Synergistic interaction of the cellulosome integrating protein (CipA) from *Clostridium thermocellum* with a cellulosomal endoglucanase

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Abstract Activity of a cellulosomal endoglucanase (endoglucanase E; EGE) from *Clostridium thermocellum* against two crystalline forms of cellulose was enhanced by combination with the cellulosome integrating protein (CipA), but CipA did not enhance EGE activity against amorphous cellulose, even though it was able to bind to it. Similarly, CipA added *in trans* to genetically truncated EGE that was unable to combine with it nevertheless enhanced EGE activity against crystalline cellulose. These results indicate that the CipA cellulose binding domain does not mediate an increase in activity solely by bringing the catalytic subunits of the cellulosome complex into intimate contact with the substrate.

Key words: Cellulase; Cellulolysis; Cellulosome; Cellulosome integrating protein; *Clostridium thermocellum*; Endoglucanase E

1. Introduction

The cellulase system of the anaerobic thermophile *Clostridium thermocellum* is integrated into a large multienzyme complex termed the cellulosome and is among the most active thus far described [1]. In addition to numerous catalytic subunits with cellulase and hemicellulase activities, the cellulosome contains a non-catalytic scaffolding protein, or cellulosome integrating protein (CipA), comprising nine homologous receptors termed cohesins of about 150 residues, to which catalytic subunits bind via a conserved docking domain termed dockerin of 24 reiterated residues. A tenth domain in CipA functions as a cellulose-binding domain (CBD; family III), mediating attachment of the complex to cellulosic substrates [1–6]. The close similarity between the sequences of the individual cohesin domains of CipA and the extremely high degree of conservation seen among the dockerin domains of the catalytic subunits indicates a lack of specificity for the interaction of the cohesin and dockerin domains. This conclusion is supported by the results of recent research. Cellulosome preparations from different strains of *C. thermocellum* contain between 13 and 50 different catalytic subunits [1,7], and it is therefore likely that the cellulosome will not have a unique composition, but will comprise a mixture of subpopulations, reflecting the relative abundance of different catalytic subunits and the random binding of these subunits to the cohesin domains of CipA. Indeed, one such subpopulation, containing predominantly six subunits has been isolated and characterised by two groups working independently [8,9].

If the cohesin/dockerin interaction lacks specificity, it also follows that it should be possible to occupy each of the nine cohesin domains of CipA with any one of the catalytic subunits of the cellulosome. Recent work showing that both cohesin domains of a greatly truncated form of recombinant CipA recognise all of the enzymatic subunits of the cellulosome, with the exception of subunit S2, suggests that this is true [10].

Notwithstanding the considerable progress that has been made in understanding the molecular basis of cellulosome assembly, several questions remain unresolved. For example, it is still largely unclear why the cellulosome is so effective in breaking down native cellulose. It is well established that the structural integrity of the complex is a prerequisite for activity against this substrate. Several reports have demonstrated that dissociation of the cellulosome, followed by reaggregation of all subunits or of defined combinations of subunits, can lead to restoration of some activity against crystalline cellulose, but the levels of activity recovered have been only a small fraction of that displayed by the native cellulosome [11,12]. There is therefore no clear view regarding the number or nature of the catalytic subunits required for maximal activity against native cellulose. Another unresolved question concerns the role of the CipA CBD in cellulolysis. It is now recognised that at least some CBDs present in microbial endoglucanases and cellobiohydrolases facilitate activity against crystalline cellulose by disrupting the ordered structure of the substrate [13]. However, it is not known whether the CipA CBD also disrupts the crystalline structure of cellulose, or simply brings the cellulosome into intimate contact with its substrate. Until now, in vitro studies of CipA function have been hampered by the difficulties associated with denaturing the cellulosome and purifying native CipA. We have circumvented these problems by expressing the *cipA* gene in *Escherichia coli*, and we describe here a method for isolating recombinant CipA. To investigate the synergistic interaction of a cellulosomal endoglucanase (endoglucanase E; EGE [14]) with recombinant CipA, and to define the roles of the respective CBDs, we have generated artificial cellulosomes containing EGE bound to CipA, and we have compared the activity of the enzyme in the free and bound states. The results show that the association of EGE with CipA affects enzyme activity significantly, and that the CipA CBD plays a pivotal role in promoting hydrolysis of crystalline cellulose by EGE.

2. Materials and methods

2.1. Bacterial strains, media and vectors

Recombinant *E. coli* JM83 and XL1-Blue were cultured in Luria-

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Bertani (LB) broth containing ampicillin (100 μg/ml). The vectors used in this study were pUC19 [15] and pKK223-3 (Pharmacia). Plasmid pRG2.0 and a second recombinant of pUC19 (pGE1.0), containing an overlapping fragment bearing the 5′ end of *cipA* and about 1.3 kbp of upstream sequence, were a generous gift from Dr U. Gerngross, Massachusetts Institute of Technology.

2.2. Recombinant DNA methods

DNA isolation, agarose-gel electrophoresis and transformation of *E. coli* were carried out as described [16]. Restriction endonucleases and other DNA modifying enzymes were used in accordance with the manufacturers instructions. Full-length *cipA* encoding the cellulosome-integrating protein CipA was constructed as follows. The 5′ region of *cipA* was excised from pGE1.0 on a 2.6 kbp *Eco*RI restriction fragment and ligated into the *Eco*RI site of pRG2.0 to generate the plasmid pCSL. Plasmid pCSL was digested with *Eco*RI and *Sma*I and a 4.8 kbp fragment containing *cipA* minus 1 kbp of 5′ sequence was ligated into *Eco*RI/*Sma*I digested pKK223-3 to generate pCA5-1. The 5′ region of *cipA* was amplified by PCR from *C. thermocellum* genomic DNA (prepared according to [17]), on a 1 kbp fragment with *Eco*RI ends, and was ligated into *Eco*RI-digested pCA5-1 to generate plasmid pCA5 which contained a full-length copy of *cipA* in-frame with the *tac* promoter of pKK223-3, but lacking the 5′ region encoding the CipA signal peptide.

2.3. Purification of CipA

E. coli XL1-Blue harbouring pCA5 was cultured for 16 h in LB broth (1 l) containing ampicillin (100 μg/ml). Harvested cells were sonicated to produce a cell-free extract which was heated to 60°C for 16 min to denature *E. coli* proteins, chilled on ice and centrifuged (12 000×*g* for 20 min at 4°C). Sigmacell (Sigma; 200 mg/ml) was added and the suspension was stirred for 1 h at 4°C before filtering (grade GF/D, Whatman). The cellulose recovered was washed three times with 0.1 M Tris-HCl, pH 7.6, resuspended in 5 ml 100% ethylene glycol for 1 h and centrifuged. The supernatant fraction, containing CipA, was dialysed against 20 mM Tris-HCl, pH 7.2 and applied to a Mono Q FPLC column (1 ml; Pharmacia). A linear gradient from 0 to 0.5 M KCl in 20 m Tris-HCl, pH 7.2 was applied to the column and CipA with M_r 198 000 eluted at 0.36 M KCl. The first 20 experimentally determined N-terminal residues of purified CipA were identical to those of the native protein.

2.4. Purification of EGE and truncated EGE

Full-length EGE was purified from a cell-free extract of *E. coli* JM83 harbouring pHGB9 by cellulose-affinity chromatography [18]. Truncated EGE consisting of the catalytic domain only (EGEtr) was expressed and purified from a culture of *E. coli* JM83 harbouring pHGB2 [14,19].

2.5. Analytical techniques

Protein was measured by dye binding [20]. SDS-PAGE gels were run essentially as described [21]; for non-denaturing PAGE, SDS was omitted. Enzymatic activity was determined as follows. All assays were performed at 60°C in 50 mM phosphate/12 mM citrate buffer, pH 6.5, containing 5 mM DTT and 10 mM $CaCl_2$. The time of incubation and amount of enzyme used with each substrate were: carboxymethylcellulose (CMC), 10 min using 150 ng of enzyme; acid swollen cellulose, 1 h using 1 μg of enzyme; Avicel, 2.5 h using 4 μg of enzyme; bacterial microcrystalline cellulose, 16 h using 17 μg of enzyme. Substrate concentrations are given in the text. Reaction rates for all substrates were linear and were determined by measuring the release of reducing sugars [22].

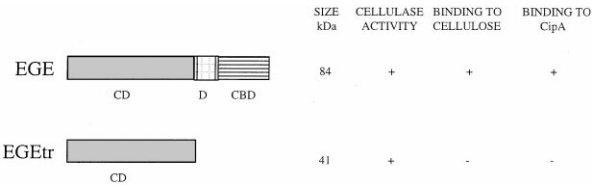

Fig. 1. Structures and properties of full-length EGE and truncated EGE. CD, catalytic domain; D, dockerin; CBD, cellulose-binding domain.

3. Results

3.1. The role of the EGE CBD

It is well established that binding of the cellulosome to cellulose is mediated by the CipA CBD [5,23]. It is also apparent that at least one cellulosomal endoglucanase, EGE, contains its own CBD [18]. To evaluate whether the EGE CBD is important in enabling the enzyme to attack different forms of cellulose, we compared the activity of full-length EGE with that of a derivative which lacks the CBD (EGEtr; Fig. 1). The data (Table 1) show that full-length EGE exhibits higher activity against Avicel and the highly crystalline substrate, bacterial microcrystalline cellulose (BMCC), when compared with EGEtr which lacks the CBD, while EGEtr displays higher activity than EGE against CMC and amorphous cellulose. These data suggest that the CBD of EGE does play a substantial role in the hydrolysis of crystalline forms of cellulose by the endoglucanase.

3.2. Binding of EGE to CipA

Non-denaturing PAGE coupled with zymogram analysis showed that full-length EGE bound to CipA producing a larger aggregate with endoglucanase activity (Fig. 2). EGEtr which lacked the dockerin domain did not aggregate with CipA, thus confirming that the 24 residue reiterated dockerin domain mediates the binding of EGE to the scaffolding protein. Previous work has indicated that the interaction between CipA cohesins and the conserved dockerin domains of the different cellulosomal enzymes lacks specificity [10]. In this study, we have generated artificial cellulosomes by mixing full-length EGE with CipA in molar ratios up to and exceeding 9:1, so as to occupy each of the CipA cohesin domains with the same endoglucanase. At molar ratios up to 9:1 (EGE:CipA), endoglucanase bound quantitatively to CipA, producing a large aggregate. At ratios exceeding 9:1 (EGE:CipA), unbound EGE was visible by non-denaturing PAGE

Table 1
Effect of CipA on the activity of truncated (EGEtr) and full-length endoglucanase E (EGE) against cellulose

Enzyme	Catalytic activity			
	1.5% CMC	2% Acid swollen cellulose	5% Avicel	0.175% BMCC
EGEtr	45.39 ± 4.27	4.31 ± 0.53	0.15 ± 0.002	0.649 ± 0.003
EGEtr:CipA	45.29 ± 3.95	4.76 ± 0.05	0.24 ± 0.002	1.206 ± 0.062
EGE	13.83 ± 3.81	2.97 ± 0.59	0.29 ± 0.050	5.360 ± 0.580
EGE:CipA	14.86 ± 3.59	2.69 ± 0.38	0.46 ± 0.060	7.690 ± 0.530
Cellulosome	53.64 ± 3.17	2.82 ± 0.18	0.84 ± 0.037	361.37 ± 11.48

With the exception of BMCC activity which is in milli-units, all activities are expressed as units per mg protein (1 unit is equivalent to 1 μmol of reducing sugar produced per minute).

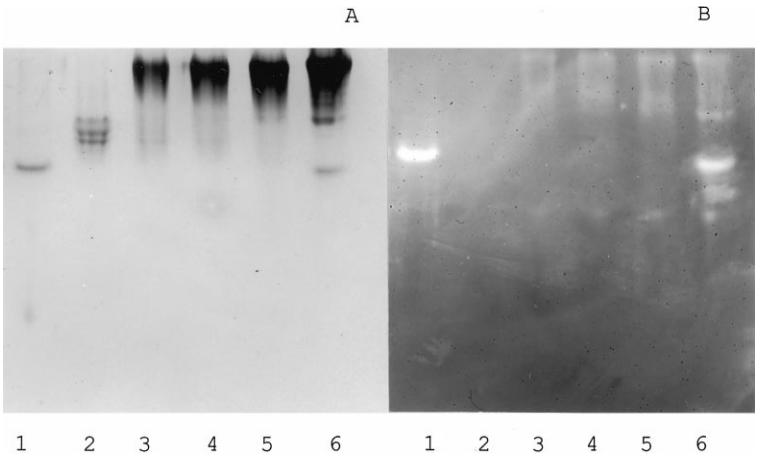

Fig. 2. Analysis of the complexes formed by binding full-length EGE to CipA. Complexes were formed by mixing CipA (6 μg: lane 2) with full-length EGE (lane 1) in molar ratios of 1:3 (lane 3), 1:6 (lane 4), 1:9 (lane 5) and 1:12 (lane 6) in PC buffer containing 5 mM dithiothreitol, 10 mM $CaCl_2$ and standing at room temperature for 45 min. Non-denaturing PAGE was carried out on a gel containing 6% acrylamide. Proteins were stained with Coomassie blue (A) and analysed for cellulase activity (B) by staining with Congo red.

(Fig. 2). These data support the notion that EGE can bind to each of the cohesin domains of CipA.

3.3. Effect of binding to CipA on the cellulase activity of EGE

Previous work has shown that dissociated cellulosomes or individual cellulosomal enzymes retain activity against amorphous cellulose, but significant hydrolysis of native cellulose is only effected by intact cellulosomes, implying a pivotal role for CipA [24]. This is supported by the observation that mutants of *C. thermocellum* which do not produce CipA have also lost the capacity to hydrolyse crystalline cellulose [25]. To investigate the mechanism whereby CipA enhances the cellulase activity of catalytic subunits of the cellulosome, we have compared the activity of free full-length EGE against recalcitrant forms of cellulose, with the activity of an equimolar mixture of EGE and CipA.

CBDs may potentiate their effect on enzyme activity either by increasing the proximity of the enzyme to its substrate, or by disrupting the structure of cellulose microfibrils. Since the latter does not require the enzyme catalytic domain and CBD to be covalently linked, we have also examined the capacity of CipA to potentiate the activity of EGEtr when added *in trans*.

The results (Table 1) indicate that when full-length EGE was bound to CipA, the complex exhibited enhanced activity against crystalline forms of cellulose, but not against amorphous cellulose or CMC. Furthermore, when added *in trans*, CipA increased the activity of EGEtr against Avicel and BMCC but not against amorphous cellulose or CMC. While these data do not discount the possibility that CipA enhances activity by improving enzyme/substrate proximity, they suggest that at least part of the effect is due to the disruption of crystalline cellulose structure by the CipA CBD.

3.4. Comparative cellulase activities of EGE/CipA aggregates and native cellulosomes

To evaluate the activity of an equimolar mixture of full-length EGE and CipA in relation to that of the native cellulosome, the hydrolysis of a range of cellulosic substrates by the two enzyme systems was investigated. The data obtained (Table 1) showed that both complexes degraded Avicel at similar rates, however, the cellulosome was approximately 100-fold more active against BMCC. This is in contrast to the results of previous studies in which purified components of the native cellulosome were recombined; complexes consisting of endoglucanases and CipA [12] or the exocellulase S8(S_8) and CipA [11] were 100–1000 times less active against Avicel than the EGE/CipA complex. The likely explanation for the low Avicelase activity, observed in [11] and [12], after recombining the catalytic subunits with CipA, is that these proteins could not be fully renatured after they had been treated with the strong protein denaturant, SDS during purification.

4. Discussion

Although CBDs have been identified in many cellulases and hemicellulases, a unifying role for these domains has not been defined. Studies by Coutinho et al. [26] have shown that the CBD of *Cellulomonas fimi* endoglucanase A (CenA) plays a pivotal role in the activity of the enzyme against crystalline cellulose, but removal of this domain did not affect the capacity of the enzyme to hydrolyse amorphous cellulose. Electron microscopy studies [13] showed that the isolated CBD of CenA could open the structure of ramie cellulose making the substrate more accessible to enzyme attack. In contrast, recent studies in our laboratories have shown that family II CBDs from a *Pseudomonas* cellulase and a xylanase potentiate catalytic activity by increasing enzyme substrate proximity, not via a disruptive effect on crystalline cellulose [27]. From the foregoing discussion it would appear that although CBDs in general can increase cellulase activity against insoluble cellulose, there is no single mechanism by which they elicit their effect.

The mechanism by which the CipA CBD increases the activity of EGE is a matter for debate. It is possible that the CBD increases cellulase activity by recruiting the enzyme onto the surface of the cellulose, enabling the resultant intimate contact between enzyme and substrate to increase the rate of bond cleavage as Mayer et al. suggested [28]. However, the observation that CipA CBD enhances the activity of EGEtr against crystalline cellulose even though the two proteins do not associate, suggests that the CBD, by binding to crystalline forms of cellulose disrupts the inter- and intra-

chain hydrogen bonding of the polysaccharide, resulting in a more amorphous structure which is susceptible to enzyme attack.

It is likely that the very much greater activity of native cellulosomes against BMCC reflects the highly crystalline nature of the substrate, and highlights the need for the synergistic interaction of both endo- and exo-acting glucanases to elicit efficient hydrolysis. It remains to be established whether CipA mediates its effect by bringing the endoglucanases and cellobiohydrolases into close contact with a CBD, or whether the close proximity of the two enzyme species is all that is required for synergistic interaction. Although the random association of catalytic subunits such as occurs in native cellulosomes would argue against the view that CipA mediates synergy between endoglucanases, the apparently high level of cellobiohydrolase S8 expression by *C. thermocellum* [29] indicates that all cellulosomes probably contain the exocellulase and an assortment of endoglucanases. If S8 can act synergistically with a range of cellulosomal endoglucanases, then it is possible that CipA, in addition to supplying a CBD, also enhances the synergistic interactions between the cellobiohydrolase and endo-acting enzymes.

To conclude, this report demonstrates that CipA CBD plays a pivotal role in cellulose hydrolysis, and demonstrates how recombinant components of the cellulosome can be used to dissect the mechanism of assembly and mode of action of the cellulosome complex.

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