The non-catalytic C-terminal region of endoglucanase E from Clostridium thermocellum contains a cellulose-binding domain

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INTRODUCTION

Clostridium thermocellum is an anaerobic thermophilic Gram-positive bacterium that rapidly degrades crystallline cellulose (Johnson et al., 1982). Hydrolysis of the polysaccharide is mediated by a large cell-associated multienzyme complex, termed the 'cellulosome', consisting of approx. 18 polypeptides, including an apparently non-enzymic protein (S1) of Mr 210000 (Lamed & Bayer, 1988). It is proposed that S1 constitutes a cellulose-binding protein which associates with C. thermocellum cellulases and xylanases to form the cellulosome, although there is no definitive proof for this role (Lamed & Bayer, 1988). By using recombinant DNA methodology, numerous C. thermocellum endoglucanase and xylanase genes have been cloned into Escherichia coli (Hazlewood et al., 1988). Individually, the encoded enzymes, some of which have been shown to be components of the cellulosome, have no significant activity towards crystalline cellulose (Hazlewood et al., 1988). This suggests that the aggregation of enzymes to form an ordered complex, or the interaction of endoglucanases with the S1 subunit, is a prerequisite for the hydrolysis of crystalline cellulose by C. thermocellum. Other cellulolytic micro-organisms, notably aerobic fungi, synthesize cellulases which do not associate into large multienzyme complexes. These enzymes often contain a cellulose-binding domain (CBD) distinct from the active site (Tomme et al., 1988). It is believed that, at least in fungi, the CBDs act in freeing the cellulose microfibrils in such a way that the enzyme can then perform the hydrolysis step (Tomme et al., 1988). Despite extensive biochemical and ultrastructural studies of the C. thermocellum cellulosome, the molecular mechanisms underlying the aggregation of the cellulosome and the association of the multienzyme complex with cellulose remain unclear. Inspection of the primary structure of the cloned C. thermocellum cellulases which are components of the cellulosome, revealed little similarity, except for a 24-amino-acid reiterated sequence, located generally at the C-terminus (Hall et al., 1988). It has been suggested that this duplicated sequence, which is not essential for catalytic activity (Hall et al., 1988; Grépinet et al., 1988), interacts directly with either S1 or cellulose (Béguin, 1990).

Our research group has recently cloned and sequenced the C. thermocellum celE gene (Romaniec et al., 1987; Hall et al., 1988) encoding endoglucanase E (EGE). The mature form of the enzyme, consisting of 780 residues with an Mr of 84016, contains the conserved duplicated sequence between residues 380 and 440. The N-terminal 339 amino acids of EGE acid of EGE constitute a functional cellulase. The aim of the present study was to investigate the function of the C-terminal domain of the enzyme. The results show that this region constitutes a CBD which is distinct from the catalytic domain (CD). The conserved sequence is not essential for catalytic activity or cellulose binding. The putative role(s) of the CBD in EGE is discussed.

MATERIALS AND METHODS

Microbial strains, vectors and culture conditions

Escherichia coli strains employed in this study were JM83 and JM101 (Norlander et al., 1983). The vectors used were pUC18, pUC19, MI3mp18, M13mp19 (Norlander et al., 1983), pMTL22p and pMTL23p (Chambers et al., 1988). Full-length celE and a truncated derivative of celE coding for only the CD of EGE were contained in recombinant plasmids pHG9B (Hazlewood et al., 1990) and pHGB2 (Hall et al., 1988) respectively. E. coli JM83 harbouring these plasmids was cultured in Luria broth (LB) supplemented with ampicillin (100 µg/ml), or on LB agar containing carboxymethylcellulose (5 mg/ml); endoglucanase activity was detected by staining with Congo Red (1%, w/v; Teather & Wood, 1982). Production of β-galactosidase activity by bacterial colonies was detected by supplementing media with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal; 4 µg/ml).

Abbreviations used: CBD, cellulose-binding domain; CD, catalytic domain; EGE, endoglucanase E; LB, Luria broth; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; dATP[35S], deoxyadenosine 5'-[α-35S]triphosphate.

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General recombinant DNA methodology

Agarose-gel electrophoresis, transformation of *E. coli*, plasmid preparation and the use of DNA-modifying enzymes were as described by Gilbert et al. (1987). DNA cloned in M13 vectors was sequenced by the dideoxy-chain-termination method (Sanger et al., 1980), except that bacteriophage-T7 DNA polymerase (Tabor & Richardson, 1987) was used instead of the Klenow fragment of DNA polymerase.

Construction of *celE* derivatives

Plasmids in the pJH series were constructed as follows: truncated *celE* encoding residues 4-367 of mature EGE (essentially the CD) was excised from pHGB2 (Hall et al., 1988) on a 1.1 kb *HindIII*–*SstI* restriction fragment and was cloned into M13mp19. By using site-directed mutagenesis in vitro, an internal *EcoRI* site at position 832 was deleted without altering the primary structure of the encoded enzyme and, in a second derivative, a translational stop was also created such that *celE* encoded residues 4-364 of mature EGE (pJH2; EGEa). The two *celE* derivatives were excised on 1.1 kb *HindIII*–*EcoRI* restriction fragments and separately cloned into pMTL22p to create pJH1 (which lacks the *EcoRI* site in *celE*) and pJH2 (which lacks the *EcoRI* *celE* site and contains a translational stop codon at position 1194 of *celE*) respectively. To construct pHJ4, pHGB9 (Hazlewood et al., 1990) was cleaved with *EcoRI*, digested with BAL31, blunt-ended with bacteriophage-T4 DNA polymerase and digested with *Sall*, which cleaves downstream of *celE*. The blunt-ended *Sall* DNA fragment of approx. 1.1 kb (encoding the 3' region of *celE*) was cloned into pHJ1 which had been digested with *EcoRI* and *XhoI*. Nucleotide sequencing of selected plasmids revealed that the 5' region of the reconstituted *celE* gene was out of frame with the 3' end of the gene. Consequently, hybrid *celE* was digested with *EcoRI*, filled in with sequenase, and re-ligated. To verify that the resultant *celE* gene now constituted a single open reading frame (encoded by pHJ4), the relevant region of *celE* was resequenced, and the cell-free extract from *E. coli* cells harbouring pHJ4 was shown to contain a thermostable (67 °C for 1 h) protein of *M*₅₇7000 that was absent from strains containing either pMTL22p alone or the plasmids harbouring the *celE* derivatives in which the 5' and 3' regions of the gene were out of phase (results not shown). Plasmid pHJ6 was constructed by cloning the 1.95 kb *SpH1–KpnI* restriction fragment from pHGB9 (Hazlewood et al., 1990) into pMTL22p. Finally, pHJ5 was synthesized by digesting pHJ6 with *SrlI* and re-ligating the 4.2 kb fragment generated. The structures of the *celE* derivatives are shown in Fig. 1.

Purification of EGE

*E. coli* JM83 harbouring truncated *celE* cloned in pMTL22p was cultured for 16 h in LB (1 litre) containing ampicillin (100 µg/ml). A periplasmic fraction which contained 70% of the total endoglucanase activity of the culture was prepared from the harvested cells as described by Hazlewood et al. (1990), and was heated at 60 °C for 10 min with gentle swirling. Denatured protein was removed by centrifuging (15000 g; 4 °C; 20 min). Endoglucanase activity in the supernatant was precipitated with (NH₄)₂SO₄ (48 % saturation), redissolved in 50 mm-Tris/HCl, pH 8.0, and dialysed against three changes of 10 mm-Tris/HCl, pH 8.0. EGE contained in the dialysed periplasmic extract was further purified by hydrophobic interaction chromatography on a Phenyl-Sepharose column (2.6 cm × 12.0 cm), essentially as described by Chauvaux et al. (1990). Column fractions were monitored by SDS/PAGE (Laemmli, 1970), and pure EGE was eluted with 8 % (v/v) ethylene glycol.

Cellulose binding studies

Cell-free extract (5 ml) prepared by sonication from *E. coli* cells (100 ml of culture) harbouring full-length or truncated forms of *celE*, was mixed with 5 ml of Avicel (PH105; FMC Corp., Philadelphia, PA, U.S.A.; 5 %, w/v) suspended in 100 mm-Tris/HCl, pH 7.5 (Buffer A). After shaking gently for 1 h at 0 or 60 °C, Avicel was recovered by filtration, washed three times with Buffer A, and bound protein was eluted with 1 ml of distilled water. The *M*₅ of proteins eluted from Avicel was determined by SDS/PAGE, using 10 % (w/v) acrylamide gels as described by Laemmli (1970).

Assays

The preparation of cell-free extracts and the assay of endoglucanase activity were as described by Romaniec et al. (1987).
Reducing sugar was determined by the dinitrosalicylic acid method of Miller (1959), and was expressed as glucose equivalents. Protein was measured as described by Lowry et al. (1951), using BSA as standard.

**Enzymes and reagents**

Restriction enzymes, bacteriophage-T4 DNA ligase and polymerase were from Bethesda Research Laboratories. Bacteriophage-T7 DNA polymerase and other reagents for DNA sequencing were from Cambridge Bioscience. The deoxyadenosine 5'-[α-3²P]triphosphate (dATP[3²P]; 1000 mCi/mmol) was supplied by Amersham International. BAL 31 was obtained from Boehringer. Unless specified, all other reagents were supplied by Sigma.

**RESULTS**

**Binding to cellulose of full-length and truncated forms of EGE**

Previous studies showed that celE encodes an endoglucanase (EGE), of Mr 84000 that exhibits some xylanase activity. The C-terminal region of the protein was not essential for catalytic activity (Hall et al., 1988). To determine the function of this non-catalytic domain of the enzyme, the capacity of full-length EGE and a truncated derivative of the endoglucanase lacking the C-terminal 416 residues (EGEa; Fig. 1) to bind crystalline cellulose (Avicel) was investigated. Results (Fig. 2) showed that cell-free extracts from E. coli harbouring pHGB9, which encodes full-length EGE, expressed a protein of Mr 84000 which bound to both Avicel and amorphous cellulose at 0 °C, but not to xylan. The protein could be eluted with distilled water, exhibited endoglucanase activity at 60 °C and was precipitated with anti-EGE antiserum. When the binding experiments were repeated at 60 °C, similar results were obtained. By contrast, E. coli cells harbouring pJH2, which encodes EGEa, failed to synthesize a protein which bound to insoluble cellulose or xylan. Similarly, purified EGEa did not interact with either polysaccharide (results not shown).

To locate more precisely the position of the CBD in EGE, derivatives of celE encoding truncated forms of the enzyme were constructed as described in the Materials and methods section (Fig. 1). The capacity of the EGE derivatives to bind cellulose was investigated. The data (Fig. 3) revealed that pJH6, which encodes EGEa, a truncated form of EGE lacking the 110 C-terminal residues, directed the synthesis of a protein of Mr 72000

**Fig. 2. Binding of EGE to polysaccharides**

Cell-free extracts of E. coli cells harbouring pHGB9 and pJH2 are shown in lanes 1 and 6 respectively. Protein eluted with distilled water from E. coli protein/Avicel mixtures, incubated at 0 °C, is shown in lanes 2 (pHGB9) and 7 (pJH2). Lane 3 contains enzyme released from a pHGB9 cell-free extract/Avicel suspension incubated at 60 °C. Bound proteins eluted with distilled water from mixtures containing E. coli (harbouring pHGB9) proteins incubated with amorphous cellulose and xylan, at 0 °C, are shown in lanes 4 and 5 respectively.

**Fig. 3. Binding of truncated derivatives of EGE to Avicel**

Enzyme eluted from Avicel with distilled water, and proteins present in cell-free extracts, are shown in lanes 1 and 2 respectively. Proteins were prepared from E. coli cells harbouring pHGB9 (a), pJH6 (b), pJH4 (c) and pJH5 (d).

**Fig. 4. Activity of native and truncated forms of EGE against β-glucan**

Native EGE (O) and the truncated derivatives EGEb (●) and EGEa (■) were assayed for β-glucanase activity at different substrate concentrations.
which bound to Avicel, could be eluted from the polysaccharide with distilled water and hydrolysed carboxymethylcellulose at 60 °C. EGEc, a derivative of EGE from which the 246 C-terminal amino acids had been deleted, did not interact with Avicel. EGEb, in which residues 367–432 of mature EGE had been deleted, bound to crystalline cellulose and could be eluted from the polysaccharide in an active form with distilled water. EGEb contains only the eight C-terminal residues of the second 24-aa duplicated sequence which is highly conserved in other C. thermocellum endoglucanases (Hall et al., 1988). These data indicate that the region of EGE which confers the ability to bind to cellulose is located between residues 432 and 671 and is distinct from the active site. Furthermore, the conserved sequence does not play an important role in either cellulose binding or catalytic activity.

Kinetic properties of native and truncated derivatives of EGE

Previous studies showed that EGE is very active against barley β-glucan. The role of the CBD in the hydrolysis of this substrate by EGE was investigated. The data (Fig. 4) revealed that EGEa, which lacks the CBD, had a specific activity of 75.4 units/mg of protein, compared with 12.96 units/mg of protein for EGE synthesized by E. coli harbouring the entire celE gene. The full-length endoglucanase displayed typical Michaelis–Menten kinetics, with an apparent $K_{m}$ of 1.2 mg/ml for β-glucan. A similar result was obtained for the enzyme EGEb, which did not contain the conserved sequence, but retained the CBD. By contrast, EGEa, which lacks the CBD, did not exhibit Michaelis–Menten kinetics at low substrate concentration, where very low activity was observed. These data suggest that the CBD plays a role in catalysis only at low substrate concentration.

DISCUSSION

It is now widely accepted that the multienzyme cellulase complex of C. thermocellum, termed the cellulosome, is composed of 14–18 endoglucanases and includes one major subunit (S1) which is apparently devoid of enzyme activity. It has been suggested (Lamed & Bayer, 1988) that this subunit is a cellulose-binding protein which functions in anchoring the other components of the cellulosome and accounts for the high affinity of the complex for crystalline cellulose. At least one study (Wu et al., 1988) has demonstrated that a component with properties very similar to S1 acts in concert with a smaller endoglucanase to slowly hydrolyse Avicel. If indeed the S1 subunit mediates the attachment of other cellulose components to cellulose, there is no obvious requirement for the individual endoglucanases of the cellulosome to contain CBDs distinct from the active site. In the present study we have shown that EGE, which is a component of the cellulosome (Hazlewood et al., 1990), contains a CBD that is distinct from the catalytic domain. Our data suggest that the binding of the cellulosome to cellulose is not the exclusive property of a single component, such as the S1 subunit, but results from the sum of the affinities for cellulose of individual enzymes like EGE. At present it is unclear how many of the cellulosome components have CBDs, but in this context, Grépinet et al. (1988) showed that the 508 N-terminal residues of xylanase Z (another proven component of the cellulosome) are not essential for catalytic activity. By analogy with EGE, it would seem entirely possible that the N-terminal domain of xylanase Z constitutes a substrate-binding domain, even though it shows no sequence similarity to the CBD of EGE.

The present study shows that deletion of the reiterated 24-aa acid sequence, conserved in other C. thermocellum endoglucanases and xylanase Z, does not affect the interaction of EGE with cellulose. This argues against the notion, suggested by Grépinet & Béguin (1986), that the conserved sequence could be a CBD.

An increase in cellulase specific activity against soluble substrates, such as we observed on removing the CBD from EGE, has been previously reported for an endoglucanase from Thermomonospora fusca (Ghangas & Wilson, 1988), but was not apparent for cellulosomes from Trichoderma reesei (Tomme et al., 1988) or Bacteroides succinogenes (McGavin & Forsberg, 1989). In vivo, there is some evidence that EGE exists both as a full-length and a truncated form (M1, 43000) which lacks the CBD (Hazlewood et al., 1990). Knowles et al. (1988) have suggested that the proteolytic removal of carbohydrate-binding domains from cellulosomes and other polysaccharide-degrading enzymes, such as glucoamylase, may be an important mechanism for the modification of their substrate specificities during the course of hydrolysis of complex insoluble substrates. Once the substrate has been degraded to short chain-length soluble oligosaccharides, enzymes with reduced affinity for the original highly polymeric substrate, but with enhanced activity against the soluble breakdown products, would be available. Micro-organisms can thus increase the diversity of enzymes expressed while maintaining a limited number of genes. Alternatively, it could be argued that the primary role of EGE in cellulolysis is to hydrolyse soluble substrates. Thus, early in cellulolysis, when the concentration of soluble substrate (cellulo-oligosaccharides) is low, possession of a CBD distinct from the active site improves the affinity of EGE for its substrate. As digestion proceeds, increased levels of soluble cellulo-oligosaccharides are released and EGE no longer requires the CBD, which is removed through proteolysis, resulting in higher activity against the depolymerized cellulose and shorter-chain intermediates.

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