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The Cellulosome: An Exocellular, Multiprotein Complex Specialized in Cellulose Degradation

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ABSTRACT: *Clostridium thermocellum* produces a highly active cellulase system that consists of a high-M_r multienzyme complex termed cellulosome. Hydrolytic components of the cellulosome are organized around a large, noncatalytic glycoprotein termed CipA that acts both as a scaffolding component and a cellulose-binding factor. Catalytic subunits of the cellulosome bear conserved, noncatalytic subdomains, termed dockerin domains, which bind to receptor domains of CipA, termed cohesin domains. CipA includes nine cohesin domains, a cellulose-binding domain, and a specialized dockerin domain. Proteins of the cell envelope carrying cohesin domains that specifically bind the dockerin domain of CipA have been identified. These proteins may mediate anchoring of the cellulosomes to the cell surface. Cellulase complexes similar to the cellulosome of *C. thermocellum* are produced by several cellulolytic clostridia. High-M_r multienzyme complexes have also been identified in anaerobic rumen fungi. The architecture of the fungal complexes also seems to rely on the interaction of conserved, noncatalytic docking domains with a scaffolding component. However, the sequence of the fungal docking domains bears no resemblance to the clostridial dockerin domains, suggesting that the fungal and clostridial complexes arose independently.

KEY WORDS: cellulase, *Clostridium thermocellum,* docking domain, protein-protein interaction, scaffolding protein.

I. INTRODUCTION

One cheerful feature of science is that seemingly mundane research areas may turn out to reveal unsuspected developments. The degradation of cellulose, whose investigation was initiated largely by utilitarian motivations, is a good example. The study of the cellulolytic bacterium *Clostridium thermocellum* has led to the identification and characterization of the cellulosome, a new type of exocellular organelle whose basic building principle is sophisticated and

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elegant. This topic has been reviewed by other authors (Felix and Ljungdahl, 1993; Bayer et al., 1994; Doi et al., 1994). The purpose of this review is to include the most recent findings and to offer our own views about the field.

Cellulose is produced by plants at a yearly rate estimated at 40 Gt. It is a major structural component of plant cell walls and the most abundant carbohydrate in the biosphere. Thus, cellulose degradation by microorganisms represents a significant part of the carbon cycle. Due to its physical properties, however, cellulose is a highly recalcitrant substrate for enzymatic hydrolysis. Cellulose molecules are built from 100 to 14,000 glucose protomers linked by β-1,4-bonds. The chains are insoluble and form cellulose fibrils, in which cellulose molecules are oriented in parallel and maintained together by interchain hydrogen bonds and van der Waals interactions between pyranose rings. Fibrils are made up of highly ordered, crystalline regions and more disorganized, amorphous regions (Figure 1). They are usually embedded in a matrix of hemicellulose and lignin, which restricts their accessibility to hydrolytic enzymes. Hemicellulose is composed of branched polysaccharides, with xylans and mannans as the main components. Lignin is a highly crosslinked, random polymer derived from the free-radical condensation of aromatic alcohols. Lignin is coupled to hemicellulose through aromatic acids such as ferulic and *p*-coumaric acid, which are linked by ester bonds to the side chains of xylans. Hemicellulose appears to act as a binding agent between cellulose fibrils and lignin. The degradation of hemicellulose involves the synergistic activity of xylanases and mannanases, which degrade the main carbohydrate backbone, as well as various enzymes acting on the side chains (acetylxylan esterases, glucuronidases, arabinofuranosidases, and ferulic acid esterases) (Biely, 1985; Coughlan and Hazlewood, 1993). The degradation of lignin is performed by few organisms, mostly fungi, and involves radical oxidation by peroxidases.

The degradation of cellulose represents a particularly interesting problem. The process cannot be adequately described by conventional enzymology, which deals with homogeneous, monophasic solutions. In some aspects, it may be compared to other heterophasic reactions such as the hydrolysis of lipids by lipases, which occurs at the interphase between the aqueous phase, containing the enzyme, and the lipid phase forming the substrate. Indeed, the interaction between hydrolytic components and the cellulose-binding factor of the cellulosome (see below) is reminiscent of the lipase-colipase interaction required for the binding of lipase to lipid-bile salt micelles (Semeriva and Desnuelle, 1976). In addition, disintegration of the compact, solid structure of the crystalline regions is required to achieve complete degradation of the substrate.

All organisms known to utilize cellulose produce a variety of hydrolytic enzymes, which act in concert to degrade cellulose and the associated hemicellulose polymers. In addition to the hydrolases required to attack hemicellulose, the degradation of native cellulose requires the action of several enzymes with different specificities. Traditionally, these have been classified as endoglucanases, cellobiohydrolases, and β-glucosidases. Endoglucanases preferentially hydrolyze the amorphous regions of the fibrils, cellobiohydrolases are exoglucanases releasing cellobiose from the end of the chains, and β-glucosidases hydrolyze cellobiose and other cellodextrins with a low degree of polymerization. For a long time, the cellulase systems produced by aerobic fungi such as *Trichoderma reesei, T. koningii,* or *Phanerochaete chrysosporium* have provided the major paradigm for the enzymology of cellulose degrada-

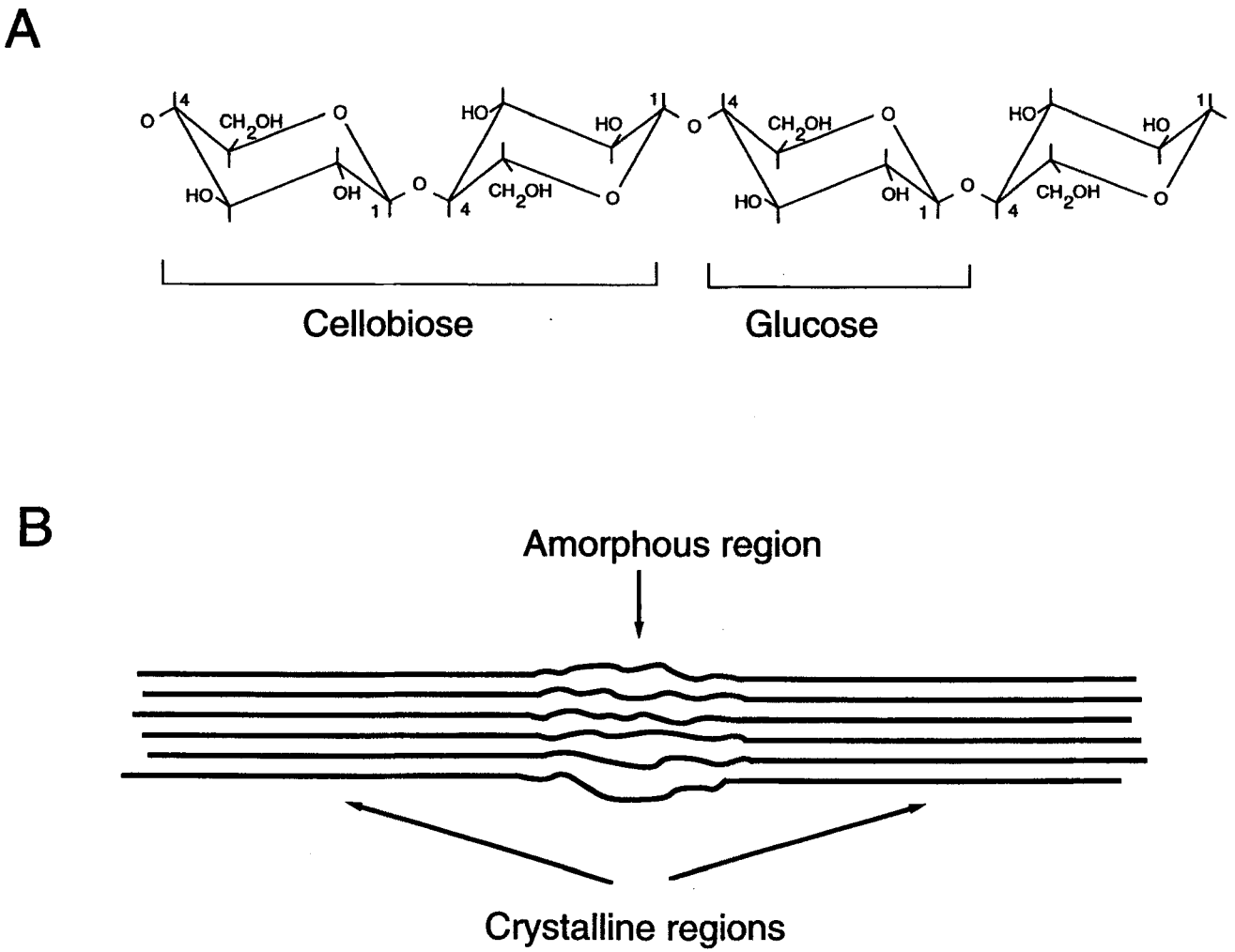

FIGURE 1. Structure of cellulose. (A) β-Glucosidic bonds; (B) schematic structure of a fibril. (From Béguin, P. and Aubert, J. P. 1992. *Ann. Inst. Pasteur/Actualités* **3:** 91–115. With permission.)

tion (Montenecourt, 1983; Wood, 1985; Coughlan, 1985; Eveleigh, 1987). These organisms often produce massive amounts of extracellular cellulases (more than 30 g/l in the case of industrial strains of *T. reesei*) (Pourquié et al., 1988). The enzymes are usually not tightly associated, which facilitates their purification and characterization. The synergism between endoglucanases and cellobiohydrolases has been proposed to involve the creation by endoglucanases of new chain termini, which are attacked by the cellobiohydrolases. β-Glucosidases prevent the build-up of cellobiose, which inhibits cellobiohydrolases. However, the model is too simplistic to explain why some endoglucanases fail to display synergism with some cellobiohydrolases (Wood and McCrae, 1978), or why some cellulase systems include two different types of cellobiohydrolases that are mutually synergistic (Fägerstam and Pettersson, 1980). In addition, the very notion that cellulases can fit into rigid categories according to their endo or exo mode of action is questionable. For example, *T. reesei* cellobiohydrolase II was convincingly shown to act as a true exoglucanase on *Valonia macrophysa* cellulose (Chanzy and Henrissat, 1985). However, the same enzyme, isolated from a recombinant yeast clone not producing any contaminating endoglucanase, is also able to generate haloes of hydrolysis on barley β-glucan stained with Congo red (Penttilä et al., 1988), a reaction that is usually ascribed to endoglucanases.

Besides cellulolytic fungi, other organisms, whose cellulase systems were much less well understood, have long been known to degrade crystalline cellulose actively. One of them is the anaerobic and (moderately) thermophilic bacterium *C. thermocellum* (McBee, 1948).

In 1982, it was shown that the bulk activity of the extracellular cellulase produced by *C. thermocellum* was comparable to the activity produced by *T. reesei*. Yet, this activity was achieved with a much lesser amount of protein released into the medium, meaning that the specific activity of the cellulase system produced by *C. thermocellum* must be very high (Johnson et al., 1982b).

Early attempts at characterizing the cellulases of *C. thermocellum* came up against the difficulty of purifying individual enzymes, which appeared to form high-molecular-weight complexes, or aggregates, and could not be dissociated by conventional methods (Aït et al., 1979). However, no special significance was attributed at first to such complexes, which were considered more as a nuisance for the biochemical analysis than as genuine biochemical entities.

II. GENESIS OF THE CELLULOSOME CONCEPT

The idea that the high-molecular-weight forms of *C. thermocellum* cellulase were in fact defined, functional entities came in a somewhat roundabout manner. The starting point was the attempt by Bayer, Lamed, and co-workers at characterizing the cellulose-binding factor responsible for the adhesion of *C. thermocellum* cells to cellulose. It turned out that an antibody fraction that agglutinated wild-type cells, but not cells from a mutant deficient in adhesion to cellulose, specifically labeled a large polypeptide of about 210 kDa. The latter, termed S1 by the authors, was present both on the surface of the cells and in the culture medium (Bayer et al., 1983). Biochemical fractionation indicated that S1 was associated with a cellulose-binding, endoglucanase-containing fraction whose bulk was eluted close to the void volume of a Sepharose 4B gel permeation column. The resulting material had a monodisperse sedimentation co-

efficient and contained, in different amounts, some 14 distinct polypeptides, which coimmunoprecipitation confirmed to be associated with S1. Electron microscopy of the preparation revealed the presence of multicomponent particles of somewhat similar apparent size (Lamed et al., 1983a).

By themselves, these results were not definite proof that the high-M_r forms of *C. thermocellum* cellulase corresponded to define complexes behaving as discrete structural and functional units. Copurification of S1 with other polypeptides meant that the aggregates were stable, not that they were specific or functional. Likewise, a monodisperse sedimentation behavior was not unexpected, as the preparation had been subjected to gel permeation chromatography. A more compelling argument was the finding that complexes with very similar polypeptide composition could be isolated from the supernatant of sonicated cells and from the culture medium (Lamed et al., 1983b). Because the two sources of complexes had very different protein compositions, random formation of aggregates having the same polypeptide composition was unlikely. Evidence that the complexes corresponded to functional entities was provided by showing that they copurified with activity against crystalline cellulose, and that this activity was strictly dependent on the presence of intact complexes (Lamed et al., 1983b; Lamed and Bayer, 1988b). All attempts to dissociate the complexes using chaotropic agents or mild proteolysis resulted in the loss of the ability to attack crystalline cellulose, although activity against amorphous cellulose was retained. In particular, the presence of the S1 subunit appeared essential to hydrolyze the crystalline substrate. Thus, the high-molecular-weight forms of *C. thermocellum* cellulase appeared as functional biological entities and were termed "cellulosomes" (Lamed et al., 1983b).

III. GENERAL PROPERTIES OF CELLULOSOMES

A. Location

The presence of cellulosomes on the surface of *C. thermocellum* can be visualized by immunocytochemical labeling and electron microscopy. Cellulosomes are located in protuberances of the outermost layer of the cell envelope, which forms a slimy, irregular coating of about 50 to 100 nm (Bayer et al., 1985; Bayer and Lamed, 1986; Nolte and Mayer, 1989). After binding to cellulose, the cellulosome-containing protuberances elongate and form filamentous protractions tethering the cells to the substrate. It has been hypothesized that these structures may also act as contact corridors channelling the diffusion of soluble degradation products from the cellulose fibers to the cells. Cellulosomes can also detach from the cells and proceed independently with the hydrolysis of the substrate (Bayer and Lamed, 1986).

B. Size and Composition

Two criteria serve as an operational definition in the purification of cellulolytic fractions termed "cellulosomes". The first one is a high affinity for cellulose, which is used for affinity chromatography. The second one is an apparent size in excess of 1 to 2 MDa by gel filtration. The estimated size of cellulosomes produced by various strains of *C. thermocellum* ranges between 2 MDa (for strains YS and JW20 (Lamed et al., 1983a; Coughlan et al., 1985; Hon-nami et al., 1986)) and 6.5 MDa (for strain ATCC 27405) (Wu et al., 1988). The cellulosomes of strain YM4 appear to be somewhat larger

than those of strain JW20 (Mori, 1992) and to contain a higher number of subunits (45 to 50 instead of 35 [Mayer et al., 1987]). However, in most cases, it is difficult to assess whether these discrepancies are due to true differences in size or to the different methods that were used to determine the molecular mass of cellulosomes (gel filtration, electron microscopy, and sedimentation velocity).

In several strains of *C. thermocellum* (but not in strain YM4) (Mayer et al., 1987), very-high-molecular-weight forms of the cellulosome ranging up to 100 MDa have been described (Lamed et al., 1983b; Honnami et al., 1986). These forms have the same bulk polypeptide composition as the 2- to 6.5-MDa species and have been termed polycellulosomes. Electron microscopy shows that the size of polycellulosomes is relatively homogeneous, and that they sometimes appear to be covered by a thin sheath. These features suggest that polycellulosomes may be more than random fragments torn off from the protuberances, and possess a defined organization (Mayer et al., 1987).

A first picture of the composition of cellulosomes can be obtained by separating cellulosomal subunits by SDS-PAGE. Fourteen different bands, labeled S1 to S14, were identified in the cellulosome of strain YS (Lamed et al., 1983b) and 26 in the cellulosome of strain JW20 (Kohring et al., 1990). Zymogram staining using carboxymethylcellulose (CMC) and Congo red shows that the majority of the bands have endoglucanase activity. Several xylanases were also detected (Kohring et al., 1990; Morag et al., 1990), in agreement with the observation that *C. thermocellum* actively degrades xylan, although it does not utilize xylose (Wiegel et al., 1985). Some of the components, particularly S1, react positively after glycoprotein staining (Lamed and Bayer, 1988b; Kohring et al., 1990). Lipids, particularly cardiolipin, have also been reported to be associated with cellulosome-containing fractions. Cardiolipin is a major component of the cell envelope of *C. thermocellum* (Bolobova et al., 1994). Thus, lipids may represent material torn off from the cell surface when cellulosomes detach from the bacteria.

C. Biochemical Properties

Although the culture supernatant of *C. thermocellum* also contains unassociated cellulases, most of the activity against crystalline cellulose can be ascribed to the cellulosome. Accordingly, the enzymatic properties of unfractionated cellulose-hydrolyzing activity coincide with those of the purified cellulosome (Lamed et al., 1985).

Binding of the cellulosome to cellulose is enhanced by increasing salt concentration, a typical feature of hydrophobic protein-carbohydrate interactions. The physical structure of cellulose strongly influences its cellulosome-binding capacity. Phosphoric acid treatment of Avicel enhances binding by more than two orders of magnitude. A yellow pigment, termed YAS for yellow affinity substance, has also been reported to enhance binding (Ljungdahl et al., 1983), but the effect is relatively modest (Lamed et al., 1985). Optimum cellulolytic activity is observed for salt concentrations (about 1 m*M* sodium acetate) that are not sufficient to promote maximal binding. Presumably, a too strong affinity reduces the activity of the complex by impairing its diffusion along the substrate (Lamed et al., 1985).

Activity against crystalline cellulose is enhanced by thiols and by Ca^{2+} (Johnson and Demain, 1984; Lamed et al., 1985; Gow and Wood, 1988) and is inhibited by cellobiose (Johnson et al., 1982a; Lamed et al., 1985). *C. thermocellum* does not secrete significant amounts of β-glucosidase. In

growing cultures, cellobiose does not accumulate because it is transported into the cells and cleaved intracellularly by cellobiose phosphorylase (Ng and Zeikus, 1982). However, cellulose degradation by acellular preparations of cellulosome is rapidly inhibited by cellobiose, which is the major product of hydrolysis. Inhibition can be prevented by adding exogenous β-glucosidase (Kadam and Demain, 1989; Lamed et al., 1991a), in which case up to 200 g/l of cellulose can be saccharified over a period of 9 d using 16 mg/g of cellulosome (Lamed et al., 1991a).

The cellulosome is quite stable. Neither high salt, nor urea, nor nonionic detergent are able to decompose the complex, and, under normal conditions, even SDS is only partially efficient unless the sample is heated (Lamed and Bayer, 1988b). However, the cellulosome can be dissociated under relatively mild conditions using 0.05% SDS at pH 5 in the presence EDTA, and reassociated by dialysis after buffer at pH >7 (Bhat and Wood, 1992).

IV. CATALYTIC COMPONENTS OF THE CELLULOSOME

With a few exceptions (Ng and Zeikus, 1981; Pètre et al., 1981; Morag et al., 1991b), most of the components characterized to date have been purified and characterized from recombinant *E. coli* clones expressing the corresponding genes. A number of groups have isolated collections of clones expressing cellulase- or hemicellulase-related activities from several *C. thermocellum* strains (Cornet et al., 1983; Millet et al., 1985; Piruzian et al., 1985; Schwarz et al., 1985; Romaniec et al., 1987; Sakka et al., 1989; Bumazkin et al., 1990; Tuka et al., 1990; Zverlov and Velikodvorskaya, 1990; Kobayashi et al., 1992). Screening for such clones was facilitated by the availability of easy plate detection tests based on staining of CMC with Congo red (Teather and Wood, 1982), or on the use of fluorogenic substrates, such as methylumbelliferyl-β-D-cellobioside and methylumbelliferyl-β-D-glucoside (van Tilbeurgh et al., 1982). With one exception (Hazlewood et al., 1988), however, these collections have not been compared with one another, so that it is often difficult to determine which of the genes isolated by one laboratory are truly different from those isolated by the others. Even within the type strain, the presence of insertion sequences may give rise to variable restriction patterns (Hazlewood et al., 1988), not to mention in different isolates of *C. thermocellum.* Nonetheless, the emerging pattern fits reasonably well with observations made by zymogram staining of cellulosome components. For example, in strain NCIB 10682 (=ATCC 27405 and DSM 1237), clones expressing 15 different endoglucanases, 2 xylanases, 2 β-glucosidases, and 2 lichenases have been described (Gräbnitz and Staudenbauer, 1988; Hazlewood et al., 1988; Schwarz et al., 1988; Schimming et al., 1991; Fontes et al., 1995). The endoglucanases differ somewhat in their catalytic properties (e.g., range of cellodextrins hydrolyzed, or randomness of bond cleavage). However, it is far from clear why such a diversity should be needed, and whether it is needed at all.

The sequences of the catalytic domains of *C. thermocellum* endoglucanases, β-glucosidases, and hemicellulases show few distinguishing features compared with similar enzymes characterized in other organisms. Referring to the classification proposed by B. Henrissat (Henrissat, 1991; Henrissat and Bairoch, 1993), β-glucosidases BglA and BglB belong to families 1 and 2, respectively, endoglucanase CelA to family 8, endoglucanases CelB, CelC, CelE, CelG, and CelH to family 5, endoglucanases CelD,

CelF, and CelI to family 9, xylanase XynZ to family 10, and lichenase LicB to family 16 of glycosyl hydrolases. Members of all these families have been encountered in a wide variety of cellulolytic organisms. Available evidence indicates that glycosyl hydrolases belonging to the same family share the same mechanism (Gebler et al., 1992) and the same overall protein fold, with conserved catalytic residues located at equivalent positions in the molecule.

It was once debated whether the cellulase system of *C. thermocellum* contained any true exoglucanase (Mayer et al., 1987). It appears now clearly that CelS (previously termed S_S or S8), one of the major components of the cellulosome, is a cellobiohydrolase belonging to the newly identified family 48 of bacterial cellobiohydrolases (Wu et al., 1988; Morag et al., 1993, 1991b; Wang et al., 1993b; Kruus et al., 1995b). In addition, two genes encoding cellobiohydrolases with properties differing from those of CelS have been cloned from *C. thermocellum* F7 (Tuka et al., 1990). One of the cellobiohydrolases, CBH3, belongs to family 9 of glycosyl hydrolases (GenBank accession No. X80993). It has been purified (Singh and Akimenko, 1993) and shown to act synergistically with other *C. thermocellum* endoglucanases to degrade Avicel and filter paper (Tuka et al., 1992; Singh and Akimenko, 1994).

The catalytic domains of several *C. thermocellum* β-glycanases have been studied in detail, yielding models for the structure and mechanism of enzymes belonging to families 5, 8, 9, and 10 (Juy et al., 1992; Dominguez et al., 1995; Alzari et al., 1996). Two basic, classical mechanisms have been proposed to account for the hydrolytic activity of glycosyl hydrolases (Koshland, 1953; Sinnott, 1990). In the first case, illustrated by *C. thermocellum* endoglucanase CelC, hydrolysis proceeds in two steps (Figure 2). Cleavage of the glycosidic bond is promoted by an acidic group (Glu-140), which donates a proton to the glycosidic oxygen. Departure of the leaving group is accompanied by the formation of an oxocarbonium intermediate, which interacts with a negatively charged carboxylate group (Glu-280) acting as a nucleophile and located on the other side of the anomeric carbon. In a second step, the proton donor is regenerated by a proton from a water molecule, and the resulting hydroxyl ion substitutes for the nucleophile at the level of the anomeric carbon (Navas and Béguin, 1992; Wang et al., 1993a; Dominguez et al., 1995). Thus, the stereochemical course of the reaction amounts to a double inversion, leading to overall retention of configuration at the level of the anomeric carbon (Gebler et al., 1992).

This mechanism is typical for cellulases and hemicellulases belonging to a large superfamily of β-glycanases grouping, among others, β-glucosidases of family 1, endoglucanases of family 5, and xylanases of family 10. All members of the superfamily are characterized by a $(\beta/\alpha)_8$ barrel structure similar to that of triose isomerase (Dominguez et al., 1995; Henrissat et al., 1995; Jenkins et al., 1995). The catalytic site is located in a cleft formed by the loops connecting strands to helices on the COOH side of the β-strands. The distance between the carboxylate residues involved in catalysis is about 5.5 Å, which is short enough to allow direct contact between the nucleophile and the substrate.

The second type of mechanism is illustrated by endoglucanase CelD. The reaction proceeds in a single step leading to inversion of configuration (Figure 3). As in lysozyme, departure of the glycosidic oxygen is promoted by a proton donor (Glu-555). However, no glycosyl enzyme intermediate is formed. The leaving group is

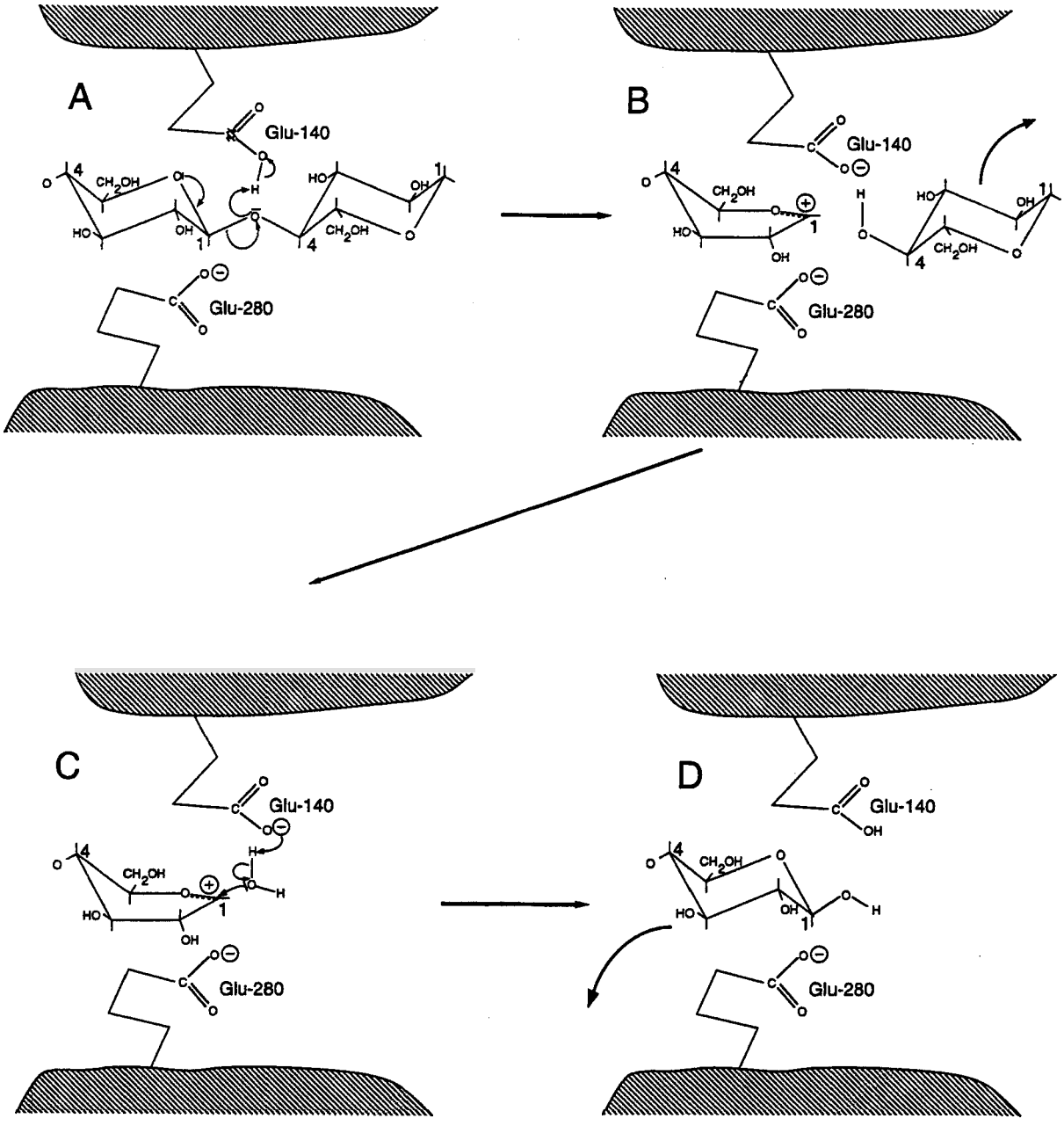

FIGURE 2. Catalytic mechanism of *C. thermocellum* endoglucanase CelC. (A) Attack of the glycosidic bond is started by the proton provided by the Glu-140 residue. (B) Splitting of the bond leads to the release of a fragment carrying a new nonreducing end group, and the formation of an oxocarbonium intermediate. The latter interacts with the Glu-280 residue. (C) A water molecule provides an OH^- ion, which reacts with the oxocarbonium ion, and an H^+ ion, which regenerates the proton lost by the Glu-140 residue. (D) The fragment carrying the new anomeric carbon is released.

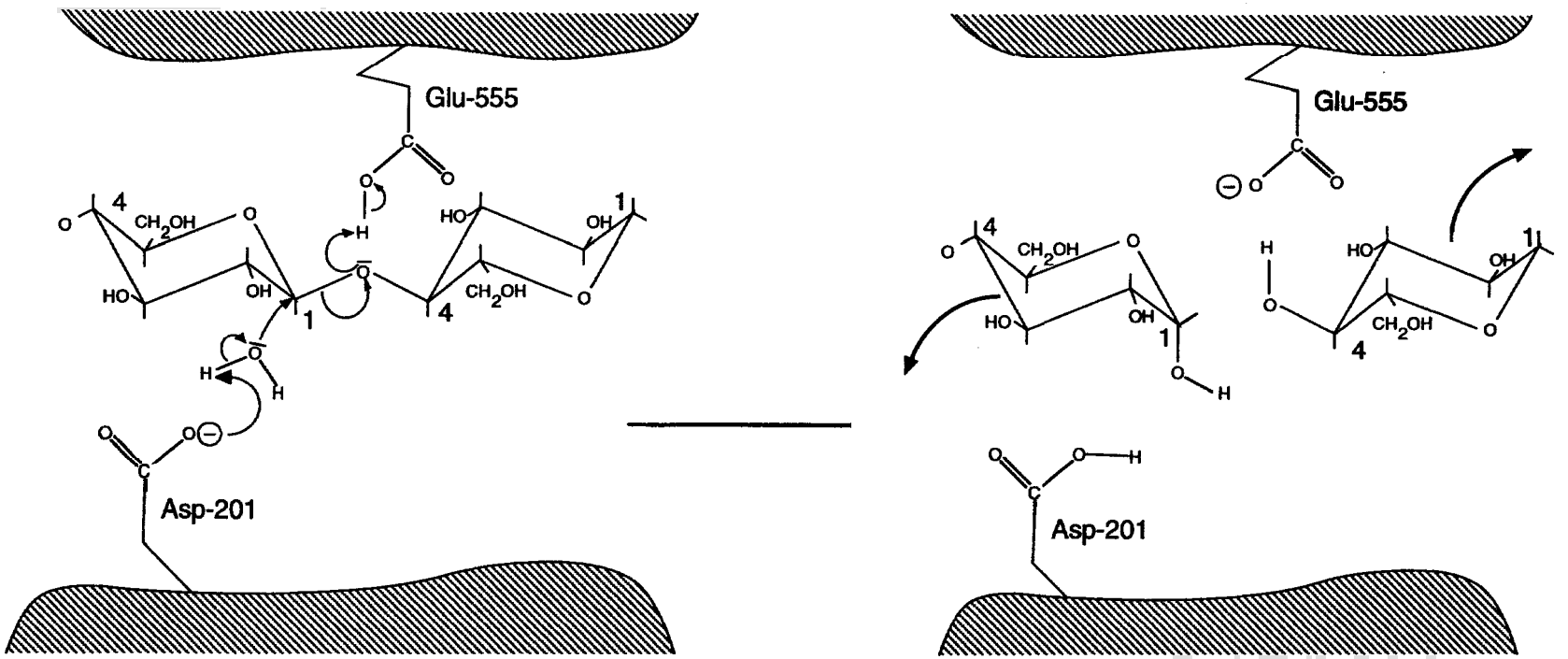

FIGURE 3. Catalytic mechanism of *C. thermocellum* endoglucanase CelD. The glucosidic bond is protonated by the Glu-555 residue. Simultaneously, the negatively charged Asp-201 residue promotes the ionization of a water molecule. The resulting OH^- ion participates in a single-step nucleophilic substitution leading to inversion of the configuration of the new anomeric carbon. (From Béguin, P. and Aubert, J. P. 1992.) *Ann. Inst. Pasteur Actualités* **3:** 91–115. With permission.)

exchanged simultaneously for a hydroxyl group, whose formation is promoted by a negatively charged nucleophile (Asp-201) located on the other side of the anomeric carbon. The distance between the two catalytic carboxylate groups is about 9.5 Å.

CelD belongs to endoglucanases of family 9 and consists of two subdomains. The COOH-terminal part of the protein folds as an α_6-α_6 barrel of about 450 residues carrying the active site, which is formed by three loops connecting six of the helices on one side of the barrel (Juy et al., 1992). The NH_2-terminal domain (about 100 residues) has a β-sheet folding pattern similar to that of the immunoglobulin modules. The latter region is absent in several β-glycanases of family 9, in particular *C. thermocellum* endoglucanases CelF and CelI.

The recently solved structure of endoglucanase CelA indicates that the inverting mechanism of family 8 endoglucanases presents an interesting variant. Although the basic framework of the protein is also an $(\alpha/\alpha)_6$ barrel, the orientation of the catalytic cleft relative to the barrel runs approximately perpendicular to that of CelD. In addition, the substrate chain appears to be kinked at the level of the scissile bond, so that one of the two adjacent residues is flipped and has the same orientation as its neighbors, instead of being rotated by 180°. As a result, the glycosidic oxygen, facing the outside of the kink, is accessible for protonation by a residue (Glu-95) lying on the same side as the nucleophile (Asp-152) (Alzari et al., 1996).

V. QUATERNARY ORGANIZATION OF THE CELLULOSOME

A first clue to the organization of the cellulosome was given by the demonstration that two components of the cellulosome could act synergistically to degrade crystalline cellulose (Wu and Demain, 1988; Wu et al., 1988). One was cellobiohydrolase CelS, then termed S_S. The other one was the largest cellulosome component, a 250-kDa glycoprotein, then termed S_L, which was shown since to correspond to the S1 component discussed above and renamed CipA (for cellulosome-integrating protein). Taken alone, CelS had some activity against CMC, but none against Avicel, and CipA had no activity against either substrate. When combined, CelS and CipA displayed significant activity against Avicel (albeit much lower than the original complex). Furthermore, CipA was shown to promote binding of CelS to cellulose. These results suggested that CipA might act as a cellulose-binding factor with which catalytic subunits would associate in order to remain adsorbed to the substrate.

A. Catalytic Components Bear Specific Docking Domains

As sequences of *C. thermocellum* endoglucanases accumulated, it became obvious that most of the enzymes included a highly conserved, noncatalytic domain of about 65 residues. This domain, located mostly at the COOH terminus of the polypeptides, contains two similar segments of 22 amino acids each. It is absent from endoglucanase CelC, which cannot be detected in the cellulosome (E. A. Bayer, personal communication), but it can be found in enzymes known to be associated with the cellulosome, such as XynZ (Grépinet et al., 1988a,b), CelE (Hall et al., 1988; Hazlewood et al., 1990), CelG (Lemaire and Béguin, 1993), or CelS (Wu et al., 1988; Wang et al., 1993b). Experiments with truncated pro-

teins lacking the duplicated segment ruled out that the latter might be directly involved in catalysis or substrate binding (Grépinet et al., 1988b; Hall et al., 1988). Western blotting using an antiserum reacting with the duplicated segment of endoglucanase CelD revealed, in the culture medium of *C. thermocellum,* a set of at least half a dozen cross-reacting polypeptides ranging from 50 to 100 kDa. These were all present in high-molecular-mass fractions binding to cellulose, indicating that they were part of the cellulosome. Furthermore, when ^{125}I-labeled forms of CelD and XynZ containing the duplicated segment were used to probe cellulosome proteins transferred to nitrocellulose, the band corresponding to CipA was labeled conspicuously. No labeling was observed when the labeled probes consisted of truncated forms of CelD and XynZ devoid of the duplicated segment (Tokatlidis et al., 1991b). Thus, the duplicated segment appears to function as a docking domain responsible for anchoring the various catalytic subunits to the scaffolding protein CipA. This domain is now termed dockerin domain (Bayer et al., 1994).

In addition to their specific function, dockerin domains enhance the hydrophobic character of proteins that harbor them. For example, the intact form of CelD comprising the dockerin domain precipitates at lower ammonium sulfate concentrations than the cognate catalytic core. The same is true for the chimeric protein CelC-Cel'D, in which the dockerin domain of CelD is fused to *C. thermocellum* CelC (Tokatlidis et al., 1993). As binding to carbohydrates is mostly mediated by hydrophobic interactions, this may explain why dockerin domains may sometimes modify the properties of cellulolytic enzymes in a fashion reminiscent of cellulose-binding domains (CBDs). Fierobe et al. (1991) compared the properties of intact and truncated forms of *C. cellulolyticum* endoglucanase CelA (formerly CelCCA), carrying or not a dockerin domain similar to those borne by the *C. thermocellum* enzymes. The intact form was somewhat more active than the truncated form on Avicel, and less active on soluble or amorphous substrates, such as CMC, barley β-glucan, or swollen Avicel. The rate of decrease in the viscosity of CMC as a function of the release of reducing sugars was lower, indicating a less random mode of action. Similar observations were made with the intact and the truncated form of endoglucanase CelC (formerly CelCCC) from the same organism, except that the intact form did not show enhanced activity toward Avicel (Fierobe et al., 1993). Another property of proteins bearing dockerin domains is an enhanced tendency to form insoluble inclusion bodies. These are clearly observed in the cytoplasm of *E. coli* cells producing large amounts CelD (Joliff et al., 1986) or CelC-Cel'D (Tokatlidis et al., 1993), but not in cells producing CelC (Tokatlidis et al., 1993) or a truncated form of CelD lacking the dockerin domain (Chauvaux et al., 1990). When cell extracts are prepared, the intact forms of CelD or CelC-Cel'D are found in the insoluble fraction, whereas the cytoplasm contains truncated forms in which the dockerin domain is partially deleted by host proteases (Tokatlidis et al., 1991a, 1993). In contrast to most other proteins forming inclusion bodies, polypeptides whose aggregation is mediated by dockerin domains are not grossly misfolded. Using a diffusible substrate, it was shown that the activity of CelD or CelC-Cel'D trapped in inclusion bodies was about as high as that of the soluble form of the enzyme extracted from the cytoplasmic supernatant (Tokatlidis et al., 1991a). Intermolecular interactions involving the dockerin domains are a likely explanation for these observations. Indeed, when a polypeptide corresponding to the dockerin domain of CelD is overproduced in *E. coli,* it also forms inclusion bodies,

which require treatment with 6 *M* guanidine hydrochloride to be solubilized. In addition, the solubilized form can only be kept in solution in buffers containing chaotropic agents such as 6 *M* guanidine hydrochloride or 6 *M* urea (unpublished data). It is tempting to speculate that the two 22-residue segments present in dockerin domains can interact with each other either within the same polypeptide, leading to hairpin formation, or between different polypeptides, leading to insoluble aggregates.

B. Structure and Function of the Cellulosome-Integrating Protein CipA

If CipA was to act as a scaffolding protein that bound a series of enzymes carrying highly similar dockerin domains, it was expected to have a multimodular structure, with reiterated receptor domains binding the dockerin domains. A CBD was also required to account for the role of CipA as a cellulose-binding factor. Mild protease treatment of CipA generated a set of discrete polypeptides, some of which bound to CelD, some to cellulose, and some to both. This suggested that distinct regions of the polypeptide were responsible for binding catalytic subunits such as CelD or for adhering to cellulose (Salamitou et al., 1992). The sequence of CipA, schematized in Figure 4, indicates that it is indeed a multifunctional protein (Gerngross et al., 1993). The polypeptide includes nine highly similar modules of about 147 residues each separated by Pro/Thr-rich linker segments, and a typical CBD of type III located between the second and the third module. The polypeptide corresponding to the seventh module was shown to bind the chimeric protein CelC-Cel'D (harboring the dockerin domain of CelD fused to CelC) with an affinity constant of $4.7 \times 10^7\ M^{-1}$ (Salamitou et al., 1994a). Similar results have been obtained for the second and third module: biotinylated polypeptides corresponding to these segments (with the adjacent CBD) were shown to bind to the catalytic subunits of the cellulosome transferred onto nitrocellulose (Yaron et al., 1995). Thus, each of the reiterated modules of CipA functions as a receptor domain responsible for binding the complementary dockerin domains borne by the catalytic subunits. These receptors are now termed cohesin domains (Bayer et al., 1994).

The interaction between cohesin and dockerin domains is enhanced in the presence of Ca^{2+} and strongly decreased in the presence of EDTA (Yaron et al., 1995). This observation can be correlated with the finding that EDTA promotes the dissociation of the cellulosome under relatively mild conditions (Bhat and Wood, 1992; Beattie et al., 1994). However, the role of Ca^{2+} in cellulosome assembly still presents unresolved issues. In particular, which — the dockerin or the cohesin domain — is responsible for binding Ca^{2+}? It was noted that part of the consensus sequence of dockerin domains resembles the EF-hand motif present in many Ca^{2+}-binding proteins (Chauvaux et al., 1990), but later studies failed to demonstrate a measurable affinity of CelC-Cel'D for Ca^{2+} (Tokatlidis et al., 1993). However, the matter deserves to be reinvestigated in view of the influence of Ca^{2+} on the cohesin-dockerin interaction. It may be that Ca^{2+} acts by binding to cohesin domains. Alternatively, binding to cohesin domains may enhance the affinity of dockerin domains for Ca^{2+} by an induced fit mechanism. There is also no clear explanation for the observation that enzymatically inactive complexes are formed upon reassociation in the presence of Ca^{2+} of dissociated cellulosome subunits. This result was interpreted by suggesting that the addi-

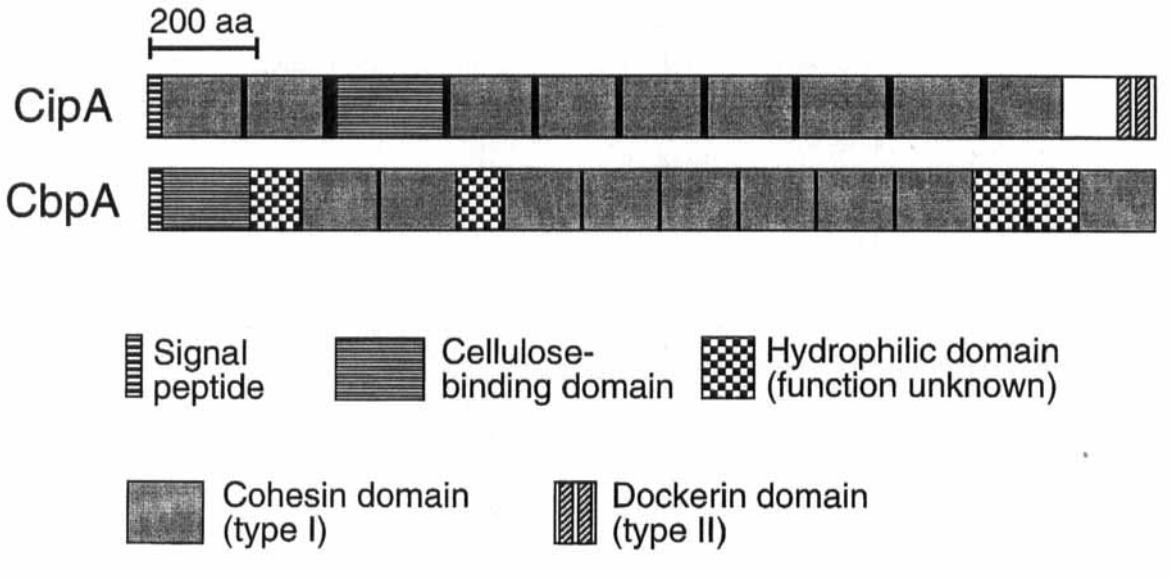

FIGURE 4. Schematic representation of the multimodular structure of *C. thermocellum* CipA and *C. cellulovorans* CbpA. The various regions identified within each polypeptide are indicated with different patterns.

tion of Ca^{2+} might prevent the formation of properly ordered complexes (Beattie et al., 1994). The hypothesis may explain why activity against native cellulose (cotton) was not recovered. However, it is not clear why almost all activity toward CMC should have been lost as well, as the latter is not supposed to depend on the formation of an ordered complex.

The DNA segment encoding the CBD has also been subcloned and expressed, and the properties of the CBD polypeptide have been investigated (Morag et al., 1995). Microcrystalline cellulose binds the purified CBD with a dissociation constant of 0.4 μM and a capacity of 0.54 μM/g. The binding capacity of amorphous cellulose is about 20-fold higher. The CBD polypeptide has been crystallized (Lamed et al., 1994). The determination of its structure will provide a paradigm for CBDs of type III, which are common among bacterial cellulases (Béguin and Aubert, 1994).

The COOH terminus of CipA consists of a duplicated segment clearly related to the dockerin domains borne by the catalytic subunits. However, this domain is unable to bind to cohesin domains of the kind borne by CipA (Salamitou et al., 1994a). Recent results indicate that it binds to another type of cohesin domain, which is borne by specific cell surface proteins (see below).

In *C. thermocellum,* CipA is heavily O-glycosylated, with an estimated carbohydrate content reaching up to 40%. Carbohydrate chains are bound to the Thr and Ser residues of the interdomain linker regions (Gerwig et al., 1993). The structure of the two major oligosaccharides has been determined. One is a β-1,4 galactopyranose dimer. The other is a branched tetrasaccharide containing D-galactopyranose, D-galactofuranose, and 3-*O*-methyl-*N*-acetyl-D-glucosamine (Gerwig et al., 1989). The function of the carbohydrate moiety is unclear. Glycosylation of the Pro-Thr-containing segment connecting the catalytic domain and the CBD of *Cellulomonas fimi* cellulases results in reduced sensitivity to

proteases (Langsford et al., 1987). Possibly the same role can be ascribed to the carbohydrate moiety of CipA. However, glycosylation does not prevent CipA from being highly sensitive to proteolytic degradation (Morag et al., 1991a; Salamitou et al., 1992).

C. Quaternary Structure and Mode of Action of the Cellulosome

The model of the cellulosome shown in Figure 5 reflects the biochemical evidence discussed above. It is also derived from a model previously proposed by Mayer and colleagues on the basis of electron micrographs of cellulosome preparations. The pictures showed structures resembling a bunch of grapes, with globular subunits linked by a thin stalk to a central string (Mayer et al., 1987). At the time, cellulosomal cellobiohydrolases had yet to be identified, and it was assumed that endoglucanases could fulfill the same role if they were clustered and poised to attack the same cellulose chain at closely spaced intervals. A set of quasisimultaneous cutting events would generate cellodextrins with a low degree of polymerization (DP). Because the latter would be held by few bonds to the crystal lattice, they could diffuse away from the reaction site, in the same manner as cellobiose generated by the action of cellobiohydrolases. However, as discussed above, there is now evidence that cellobiohydrolases are produced by *Clostridium thermocellum.* Indeed, *in vitro* reconstitution experiments suggest that both classical endoglucanase/cellobiohydrolase synergism and enhanced activity due to clustering may contribute to the efficiency of the cellulosome. Bhat and colleagues isolated an endoglucanase, two cellobiohydrolases, and CipA by preparative electrophoresis of cellulosome components, and studied their effect alone and in combination (Bhat et al., 1994). Each pairwise combination of components had a higher cellulolytic activity than the sum of the activities of individual subunits. Thus, the endoglucanase showed synergism with each of the cellobiohydrolases, and the cellobiohydrolases showed synergism with each other. Adding the three catalytic components resulted in an even higher enhancement of activity. The same situation prevails with the unassociated cellulase systems of aerobic fungi. However, adding CipA to any of the individual catalytic components or to any combination of the three enzymes resulted in a 2.4- to fourfold increase in the release of total soluble sugars. In the case of the endoglucanase, the increase was even more striking (ninefold) when considering the release of soluble reducing sugars. This indicates that combining the endoglucanase with CipA induced more closely spaced cutting events and generated cellodextrins with a lower DP. At least 60% of each enzyme was able to bind by itself to cellulose, and binding was not significantly influenced by the addition of CipA. Thus, the enhancement of hydrolysis observed upon adding CipA cannot be accounted for by improved adsorption of the catalytic components to the substrate. However, it is likely that CipA induces clustering of the enzymes on the surface of the substrate. Even if the original multicutting event model is no longer to be taken literally, clustering probably facilitates the degradation process by allowing enzymes to act on regions whose structure has already been disrupted by previous attacks. Indeed, contrary to fungal cellulase systems, hydrolysis by the cellulosome induces very little change in the DP of the residual substrate, indicating that degradation is highly processive (Puls and Körner, 1987).

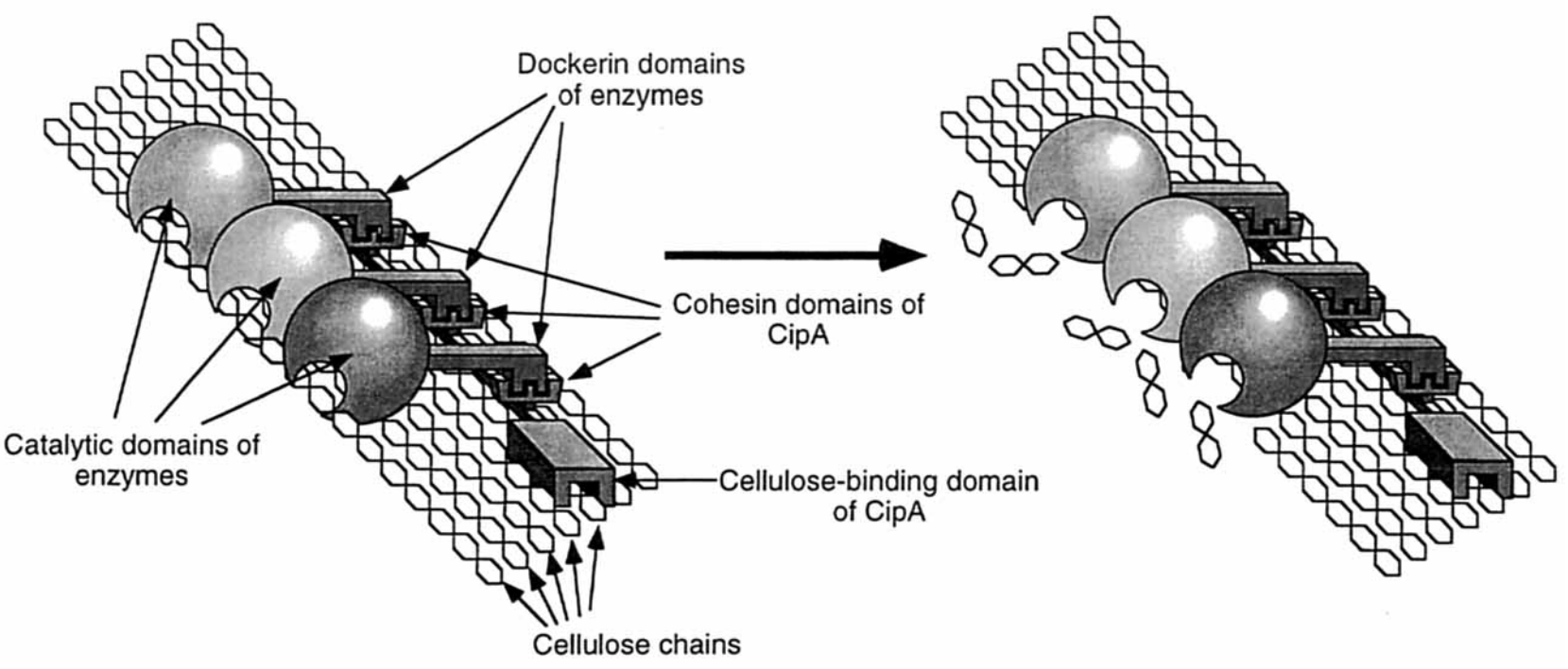

FIGURE 5. Model for the structural organization of subunits within the cellulosome. The diagram shows a portion of the complex, with the CBD of CipA and three of the CipA cohesin domains, to which catalytic components are bound by means of their dockerin domains. Glucose residues are not drawn to scale. (From Béguin, P., Millet, J., and Aubert, J.-P. 1992. *FEMS Microbiol. Lett.*, **100:** 523–528. With permission.)

D. Heterogeneity of the Cellulosome

The term cellulosome may conjure up, by analogy with the ribosome, the picture of a well-defined structure in which each of the subunits has a unique position and stoichiometry. Several arguments make such a model unlikely. That cellulosomes can assume different shapes or apparent sizes has long been known from sedimentation, gel filtration, and electron microscopic studies (Lamed et al., 1985; Mayer et al., 1987). In particular, cellulosomes isolated at early stages of cultivation appear as densely packed complexes, whereas at later stages, cellulosomal particles display a looser appearance. In aging cultures, many free subunits are detected, suggesting that the complexes undergo partial dissociation (Mayer et al., 1987). In addition, assembly of the cellulosome probably does not occur according to a strictly defined pattern. The CipA molecule contains only nine cohesin domains, and 12 polypeptides containing dockerin domains have already been identified by DNA sequencing. Obviously, each dockerin-bearing polypeptide cannot be assigned to a unique cohesin domain of CipA. In addition, cohesin domains are extremely similar (>95% identical residues for domains 4 to 8), leaving little freedom for modulating binding specificity for defined dockerin domains. Indeed, even cohesin domains 2 and 3 were found to bind equally well to the various cellulosome subunits transferred to nitrocellulose, although the two domains are only 75% identical (Yaron et al., 1995). Furthermore, the affinities of the dockerin domain of endoglucanase CelD for the seventh cohesin domain of CipA and for the cohesin domain borne by the OlpA protein (see below) differed by less than one order of magnitude, although the two domains share less than 35% identical residues (Salamitou et al., 1994a). Another argument is that no preferred neighborhood relationship could be revealed between catalytic cellulosome components using various bifunctional cross-linking reagents. For all catalytic subunits tested, specific cross-linking was only observed with CipA (L. Mels and M. Claeyssens, personal communication). These considerations are backed up by evidence showing that cellulosome preparations comprise complexes having different subunit compositions. Subpopulations of complexes containing only subsets of subunits and having higher activity on crystalline cellulose can be purified by ion-exchange (Ali et al., 1995a) or lectin affinity chromatography (Kobayashi et al., 1990). The same observation was made in the case of the cellulolytic complexes produced by *C. papyrosolvens* C7, which are probably built according to the same principle as the *C. thermocellum* cellulosome. These complexes were fractionated into subpopulations having similar size, but different subunit composition, activity pattern, and electron microscopic morphology (Pohlschröder et al., 1994, 1995). Interestingly, synergism appears to exist not only between the subunits within the complexes, but between the complexes themselves (Pohlschröder et al., 1994).

However, the heterogeneity of cellulosome populations does not mean that their organization is totally random. It is quite possible that preferred neighborhood relationships exist between defined subunits, particularly if their catalytic domains tend to associate with one another. A case in point is endoglucanase CelG, whose catalytic domain associates to form dimers (Lemaire and Béguin, 1993).

VI. THE CELLULOSOME AND THE CELL SURFACE OF *C. THERMOCELLUM*

The cellulosome can hardly be described adequately without referring to the cell en-

velope of *C. thermocellum*. Indeed, the complex is probably assembled from components bound to the cell envelope. Because all known sequences of cellulosome polypeptides, including CipA, start with a typical signal peptide, they are most likely secreted individually through the general secretion pathway. Furthermore, no free CipA has ever been detected in the culture medium. Thus, attachment of catalytic components to CipA probably occurs on the surface of the cells. Investigating the composition and organization of the cell envelope of *C. thermocellum* is therefore essential to understand how the cellulosome is assembled and how it is anchored to the cell surface.

A. Morphology of the Cell Envelope

Electron microscopy of thin sections of *C. thermocellum* cells reveals that the cell envelope is composed of several layers. These include the cytoplasmic membrane, the peptidoglycan, an S-layer (Nolte, 1992; Lemaire, 1995), and an amorphous outer layer (Bayer et al., 1985; Bayer and Lamed, 1986; Nolte and Mayer, 1989; Salamitou et al., 1994b; Lemaire et al., 1995).

The thickness of the peptidoglycan does not exceed 10 nm. Its composition is typical of that found for numerous other clostridia (Lemaire et al., 1995). The S-layer consists of a hexagonal network formed by a 130-kDa promoter (Lemaire, 1995). The latter appears identical to the 130-kDa glycoprotein characterized by Lamed and co-workers (Lamed and Bayer, 1988a). The S-layer promoter is the major component of the cell envelope fraction after washing with Triton X-100. It is noncovalently bound to the peptidoglycan and can be solubilized by SDS, guanidine hydrochloride, or by digesting the peptidoglycan with lysozyme (Lemaire, 1995). The outer layer forms an amorphous, irregular coating on the outside of the cells, with cellulosome-containing protuberances ranging up to 150 to 200 nm. It is apparent in whole-cell mounts negatively stained with ammonium molybdate (Salamitou et al., 1994b; Lemaire et al., 1995) or in thin sections stained with ruthenium red (P. Gounon, personal communication) or cationized ferritin (Bayer et al., 1985; Bayer and Lamed, 1986; Salamitou et al., 1994b; Lemaire et al., 1995). The AD2 mutant selected by Bayer and co-workers for its failure to adhere to cellulose (Bayer et al., 1983) still possesses an outer layer stained by cationized ferritin, but the layer is thin and regular and is devoid of cellulosome-containing protuberances (Bayer et al., 1985).

B. Cellulase-Binding Components of the Cell Envelope

Several genes encoding cell surface proteins that bind either individual cellulolytic components or CipA have been identified recently. Three of the genes, *olpB*, ORF2, and *olpA*, form a cluster located downstream of CipA (Fujino et al., 1993a). A fourth gene, *sdbA*, is not closely linked with the cluster (Leibovitz and Béguin, 1996). The corresponding polypeptides are composed of multiple domains (Figure 6). The four proteins contain a COOH-terminal triplicated motif, which is similar to sequence segments present in the S-layer proteins of several bacteria, and which have been termed SLH domains (for S-layer homology) (Lupas et al., 1994). The NH_2-terminal regions of OlpB and Orf2p consist of highly conserved modules of 150 to 160 residues, which are reiterated four- and twofold, respectively,

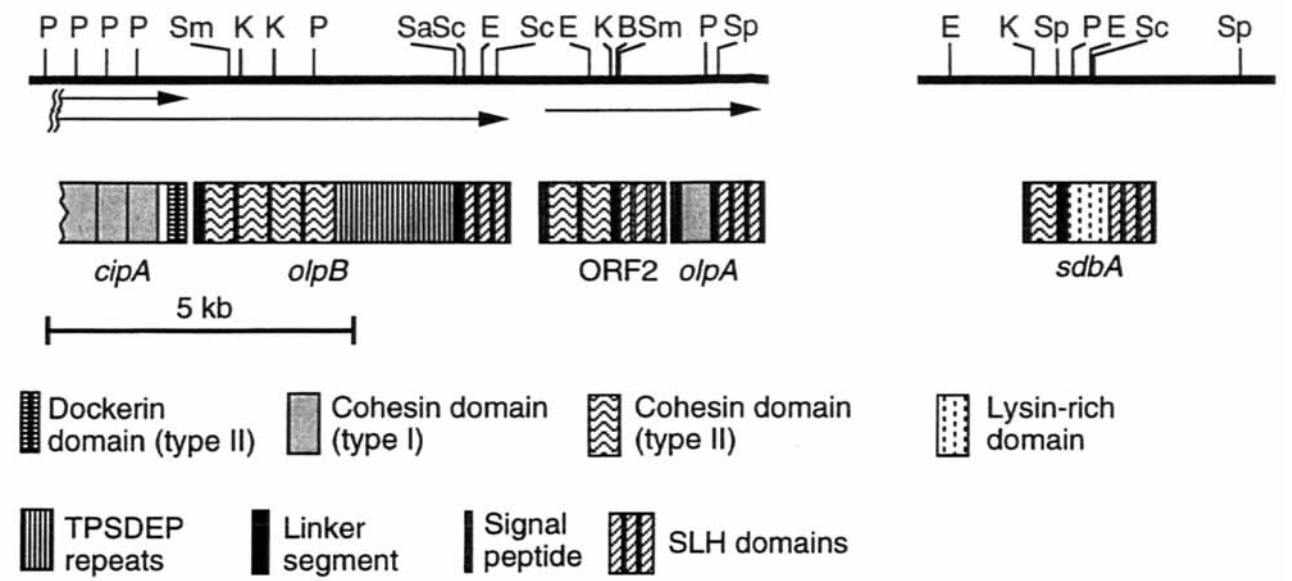

FIGURE 6. Genes encoding cell surface proteins with cohesin domains of type II, which are probably involved in anchoring cellulosome and cellulosome components to the surface of *C. thermocellum*. The genes *cipA, olpB,* ORF2, and *olpA* are contiguous. *sdbA* maps in a different region of the chromosome. The position and orientation of the *cipA-olpB* and ORF2-*olpA* transcripts are indicated by arrows. The positions of the segments encoding the various regions identified within each polypeptide are indicated with different patterns. E, *Eco*RI; K, *Kpn*I; P, *Pst*I; Sa, *Sal*I; Sc, *Sac*I; Sm, *Sma*I.

in the two polypeptides. A single copy of a similar module is present in SdbA. The NH_2-terminal sequence of OlpA is related to the cohesin domains of CipA, although it is more distant from the consensus. In OlpB, Orf2p, and OlpA, the NH_2- and COOH-terminal regions are separated by linker segments rich in proline, glycine, and hydroxy amino acids. The central segment of OlpB is quite remarkable: the motif TPSDEP is repeated 73-fold.

The presence of SLH domains suggested that these polypeptides were located in the cell envelope of *C. thermocellum.* Indeed, electron microscopy showed that antibodies directed against OlpA and OlpB bind to antigens located on the cell surface (Salamitou et al., 1994b; Lemaire et al., 1995). In addition, the biochemical properties of SLH domains are consistent with the hypothesis that they mediate the attachment of proteins to the cell envelope. Chimeric MalE protein harboring the SLH domains of OlpB binds to peptidoglycan. SLH domains can also interact with each other, possibly leading to the formation of a network within the outer layer (Lemaire et al., 1995). The latter phenomenon would be more consistent with the observation that OlpA and OlpB can be found in protuberances, at a distance precluding direct contact with the peptidoglycan layer.

At first, the cohesin domain present in OlpA appeared as a good candidate for binding the dockerin-like sequence at the COOH terminus of CipA, which would provide an attractive model for the attachment of the cellulosome to the cell surface. However, while OlpA is able to bind the dockerin domain of CelD, it cannot bind the dockerin domain of CipA (Salamitou et al., 1994a). Thus, the role of OlpA is more likely to bind individual cellulolytic components.

Anchoring of cellulosomes to the surface of the cells appears to be mediated by the interaction between the COOH-terminal dockerin domain of CipA and the domains located at the NH_2-terminal end of OlpB, ORF2p, and SdbA. SdbA was cloned on the basis of its affinity for the dockerin domain of CipA, which was grafted onto endoglucanase CelC (Salamitou et al., 1994a; Leibovitz and Béguin, 1996). Subcloning and expression of the region encoding the NH_2-terminal domain revealed that the latter was responsible for the binding of the probe. Subsequently, the same result was obtained with one of the NH_2-terminal repeats of OlpB. The other highly similar modules present in OlpB and in the polypeptide encoded by ORF2 can reasonably be expected to have the same binding properties. Based on the difference in binding specificity, it has been proposed to distinguish between cohesin and dockerin domains of type I and II. Cohesin domains of type I are represented by the cohesin domains of CipA and OlpA, and the cognate dockerin domains by the duplicated segments borne by catalytic components. Cohesin domains of type II, represented by the NH_2-terminal modules of OlpB, SdbA, and ORF2p, recognize the dockerin domain of type II (so far the only one of its kind) borne by CipA (Leibovitz and Béguin, 1996).

It may be noted that some hydrolytic enzymes may also be bound directly to the surface: xylanase XynX contains SLH segments, but no dockerin domain. In this, XynX resembles some of the hydrolases described in other organisms, such as the endoglucanase of *Bacillus* sp. KSM-635 (Ozaki et al., 1990), the xylanase of *Thermoanaerobacterium saccharolyticum* B6A-RI (Lee et al., 1993), and the pullulanase of *Thermoanaerobacter thermosulfurigenes* EM1 (Matuschek et al., 1994). The latter enzyme has indeed been demonstrated to be localized on the bacterial cell surface (Antranikian et al., 1987).

Thus, the cell envelope of *C. thermocellum* appears well equipped to retain indi-

vidual cellulolytic enzymes, as well as cellulosomes, on the surface of the bacteria. Moreover, the function of "carrier" proteins such as OlpB, ORF2p, SdbA, and OlpA may be broader than the mere presentation of enzymes on the surface of the cells. By trapping CipA and the catalytic subunits of the cellulosome before they have a chance to escape into the culture medium, they might serve as "extracellular chaperones" participating in cellulosome formation. In addition, multivalent carrier proteins, such as OlpB, which could furthermore associate by means of their SLH domains, could potentially be involved in the architecture of polycellulosomes.

VII. REGULATION

In the absence of tools for genetically manipulating *C. thermocellum,* knowledge about the regulation of genes involved in cellulose degradation is somewhat preliminary. In contrast to *T. reesei* and *Cellulomonas,* there is no evidence suggesting that cellulase synthesis in *Clostridium thermocellum* requires induction by oligosaccharides originating from cellulose degradation. Cellulase is produced in the presence of substrates, such as sorbitol or fructose, that are not derived from cellulose hydrolysis (Johnson et al., 1985; Nochur et al., 1993). The production of cellulase by *C. thermocellum* relative to that of other cell proteins increases dramatically when the bacterium is grown under carbon source limitation. Such conditions prevail when the substrate is fed in small batches or inefficiently metabolized, such as fructose when fed to cellobiose-adapted cells (Johnson et al., 1985; Nochur et al., 1993). This suggests that cellulase biosynthesis is regulated by a mechanism analogous to catabolite repression. A positive correlation has been established between specific cellulase activity and parameters related to energy metabolism, such as ATP content and proton motive force (Nochur et al., 1993). It must be noted, however, that measurements of cellulase activity as a function of dry cell weight do not provide an estimate of the differential rate of synthesis, which would require measuring the increase in activity as a function of time and cell mass.

Expression of specific genes was also studied at the level of mRNA synthesis. Most of the genes are scattered, and their mRNA is monocistronic. A notable exception is the *cipA-olpB*-ORF2-*olpA* cluster, in which *cipA* and *olpB* on one hand, and Orf2 and *olpA* on the other hand, are cotranscribed (Fujino et al., 1993a). Start sites have been determined for the transcripts of *celA, celD,* and *celF.* Transcription of *celA* and *celD* starts at two sites, with the distal and proximal sites resembling *Bacillus subtilis* σ^A and σ^D promoters, respectively (Béguin et al., 1986; Mishra et al., 1991). A time course dot-blot analysis of mRNA content indicated that for *celA, celD,* and *celF,* transcripts were most abundant when cells were about to reach the stationary phase due to carbon source limitation. Some delay was observed for the appearance of *celC* mRNA. Early *celD* transcription started from the σ^D-like promoter and shifted to the σ^A-like promoter once the cells reached the stationary phase (Mishra et al., 1991).

VIII. RELATED CELLULOLYTIC SYSTEMS IN OTHER ORGANISMS

A. Cellulase Complexes in Other Clostridia

The best documented evidence for complexes similar to *C. thermocellum* cellulosomes has been described for mesophilic cellulolytic clostridia, particularly *C. cellulo-*

vorans and *C. cellulolyticum.* For both organisms, the composition of the complexes appears somewhat simpler than in the case of *C. thermocellum.* A size of 900 kDa was reported for the *C. cellulovorans* cellulosome (Shoseyov and Doi, 1990). The high-molecular-weight cellulase fraction isolated from *C. cellulolyticum* eluted in the exclusion volume of a Sepharose 4B column, suggesting that it was composed of polycellulosomes (Madarro et al., 1991). In both cases, catalytic components are organized around a 160- to 170-kDa component, which contains a CBD and reiterated cohesin domains (Shoseyov and Doi, 1990; Madarro et al., 1991; Shoseyov et al., 1992) (A. and J.-P. Belaich, personal communication). The scaffolding components of *C. cellulovorans* and *C. cellulolyticum* have been termed CbpA and CipC, respectively. The CBD and the cohesin domains of both proteins display sequence similarities with the corresponding domains of *C. thermocellum* CipA. Indeed, the binding parameters of the CBDs of CipA and CbpA are very similar (Goldstein et al., 1993; Morag et al., 1995). However, the scaffolding proteins of the two mesophilic clostridia differ from CipA in two respects. Neither polypeptide possesses a C-terminal dockerin domain like the dockerin domain of type II borne by CipA. By contrast, the two proteins include hydrophilic modules, which are present in four copies in CbpA (Figure 4). As yet, no function has been assigned to these modules, which are also present in two copies in CelZ, a cellulase from *C. stercorarium* (Jauris et al., 1990). It would be of interest to investigate whether they fulfill the same anchoring role as the type II dockerin domain of CipA.

The genes encoding several catalytic subunits of the cellulase system of *C. cellulolyticum* have been cloned and sequenced. Two enzymes, CelA and CelD, belong to the family 5 glycosyl hydrolases and are encoded by isolated genes (Faure et al., 1989; Shima et al., 1991). A large cluster comprising at least five genes encodes the remaining components identified so far (Bagnara-Tardif et al., 1991): CipC (the scaffolding protein) (A. and J.-P. Belaich, personal communication), CelF (a family 48 cellobiohydrolase), CelC (a family 8 endoglucanase), and CelG and CelE (both family 9 endoglucanases). The biochemical properties of CelA and CelC have been characterized (Fierobe et al., 1991, 1993), and the three-dimensional structure of CelA has been determined (Ducros et al., 1995). Because *C. cellulolyticum* CelA and *C. thermocellum* CelC belong to the same family, but possess distinct enzymatic properties, comparison between the structures of the two enzymes should provide interesting insights about structural determinants influencing catalytic specificity.

All of the known sequences of *C. cellulolyticum* cellulases contain a COOH-terminal repeat clearly related to the dockerin domains of *C. thermocellum* enzymes. It was shown recently that this domain specifically interacts with the dockerin domains borne by CipC (A. and J.-B. Belaich, personal communication). This supports the contention that the organization of the *C. cellulolyticum* cellulase complex is similar to that of the *C. thermocellum* cellulosome.

Five genes encoding endoglucanases have been cloned from *C. cellulovorans* (Doi et al., 1994). In addition, the cellulase complex contains a major 70-kDa polypeptide with low activity against CMC, which may correspond to a family 48 cellobiohydrolase. Two of the genes, *engB* and *engD,* have been sequenced and found to encode endoglucanases of family 5. However, while EngB possesses a COOH-terminal duplicated sequence related to *C. thermocellum* dockerin domains, in EngD this region is replaced by a CBD of type I (Hamamoto et al., 1992). Surprisingly, EngD appears none-

theless to be a component of the *C. cellulovorans* cellulosome (Foong and Doi, 1992), and the protein binds *in vitro* to one of the cohesin domains of the scaffolding protein CbpA with about the same affinity as EngB (Takagi et al., 1993). Possibly, the *C. cellulovorans* complex represents a less specific form of cellulosome, in which the binding specificity of the cohesin domains is not restricted to complementary dockerin domains. Another intriguing property of the *C. cellulovorans* system is that on growth on cellobiose, the components of the cellulosome are secreted into the medium, but fail to associate: when immunoaffinity chromatography is performed on the culture supernatant using antibodies directed against specific subunits, other subunits fail to copurify. However, the addition of cellulose to the culture supernatant appears to trigger the formation of complexes. Analysis of material bound to cellulose indicates that it contains CbpA and two other major components of the cellulosome, P100 and P70. The latter polypeptides must be bound to CipA because they fail to bind to cellulose by themselves (Matano et al., 1994). No such observations were made with *C. thermocellum,* which readily synthesizes cellulosomes when grown on cellobiose (Bayer et al., 1985). These differences may be correlated to the fact that *C. cellulovorans* belongs to a phylogenetic cluster that is quite distinct from the group including *C. thermocellum, C. cellulolyticum,* and a number of other cellulolytic clostridia (Lin et al., 1994; Rainey and Stackebrandt, 1994).

Although the evidence is not as strongly documented as for the organisms discussed above, several bacteria belonging to the clostridial assemblage probably produce cellulolytic complexes related to the cellulosome. *C. josui* possesses a cluster of cellulase genes that are highly similar to those of *C. cellulolyticum* and are organized in the same order. The encoded polypeptides include typical dockerin domains (Fujino et al., 1993b). Biochemical evidence for the presence of associated cellulases is suggested by the fact that the purification of one of the endoglucanases present in the *C. josui* culture medium was reported to require the presence of 6 *M* urea (Fujino et al., 1989). The same procedure was required for the purification of *C. thermocellum* endoglucanase CelA (Pètre et al., 1981).

C. papyrosolvens strain C7 produces a set of cellulolytic complexes of about 700 kDa having different polypeptide compositions and different substrate specificities (Pohlschröder et al., 1994) (see above). A 125- to 130-kDa polypeptide showing immunological cross-reactivity with *C. thermocellum* CipA is present in all complexes (Cavedon et al., 1990b; Pohlschröder et al., 1994). This component was not produced in a mutant in which CMCase activity was retained, but Avicelase activity was lost. The mutant was found to produce unassociated cellulases (Cavedon et al., 1990a). This suggests that the 125- to 130-kDa polypeptide may be the scaffolding component of the complex.

Bacteroides cellulosolvens has been recently recognized on the basis of 16 S RNA analysis as a close relative of mesophilic cellulolytic clostridia such as *C. cellulolyticum* and *C. papyrosolvens* (Lin et al., 1994). This finding is in agreement with previous studies showing that *B. cellulosolvens* also produces a 700-kDa cellulolytic complex containing a 230-kDa glycoprotein, which cross-reacts with an antiserum directed against *C. thermocellum* CipA (Lamed et al., 1991b).

In many other bacteria degrading either cellulose or hemicellulose, hydrolytic enzymes appear to be clustered in cell surface protuberances (Lamed et al., 1987; Bayer et al., 1994). This is the case, for example, for rumen bacteria such as *Ruminococcus albus* (Stack and Hungate, 1984), *Butyrivibrio*

fibrisolvens (Lin and Thomson, 1991), or *Fibrobacter succinogenes* (Forsberg et al., 1981). However, although several β-glycosyl hydrolase genes have been cloned from these organisms, none of the encoded proteins contains sequence elements reminiscent of the clostridial cohesin domains. Thus, it is open to question whether enzymes present in protuberances of these organisms are organized in complexes of the same type as the clostridial cellulosomes. In some cases, at least, it seems that the high M_r, sedimentable cellulase fraction present in the culture medium derives from fragments of the cell envelope bearing cell-bound enzymes (Forsberg et al., 1981).

B. High-Molecular-Mass Complexes in Anaerobic, Cellulolytic Fungi

Among the rumen microflora, some of the most active cellulolytic microorganisms are obligate anaerobic chytridiomycete fungi. The zoospore stage of these organisms was long mistaken for flagellates, until Orpin (1975) recognized the fungal nature of *Neocallimastix frontalis*. To date, the most intensively studied species are *N. frontalis*, *N. patriciarum*, and *Piromyces* sp. (for a review, see Teunissen and Op den Camp [1993]).

N. frontalis and *Piromyces* sp. produce high-M_r complexes that can be isolated by affinity chromatography. The molecular mass of the *N. frontalis* complex was estimated at 670 kDa (Wilson and Wood, 1992a). Depending on the culture conditions, only 4 to 50% of the total endoglucanase, and 1 to 17% of the β-glucosidase, but none of the xylanase activity of *N. frontalis* was associated with the high-M_r species (Wilson and Wood, 1992b). These complexes are very active against crystalline cellulose, whereas enzymes present in the unassociated, low-M_r fraction, are active only against amorphous cellulose (Wilson and Wood, 1992a,b; Ali et al., 1995b).

The distribution of the *Piromyces* complexes is polydisperse, with a major fraction of about 400 kDa and larger species migrating between the void volume and the 670-kDa marker on a Sepharose CL-4B column. The associated forms include more than 80% of the total endoglucanase, xylanase, and mannanase activities (Ali et al., 1995b). Some of the polypeptides occur both in free and associated form (e.g., the xylanase purified by Teunissen et al., 1993).

cDNAs encoding various cellulolytic and hemicellulolytic components have been cloned from *N. frontalis* (Reymond et al., 1991), *N. patriciarum* (Gilbert et al., 1992; Xue et al., 1992a,b; Zhou et al., 1994), and *Piromyces* sp. (Ali et al., 1995b; Fanutti et al., 1995). These include endoglucanases, cellobiohydrolases, xylanases, and mannanases. Genes encoding bifunctional xylanases, with two domains belonging to the family 11 of glycosyl hydrolases, have been characterized in *N. patriciarum* (Gilbert et al., 1992) and *Piromyces* sp. (Fanutti et al., 1995). However, none of the β-glycanases encoded by the 17 different genes cloned from *Piromyces* sp. binds to cellulose (Ali et al., 1995b). Because at least some of them would be expected to be associated with the large, cellulose-binding complexes, this led the authors to suggest that the complexes may consist of catalytic subunits associated with at least one protein that possesses a CBD. Recent data support the convergence between the clostridial cellulosomes and the cellulolytic complexes of *Piromyces* sp. and *N. patriciarum*. An endoglucanase, two xylanases, and a mannanase from these organisms have been shown contain a noncatalytic, conserved segment of about 40 residues. This segment

is present in three copies in the *Piromyces* mannanase and in two copies in the other enzymes. The duplicated segment of *Piromyces* xylanase fused to glutathione *S*-transferase conferred on the resulting polypeptide the capacity to bind specifically to 97- and 116-kDa components of the *N. patriciarum* and *Piromyces* complexes, respectively. The same property was observed for the duplicated segment isolated after proteolytic cleavage of the fusion protein (Fanutti et al., 1995). Once the gene encoding the scaffolding component is cloned and sequenced, it will be of interest to analyze whether it is composed of reiterated receptor modules like CipA and related proteins in clostridia. Thus, the organization principle of glycosyl hydrolase complexes appears remarkably similar in the rumen fungi and in the cellulosome-producing clostridia. In both cases, enzymes bind to a scaffolding component by means of a conserved docking domain. However, the reiterated segments of the fungal enzymes bear no sequence similarity with the dockerin domains of clostridia. This suggests that the two types of complexes probably evolved through independent convergence, and that their organization is particularly well adapted to the task of degrading a recalcitrant substrate like cellulose.

IX. CONCLUSIONS AND PERSPECTIVES

In the past 2 decades, research on cellulose degradation has experienced several conceptual advances. One was the demonstration of synergistic effects between endoglucanases and cellobiohydrolases and between different types of cellobiohydrolases in the nonassociated cellulase systems of aerobic fungi. Another was the discovery that enzymes participating in the degradation of crystalline cellulose possess specific CBDs that are distinct from the catalytic domains (van Tilbeurgh et al., 1986; Langsford et al., 1987; Gilkes et al., 1988; Tomme et al., 1988). Cellulose-binding domains are also present in several hemicellulases, which degrade the cell wall polysaccharides associated with cellulose (Kellett et al., 1990; Hazlewood and Gilbert, 1992). The cellulosome combines the two previous concepts: the catalytic components act synergistically, and they are bound, albeit noncovalently, to an independent cellulose-binding domain borne by the scaffolding component. In addition, it introduces the notion that the spatial integration of the hydrolases into a multienzymatic complex potentiates the efficiency with which crystalline cellulose is degraded.

One of the tasks for future research is to assess the relative contribution to cellulosome activity of synergism, cellulose-binding, and spatial integration to the cellulolytic activity of the cellulosome. The genes encoding the scaffolding component and a large number of catalytic components are now available for constructing and studying a wide variety of artificial complexes. Various combinations of catalytic subunits can be tested for synergism. In addition, genetic engineering offers the opportunity to prepare versions of the scaffolding subunit, which may either be devoid of CBD or consist of a CBD linked to a single cohesin domain (Kruus et al., 1995a). Using such truncated proteins in reconstitution experiments should help determine the respective contribution of the presence of a CBD and of the clustering of the catalytic components to the catalytic efficiency of the cellulosome.

Another task is to elucidate the pathway of assembly of the complex on the cell surface. In this respect, characterization of cell surface components such as OlpA, OlpB, SdbA, and others to be discovered may sug-

gest plausible ideas. However, testing the validity of hypotheses will remain difficult as long as genetic tools remain unavailable for knocking out the respective genes and analyzing the phenotype of the resulting mutants.

Cellulose degradation is far from being the only biochemical pathway in which enzymes are integrated in multifunctional complexes. Well-known examples include tryptophan synthetase (Hyde et al., 1988) and the synthesis of fatty acids and polyketide antibiotics (for reviews, see Wakil [1989]; Katz and Donadio [1993]). In addition, some artificial bifunctional enzymes obtained through gene fusion show superior properties to mixtures of single enzymes for performing coupled reactions (Bülow and Mosbach, 1991; Olsen et al., 1996). Presumably, spatial association of catalytic centers favors the channeling of reaction intermediates. In this respect, the cellulosome offers a versatile opportunity for coupling several enzymes in order to form multifunctional complexes having interesting properties. Contrary to most other complexes containing multiple polypeptides, the organization of subunits around CipA appears remarkably flexible. It is likely that for polypeptides not exceeding 100 kDa, the only requirement for integration into artificial complexes would be the presence of a dockerin domain, which can be grafted by genetic engineering (Tokatlidis et al., 1993). The potentialities of such complexes for biotechnology have been reviewed by Bayer et al. (1994). Their realization would provide another example of applied science depending on basic research and serendipity.

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