Dissociation of the Cellulosome of *Clostridium thermocellum* in the Presence of Ethylenediaminetetraacetic Acid Occurs with the Formation of Truncated Polypeptides[†,‡]

Sang Ki Choi[§] and Lars G. Ljungdahl*

Department of Biochemistry and Molecular Biology, Center for Biological Resources Recovery, University of Georgia, Athens, Georgia 30602-7229

Received October 17, 1995; Revised Manuscript Received February 8, 1996[⊗]

ABSTRACT: The cellulosome of *Clostridium thermocellum* JW20 consists of 14−26 different polypeptides as determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis. The intact cellulosome hydrolyzes crystalline cellulose in the presence of Ca^{2+} and thiols. This activity is inhibited by ethylenediaminetetraacetic acid (EDTA). Ca is incorporated into the cellulosome and is tightly bound as demonstrated using ^{45}Ca added to the growth medium. Upon incubation in 50 mM Tris (pH 7.5), 0.1 M NaCl, and 5 mM EDTA at 37 °C, Ca is released from the cellulosome, which disintegrates into polypeptides. The SDS−PAGE pattern of cellulosomal polypeptides is remarkably different after the EDTA treatment when compared to this pattern of untreated cellulosomes. Polypeptide bands corresponding to molecular masses of 160, 98, 76, and 54 kDa disappear, and new bands of masses 150, 132, 91, 71, 57, and 46 kDa appear. N-terminal analyses of the 98, 76, 91, and 71 kDa polypeptides show that the 91 and 71 kDa polypeptides are truncated products of the 98 and 76 kDa polypeptides, respectively. The 76 and 71 kDa polypeptides correspond to CelS [Wang, W. K., Kruus, K., & Wu, J. H. D. (1993) *J. Bacteriol. 175*, 1293−1302]. The 71 kDa polypeptide is formed from the 76 kDa polypeptide during the EDTA treatment, by a cleavage that occurs at asparagine residue 681. It involves the removal of 60 amino acid residues from the C-terminal end. All catalytic subunits so far characterized contain an asparagine residue corresponding to residue 681 of CelS. This residue is part of the conserved duplicated region found in catalytically active subunits, and it is postulated that several of these subunits also are truncated by the EDTA treatment. The polypeptides truncated by the EDTA treatment had reduced Ca binding capacities compared to their native subunits, indicating a Ca-binding site within the conserved duplicated region. This region has been implicated in the binding of the catalytic peptides to the scaffolding polypeptide CipA, which is a structural protein of the cellulosome and has a strong affinity for calcium.

Cellulolytic enzymes have been intensely studied for utilization of naturally abundant cellulose resources for the production of valuable fuels and chemicals (Eriksson et al., 1990; Béguin & Aubert, 1994). Cellulolytic fungi and aerobic bacteria secrete several different cellulolytic enzymes which exist separately (Coughlan & Ljungdahl, 1988; Coughlan & Mayer, 1992). However, cellulolytic anaerobic bacteria tend to secrete complexes of cellulases observable on the cell surface (Lamed et al., 1987). An example is the cellulase complex or cellulosome produced by the thermophilic anaerobic bacterium *Clostridium thermocellum* (Bayer & Lamed, 1986; Mayer et al., 1987). It is composed of perhaps as many as 26 different subunits (Kohring et al., 1990). Its mass has been estimated to be from 2×10^6 to 6.5×10^6 Da (Lamed et al., 1983; Wu et al., 1988). The cellulosome exists also as a polycellulosome with a mass calculated from 50×10^6 to 80×10^6 Da (Coughlan et al., 1985). The hydrolysis of crystalline cellulose by the cellulosome is dependent on dithiothreitol (DTT) and calcium ions (Johnson & Demain, 1984; Hon-nami et al., 1986).

The cellulosome is too stable to be disrupted by urea, guanidine hydrochloride, and various detergents (Lamed et al., 1983; Ljungdahl et al., 1988). Treatment of the cellulosome with sodium dodecyl sulfate (SDS)[1] in the presence of EDTA and thiols has been found to be the most effective way to dissociate it (Bhat & Wood, 1992; Mori, 1992; Wu et al., 1988). The difficulty of dissociation of the cellulosome has hindered the isolation of its subunits and the study of its quaternary structure. It must be noted that treatment with SDS leads to almost complete loss of ability to hydrolyze crystalline cellulose, but with little or no loss of endoglucanase activity. A few polypeptides have been obtained from the cellulosome after SDS treatment. One of them, CelA, which has endoglucanase activity has been purified in a form

[†] Supported by Contract DE-FG05-93ER20127 from the U.S. Department of Energy. L.G.L. was supported by the Georgia Power Company through a Distinguished Professorship in Biotechnology.

[‡] A preliminary abstract has been published in *Abstracts Am. Soc. Microbiol. 94th Gen. Meeting*, May 1994, p 295, abstract K-110.

* Corresponding author's address: Department of Biochemistry and Molecular Biology, Center for Biological Resource Recovery, University of Georgia, Life Sciences Building, Athens, GA 30602-7229. Phone: (706) 542-7640. Fax: (706) 542-2222. E-mail: Ljungdah@bscr.uga.edu.

[§] Present address: Laboratory of Molecular Genetics, Building 6B, Room 326, NICHD, National Institutes of Health, Bethesda, MD 20892-2785.

[⊗] Abstract published in *Advance ACS Abstracts,* April 1, 1996.

[1] Abbreviations: CDR, conserved duplicated region (Dockerin); CMC, carboxymethylcellulose; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; IRE, internally repeated element (Cohesin); SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; YAS, yellow affinity substance.

0006-2960/96/0435-4897$12.00/0

which shows anomalous migration on SDS gels (Mori, 1992). Another cellulosomal polypeptide is CelS which has been isolated in a truncated, but catalytically active, form after treatment of the cellulosome with protease K (Morag et al., 1991). CelS, also known as S_s, has, in addition, been obtained in a protein aggregate from SDS-treated cellulosomes (Wu et al., 1988). This aggregate combined with the largest polypeptide of the *C. thermocellum* cellulosome, previously designated S_L but now called CipA (cellulosome-integrating protein), has some activity toward hydrolysis of crystalline cellulose (Wu et al., 1988). It was the first successful demonstration of the ability of two cellulosomal fractions interacting to degrade crystalline cellulose cooperatively.

A structure proposed for the cellulosome was initially based on electron microscopic observations (Mayer et al., 1987; Mayer, 1988). They showed the cellulosome to consist of several rows of subunits, which were parallel to the major axis. The subunits seem to be of similar size and aligned with similar space between them. It was proposed that, when the cellulosome binds to cellulose, subunits arranged in a row cut the cellulose chain simultaneously at several places, forming cellodextrines. This proposal has recently obtained support by the result that CipA is a structural but nonenzymatic protein of the cellulosome and that it binds several catalytically active subunits (Fujino et al., 1993). Fibers, which were observed in electron microscopic projections to be interconnecting the active subunits, are now considered to constitute CipA or other scaffolding proteins. CipA has been sequenced (Gerngross et al., 1993). It has a mass of 196 800 Da and contains one cellulose binding domain (CBD) and nine internally repeated elements (IREs), each of which is composed of 146 amino acid residues. The IREs are involved in the interaction with the catalytically active cellulosomal subunits (Fujino et al., 1992). CipA interacts also with a protein called ORF3p, which may bind CipA to the cell surface of *C. thermocellum* (Fujino et al., 1993).

In addition to CipA and ORF3p, several genes encoding cellulolytically active polypeptides of the *C. thermocellum* cellulosome have been cloned into *Escherichia coli* and sequenced (Béguin, 1990; Felix & Ljungdahl, 1993). Many of the gene products have been expressed and characterized and have been found to be components of the cellulosome. They include CelA (Béguin et al., 1985), CelB (Grepinet & Béguin, 1986), CelD (Joliff et al., 1986), CelE (Hall et al., 1988), CelF (Navarro et al., 1991), CelG (Lemaire & Béguin, 1993), CelH (Yagüe et al., 1990), CelS (Wang et al., 1993), CelX (Hall et al., 1988), and the xylanase XynZ (Grepinet et al., 1988). All of these cellulosomal subunits have conserved duplicated regions, CDR1 and CDR2, which are each composed of 22−24 amino acid residues and are linked together by 8−15 amino acid residues (Béguin, 1990). In most of the subunits, the CDRs are close to the C-terminal, although in CelE and XynZ, they are in the middle of the protein sequences. Evidence that binding of the catalytic subunits to CipA involves the CDR and IRE domains has been presented (Tokatlidis et al., 1991; Fujino et al., 1993; Salamitou et al., 1994). The CDR domain is not essential for the catalytic activity of the subunits (Hall et al., 1988). It should be noted that an endoglucanase, CelC of *C. thermocellum*, does not contain the CDR domain and that it apparently is not a part of the cellulosome (Schwarz et al., 1988). Bayer et al. (1994) have named the CDR domain "Dockerin", the IRE domain "Cohesin", and the CipA type of subunit "Scaffoldin".

In this paper, we report that the cellulosome dissociates (disintegrates) with elimination of calcium by treatment with EDTA. Several of the dissociated proteins were obtained in catalytically active forms, but truncated, missing part of the CDR1 and the complete CDR2 sequences. We propose that calcium plays an important role in the integrity of the cellulosome. This is discussed further in the following paper (Choi & Ljungdahl, 1996).

MATERIALS AND METHODS

Bacterial Growth. *C. thermocellum* JW20, described by Freier et al. (1988), was grown at 60 °C under an atmosphere of nitrogen in prereduced medium (Wiegel & Dykstra, 1984) but with the modification that 1.5 g/L $NaHCO_3$ was used instead of 0.5 g/L. Avicel PH-101 (FMC Corp., Newark, DE) (1%, w/v) was used as a carbon source. Cultures were harvested after 3 days of growth when the cellulose particles were coated with the yellow affinity substance (YAS), forming YAS−cellulose (Ljungdahl et al., 1988). To label the cellulosome with ^{45}Ca, 2.23 mg of $CaCl_2$ containing 1 mCi ^{45}Ca (Amersham Life Science, Arlington Heights, IL) was added per 7 L of calcium-free medium.

Cellulolytic Activities. *endo-*β-1,4-Glucanase (EC 3.2.1.4) [carboxymethyl cellulase (CMCase)] activity was assayed at 60 °C with a solution of 1% (w/v) CMC (low viscosity) (Sigma, St. Louis, MO) in 50 mM Bis-Tris (pH 5.5). Reducing sugars produced were assayed by the method of Miller et al. (1960), using glucose as a standard. Crystalline cellulose hydrolyzing activity (avicelase) under aerobic conditions was determined by incubation at 60 °C for 3−5 h of 100 μL of enzyme solution with 900 μL of 1% (w/v) Avicel in 50 mM Bis-Tris (pH 5.5) containing 7 mM $CaCl_2$. Reducing sugars produced were determined with tetrazolium blue reagent, using glucose as a standard (Jue & Lipke, 1985). Avicelase activity under anaerobic conditions was measured as described by Nolte and Holzenburg (1990). The enzyme solution was added to a suspension of 4% Avicel in 100 mM Bis-Tris (pH 5.5) containing 7 mM $CaCl_2$ and 10 mM DTT. The assay mixture was flushed with nitrogen for 5 min and then incubated at 60 °C for 3−5 h. Cellobiose and glucose, the major products, were determined spectrometrically using β-glucosidase (Boehringer, Mannheim) and glucose dehydrogenase (Sigma) in a coupled assay. Cellobiose was used as a standard. One unit of enzyme activity is the amount that releases 1 μmol of glucose equivalent per minute at 60 °C.

Purification of the Cellulosome. Cellulosomes were purified as previously described (Ljungdahl et al., 1988). YAS−cellulose particles in the culture were allowed to settle. The culture fluid containing bacteria and soluble proteins was siphoned off and discarded. The YAS−cellulose with bound enzyme was washed with 100 mM triethanolamine maleate (pH 6.85) to remove residual bacteria and proteins. The washed YAS−cellulose with the cellulosome complex attached was packed to form a column, and the cellulosomes were eluted with distilled water. Fractions with avicelase activity were pooled and concentrated with an Amicon ultrafiltration apparatus (XM50). The crude cellulosome fraction was centrifuged at 6000*g* for 60 min and further purified using a Sepharose 4B (2.6 × 85 cm) column

(Pharmacia), equilibrated with 50 mM Tris (pH 7.5) containing 0.1 M NaCl. Cellulosomes labeled with ^{45}Ca were purified with the same method. Radioactivity of ^{45}Ca was determined with a scintillation counter (Beckman).

Dissociation of the Cellulosome Using EDTA. The cellulosome was dissociated aerobically by incubation in a solution containing 50 mM Tris (pH 7.5), 0.1 M NaCl, and 5 mM EDTA at 37 °C for 4−144 h. The extent of dissociation was followed by measurement of the disappearance of the intact cellulosome eluting on a Superose 6 column (10 × 30 cm, Pharmacia), by SDS−PAGE, and by measurement of avicelase activity. The effect of protease inhibitors on the dissociation was tested by addition of from 0.1 to 1 mM phenylmethanesulfonyl fluoride (PMSF), benzamidine, pepstatin, aprotinin, or antipain (Boehringer) together with 5 mM EDTA to the cellulosome solution. Protease activity of the cellulosome was checked by a protease detection kit using resorufin-labeled casein (Calbiochem, San Diego, CA) as the substrate. Reassociation of the dissociated cellulosome was tested by addition of $CaCl_2$ or $MgCl_2$ to the dissociated cellulosome and by incubation for several days. Two proteins of 46 and 71 kDa with cellulolytic activities were purified from the dissociated cellulosome by conventional methods using gel filtration and ion exchange chromatography (Choi & Ljungdahl, 1996).

Molecular Mass Estimation by Mass Spectrometry and Metal Analysis. The 71 kDa protein was analyzed by electrospray ionization mass spectrometry (ESIMS) on a Finnigan TSQ700 triple quadruple mass spectrometer (Harvard Microchem facility). Metal content in the cellulosome was determined with plasma emission spectrophotometry (Jarrell-Ash 965 ICP).

Determination of C-Terminal and N-Terminal Amino Acid Sequences. For determination of the C-terminal sequence of the 71 kDa protein, a 420 pmol aliquot was derivatized with iodoacetamide and then digested with trypsin (enzyme: substrate ratio of 1:20, for 12 h at 37 °C) according to described protocol (Stone et al., 1989). Digests were checked for complete hydrolysis using a reverse phase HPLC RP-300 cartridge from Brownlee Lab Aquapore (Foster City, CA). Elution was with a gradient from 0.1% trifluoroacetic acid (TFA) in water to 0.08% TFA in acetonitrile for 60 min at a flow rate of 0.2 mL/min. The trypsin-digested 71 kDa protein (200 μL total volume, pH adjusted to 5.0) was loaded onto a 1 mL anhydrotrypsin agarose column (Takara Biochemicals Inc., Berkeley, CA) equilibrated with 50 mM sodium acetate (pH 5.0) containing 20 mM $CaCl_2$. The column was washed using 20 mL of the same buffer. Anhydrotrypsin is catalytically inert but selectively binds peptides that have Arg or Lys at their C-terminal. Therefore, the wash contained only the C-terminal peptide as confirmed by high-performance liquid chromatography (HPLC). The sequence of the peptide was determined.

To determine N-terminal sequences of the 98 and 76 kDa polypeptides of the cellulosome, and the 91 and 71 kDa polypeptides of the dissociated cellulosome, the proteins on SDS−polyacrylamide gel were transblotted to a polyvinylidene difluoride (PVDF) membrane in a Bio-Rad transblot apparatus at 30 V for 16 h at 4 °C with transfer buffer containing 192 mM glycine, 25 mM Tris (pH 8.3), and 20% (v/v) methanol (Towbin et al., 1979). The polypeptides were excised with a razor blade after being stained with Ponceau S. N-terminal amino acid sequence analyses of these samples were performed with an Applied Biosystems 477 A gas phase sequencer equipped with an automatic on-line phenylthiohydrantoin derivative analyzer.

Table 1: Elements Present in the Cellulosome of *C. thermocellum*[a]

element	cellulosome (mol/mg)
Ca	47 (193)
Cu	1 (4)
Si	3 (12)
Zn	4 (16)
Fe	1 (6)
P	768 (3075)

[a] Cellulosomes (1.4 mg/mL) were dialyzed against three changes of 1 L of 50 mM Tris-HCl (pH 7.5) at 4 °C for 19 h. Element content was determined by plasma emission spectroscopy. The number in parentheses represents the moles of metal per mole of cellulosome when the molecular mass of the cellulosome was estimated as 4 million. The results represent determinations of three different cellulosome preparations. The variation was less than 10%.

Protein Determination and Gel Electrophoresis. Protein was measured using either the Micro bicinchonic acid (BCA) method (Pierce, Rockford, IL) or the Rose Bengal method (Elliot & Brewer, 1978) using bovine serum albumin as a standard. SDS−polyacrylamide gel electrophoresis (SDS−PAGE) was performed by the method of Laemmli (1970).

Calcium Binding to Immobilized Protein. To detect Ca binding to protein, a ^{45}Ca-blotting technique was used (Maruyama et al., 1984). Proteins from the SDS−PAGE (7.5%) were transferred by electrophoresis to a PVDF membrane as described before. The membrane was washed with 10 mM imidazole (pH 6.8), 60 mM KCl, and 5 mM $MgCl_2$ for 1 h and then incubated with ^{45}Ca (1 μCi/mL) for 10 min in the same buffer. The blot was washed three times with deionized water for 5 min, allowed to dry in air for 3 h at room temperature, and then exposed to X-ray film for 24 h.

RESULTS

Purification of the Cellulosome and Its Composition. C. thermocellum when growing on cellulose produces a yellow compound (YAS) which attaches to the cellulose, forming YAS−cellulose. In the presence of 0.005 mM or higher concentrations of salt or buffers, the cellulosome has a high affinity for YAS−cellulose. Residual YAS−cellulose from 2−3-day-old cultures is therefore a very convenient source of cellulosomes which can be easily eluted from the YAS−cellulose using distilled water as the solvent. The process described earlier (Ljungdahl et al., 1988) yields an essentially pure cellulosomal preparation at a yield of about 5 mg per 17 L of culture when 1% cellulose is the substrate. The cellulosomes were further purified by Sepharose 4B column chromatography. They emerged as a single protein complex with a mass estimated between 2 and 5 million Da. Analysis using SDS−PAGE revealed the usual pattern for pure cellulosomes of 14 major bands, plus at least 10 minor bands confirming previous results (Lamed et al., 1983; Kohring et al., 1990). The 197, 98, and 76 kDa polypeptides represent the predominant subunits. The 197 kDa CipA is the largest subunit. It appeared as a broad band on SDS−PAGE, maybe due to a high content of carbohydrate (Gerwig et al., 1989).

The cellulosome contains Ca, Cu, Si, Zn, Fe, and P as determined with plasma emission spectroscopy (Table 1). Calcium, known to be required for crystalline cellulase

Table 2: Effect of Chelating Agents on Cellulolytic Activities of the Cellulosome[a]

chelating agent	concn (mM)	CMCase (%)	avicelase (%)
o-phenanthroline	2	90	96
	10	73	76
8-hydroxyquinoline	2	102	100
	10	87	84
EDTA	1	106	32
	5	100	28
EGTA	1	115	28
	5	112	28
2,2′-dipyridyl	5	98	80
	20	90	92

[a] Cellulosomes (1.4 mg/mL) were incubated aerobically in 50 mM Tris (pH 7.5) and 0.1 M NaCl including chelating agents as given for 20 h at 37 °C. Endoglucanase (CMCase) activity and crystalline cellulolytic (avicelase) activity were determined under aerobic conditions. Enzyme activity is expressed as percent of activity obtained without a chelating agent.

activity (Johnson & Demain, 1984; Hon-nami et al., 1986), was the major metal present. Assuming the mass of the cellulosome to be 4×10^6 Da, it contained about 190 mol of Ca per mole of cellulosome. Most of the Ca, 73%, was tightly bound to the cellulosome. This was ascertained by incubation of ^{45}Ca-labeled cellulosomes with 14 mM unlabeled $CaCl_2$ at pH 7.5 and 37 °C for 24 h. During the first hour, 27% of the ^{45}Ca exchanged with unlabeled Ca. The rest of the ^{45}Ca was stable and did not exchange during the 24 h of incubation. The presence of phosphorus was unexpected and not further investigated. It could be present as a counterion, but it has been reported that cardiolipin is present in the cellulosome (Bolobova & Zhukov, 1991).

Cellulolytic Activity of the Cellulosome and Effect of Chelating Agents. The cellulolytic activity of the cellulosome is inhibited by EDTA (Johnson et al., 1982). The effects of preincubation of the cellulosome at pH 7.5 for 20 h at 37 °C with several chelating agents, including EDTA, are shown in Table 2. Although *o*-phenanthroline and 8-hydroxyquinoline at high concentrations are slightly inhibitory, they did not affect endoglucanase and avicelase activities very much. The endoglucanase activity was not, or very little, affected by EDTA and EGTA. In contrast, the activity toward crystalline cellulose was decreased 70% by the treatment with EDTA and EGTA. Given that Ca is the most prominent metal in the cellulosome and that *o*-phenanthroline has high affinity for iron and zinc, 2,2′-dipyridyl for iron and 8-hydroxyquinoline for copper, iron, and zinc (Sillen & Martell, 1964), it appears that the target of EDTA and EGTA was calcium which is chelated by these agents.

A time course of the effect of EDTA on the cellulolytic activities of the cellulosome is given in Figure 1. It shows that the endoglucanase activity was not affected even after 48 h of EDTA treatment. The avicelase activity, however, was decreased immediately upon addition of EDTA to 70% and then further to about 20% of the original activity during the next 4 h of incubation. As shown in Figure 1, when the avicelase was assayed under anaerobic conditions, a higher activity was obtained compared with that obtained under aerobic assay conditions. Still, only 40% of the original activity remained, after about 12 h of EDTA treatment. It should be noticed that during the first 3 h of the treatment there was almost complete recovery of the avicelase activity when assays were done anaerobically.

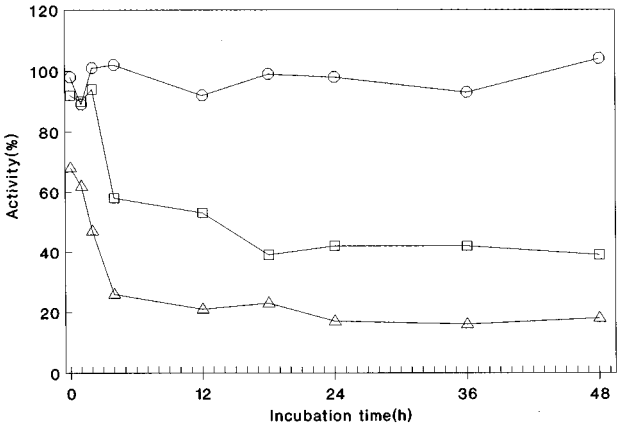

FIGURE 1: Cellulase activity of EDTA-treated cellulosomes. Cellulosomes were incubated at 37 °C in 50 mM Tris, 0.1 M NaCl, and 5 mM EDTA. Samples were taken at times indicated for assays of cellulase activities. CMCase activity (○); avicelase activity was measured using aerobic conditions in the presence of 7 mM $CaCl_2$ (△) and under anaerobic conditions in the presence of 7 mM $CaCl_2$ and 10 mM DTT (□).

The EDTA treatment strikingly changed the SDS−PAGE pattern of the cellulosome as shown in Figure 2. This change seems to correlate with the time course of the decrease in crystalline cellulolytic activity as was shown in Figure 1. Several protein bands disappeared, and new bands of lower masses appeared. Most noticeable was the disappearance of bands corresponding to masses of 160, 98, 76, and 54 kDa and the appearance of new bands of masses 150, 132, 91, 57, and 46 kDa. It should also be noted that some new bands of masses, smaller than 46 kDa, appeared. The disappearance of the two major subunits (98 and 76 kDa) seems to correlate with the appearance of two new major subunits (91 and 71 kDa). The process is slow and proceeds over a period of 48 h. Changes in other polypeptides (54 and 46 kDa) may occur within 4 h of the EDTA treatment.

The decrease in the mass of several of the polypeptides during the EDTA treatment could be caused by proteolysis. A protease could be a contaminant or constituent of the cellulosome. To test this, the EDTA treatment was carried out in the presence of several protease inhibitors: PMSF, benzamidine, pepstatin, aprotinin, and antipain. None of these affected the EDTA treatment. In addition, we were not able to detect a cellulosomal protease during incubation of the cellulosome with resorufin-labeled casein and EDTA for 48 h. These results suggest that there is no cleavage reaction by a protease during the EDTA treatment.

Effect of Chelation of Calcium on Dissociation of the Cellulosome. The decrease of the cellulosomal activity in the hydrolysis of crystalline cellulose by the EDTA treatment could be due to a removal of Ca, or the fact that the cellulosomes may have dissociated, or both. To test this, cellulosomes containing ^{45}Ca were prepared by growing *C. thermocellum* on 1% cellulose in medium with $^{45}CaCl_2$. The cellulosomes and EDTA-treated cellulosomes were chromatographed on a Superose 6 column (Figure 3). It is shown in Figure 3A that the cellulosomes before the EDTA treatment eluted as an intact protein (OD_{280}) complex coinciding with crystalline cellulase activity, and ^{45}Ca. After incubation of the cellulosomes in 50 mM Tris (pH 7.5), 0.1 M NaCl, and 5 mM EDTA at 37 °C for 12 h, the elution profile on the Superose column changed noticeably (Figure 3B). The protein fraction b eluted at a place very close to

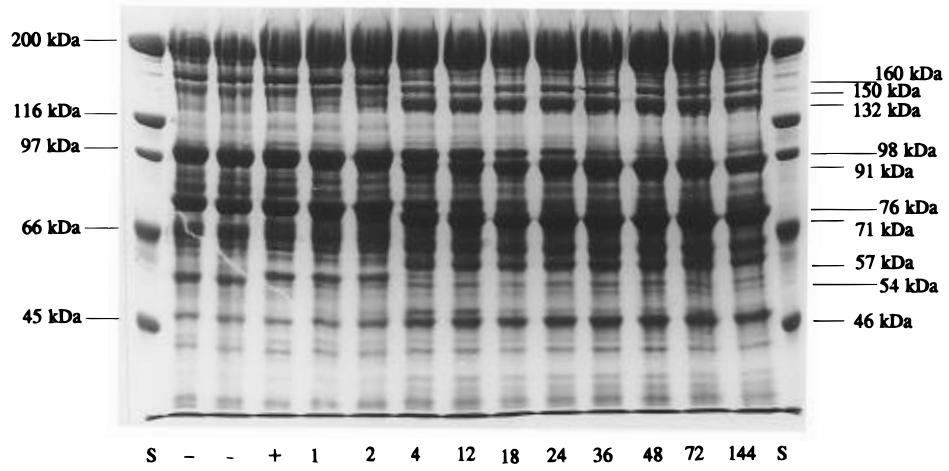

FIGURE 2: Analysis by SDS−PAGE (7.5%) of cellulosomal components of samples obtained as indicated during incubation with EDTA. The samples were those of Figure 1. Staining was with Coomassie blue. The numbers at the bottom of the gel represent the incubation time (in hours). + indicates cellulosome containing EDTA without incubation; − indicates control samples without EDTA (two samples were used, one stored frozen at 20 °C for 1 month and the second stored at room temperature for 2 weeks); S indicates molecular marker proteins. Long lines at the right side of the gel indicate bands disappearing during incubation; short lines indicate new bands appearing during incubation.

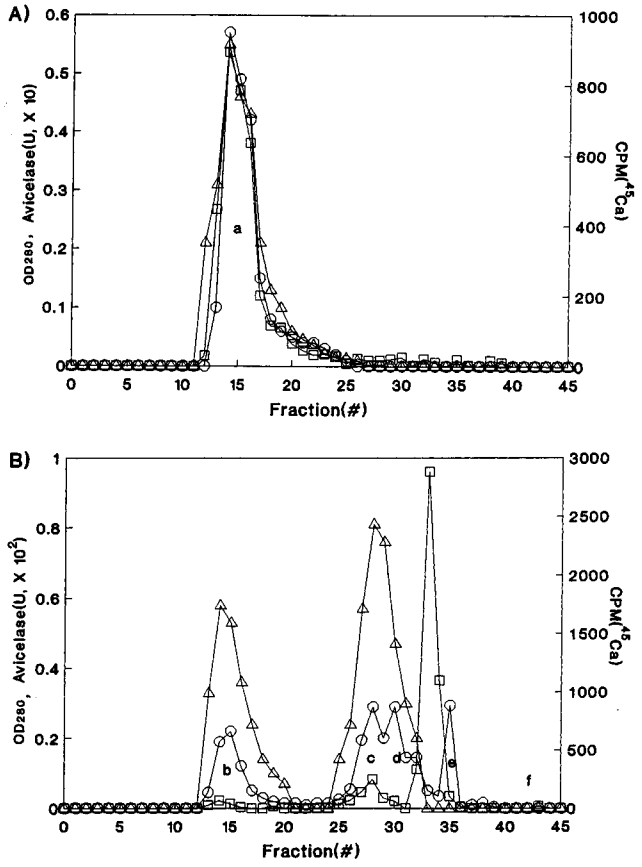

FIGURE 3: Gel filtration profiles of native cellulosomes and EDTA-treated cellulosomes. ^{45}Ca-labeled cellulosomes were fractionated on a Superose 6 column with 50 mM Tris (pH 7.5) and 0.1 M NaCl at a flow rate of 0.5 mL/min and a fraction size of 0.6 mL. The protein elution profile was monitored by A_{280} absorbance (○): ^{45}Ca (□) and avicelase activity (△). (A) Elution profile of ^{45}Ca-labeled native cellulosomes (280 μg). (B) Elution profile of EDTA-treated ^{45}Ca-labeled cellulosomes (280 μg). In panel B, the cellulosomes had been incubated for 12 h in elution buffer with 5 mM EDTA at 37 °C before being applied to the Superose 6 column. An enzyme unit is expressed in micromoles of reducing sugar formed per minute.

that of the cellulosomes (fraction a, Figure 3A). It contained 11% of the avicelase activity of the intact cellulosomes, about

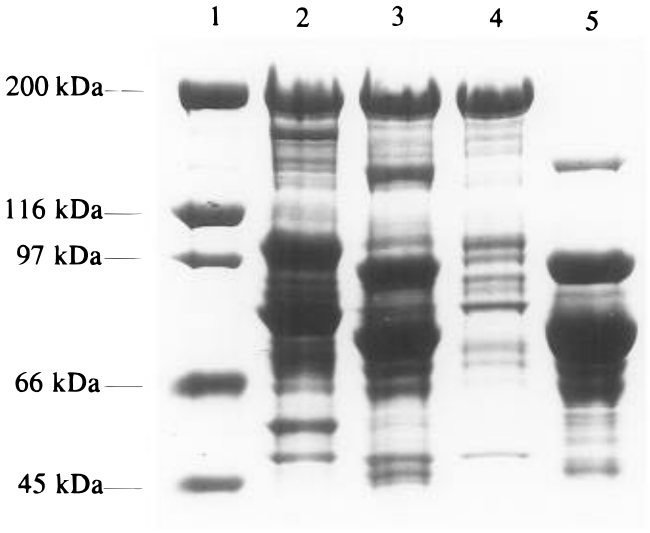

FIGURE 4: SDS−PAGE (7.5%) of fractions corresponding to peaks b−d separated by gel filtration chromatography as performed in Figure 3B: lane 1, molecular mass markers; lane 2, cellulosomes before EDTA treatment; lane 3, EDTA-treated cellulosomes; lane 4, concentrated fractions (13−19); lane 5, concentrated fractions (26−31).

30% of the protein, and almost no ^{45}Ca. Several new protein fractions appeared. Fractions c and d were mixtures of polypeptides. Combined they contained the major part of the protein, about 16% of the original avicelase activity, and a small amount of ^{45}Ca. About 90% of ^{45}Ca was recovered after peak d and was shown to be an EDTA−^{45}Ca complex. Free ^{45}Ca would have eluted at place f as indicated. Fraction e consisted of a mixture of smaller polypeptides. As will be discussed later, they may be C-terminal portions of truncated proteins of catalytically active subunits formed during the EDTA treatment.

The fractions obtained from the Superose 6 column of Figure 3B were analyzed using SDS−PAGE (Figure 4). Lane 2 represents the intact cellulosome before the EDTA treatment and lane 3 the cellulosome after this treatment. The results are completely in agreement with those previously shown in Figure 2. The two major bands of the intact cellulosome representing subunits of masses 98 and 76 kDa

disappeared, whereas two new major bands of masses 91 and 71 kDa appeared. The SDS−PAGE analysis of peak b in Figure 3B shows that it no longer represents the intact cellulosome (lane 4, Figure 4). It consists mainly of the 197 kDa subunit CipA, plus low amounts of other polypeptides. It should be noted that the 197 kDa subunit appears unaltered by the EDTA treatment. The Superose 6 fractions c and d, when combined, contain the 91 and 71 kDa subunits (lane 5, Figure 4) as major components and other subunits. The CipA subunit is not present in this fraction. The results shown in Figures 3 and 4 clearly show that the EDTA treatment involves the breaking up of the cellulosome, yielding a mixture of polypeptides that can be purified and characterized. The observation that the EDTA treatment leads to proteins of apparent lower masses suggests that the cellulosomal subunits have been truncated or structurally changed.

The conditions required for the dissociation of the cellulosome by the EDTA treatment were investigated by using fractionation on the Superose 6 column. Variations in concentrations of EDTA (1−20 mM), ionic strength [5−750 mM Tris-HCl (pH 7.5)], and pH (6.8−8.8) did not change significantly the result of the EDTA treatment. However, incubation temperature had a significant effect. The dissociation occurred well at 37 or 48 °C and very slowly at 4 °C, and it was dependent on aerobic conditions. It did not proceed anaerobically. Addition of Mg^{2+} (2−5 mM) or Ca^{2+} (2−5 mM) did not lead to any reassociation of the cellulosomes after dissociation with EDTA.

Identification of Dissociated Proteins by N-Terminal and C-Terminal Sequencing and the Demonstration That the 76 kDa Polypeptide Had Lost a Peptide of 60 Amino Acid Residues at the C-Terminal during the EDTA Treatment. The dissociation phenomenon could involve the formation of truncated polypeptides. To investigate this, we compared the 98 and 76 kDa polypeptides of the cellulosome with the 91 and 71 kDa proteins of the dissociated cellulosome. As shown in Figure 2, it seems that the 98 and 76 kDa polypeptides shift to the 91 and 71 kDa polypeptides, respectively, during the EDTA treatment. This conclusion is based on the gradual change of band densities upon SDS−PAGE with time of EDTA treatment. Therefore, N-terminal sequences of the four proteins were determined. Results are given in Figure 5, which shows that the 98 and 91 kDa proteins have the same N-terminal sequence, as have the 76 and 71 kDa proteins. This strongly indicates the conversion of the 98 and 76 kDa proteins to the 91 and 71 kDa proteins, respectively. Recently, the CelS component of the *C. thermocellum* ATCC 27405 cellulosome was cloned and sequenced. As shown in Figure 5, it has the same N-terminal sequence as the 76 and 71 kDa subunits (Wang et al., 1993). The molecular mass of CelS is 80 670 Da as deduced from the amino acid sequence. We feel that the CelS polypeptide corresponds to the 76 kDa subunit of our cellulosomal preparation. A truncated protein (S8-tr) which was identified as cellobiohydrolase has been isolated from the cellulosome after treatment with protease K (Morag et al., 1993). The S8-tr protein has been identified as a product of CelS by peptide mapping (Morag et al., 1993). The CelS obtained as a recombinant protein from *E. coli* was recently shown to be an exoglucanase (Kruus et al., 1995).

The 98 kDa polypeptide, like the 76 kDa CelS protein, is a major component of the cellulosome. A search for the

Protein		Sequence
98 kDa[a]		L E D K S(S)K L P D Y K N D L(L)Y E
91 kDa[b]		L E D K S(S)K L P D Y K N
76 kDa[a]		G P T K A P T K D(G)
71 kDa[b]		G P T K A P T K D G T X Y K D L F X E
CelS[c]		G P T K A P T K D G T S Y K D L F L E
46 kDa[b]		A G V P F N T X Y P Y G P X
CelA[d]		A G V P F N T K T P Y G P T
71 kDa[e]	(674)	T K L Y G D V N (681)
CelS[e]	(674)	T K L Y G D V N D D G K V N S T D
		A V A L K R Y V L R S G I S I N T
		D N A D L N E D G R V N S T D L G
		I L K R Y I L K E I D T L P Y K N (741)

FIGURE 5: N-terminal and C-terminal sequences of cellulosomal proteins. Footnotes: a, protein obtained from intact cellulosomes; b, protein obtained from EDTA-treated cellulosomes; c, CelS is a 80 670 Da protein from *C. thermocellum* ATCC 27405 (Wang et al., 1993); d, CelA consists of 488 amino acids with a mass of 52 503 Da [it was obtained from *C. thermocellum* NCIB 10682 (Béguin et al., 1985)]; e, C-terminal amino acid residues of the 71 kDa protein (Thr-674 to Asn-681) and CelS (Thr-674 to Asn-741). Parentheses represent uncertainty, and X represents an undetermined amino acid.

N-terminal sequence of the 98 and 91 kDa proteins using the Swiss-Prot and other data banks failed to find an absolute matching sequence among the proteins of *C. thermocellum* that have been sequenced. Thus, it is possible that the 98 kDa polypeptide has not been recognized during the extensive genetic work with *C. thermocellum* (Hazlewood et al., 1988; Béguin, 1990; Gilkes et al., 1991) and may constitute an additional subunit of the cellulosome. However, a 1,4-β-cellobiosidase from *C. thermocellum* (GenBank accession no. X80993 submitted by V. V. Zverlov, unpublished) contains the sequence LEDNSSTLPPYKNDLLYE, which is similar to the N-terminal of the 98 kDa subunit (Table 2). The exceptions are three amino acids underlined in the sequence. From the sequence data, this cellobiosidase has a mass of 138 077 Da. It is therefore unlikely that the 98 kDa subunit is the same as the 138 kDa cellobiosidase.

Figure 2 shows that it is likely that the 54 kDa subunit is converted to the 46 kDa subunit during the EDTA treatment. The N-terminal sequence of the 46 kDa subunit matches that of CelA (Figure 5) (Béguin et al., 1985). CelA has previously been isolated from the broth of *C. thermocellum* culture (Petre et al., 1981) and also from the cellulosome (Mori, 1992). The *cel*A gene has been sequenced and found to encode a protein of 488 amino acids with a mass of 52 500 Da (Béguin et al., 1985). The mass reported for CelA obtained by SDS−PAGE analysis varies between 48 and 56 kDa. We feel that the 54 kDa protein observed on the SDS−PAGE of Figure 2 corresponds to CelA and that the 46 kDa is a truncated form of CelA.

Upon examination of Figure 2, it is noticeable that, when a protein band disappears as a result of the EDTA treatment, there follows the appearance of a new protein band of from 5 to 8 kDa less mass. The results indicate that many of the cellulosomal subunits undergo a similar degradation during the EDTA treatment. The results of the N-terminal analysis (Figure 5) demonstrate that a cleavage does not involve the N-terminal part.

To investigate if a cleavage occurs at the C-terminal end, the 71 kDa protein was purified and analyzed by mass spectrometry to accurately assess its molecular mass. It was

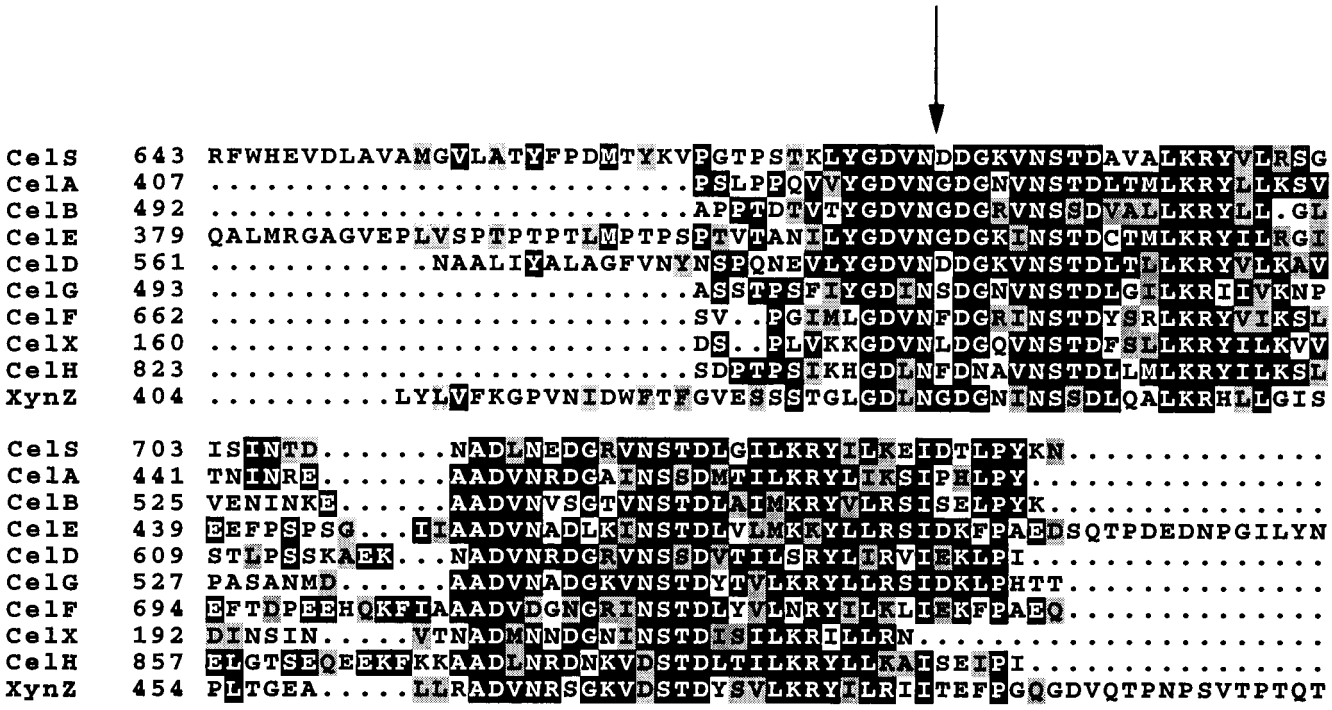

FIGURE 6: Alignment of the conserved duplicated regions (CDRs) of enzymatically active subunits of the cellulosome of *C. thermocellum*. For CelS, the CDR1 includes residues 676−700 and the CDR2 residues 710−734. The cellulosomal subunits are assumed to be cleaved during dissociation by treatment of EDTA at a specific site after N-681 as found with CelS. The cleavage site of CelS is marked with an arrow. Lightly shaded residues represent residues having similar chemical properties, and darkly shaded residues are identical

found to be 73 694 Da, indicating, by subtraction from the native CelS having a mass of 80 670 Da, that a peptide of 6976 Da was cleaved off. This was checked by determination of the C-terminal end of the 71 kDa protein. Its sequence was L-Y-G-D-V-N, which corresponds to the amino acid sequence 676−681 of CelS. Thus, the sequence data demonstrate that the carboxyl end of the 71 kDa protein is 60 amino acid residues shorter than that of CelS as was deduced from the gene sequence and that the C-terminal cleavage occurred after Asn-681 (Figure 5). This 60-amino acid sequence corresponds to a mass of 6645 Da, which fits well with the assays of the molecular masses for the 76 and 71 kDa polypeptides.

Genetic analyses of a number of genes encoding cellulosomal proteins from *C. thermocellum* have revealed that these proteins, which are enzymatically active subunits, have the two reiterated domains CDR1 and CDR2, at the C-terminal end (Figure 6). For CelS, CDR1 includes amino acid residues 676−700 and CDR2 residues 710−734. These domains have been postulated to be involved in binding the catalytically active subunits to the scaffolding subunit CipA (Tokatlidis et al., 1993; Salamitou et al., 1994; Yaron et al., 1995). The cleavage of CelS at the C-terminal end by the EDTA treatment occurs at Asn-681. This is a residue of the CDR1 domain which is conserved in all cellulosomal polypeptides listed in Figure 6. We postulate that the EDTA treatment affects all of these subunits forming truncated polypeptides of from 59 to 78 amino acids shorter than the corresponding peptides of the intact cellulosome. It was mentioned earlier that after the EDTA treatment many subunits appear to have from 5 to 8 kDa less mass than when analyzed without the EDTA treatment. Thus, the EDTA treatment causes the cellulosome to disintegrate rather than dissociate. Apparently, it involves a cleavage to form truncated subunits. That this cleavage occurs within the CDR1 domain supports the idea that the CDR domains are involved in the binding of the subunits to the CipA. The cleavage may require the intact cellulosome or at least that the subunits are bound to CipA. Thus, the 76 and 98 kDa subunits obtained from SDS−PAGE by electroelution when treated with EDTA did not undergo cleavage.

A search was done to find the C-terminal ends cleaved off from the cellulosomal polypeptides during the EDTA treatment. The fraction representing peak e in Figure 2B could harbor these C-terminal ends. It was concentrated and subjected to SDS−PAGE (10 to 20% gradient). Western blots were performed with anti-bCDR1 and anti-bCDR2 antibodies prepared as described by Choi and Ljungdahl (1996). With anti-bCDR2, five bands having masses 18, 25, 30, 38, and 46 kDa were detected. With anti-bCDR1, two bands with masses of 25−38 kDa were found. It was expected that these peptides should be about 7 kDa, but they are larger. It should be noted that the difference in mass between the peptides is about 7 kDa, which may suggest that they interact and form aggregates. Although these results do not confirm the formation of C-terminal peptides of 7 kDa by the EDTA treatment, they demonstrate the presence of small peptides in the cellulosome after the treatment. However, the analyses using N-terminal and C-terminal sequences provide the evidence of formation of 7 kDa peptides due to the treatment with EDTA.

Calcium Binding Properties of Cellulosome Components. The treatment of the cellulosome with EDTA disintegrates the cellulosome with formation of truncated subunits. It also involves the removal of calcium. The possibility that the truncated subunits were less prone to bind calcium was investigated. The results are shown in Figure 7. Although the truncated subunits bind calcium as demonstrated in lane 4, it is obvious by comparison of lanes 3 and 4 that the polypeptides after the EDTA treatment bind less Ca. This

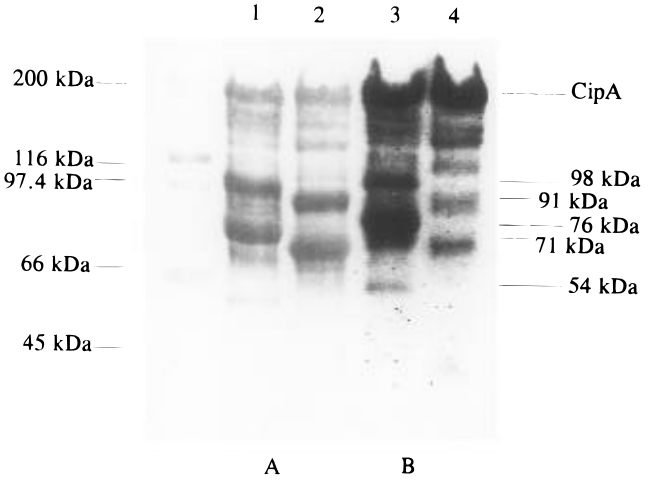

FIGURE 7: Calcium binding by cellulosomal components of the cellulosome before and after EDTA treatment. Proteins separated by SDS gel (7.5%) were transblotted to PVDF membranes and overlaid with 0.1 mCi ^{45}Ca per 100 mL of buffer as described in Materials and Methods. Lanes 1 and 3 represent the cellulosome before EDTA treatment and lanes 2 and 4 the cellusome after this treatment for 12 h. (A) Coomassie blue staining of the SDS gel. (B) Autoradiography of the ^{45}Ca-overlaid membrane. Long lines indicate CipA, 98, 76, and 54 kDa bands of the cellulosome before EDTA treatment, and short lines represent the 91 and 71 kDa bands of the dissociated cellulosome.

is especially seen when the 98 and 76 kDa subunits of lane 3 are compared with the 91 and 71 kDa truncated subunits. The results demonstrate that the CDR domains have Ca-binding properties. That the truncated subunits bind Ca was not surprising since it has been shown that endoglucanase CelD from *C. thermocellum* contains three Ca-binding sites which do not involve the CDR domains (Juy et al., 1992; Chauvaux et al., 1995). All of our attempts to reassociate the cellulosome after the extensive EDTA treatment failed. This can now be explained since both the CDR domain and Ca would be involved in the attachment of the catalytically active subunit to the scaffolding subunit CipA. It should be noticed that the CipA subunit apparently binds as much Ca after the EDTA treatment as it does before this treatment. In addition, some other subunits larger than 116 kDa are shown to bind a substantial amount of Ca. The roles of these subunits of the cellulosome have not been determined, but it is possible that they serve a function similar to that of CipA.

DISCUSSION

Electron microscopic (Mayer et al., 1987; Mayer, 1988) and genetic studies (Fujino et al., 1993; Gerngross et al., 1993) have revealed that the cellulosome from *C. thermocellum* has a highly ordered structure. Structural proteins and catalytically active subunits form a very stable complex, which withstands most denaturing agents and conditions (Lamed et al., 1983; Ljungdahl et al., 1988). This has made it difficult to obtain individual subunits from the cellulosome for studies of their catalytic and other properties. Only CelA and a truncated form of CelS have been directly obtained from the cellulosome (Mori, 1992; Morag et al., 1993). Other cellulosomal components have been obtained as extracellular protein aggregate (Wu et al., 1988) or as recombinant proteins expressed in *E. coli*, other bacteria, or yeast.

One goal of this investigation was to find a mild method for dissociation of the cellulosome into subunits or subcellulosomal complexes. We considered the possibility that Ca could be involved in the stabilization of the cellulosome. This was based on reports that the hydrolytic activity toward crystalline cellulose is dependent on Ca^{2+} and is almost completely inhibited by EDTA and that this inhibition is reversed more effectively by Ca than by any other metal (Johnson et al., 1982, 1984; Hon-nami et al., 1986) A further indication was the demonstration of Ca-binding sites of CelD (Juy et al., 1992; Chauvaux et al., 1995). Our findings that Ca is the major metal of the cellulosome (Table 1) and that it is tightly bound support the idea that Ca plays a structural role in the cellulosome. It may in addition have a catalytic role, since free Ca^{2+} greatly stimulated crystalline cellulase activity.

Treatment of the cellulosome with EDTA released the tightly bound Ca of the cellulosome which then dissociated with the formation of several truncated polypeptides. The formation of truncated polypeptides was unexpected. It shows that the EDTA treatment with removal of Ca is not a simple dissociation. It is an irreversible process involving the disintegration of the cellulosome. The SDS−PAGE analyses of the polypeptides of the cellulosome after the EDTA treatment demonstrated that several of them moved on the gel with a lower molecular mass of about 7 kDa (Figure 2). Prominent examples were the polypeptides of 98 and 76 kDa, which after the EDTA treatment moved as 91 and 71 kDa polypeptides, respectively. The 76 kDa polypeptide identified as CelS after N-terminal sequencing (Wang et al., 1993) was cleaved at the C-terminal after asparagine residue 681, yielding the 71 kDa polypeptide, which was 60 amino acid residues shorter than the 76 kDa subunit. The asparagine 681 is part of the CDR1 domain which has been suggested to be involved in the binding of the catalytic subunits of the cellulosome to the scaffolding subunit CipA (Fujino et al., 1993; Salamitou et al., 1994; Yaron et al., 1995). As shown in Figure 5, an asparagine residue corresponding to asparagine residue 681 of CelS is present in all of the catalytically active subunits of the cellulosome so far analyzed. We suggest that all of them are cleaved by the EDTA treatment in a way similar to that of CelS.

At the present time, we do not know the mechanism for the cleavage reaction. It occurs in the presence of several proteinase inhibitors, and a search for a proteinase was negative. Thus, we feel the cleavage reaction which occurs aerobically is nonenzymatic. There are examples of specific cleavages of proteins catalyzed by metal−EDTA complexes. These reactions require O_2 and are thought to involve oxygen or hydroxyl radicals (Kim et al., 1985; Ermácora et al., 1992; Stadtman, 1993). These radicals can be generated by the oxidation of a transition metal via the Fenton reaction. Calcium is not considered a transition metal, and if involved, it would be a first example of a protein cleavage involving an EDTA−calcium complex. The possibility still exists that copper, iron, or zinc which are present in low amounts in the cellulosome (Table 1), could be involved in the cleavage reaction. However, the truncated polypeptides have less Ca-binding capacity than their parental subunits, which shows that the C-terminal ends with the CDR domains have Ca-binding sites.

From the results presented by Wu et al. (1988), it has been suggested that CipA interacts with the catalytic subunits and is required for the hydrolysis of crystalline cellulose by the

cellulosome. As shown in Figure 3B, Avicel is hydrolyzed, albeit at a lower rate than the intact cellulosome, by fractions c and d which do not contain CipA. This indicates that CipA is not required for the hydrolysis of crystalline cellulose. It is possible, however, that fractions c and d may contain scaffolding proteins other than CipA.

ACKNOWLEDGMENT

The authors thank Professor Carlos R. Felix, Universidade de Brasília, Dr. Nanda Menon, Dr. Alan Przybyla, and Dr. Xinliang Li for valuable advice during the investigation, and also Dr. John K. Wunderlick for N-terminal sequencing.

REFERENCES

Bayer, E. A., & Lamed, R. (1986) *J. Bacteriol. 167*, 828−836.
Bayer, E. A., Morag, E., & Lamed, R. (1994) *Trends Biotechnol. 12*, 379−386.
Béguin, P. (1990) *Annu. Rev. Microbiol. 44*, 219−248.
Béguin, P., & Aubert, J.-P. (1994) *FEMS Microbiol. Rev. 13*, 25−58.
Béguin, P., Cornet, P., & Aubert, J.-P. (1985) *J. Bacteriol. 162*, 102−105.
Bhat, K. M., & Wood, T. M. (1992) *Carbohydr. Res. 227*, 293−300.
Bolobova, A. V., & Zhukov, A. V. (1991) *Biokhimiya 56*, 1304−1311.
Chauvaux, S., Souchon, H., Alzari, P. M., Chariot, P., & Béguin, P. (1995) *J. Biol. Chem. 270*, 9757−9762.
Choi, S. K., & Ljungdahl, L. G. (1996) *Biochemistry 35*, 4906−4910.
Coughlan, M. P., & Ljungdahl, L. G. (1988) *FEMS Symp. 43*, 11−30.
Coughlan, M. P., & Mayer, F. (1992) in *The Prokaryotes* (Balows, A., Trüper, H. G., Dworkin, M., Harder, W., & Schleifer, K.-H., Eds.) 2nd ed., pp 460−516, Springer-Verlag, Berlin.
Coughlan, M. P., Hon-nami, K., Hon-nami, H., Ljungdahl, L. G., Paulin, J. J., & Rigsby, W. E. (1985) *Biochem. Biophys. Res. Commun. 130*, 904−909.
Elliott, J. I., & Brewer, J. M. (1978) *Arch. Biochem. Biophys. 190*, 351−357.
Eriksson, K.-E. L., Blanchette, R. A., & Ander, P. (1990) *Microbial and Enzymatic Degradation of Wood and Wood Components*, Springer-Verlag, Berlin.
Ermácora, M. R., Delfino, J. M., Cuenoud, B., Schepartz, A., & Fox, R. O. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 383−387.
Felix, C. R., & Ljungdahl, L. G. (1993) *Annu. Rev. Microbiol. 47*, 791−819.
Freier, D., Mothershed, C. P., & Wiegel, J. (1988) *Appl. Environ. Microbiol. 54*, 204−211.
Fujino, T., Beguin, P., & Aubert, J.-P. (1992) *FEMS Microbiol. Lett. 94*, 165−170.
Fujino, T., Beguin, P., & Aubert, J.-P. (1993). *J. Bacteriol. 175*, 1891−1899.
Gerngross, U. T., Romaniec, M. P. M., Kobayashi, T., Huskisson, N. S., & Demain, A. L. (1993) *Mol. Microbiol. 8*, 325−334.
Gerwig, G. J., de Waard, P., Kamerling, J. P., Vliegenthart, J. F. G., Morgenstern, E., Lamed, R., & Bayer, E. A. (1989) *J. Biol. Chem. 264*, 1027−1035.
Gilkes, N. R., Henrissat, B., Kilburn, D. G., Miller, R. C., Jr., & Warren, R. A. J. (1991) *Microbiol. Rev. 55*, 303−315.
Grépinet, O., & Béguin, P. (1986) *Nucleic Acids Res. 14*, 1791−1799.
Grépinet, O., Chebrou, M.-C., & Béguin, P. (1988) *J. Bacteriol. 170*, 4582−4588.
Hall, J., Hazlewood, G. P., Barker, P. J., & Gilbert, H. J. (1988) *Gene 69*, 29−38.
Hazlewood, G. P., Romaniec, M. P. M., Davidson, K., Grepinet, O., Beguin, J., Millet, J., Raynaud, O., & Aubert, J.-P. (1988) *FEMS Microbiol. Lett. 51*, 231−236.
Hon-nami, K., Coughlan, M. P., Hon-nami, H., & Ljungdahl, L. G. (1986) *Arch. Microbiol. 145*, 13−19.
Johnson, E. A., & Demain, A. L. (1984) *Arch. Microbiol. 137*, 135−138.
Johnson, E. A., Sakajoh, M., Halliwell, G., Madia, A., & Demain, A. L. (1982) *Appl. Environ. Microbiol. 43*, 1125−1132.
Joliff, G., Béguin, P., & Aubert, J.-P. (1986) *Nucleic Acids Res. 14*, 8605−8613.
Jue, C. K., & Lipke, P. N. (1985) *J. Biochem. Biophys. Methods 11*, 109−115.
Juy, M., Amit, A. G., Alzari, P. M., Poljak, R. J., Claeyssens, M., Béguin, P., & Aubert, J.-P. (1992) *Nature 357*, 89−91.
Kim, K., Rhee, S. G., & Stadtman, E. R. (1985) *J. Biol. Chem. 260*, 15394−15397.
Kohring, S., Wiegel, J., & Mayer, F. (1990) *Appl. Environ. Microbiol. 56*, 3798−3804.
Kruus, K., Wang, W. K., Ching, J., & Wu, J. H. D. (1995) *J. Bacteriol. 177*, 1641−1644.
Laemmli, U. K. (1970) *Nature 227*, 680−685.
Lamed, R., Setter, E., & Bayer, E. A. (1983) *J. Bacteriol. 156*, 828−836.
Lamed, R., Naimark, J., Morgenstern, E., & Bayer, E. A. (1987) *J. Bacteriol. 169*, 3792−3800.
Lemaire, M., & Béguin, P. (1993) *J. Bacteriol. 175*, 3353−3360.
Ljungdahl, L. G., Coughlan, M. P., Mayer, F., Mori, Y., & Hon-nami, K. (1988) *Methods Enzymol. 160*, 483−500.
Maruyama, K., Kikawa, T., & Ebashi, S. (1984) *J. Biochem. 95*, 511−519.
Mayer, F. (1988) *Electron Microsc. Rev. 1*, 69−85.
Mayer, F., Coughlan, M. P., Mori, Y., & Ljungdahl, L. G. (1987) *Appl. Environ. Microbiol. 53*, 2785−2792.
Miller, G. L., Blum, R., Glennon, W. E., & Burton, A. L. (1960) *Anal. Biochem. 2*, 127−132.
Morag, E., Halevy, I., Bayer, E. A., & Lamed, R. (1991) *J. Bacteriol. 173*, 4155−4162.
Morag, E., Bayer, E. A., Hazlewood, G. P., Gilbert, H. J., & Lamed, R. (1993) *Appl. Biochem. Biotechnol. 43*, 147−151.
Mori, Y. (1992) *Biosci., Biotechnol., Biochem. 56*, 1198−1203.
Navarro, A., Chebrou, M.-C., Béguin, P., & Aubert, J.-P. (1991) *Res. Microbiol. 142*, 927−936.
Nolte, A., & Holzenburg, A. (1990) *FEMS Microbiol. Lett. 72*, 201−208.
Petre, J., Longin, R., & Millet, J. (1981) *Biochimie 63*, 629−639.
Salamitou, S., Raynaud, O., Lemaire, M., Coughlan, M., Béguin, P., & Aubert, J.-P. (1994) *J. Bacteriol. 176*, 2822−2827.
Schwarz, W. H., Schimming, S., Rücknagel, K. P., Burgschwaiger, S., Kreil, G., & Staudenbauer, W. L. (1988) *Gene 63*, 23−30.
Sillen, L. G., & Martell, A. E. (1964) in *Stability constants of metal ion complexes*, Chemical Society, London.
Stadtman, E. R. (1993) *Annu. Rev. Biochem. 62*, 797−821.
Stone, K. L., LoPresti, M. B., Crawford, J. M., DeAngilis, R., & Williams, K. R. (1989) in *A Practical Guide to Protein and Peptide Purification for Microsequencing.* (Matsudaira, P. T., Ed.) pp 31−47, Academic Press, New York.
Tokatlidis, K., Salamitou, S., Béguin, P., Dhurjati, P., & Aubert, J.-P. (1991) *FEBS Lett. 291*, 185−188.
Tokatlidis, K., Dhurjati, P., & Béguin, P. (1993) *Protein Eng. 6*, 947−952.
Towbin, J., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350−4354.
Wang, W. K., Kruus, K., & Wu, J. H. D. (1993) *J. Bacteriol. 175*, 1293−1302.
Wiegel, J., & Dykstra, M. (1984) *Appl. Microbiol. Biotechnol. 20*, 59−65.
Wu, J. H. D., Orme-Johnson, W. H., & Demain, A. L. (1988) *Biochemistry 27*, 1703−1709.
Yagüe, E., Béguin, P., & Aubert, J.-P. (1990) *Gene 89*, 61−67.
Yaron, S., Morag, E., Bayer, E. A., Lamed, R., & Shoham, Y. (1995) *FEBS Lett. 360*, 112−124.

BI9524629