Characterization of a Cellulose-Binding, Cellulase-Containing Complex in *Clostridium thermocellum*

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The isolation and biochemical characterization of the extracellular form of a cellulose-binding factor (CBF) from *Clostridium thermocellum* is described. The CBF was isolated from the culture supernatant by a two-step procedure which included affinity chromatography on cellulose and gel filtration on Sepharose 4B. The isolated CBF was homogeneous as determined by immunoelectrophoresis, polyacrylamide gel electrophoresis, gel filtration, and analytical ultracentrifugation analysis. The CBF was found to form a complex which exhibited a molecular weight estimated at 2.1 million. Electron microscopic analysis of negatively stained preparations of the isolated CBF revealed a particulate, multisubunit entity of complicated quaternary structure. The molecule appeared to be about 18 nm in size. Although urea failed to break the complex into its component parts, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate resolved the CBF complex into 14 polypeptide bands. Immunoprecipitation experiments confirmed that these polypeptides indeed formed part of the same complex. Interestingly, by using the whole-cell immunization procedure described in the accompanying article (Bayer et al., J. Bacteriol., 156:818–827, 1983) only one CBF subunit (*M*₂ = 210,000) was found to be antigenically active. By using a gel-overlay assay technique, at least eight of the remaining CBF-associated polypeptide components were shown to exhibit cellulolytic activity. The results are consistent with the contention that the CBF comprises a discrete, multisubunit complex or group of closely related complexes which exhibit separate antigenic and multiple cellulase activities in addition to the property of cellulose binding. It appears that the CBF is not only responsible for the adherence of the cells to cellulose but also constitutes a major part of the cellulolytic apparatus of this organism.

The potential of *Clostridium thermocellum* for industrial fermentation of cellulosic substrates has been recognized for many years. This thermophilic anaerobe is known to excrete large amounts of cellulolytic enzymes of particularly high specific activity (19). Of note is the relatively high activity of this enzyme system on α-crystalline cellulose (11).

In the accompanying article (3), we have shown that the adherence of cells of *C. thermocellum* to their insoluble cellulose substrate is apparently mediated by an antigenically active cellulose-binding factor (CBF) located on the cell surface and in the extracellular medium. Cellulolytic activity in the form of a carboxymethylcellulase (CMCase) was found to be closely associated with the CBF. It was not clear, however, to what extent the antigenic, cellulolytic, and cellulose-binding activities were connected. It was therefore of interest to determine whether these activities reflect the same macromolecular entity or whether they derive from two (or more) separate subunits which are but part of a greater cellulase complex.

To approach this question, we proceeded to isolate the CBF based on the evidence obtained in the accompanying article (3). Since both the cell-associated and extracellular forms of the factor appeared to be immunochemically similar, the CBF was isolated from the cell-free culture supernatants. The factor was isolated by a two-step procedure which included affinity chromatography directly on microcrystalline cellulose and further purification by gel filtration. Various biochemical and biophysical techniques were employed to investigate the molecular nature of the isolated CBF. The evidence supports the notion that the CBF is a defined, multisubunit complex and that the antigenic and multiple cellulolytic activities of the complex correspond to the action of separate subunits.
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MATERIALS AND METHODS

Organism and fermentation conditions. C. thermocellum YS, LQRI, and J1 were those described in the accompanying article (3). Comparative studies on the extracellular cellulase were performed on culture supernatants of the respective cells grown in serum bottles as reported. For purification of the CBF, a 50-liter culture of C. thermocellum YS was grown in a fermentor (Biotec). The culture medium contained the following additives per 50 liter of deionized water: 25 g of MgCl₂, 65 g of (NH₄)₂SO₄, 1.75 liter of 1 M potassium phosphate (pH 7.4), 250 g of yeast extract, 100 mg of resazurin, and 250 g of microcrystalline cellulose (Merck & Co., Inc.). A solution (100 ml) of cysteine-hydrochloride (15% [wt/vol]) (pH 8.5; autoclaved separately), was added immediately before inoculation. After being flushed with a stream of nitrogen gas (continued at a rate of 100 ml/min throughout the fermentation), a 2-liter inoculum (grown as described in the accompanying paper [3]) was introduced. Cells were grown for 30 h at 60°C under constant stirring at 100 rpm. The cells were centrifuged in a Sharplies super-centrifuge (Sharplies Centrifuges Ltd.). The supernatant was brought to a final concentration of 0.02% with NaCl and stored at 4°C until processed further.

Gel chromatography. Preparative gel chromatography was carried out at room temperature on a Sepharose 4B column. The column dimensions were 2.5 by 80 cm, and the column was equilibrated and eluted with 50 mM Tris-hydrochloride buffer (pH 7.7) (Tris buffer), containing 0.05% NaCl. The flow rate was 40 ml/h and fractions of 6.5 ml were collected. The same column and conditions were used for analysis of crude supernatant and purified CBF. Analytical gel chromatography was performed on a Sepharose 2B column (1.5 by 62 cm) or on a Sephadex S-300 column (1.1 by 52 cm) performed with the same buffer. Flow rates for these columns were 10 and 5 ml/h, respectively.

Analytical ultracentrifugation. The sedimentation coefficient was determined in a Spinco model E analytical ultracentrifuge (48,000 rpm) at 20°C. Various concentrations of protein, ranging from 0.7 to 6.3 mg/ml in Tris buffer were analyzed, and the intrinsic sedimentation and diffusion coefficients (s₂₀,w and D₂₀,w, respectively) were determined.

Slab gel electrophoresis. The method of Laemmli (13) or Weber and Osborn (23) for slab gel electrophoresis was used. Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) was performed for 3 h at 120 V with 3% stacking gels and either 6% separating gels or 6 to 10% continuous gradients. The running buffer consisted of 0.1% SDS in 25 mM Tris-glycine buffer (pH 8.9). Samples were diluted with one-half volume of sample buffer which contained 3% (wt/vol) SDS, 10% (vol/vol) glycerol, 5% (vol/vol) β-mercaptoethanol, and 0.2% (wt/vol) bromophenol blue in 62.5 mM Tris-hydrochloride buffer (pH 6.8), and the samples were boiled for 3 min before electrophoresis. The gel was then removed from the mold, stained with Coomassie brilliant blue R250, destained, and photographed. A mixture of high-molecular-weight markers (Sigma Chemical Co.) was used as a standard. Polyacrylamide gels not containing SDS were 1.5-mm thick and consisted of 3% acrylamide in the stacking gel and 5% acrylamide in the separating gel.

Crossed immunoelectrophoresis. Crossed immunoelectrophoresis of the CBF subunits was performed on SDS gels by a modification of the "molding-in" procedure (5, 14). SDS-PAGE was carried out on the isolated CBF (10 μg) as described above. The desired lane (a 7-mm-wide strip) was excised from the gel, washed with three changes of Tris buffer, and washed twice with barbital buffer (3). The gel was placed length-wise on a rectangular glass plate (76 by 51 mm. Chance Bros. Ltd.) 8 mm from the edge of the plate. The SDS gel was then embedded in a 3-mm-thick agarose gel (1% agarose in barbital buffer). The agarose gel was cut 5 mm from the edge of the SDS gel (20 mm from the edge of the plate) to allow space (31 mm) for the antibody-containing gel. The latter consisted of 70 μl of CBF-specific antibody preparation in 5 ml of barbital buffer containing 1% agarose. Electrophoresis was performed for 3 h at 200 V.

On-plate CMCase overlay. Several enzymes have previously been shown to be renaturable after SDS-PAGE (12). The on-plate CMCase assay described in the accompanying article for rocket immunoelektrophoresis (3) was therefore modified for SDS-PAGE gels. To regenerate the denatured enzyme, the gel was rinsed gently but thoroughly with three changes of Tris buffer and twice with 0.1 M sodium acetate buffer (pH 5) (acetate buffer). The gel was then placed in a mold, and a 1.5-mm overlay (1% agarose and 1.4% carboxymethyl cellulose [CMC] in acetate buffer, incubated at 50°C) was poured onto the gel. Replicate gels were subjected to various periods of incubation (3 to 20 h) at 40°C, cooled to room temperature, and immersed in 2-propanol. After 20 min, the agarose overlay was carefully separated from the polyacrylamide gel, and the latter was stained as above with Coomassie brilliant blue. The agarose gel was developed further with 2-propanol until the zones of clearing stabilized (usually 2 to 3 h). Gels were then photographed but could also be stored in 2-propanol for long periods of time (weeks). Photography was performed by lateral illumination.

Immunoprecipitation. Samples (25 μg) of the isolated CBF were treated with various amounts (ranging from 100 μg to 2 mg) of the CBF-specific antibodies and brought to 250 μl (final volume) with Tris buffer. After incubation overnight at 4°C, a portion (32 μg) in 50 μl of buffer of purified goat anti-rabbit immunoglobulin G (IgG) was added to enhance precipitation. After an additional 2-h incubation period, samples were centrifuged at 10,000 × g for 30 min. The CMCase activity of the supernatant fractions was determined as described previously (16). The pellet was washed once with 1 ml of 1% Triton X-100 in 1 M NaCl and twice with the same detergent in Tris buffer. The resultant detergent-washed immunoprecipitate was dissolved in SDS-containing sample buffer (75 μl), and SDS-PAGE was performed on 15-μl samples as described above.

Electron microscopy. Samples of the isolated CBF were diluted to 50 μl/ml and brought to 5 μl/ml with bacitracin. The solution was applied to 400-mesh copper grids, which contained a layer of Formvar bearing...
small (about 5- to 10-μm) holes for enhanced resolution. Specimens were stained with 0.5% uranyl acetate and viewed in a Philips 300 transmission electron microscope at 80 V.

**MISCELLANEOUS MATERIALS AND METHODS.** Reference (anti-whole cell) antibodies and CBF-specific (mutant AD2-adsorbed) antibodies were prepared as described in the accompanying article (3). Rocket immunoelectrophoresis and in vitro measurement of cellulase activity were also performed as previously reported (3). Autoradiography was performed by the method of Adair et al. (1) on gels which had previously undergone successive treatments with CBF-specific antibodies and 125I-labeled protein A. Protein content was measured in vitro by the Bradford method (4).

**RESULTS**

**Isolation of the CBF.** The steps designed for isolating the CBF from the extracellular medium are summarized in Table 1. A 50-liter culture of *C. thermocellum* was grown on cellulose under conditions where the cellulose was completely hydrolyzed. The cells were separated from the culture medium by centrifugation. Specific adsorption of the cellulose-binding factor was achieved by interacting the cell-free supernatants with insoluble microcrystalline cellulose. A portion of the supernatant (fraction 1, Table 1), consisting of 23 liters, was brought to pH 7.7 with 1 N NaOH, and Whatman cellulose CC31 (276 g) was added in bulk. The suspension was stirred mechanically for 1 h at room temperature, and the cellulose was allowed to settle by gravity for a period of 2 h. The supernatant (fraction 2, Table 1) was removed and saved for subsequent studies. The cellulose was washed through a Büchner funnel with 3 liters of 20 mM Tris-hydrochloride buffer (pH 7.7) (Tris buffer). The cellulose-binding factor was eluted from the cellulosic matrix with 1.5 liters of a 1% solution of triethylamine (TEA), and the eluent was immediately neutralized with 10% acetic acid (TEA-eluent; fraction 3, Table 1). To concentrate the protein, acetone (2.5 liters) was added, and the precipitate was dissolved in 150 ml of Tris buffer (fraction 4, Table 1). A sample (7 ml, 24.1 mg of protein) was applied to a Sepharose 4B column. The major peak (Fig. 1) appeared shortly after the position of the void volume. Closely associated with this peak was the bulk of both the cellulolytic and antigenic activities applied to the column. The major peak fractions (42 ml) were pooled (fraction 5, Table 1) and brought to 60% acetone. The precipitate was redissolved in 5 ml of Tris buffer (fraction 6, Table 1) to a final protein concentration of 2.2 mg/ml. Unless otherwise stated, fraction 6 (Table 1) was used for additional characterization studies.

The isolation method described above did not result in extensive purification of the CBF with respect to either CMCase or antigenic activity. Under the described conditions, only partial recovery of the CBF could be obtained. Specific adsorption of the CBF to cellulose resulted in the removal from the supernatant of 60 and 55% of the respective cellulolytic and antigenic activities.

Of the various elution conditions attempted, including 0.1 M acetic acid, 0.1 M NaOH, Triton X-100 (0.1 to 1%), SDS (0.1 to 1%), high salt (3 M KCl), and 8 M urea, the most effective method was found to be with a 1% solution of TEA. TEA-elution released 58, 23, and 30% of the respective amounts of total protein, CMCase, and antigenic activities originally bound to the cellulose. It is not clear at this point whether the loss in specific CMCase and antigenic activities results from nonspecific elution.

**Table 1. Isolation of the cellulose-binding complex**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Vol (ml)</th>
<th>Protein (mg)</th>
<th>CMCase activity*</th>
<th>Antigenic activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total (U)</td>
<td>Specific (U/mg)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Culture supernatant</td>
<td>23,000</td>
<td>3,680</td>
<td>42,600</td>
<td>11.6</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 Cellulose supernatant</td>
<td>23,000</td>
<td>2,800</td>
<td>16,200</td>
<td>5.7</td>
</tr>
<tr>
<td>(unbound fraction)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 TEA-eluent</td>
<td>1,500</td>
<td>510</td>
<td>6,000</td>
<td>11.8</td>
</tr>
<tr>
<td>4 Acetone (60%)</td>
<td>495</td>
<td></td>
<td>5,800</td>
<td>11.7</td>
</tr>
<tr>
<td>5 Sepharose 4B</td>
<td>42</td>
<td>12.7</td>
<td>235</td>
<td>18.5</td>
</tr>
<tr>
<td>6 Acetone (60%)</td>
<td>5</td>
<td>11.0</td>
<td>201</td>
<td>18.3</td>
</tr>
</tbody>
</table>

*One unit of CMCase activity is defined as the amount of reducing sugar (micromoles of glucose) liberated per minute.

*One unit of antigenic activity corresponds to a 1-cm rocket generated by the Laurell technique (with CBF-specified antibodies) under the conditions described in the text.

*Cell-free supernatant after adsorption of the CBF with cellulose and removal by filtration.

*A sample (7 ml, 24.1 mg) from fraction 4 (corresponding to 283 units of CMCase activity and 122 units of antigenic activity) was applied to the Sepharose 4B column, and the pooled fractions from Fig. 1 were analyzed.
Upon electrophoresis, the CBF failed to enter 5% polyacrylamide gels; the band remained at the boundary between the upper (3% polyacrylamide) and lower gels. A single broad protein band was obtained upon electrophoresis of the CBF in agarose gel. Gel filtration on a Sephacryl S-300 column yielded a single protein peak at the void volume. Interestingly, chromatography of the CBF in the presence of 8 M urea failed to alter the position of this peak. Gel filtration of the CBF on columns of Sepharose 4B and Sepharose 2B yielded single symmetrical protein peaks with selectivity coefficients ($K_{av}$) of 0.23 and 0.52, respectively, corresponding to an apparent molecular weight of about 2 million.

Negatively stained preparations of the isolated CBF (Fig. 4) revealed particulate structures of relatively uniform size (approximately 18 nm). Close inspection of the micrographs indicated that the CBF was composed of several different subunit types. In some cases, the CBF appeared to be irregular in shape. The reasons for the observed multiplicity in form could be explained by an innate or procedure-induced flexibility of the CBF structure. Alternatively, the multiple forms may reflect different orientations of a complicated quaternary structure. The molecule may thus consist of an organized conglomerate of different subunits. In some cases, or adverse effects of the TEA treatment. In a subsequent experiment, purification of a small amount of material by gel filtration alone resulted in a CBF preparation exhibiting similar biochemical properties.

Homogeneity of the isolated CBF. Rocket immunoelectrophoresis of the CBF revealed a single precipitin peak in the intermediate gel (Fig. 2). No extraneous peaks remained in the upper gel, indicating that the isolated CBF was antigenically pure vis-à-vis the CBF-specific antibody.

Sedimentation velocity studies (Fig. 3) of the TEA-eluent (fraction 4, Table 1) revealed one major and two minor peaks. The contaminants were removed by chromatography on Sepharose 4B, and the resultant preparation (fraction 6, Table 1) afforded a single symmetrical peak in the analytical ultracentrifuge. A molecular weight of 2.1 million was calculated from the intrinsic sedimentation coefficient ($s^*_{20,w} = 22.0$) and the intrinsic diffusion coefficient ($D^*_{20,w} = 1.02 \times 10^{-7} \text{cm}^2/\text{sec}$), assuming a partial specific volume ($\nu$) of 0.75 ml/g.

FIG. 1. Sepharose 4B gel chromatography of crude C. thermocellum YS supernatant (A) and partially purified, acetone-concentrated TEA-eluent (B). The elution positions of blue dextran and catalase are designated by arrows. Pooled fractions used for further experiments are indicated in (B).

FIG. 2. Rocket immunoelectrophoresis pattern of the crude supernatant (lane A), the isolated CBF (lane B), and the cellulose supernatant (lane C) (unbound fraction, Table 1). The intermediate gel contained the CBF-specific antibody, and the upper gel contained the reference antibody preparation. Samples were applied to the well in the bottom gel, and electrophoresis was performed with the anode at the top. Note that owing to the log-log relationship between peak height and amount of antigen, the residual CBF in lane C represented only about 40% of that in lane A.
FIG. 3. Sedimentation pattern of the acetone-concentrated, TEA-eluent fraction (upper pattern) and the isolated CBF (lower pattern) after Sepharose 4B column chromatography. Note that the extraneous peaks (arrows) in the upper pattern were eliminated after the gel chromatography step. The lower pattern is characterized by a single symmetrical peak.

FIG. 4. General view of a field of a negatively stained preparation of the isolated CBF and characteristic particle images (inserts at bottom). Structures are particulate in nature and appear to be composed of several smaller subunits. In some instances, higher-order aggregates can be observed. A variety of different particle forms are visible. Higher magnification of some characteristic particle images are shown in the inserts at the bottom of the figure. General view. ×210,000; inserts. ×400,000.

the stain clearly penetrated to the core of the molecule, and a central cleft or channel could be distinguished.

**Analysis of protein composition.** At least 14 distinct polypeptides could be consistently discerned upon SDS-PAGE of the isolated CBF. Enumeration of the CBF subunits and assignment of the respective molecular weight values are listed in Fig. 5. The apparent molecular weights (Mr) ranged from 48,000 to 210,000. Quantitatively, the five major subunits (as estimated by integration of densitometric tracings of gels stained with Coomassie brilliant blue R250) were S8, S1, S3, S2, and S5, corresponding to respective Mr values of 75,000, 210,000, 150,000, 170,000, and 98,000.

To determine the extent of antigenicity of the various subunits, the isolated CBF was subjected to SDS-PAGE, followed by crossed immunoelectrophoresis of the separated subunits (Fig. 6). Interestingly, only one subunit, S1 (Mr = 210,000), exhibited antigenic activity with the CBF-specific antibodies. The sole antigenic nature of this subunit was further supported by subjecting the gels to successive incubations with antibody and 125I-labeled protein A; subsequent autoradiography revealed a single band at an Mr of 210,000 (data not shown).

Enzyme activity of the various subunits was determined directly on the gels by a CMC-
are markers in A) and the isolated ing of weights 156, VOL. overlay technique under renaturing conditions as outlined above. By comparing the resultant pattern of CMCase activity with the position of protein bands on control gels (Fig. 7), it was determined that subunits S2 and S8 (Mr = 170,000 and 75,000, respectively) exhibit the highest levels of renatured cellulolytic activity. Other subunits, including S5, S11, S13, and S14, also appear to be cellulases, and some cellulase activity may also be associated with subunits S3, S6 or S7, and S4. In short, with the exception of the antigenic subunit, S1, most of the other protein bands identified with the CBF complex seem to be cellulases. It should be noted that the extent of CMCase activity visible by means of the gel overlay can be considered only in terms of a qualitative estimate of enzyme activity; since these samples were subjected to extremely harsh conditions (boiling in SDS and electrophoresis at 120 V) before they were assayed.

To examine further whether the observed components are indeed physically associated within the same complex, immunoprecipitation of the isolated CBF was performed with the CBF-specific antibodies. As shown above (Fig. 6), this antibody preparation appears to be specific exclusively for the S1 subunit. SDS-PAGE of the detergent-washed immunoprecipitate revealed that the pattern of polypeptide subunits in the precipitate (Fig. 8) was strikingly similar to that of the characteristic polypeptide composition of the CBF (Fig. 5). As the amount of added antibody increased, the relative proportions of the immunoprecipitated components did not appear to differ significantly. Only the S6 component appeared to be missing. Components S12 through S14 were masked on the gels by the appearance of the precipitated IgG (heavy chain), and hence, these particular experiments could neither confirm nor deny their involvement in the CBF.

The contention that the CMCase activity is associated with the complex is further supported by the finding that after immunoprecipitation, little cellulolytic activity was found in the supernatant fluids. Using greater than 200 µg of anti-CBF antibodies per 25 µg of isolated CBF resulted in over 97% removal of the CMCase activity from the sample.
170K
75K
were glycogen complex
were structural of phosphatase phosphorolase revealed the polypeptides (and lose-grown cells filtration analyses accompanying fraction previously peak, as immunochemical analysis at a eluted molecular-weight subunit) in YS 10 million) proteins. In the lane containing low levels of protein (lane B), only two cellulolytically active components could be distinguished (at positions corresponding to subunits S2 and S8). Increasing the quantity of protein or the reaction time or both revealed additional enzymatically active bands including S3, S4, S5, S6 or S7, S11, S13, and S14.

FIG. 7. SDS-PAGE of the isolated CBF stained for protein (lanes A and C) or processed for cellulolytic activity by the CMC-overlay technique (lanes B and D). Samples applied to lanes A and B contained 10 μg of protein, and those samples in lanes C and D had 50 μg of protein. The enzymatic reaction was carried out for 10 h. In the lane containing low levels of protein (lane B), only two cellulolytically active components could be distinguished (at positions corresponding to subunits S2 and S8). Increasing the quantity of protein or the reaction time or both revealed additional enzymatically active bands including S3, S4, S5, S6 or S7, S11, S13, and S14.

CBF complex in other strains. Comparative gel filtration analyses of the supernatants of cellulose-grown cells of C. thermocellum LQRI and J1 were strikingly similar to that shown for strain YS in Fig. 1. In each case, the predominant fraction (>70% of the CMCase activity was eluted at a position equivalent to that of high-molecular-weight (>1 million) proteins. Interestingly, immunochemical analysis and SDS-PAGE revealed the presence of the CBF antigenic peak, as well as the spectrum of CBF-related polypeptides (and in particular, the 210,000-molecular-weight subunit) in all three strains tested. The presence of the CBF peak in sonic extracts of the three strains was reported in the accompanying paper (3).

DISCUSSION

The arrangement of specific enzymes as part of multienzyme complexes has been demonstrated previously in many systems. For example, enzymes involved in the phosphorolysis of glycogen were isolated from rabbit muscle as a specific structural entity in the form of a protein-glycogen complex (15). Regulatory conditions on phosphorylase, phosphorylase kinase, and phosphorylase phosphatase contained in the complex were found to simulate those of the in vivo system, whereas the disrupted complex or the purified enzymes exhibited altered regulatory states (9, 10). A second example of multienzyme complexes involves the physical association of different aminoacyl-tRNA synthetases within the same macromolecular entity (17).

The degradation of cellulose by various microorganisms is accomplished by the concerted action of a group of enzymes collectively termed cellulases. It has been previously suggested that the cellulase system of C. thermocellum may also comprise an aggregate or complex (2, 21). Most studies of this particular enzyme system have concentrated on activity analyses of crude enzyme preparations (6, 11, 18, 22) or have been devoted to the isolation of purified monomeric cellulolytic forms (20, 21). In the present study, we isolated a multiprotein complex which was associated with cellulolytic activity as well as the adherence of the bacterium to the insoluble substrate.

Several complementary lines of evidence favor the notion that the isolated complex serves as a discrete structural and functional unit. The complex appeared immunochemically pure and of homogeneous size. Moreover, electron microscopic analysis of negatively stained preparations of the isolated CBF revealed recurrent molecular forms which consisted of large, apparently flexiform, multisubunit structures of rela-
tively uniform size. The purified product was not merely an artifact of the isolation procedure; since (i) gel filtration alone (without the affinity chromatography step) yielded a similar polypeptide pattern in the pooled high-molecular-weight fractions, and (ii) purification of the cell surface CBF (from cell sonic extracts by the procedure described above) resulted in a polypeptide profile similar to that of the extracellular CBF (unpublished data). The rigorous co-isolation of the polypeptide components mentioned above was further corroborated by immunoprecipitation experiments. Although the antibodies used were directed against a single polypeptide derivative of the complex (the S1 component), immunoprecipitation resulted in the coprecipita-
tion of practically all of the identified CBF-associated polypeptides as well as the cellulolytic activity. The S1 component thus appears to be a common denominator among the other components of the complex. Furthermore, the S1 subunit apparently represents the same polypeptide band ($M_r = 210,000$) present in wild-type sonic extracts but absent in mutant strain AD2 (3).

It is somewhat surprising that the S1 component appears to be the sole antigenic species recognized by the CBF-specific antibody preparation. This could imply that the S1 component is either more immunogenic than the other components of the complex or that it is more exposed on the cell surface. It is also not yet clear whether this phenomenon reflects the singular lack or alteration of the S1 component on the mutant cell surface or whether the entire CBF complex is lacking or modified. It should be emphasized here that an alternative explanation is also possible in that the concentration of antibodies specific for other bands may simply be lower than that for the S1 subunit or that SDS-PAGE had a deleterious effect on the antigenic properties of the other subunits. In any event, additional experiments comparing the characteristics of the mutant-derived components with those of the CBF should clarify these considerations.

With the striking exception of the antigenic S1 subunit, the majority of the other components of the complex appear to be cellulases. The spectrum of various molecular sizes of these cellulases, together with their apparent nonuniform action on the CMC overlay (Fig. 7), suggests that these are indeed separate cellulolytic enzymes. Moreover, we have found in preliminary experiments that cellobiose essentially comprises the sole product of the CBF-mediated cellulose hydrolysis; virtually no celloctriose and only limited (<5%) amounts of glucose could be detected by thin-layer chromatography. This profile is typical either of a cellobiohydrolase alone or of a combination of endocellulase(s) together with cellobiohydrolase. Since the CMC case activity of C. thermocellum is reflective of the action of endocellulases, our data support the presence of the combined enzymatic system. This phenomenon may provide this thermophilic anaerobe with the required mecha-
nism for highly efficient cellulolysis.

In theory, anaerobic bacteria would be expected to exhibit greater hydrolytic efficiency at this early premetabolic step, because the total energy yield of subsequent catabolic pathways is much lower than that of aerobes. The tendency of anaerobic cellulolytic bacteria to form high-

molecular-weight, multisubunit cellulases may be a more general feature of these organisms. For example, Yu and Hungate (24) found multiple high-molecular-weight forms of cellulases in Ruminococcus albus. In addition, Gawthorne (7) has reported that most rumen cellulase activity is associated with high-molecular-weight (perhaps membrane-bound) material. In the gram-
negative bacterium, Bacteroides succinogenes, most of the extracellular CMCase activity appeared to be associated with sedimentable membranous fragments (8). The exact mechanistic nature of the association between multiple cellu-

lase subunits may therefore differ from species to species.

Our current perception of the CBF in C. thermocellum can thus be viewed as a large, discrete, multisubunit complex(es) which exhibits both antigenic and cellulolytic activities. It is clear that the antigenic and cellulolytic activities reside in separate components on the complex. The complex apparently comprises various dif-

ferent forms of cellulases, each of which may bear separate specificities toward different qua-

ternary structures on the complex cellulose sub-
strate. The major organizational role of this complex might be designed for effective delivery to the substrate as well as to bring into proximity the various complementary enzymes (e.g., exo-
and endocellulases). In addition, the complex may be structured in such a way as to enable the protection of various product intermediates and to facilitate their transfer to other cellulase components for further hydrolysis. In any event, the cellulase subunits seem to be arranged within the CBF complex in a defined supramolecular fashion designed for highly efficient cellulose degradation.

It is as yet unknown whether the cellulose-

binding activity, which results in the specific binding of the entire bacterial cell (3) as well as the CBF complex itself to the cellulosic sub-
strate, is mediated mainly through the action of a single subunit (i.e., a β-glucoside-specific or higher order celloxidrin-specific lectin) of the complex or whether the combined action of the various cellulases (which, as enzymes, would be
expected to exhibit high levels of binding specificity toward the substrate) contributes in a cooperative manner to effect this binding. Further characterization of isolated CBF subunits should resolve this distinction.

ACKNOWLEDGMENTS
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