Calcium-binding affinity and calcium-enhanced activity of *Clostridium thermocellum* endoglucanase D

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Clostridium thermocellum endoglucanase D (EC 3.2.1.4; EGD), which is encoded by the *celD* gene, was found to bind Ca^{2+} with an association constant of $2.03 \times 10^6 \text{ M}^{-1}$. Ca^{2+} stimulated the activity of EGD towards swollen Avicel by 2-fold. In the presence of Ca^{2+} , the K_d of the enzyme towards *p*-nitrophenyl- β -D-cellobioside and carboxymethylcellulose was decreased by 4-fold. Furthermore, Ca^{2+} increased the half-life of the enzyme at 75 °C from 13 to 47 min. Since the 3' sequence of *celD* encodes a duplicated region sharing similarities with the Ca^{2+} -binding site of several Ca^{2+} -binding proteins, a deleted clone was constructed and used to purify a truncated form of the enzyme which no longer contained the duplicated region. The truncated enzyme was very similar to EGD expressed from the intact gene with respect to activity, Ca^{2+} -binding kinetics and Ca^{2+} effects on substrate binding and thermostability. Thus the latter parameters do not appear to be mediated through the duplicated conserved region.

INTRODUCTION

Clostridium thermocellum, a Gram-positive thermophilic bacterium, ranks among the most efficient cellulolytic micro-organisms (Johnson *et al.*, 1982). The cellulose-degrading activity resides in a multienzyme complex, the cellulosome (Lamed *et al.*, 1983). This thermostable cellulase complex has an M_r of 2.1×10^6 and contains 14–18 different polypeptides.

Ca^{2+} is known to increase by 9-fold the activity of the cellulosome with microcrystalline cellulose, and by 3-fold the activity with amorphous cellulose (Lamed & Bayer, 1988). Furthermore, the predicted sequences of *C. thermocellum* endoglucanases A, B, D, E and xylanase Z all contain conserved, duplicated segments of 24 amino acids each (Grépinet *et al.*, 1988; Hall *et al.*, 1988). The first 12 residues of the conserved segments bear significant resemblance to the EF-hand class of Ca^{2+} -binding sites (Kretsinger, 1980) of various Ca^{2+} -binding proteins (Fig. 1).

Therefore it was of interest to investigate whether Ca^{2+} affects individual cellulosome components, and, if so, whether Ca^{2+} effects are mediated through the conserved, duplicated segment which is similar to the Ca^{2+} -binding site of Ca^{2+} -binding proteins.

This paper reports that endoglucanase D (EC 3.2.1.4; EGD), which is known to be part of the cellulosome (Lamed & Bayer, 1988), showed strong binding to Ca^{2+} , and that properties such as activity towards swollen Avicel, the K_d with *p*-nitrophenyl- β -D-cellobioside (*p*-NPC) and carboxymethylcellulose (CMC), and thermostability were influenced by Ca^{2+} . However, very similar properties were also found for a truncated form of EGD which was purified from a clone whose conserved, duplicated sequence had been deleted.

Fig. 1. Comparison between the first 12 amino acids of the duplicated, conserved segments in endoglucanases A, B, D and E and xylanase Z of *C. thermocellum* and EF hand-type Ca^{2+} -binding sites

A, consensus of repeated segments in *C. thermocellum* enzymes (Grépinet *et al.*, 1988); B, α -b chain of sarcoplasmic reticulum Ca^{2+} -binding protein from penaeid shrimp (Takagi & Konishi, 1984); C, myosin EDTA regulatory light chain from scallop (Kendrick-Jones & Jakes, 1977); D, human calpain (Ohno *et al.*, 1986); E, spinach calmodulin (Lukas *et al.*, 1984). Residues of the *C. thermocellum* consensus that are present in the majority of the duplicated segments are in capitals. Minority residues of the consensus which are present at least twice are in lower case. The shaded areas include residues identical with the *C. thermocellum* consensus; the lighter areas include residues with similar chemical properties (N, Q; R, K; T, S; E, D).

Abbreviations used: EGD, endoglucanase D; *p*-NPC, *p*-nitrophenyl- β -D-cellobioside; CMC, carboxymethylcellulose; PMSF, phenylmethanesulphonyl fluoride; PAGE, polyacrylamide-gel electrophoresis.

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MATERIALS AND METHODS

Bacterial strain

Escherichia coli TG1 (Wain-Hobson *et al.*, 1985) carrying plasmid pCT603 or pCT605/607/608 was grown in Luria Bertani medium (Maniatis *et al.*, 1982) at 37 °C in the presence of 100 µg of carbenicillin/ml.

Plasmid constructions

All restriction endonucleases were purchased from Amersham or Boehringer-Mannheim and T4 DNA ligase was from Boehringer-Mannheim. The Klenow fragment of DNA polymerase was from Amersham, and was used as recommended by the suppliers. The *Hind*III fragment of the pCT603 plasmid (Joliff *et al.*, 1986a) was re-cloned in pUC19 to yield pCT605 (Fig. 2). Like pCT603, pCT605 carried the coding sequence of *celD* fused in frame with the first codons of the *lacZ* gene present in the vector. The *Hinc*II fragment containing the 3' end of *celD* carrying the duplicated, conserved segment was deleted from pCT605 to yield pCT607. In order to restore a stop codon close to the 3' end of the truncated gene, pCT607 was cleaved with *Sal*I, the 3' recessed ends were filled with the Klenow fragment of DNA polymerase and the plasmid was recircularized to yield pCT608. The frame shift occurring at the level of the filled-up *Sal*I site

Fig. 2. Construction of *celD* plasmids

MCS, multiple cloning site; E, *Eco*RI; H3, *Hind*III; H2, *Hinc*II; Sa, *Sal*I; PI, *Pvu*I. The conserved, duplicated region is hatched. The name of each plasmid is underlined. The nucleotide sequence of the *Hinc*II site in *celD* gene carried by pCT605 and of the 3' end of *celD* carried by pCT607 and pCT608 is indicated on the right. Nucleotides corresponding to the *celD* sequence are in bold type.

(which became a *Pvu*I site) placed a TAG stop codon in frame with *celD* 10 bp downstream from the end of the truncated gene (Fig. 2). The construction was verified by cleaving with *Pvu*I and by dideoxynucleotide sequencing (Chen & Seeburg, 1985).

Purification of EGD and of C-terminal-deleted EGD

Except for the heat treatment, all operations were performed at 4 °C. EGD was isolated from cytoplasmic granules of *E. coli* TG1 (pCT603) as described previously (Joliff *et al.*, 1986b). For the purification of truncated EGD from *E. coli* TG1 (pCT608), the procedure described for the cytoplasmic fraction of EGD (Joliff *et al.*, 1986b) was used, up to and including heat treatment at 60 °C, except that the crude extract contained 20 µg of phenylmethanesulphonyl fluoride (PMSF)/ml. The heat-treated extract was adjusted with 1 M-potassium phosphate buffer (pH 6.6) to 0.5 M-potassium phosphate. The extract was then loaded on a Phenyl-Sepharose (Pharmacia) column (3 cm × 10.5 cm) previously equilibrated with 0.5 M-potassium phosphate buffer (pH 6.6). The resin was washed with 0.15 M-potassium phosphate (pH 6.6) until the A_{280} returned to the baseline level, and was then eluted with 10 mM-potassium phosphate/30% (v/v) ethylene glycol at a flow rate of 20 ml/h. Active fractions were pooled and concentrated by ultrafiltration on an Amicon YM 10 membrane. The truncated EGD was further purified by gel filtration. A column (1.5 cm × 85 cm) was filled with Ultrogel AcA44 (LKB) and equilibrated with 40 mM-Tris/HCl buffer (pH 7.1). The resin was eluted with the same buffer at a flow rate of 12 ml/h. Active fractions were analysed by SDS/polyacrylamide-gel electrophoresis (PAGE) (Laemmli, 1970), and fractions showing the highest purity were pooled.

Enzyme assays

The protein concentration was determined by using the Coomassie Blue G-250 binding assay (Sedmak & Grossberg, 1977) with bovine serum albumin as a standard. Activity towards *p*-NPC (Sigma) was measured by incubating the enzyme at 60 °C in 0.75 ml of PC buffer (50 mM- K_2HPO_4 /12.5 mM-citric acid, pH 6.3) containing 1.1 mM-*p*-NPC. The reaction was stopped by adding 0.25 ml of 1 M- Na_2CO_3 , and the release of *p*-nitrophenol (*p*-NP) was measured at 410 nm. One unit of activity is defined as the amount of enzyme liberating 1 µmol of *p*-NP/min. Carboxymethylcellulase was measured by incubating endoglucanases in 50 mM-Mes buffer, pH 6.0, containing 0.5% (w/v) CMC (Sigma, medium viscosity) for 15 min at 60 °C. The reaction was stopped by the addition of 2.0 ml of Somogyi reagent (Somogyi, 1952) and the mixtures were then boiled for 15 min. After cooling, 2 ml of Nelson reagent (Nelson, 1944) was added and mixed with care, followed by 4.0 ml of water. Reaction mixtures were centrifuged at 5000 g_{max} for 5 min before reading the absorbance of the supernatant at 520 nm. Glucose (0–200 µg) was used as standard. One unit of activity corresponds to one µmol of glucose equivalent liberated/min. Hydrolysis of phosphoric acid-swollen Avicel (Wood, 1971) was measured by incubating EGD in 10 ml of 50 mM-Mes, pH 6.0, containing 3 mg of acid-swollen Avicel (grade PH 101) at 60 °C in the presence or absence of 7 mM- $CaCl_2$. The decrease in turbidity (%) was measured by reading the absorbance at 660 nm. Total sugar in the supernatant was estimated

by the phenol/H₂SO₄ method, with glucose as a standard (Dubois *et al.*, 1956).

***K_d* and *V_{max}* values towards *p*-NPC and CMC**

The *K_d* and *V_{max}* values of purified enzymes were measured at different concentrations of *p*-NPC in 50 mM-Mops buffer, pH 6.3, containing 10 mM-EGTA or 1 mM-CaCl₂. The *K_d* and *V_{max}* towards CMC were determined in 50 mM-Mes buffer, pH 6.0, in the presence or absence of 7 mM-CaCl₂. A concentration of 1 mM-CaCl₂ was found to be saturating with *p*-NPC, but not with CMC (results not shown), presumably because the latter acts as a chelator.

Thermostability

Purified enzyme was incubated at 75 °C in 50 mM-Mops buffer, pH 6.3, containing 1 mM-EGTA or 1 mM-CaCl₂. Samples were taken at different times and assayed for activity at 60 °C in the presence of 1.1 mM-*p*-NPC/1 mM-CaCl₂/50 mM-Mops buffer, pH 6.3. The half-life of the enzyme was determined from semi-logarithmic plots.

Ca²⁺-binding assay

Ca²⁺ binding was assayed using a chelating resin (Wasserman *et al.*, 1968). Aliquots (10 mg) of Chelex-100 400 mesh resin (Bio-Rad) were equilibrated with increasing volumes (0.1–0.8 ml) of 50 mM-Tris/HCl, pH 7.5, containing 0.1 μM-CaCl₂ and 10⁶–10⁷ c.p.m. of ⁴⁵Ca/ml (Amersham, 500–700 Ci/mol). For a duplicated assay, 4 × 25 μl of the suspension were used. The buffer was eliminated by centrifugation. Purified protein (20 μl of 1–2 mg/ml solution) was added to two samples and 20 μl of Tris/HCl buffer was added to the two others. After 5 min at room temperature, the tubes were centrifuged for 2 min at 10000 *g_{max}*. Free ⁴⁵Ca was counted in the supernatant of tubes containing buffer, and total (bound + free) ⁴⁵Ca was counted in the supernatant of tubes containing protein. The number of sites and the binding constant were calculated from Scatchard plots.

Computer methods for detecting similarities with EF-hand calcium-binding domain

The sequences of endoglucanases A, B, D and E and xylanase Z were scanned using the PROSITE program (Bairoch, 1989) from the PC/Gene sequence analysis package (available from Intelligenetics Inc. or Genofit SA, Geneva, Switzerland).

RESULTS

Purification of truncated endoglucanase D

The purification of the truncated EGD was achieved starting from *E. coli* TG1 (pCT608) carrying the *celD* gene with a deletion of 233 bp at the 3' end and a terminator codon close to the new 3' end. The purification scheme previously devised for the purification of EGD expressed from the intact gene carried by pCT603 (Joliff *et al.*, 1986b) had to be modified. Extraction of cytoplasmic granules with urea followed by dialysis failed to yield active enzyme. Furthermore, the truncated form of EGD lost activity after ammonium sulphate precipitation. Hence the enzyme was purified from the cytoplasmic supernatant and the ammonium sulphate precipitation step was replaced by hydrophobic chromatography on Phenyl-Sepharose followed by a gel filtration on AcA44. Specific activities and yields at various stages of purification are summarized in Table 1, and analysis of the various fractions by SDS/PAGE is shown in Fig. 3. About 75% of *E. coli* TG1 (pCT608) proteins were eliminated by the heat treatment. Specific activity increased by 3-fold after the Phenyl-Sepharose step. After gel filtration on AcA44, a single band of *M_r* 62000 was detectable by gel electrophoresis. Western blotting with anti-EGD antiserum confirmed that the *M_r*-62000 band was the truncated EGD (results not shown). With 1.1 mM-*p*-NPC as substrate, the purified truncated EGD had a specific activity of 1.57 units/mg, similar to that of purified EGD (1.52 units/mg). The *M_r* of 62000 found for the truncated EGD was close to the *M_r* expected from the sequence of the truncated gene (63000). However, the *M_r* of 65000 of EGD expressed from *E. coli* TG1 (pCT603) was significantly lower than the *M_r* of 71300 expected from the sequence of the whole gene (Joliff *et al.*, 1986a,b). The C-terminal analysis of the *M_r*-65000 EGD indicated that the enzyme undergoes proteolytic cleavage, with the major proteolysis site occurring between Leu-605 and Lys-606 (results not shown). EGD ending at Leu-605 (referred to as EGD in the following) had an expected *M_r* of 66400 and contained most of the first duplicated segment of the conserved sequence, including one of the putative Ca²⁺-binding sites. The latter was absent from the sequence of the truncated EGD encoded by pCT608.

Comparative properties of EGD and truncated EGD

Fig. 4 shows Scatchard plots for the binding of Ca²⁺ to

Table 1. Purification of truncated EGD produced by *E. coli* TG1 (pCT608)

Activity was assayed in PC buffer (50 mM-K₂HPO₄/12.5 mM-citric acid, pH 6.3) containing 1.1 mM-*p*-NPC as a substrate.

Step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Crude extract	118	1097	114	0.10	100
Streptomycin sulphate supernatant	110	792	108	0.14	95
Heat-treated extract	94	197	76	0.39	67
Phenyl-Sepharose column effluent	32	92	116	1.27	102
Phenyl-Sepharose column effluent (concentrated)	1.6	51	45	0.88	39
AcA44 column effluent	11	30	47	1.57	41

Fig. 3. Electrophoretic monitoring of the purification of the truncated EGD

Electrophoresis was performed in a 10% polyacrylamide gel in the presence of 0.1% SDS. Lane a, crude extract (33 µg of protein); lane b, streptomycin sulphate supernatant (32 µg of protein); lane c, heat-treated extract (11 µg of protein); lane d, Phenyl-Sepharose column effluent (6 µg of protein); lane e, Phenyl-Sepharose column effluent, concentrated (6 µg of protein); lane f, AcA44 column effluent (5 µg of protein). Standard proteins of known M_r were as follows: phosphorylase *b*, 92 500; EGD expressed from *E. coli* TG1 (pCT603) carrying the intact *celD* gene, 65 000; ovalbumin, 45 000; carbonic anhydrase, 31 000; soybean trypsin inhibitor, 21 500; lysozyme, 14 300.

Fig. 4. Scatchard analysis for the equilibrium Ca^{2+} -binding data of EGD ($\square$) and truncated EGD ($\blacklozenge$)

Each point is the average of duplicate determinations. For both enzymes, the number of Ca^{2+} -binding sites per molecule is 1.9 and the association constant for calcium is $K_a = 2.03 \times 10^6 \text{ M}^{-1}$.

EGD expressed from intact or truncated *celD*. From the linear portion of the plot, it was calculated that both enzymes bound 1.9 Ca^{2+} ions per molecule, with an association constant of $2.03 \times 10^6 \text{ M}^{-1}$. Kinetic properties of EGD and truncated EGD are reported in Table 2. For both enzymes, activity on swollen Avicel was similar and was increased by about 2-fold in the presence of Ca^{2+} . EGD and truncated EGD had similar K_d and $V_{\max}$ values with both *p*-NPC and CMC. Their affinity for *p*-NPC and CMC was enhanced by about 4-fold by Ca^{2+} , with little effect on the $V_{\max}$. Furthermore, Ca^{2+} stabilized both forms of EGD at 75 °C, the thermostability being somewhat lower with the truncated enzyme in presence of Ca^{2+} .

DISCUSSION

Ca^{2+} was found to bind to EGD with high affinity. Using bovine serum albumin as standard to determine

Table 2. Effects of Ca^{2+} on activity parameters of EGD and truncated EGD

Activity parameters were assayed as described in the Materials and methods section. Hydrolysis of swollen Avicel was performed for 18 h in the presence of one carboxymethylcellulase unit of enzyme.

Enzyme	Substrate...	Swollen Avicel		<i>p</i> -NPC		CMC		
		ΔA_{660} (%)	Soluble sugars released (µg)	K_d (mM)	$V_{\max}$ (units/mg)	K_d (mM)	$V_{\max}$ (units/mg)	Half-life at 75 °C (min)
EGD	− Ca^{2+}	16.2	249	10.7	17.2	0.68	520	13
	+ Ca^{2+}	31.3	531	2.7	14.0	0.17	692	47
Truncated EGD	− Ca^{2+}	15.9	278	11.1	17.9	0.72	515	14
	+ Ca^{2+}	29.5	492	2.5	17.9	0.16	594	26

EGD concentration, about two Ca²⁺-binding sites were found per molecule. Ca²⁺ increased the thermostability of EGD and decreased its K_d for *p*-NPC and CMC. Since the enzyme retained activity even in the absence of Ca²⁺, the simplest explanation of these effects is that Ca²⁺ stabilizes a conformation having a higher affinity for the substrate and a higher thermostability. Among other endoglucanases, the carboxymethylcellulase activity of EGC, but not EGA or EGB, was found to be stimulated by 1.5-fold in the presence of Ca²⁺ (L. A. Gow, unpublished work).

Deletion of the conserved, duplicated region of EGD did not affect the activity of the enzyme towards swollen Avicel, *p*-NPC or CMC. Similar results were obtained for EGE (Hall *et al.*, 1988) and xylanase Z (Grépinet *et al.*, 1988), showing that the conserved, duplicated domain is not involved in the catalytic site.

Furthermore, truncated EGD was found to have similar Ca²⁺-binding properties and to be influenced by Ca²⁺ in the same manner as EGD. Therefore, Ca²⁺-binding and Ca²⁺ effects on thermostability and substrate-binding affinity are not mediated by the portion of the conserved region remaining in EGD expressed from *E. coli* TG1 (pCT603). The latter still contains one of the putative Ca²⁺-binding sites. However, since the full-length gene product of *celD* could not be isolated, it cannot be excluded that the full integrity of the sequence is required for the proper folding of the putative Ca²⁺-binding sites. Therefore the data do not preclude the possibility that the two conserved segments might function as additional Ca²⁺-binding sites in the intact protein.

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