number of easily accessible sites (Figure 4). The addition of

Figure 4 Hydrolysis of Avicel by CelD and by the CelD–Cip14 complex after fresh substrate addition

A 190 pmol portion of CelD, or a mixture of 190 pmol of CelD plus 340 pmol of Cip14, was incubated in duplicate with 1 ml of 0.5% (w/v) Avicel for 48 h. Then another 1 ml of 0.5% Avicel was added to one pair of tubes, while the other pair served as a control. Curve 1, CelD, control; curve 2, CelD, fresh Avicel added; curve 3, CelD–Cip14 complex, control; curve 4, CelD–Cip14 complex, fresh Avicel added.

Cip14 or Cip16 appeared to increase the percentage of the substrate available for degradation.

CelD, Cip7 and Cip6

No significant stimulation of Avicel hydrolysis was observed when Cip7 or Cip6, which contain no CBD, was added to CelD

Figure 5 Hydrolysis of Avicel (A and B) and amorphous cellulose (C and D) by CelD and by complexes of CelD with Cip7 (A and C) or Cip6 (B and D)

Hydrolysis was performed in 2 ml of 0.5% (w/v) Avicel or in 1 ml of 0.5% (w/v) amorphous cellulose. (A) Hydrolysis of Avicel by CelD and Cip7. Curve 1, 1800 pmol of Cip7; curves 2–6, 190 pmol of CelD plus 0 pmol (curve 2), 73 pmol (curve 3), 180 pmol (curve 4), 535 pmol (curve 5) or 1800 pmol (curve 6) of Cip7. (B) Hydrolysis of Avicel by CelD and Cip6. Curve 1, 1380 pmol of Cip6; curves 2–6, 190 pmol of CelD plus 0 pmol (curve 2), 34 pmol (curve 3), 68 pmol (curve 4), 250 pmol (curve 5) or 1380 pmol (curve 6) of Cip6. (C) Hydrolysis of amorphous cellulose by CelD and Cip7. Curve 1, 910 pmol of Cip7; curves 2–5, 95 pmol of CelD plus 0 pmol (curve 2), 91 pmol (curve 3), 270 pmol (curve 4) or 910 pmol (curve 5) of Cip7. (D) Hydrolysis of amorphous cellulose by CelD and Cip6. Curve 1, 690 pmol of Cip6; curves 2–4, 95 pmol of CelD plus 0 pmol (curve 2), 34 pmol (curve 3) or 690 pmol (curve 4) of Cip6.

Figure 6 Hydrolysis of Avicel by CelD and CipA

Hydrolysis was performed in 2 ml of 0.5% (w/v) Avicel. Curve 1, 180 pmol of CipA; curves 2–6, 190 pmol of CelD plus 0 pmol (curve 2), 36 pmol (curve 3), 72 pmol (curve 4), 180 pmol (curve 5) or 360 pmol (curve 6) of CipA.

(Figures 5A and 5B). The hydrolysis of amorphous cellulose was enhanced. However, the effect was less pronounced than with Cip14 or Cip16, particularly at concentrations of Cip7 and Cip6 near the equivalence point (Figures 5C and 5D). The presence of an additional cohesin domain in Cip6 did not increase the synergistic action of this protein significantly compared with Cip7.

CelD and CipA

The activities of complexes composed of CelD and Cip14 or Cip16 were compared with that of complexes formed between

CelD and CipA (Figure 6). The hydrolysis of Avicel increased with increasing concentrations of CipA, and reached a maximum at about 1 mol of CipA per 1–2 mol of CelD. At higher CipA/CelD ratios, the efficiency of Avicel hydrolysis decreased. In the latter case, all components were bound to cellulose (I. Kataeva, unpublished work), ruling out the possibility that excess CipA might compete for binding sites on the substrate. The rate of Avicel hydrolysis decreased somewhat more slowly when CelD was combined with CipA than with Cip14 or Cip16, so that soluble reducing sugars increased significantly for up to 5 days. However, even at an optimal ratio of components, the synergism of CelD with CipA was less efficient than that with Cip14 or Cip16, particularly for incubation times shorter than 24 h.

DISCUSSION

Previous results had shown that CipA potentiates the activity of endoglucanase CelD towards Avicel [14]. The experiments reported in the present paper were designed to test whether this effect was due to attachment of the enzyme to a component bearing a CBD, or to a clustering effect that allowed simultaneous attack by more than one enzyme acting at the same site of the substrate. The results clearly indicate that the presence of a CBD on the mini-CipA component is critical for stimulation of Avicel hydrolysis. No enhancement of Avicel hydrolysis was observed with Cip7 and Cip6, which lack a CBD. The effects of Cip14 and Cip16 were not due merely to an increase in the fraction of CelD bound to the substrate, since, under the conditions of the assay, most CelD was adsorbed to the substrate even when uncomplexed. As observed previously for CipA [14], the effects of Cip14 and Cip16 are to improve the efficiency of the bound enzyme. In particular, the fraction of the substrate that can be hydrolysed is

increased. Two explanations, which are not mutually exclusive, may account for the effects of Cip14 and Cip16. When complexed with Cip14 or Cip16, the catalytic domain of CelD may be more adequately poised to attack the substrate than when the enzyme is bound by itself. In addition, the CBD may not only promote productive binding of the enzyme but also participate in the destructure of the substrate. Indeed, the CBD of *Cellulomonas fimi* endoglucanase CenA disrupts the surface of cotton and ramie cellulose fibres and releases small particles from them [35]. Enhancement of CenA activity by the CBD was observed even when added separately to the core enzyme [36].

Clustering of endoglucanases within the same complex was predicted to enhance their activity towards crystalline cellulose by allowing a set of quasi-simultaneous multicutting events along the same cellulose chain [18]. This prediction was tested by comparing results obtained with CelD complexed with mono- or bi-valent mini-CipA molecules or with intact CipA. A prerequisite for the interpretation of the data was to ascertain the stoichiometry of the complexes. Sedimentation analysis established unequivocally that Cip14 and Cip16 bound one and two molecules respectively of CelD. This result validates the previous determination of affinity constants between cohesin and dockerin domains [7], which assumed, but did not demonstrate, a 1:1 stoichiometry. The binding of CelD to the two sites of Cip16 was co-operative. The significance of this finding for the process of cellulosome assembly remains to be investigated. It may be noted that, if relative amounts of cellulosome components are computed from densitometric scannings of SDS/PAGE gels [1], CipA amounts to one-ninth or one-tenth of all components on a per mol basis. This is compatible with full occupancy of the nine cohesin domains of CipA within the cellulosome. Accordingly, a 2.1 MDa cellulosome [37] should comprise two fully decorated CipA molecules.

Cip16 enhanced Avicel hydrolysis to the same extent as did Cip14, even though it bound two CelD molecules. Conversely, no increase in activity against Avicel was observed with Cip6, which carries two cohesin domains but no CBD. Likewise, CipA proved to be less efficient, if anything, than Cip14 or Cip16 in stimulating the hydrolysis of Avicel by CelD. In addition, optimum synergism between CipA and CelD occurred at ratios for which the cohesin domains of CipA were far from saturated. Thus the physical association of two or more molecules of endoglucanase CelD did not significantly enhance the degradation of Avicel, as would have been expected from the basic version of the multicutting event model [18].

The enhancement of the hydrolysis of amorphous cellulose observed when Cip7 or Cip6 was added to CelD was unexpected. Both Cip7 and Cip6 decrease rather than increase the adsorption of CelD to the substrate. It is possible that this reduced affinity may increase the desorption of improperly bound enzyme, the active site of which would not have access to the substrate. However, maximum enhancement requires more Cip7 and Cip6 than would be necessary to bind all CelD. This would suggest that Cip7 and Cip6 act by modifying the substrate, but this would not normally be expected for molecules not containing a CBD.

In spite of the enhancement of CelD activity by Cip14, Cip16 or CipA, artificial complexes tested in the present study hydrolysed only a small fraction of the Avicel, whereas degradation of the same substrate by the native cellulosome proceeds to near completion [12,38,39]. One obvious reason is that, most likely, more than one kind of enzyme has to be incorporated into the complex. Estimates of the number of different polypeptides within the cellulosome range up to 26 [40]. Re-association experiments performed with subunits isolated from the cellu-

losome (one endoglucanase, two exoglucanases and CipA) demonstrated that, for maximum activity, the presence of all four components was necessary [13]. CelD was chosen for the present study because it is easily purified in intact and active form from inclusion bodies produced in recombinant *E. coli* clones [27,41]. Many cellulosome components are difficult to isolate from recombinant *E. coli* clones due to the degradation of dockerin domains by host proteases. Devising strategies for obtaining such components in intact form will extend the range of combinations that can be tested, which may improve the efficiency of artificial complexes made from proteins originating from recombinant clones.

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