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Carbohydrate Binding Modules: Biochemical Properties and Novel Applications

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INTRODUCTION

In the late 1940s, it was proposed that the initial stage in the enzymatic degradation of crystalline cellulose involves the action of an unknown nonhydrolytic component, termed C_1. This component was thought to be responsible for destabilization (nonhydrolytic disruption) of the cellulose structure, making the substrate accessible to the enzyme, the C_x component (161). The proteolytic susceptibility of the connecting linker between the carbohydrate binding module (CBM) moiety and the enzyme facilitated isolation of the individual domain, leading to the first CBM isolation of the fungus *Trichoderma reesei* and the bacterium *Cellulomonas fimi* (69, 194, 201). While this model is still controversial, the first C_1 component was cloned from *Clostridium cellulovorans* and *Cellulomonas fimi* (49, 74, 172, 173).

CBMs were initially classified as cellulose binding domains (CBDs), based on the initial discovery of several modules that bind cellulose (69, 194, 201). However, more and more modules in carbohydrate-active enzymes that bind carbohydrates other than cellulose are being found. These findings prompted the need to reclassify these polypeptides with more-comprehensive terminology. A CBM is defined as a contiguous amino acid sequence within a carbohydrate-active enzyme with a discrete fold having carbohydrate binding activity (22, 23, 43). To date, more than 300 putative sequences in more than 50 different species have been identified, and the binding domains have been classified into 43 different families based on amino acid sequence, binding specificity, and structure (for reviews, see references 26, 45, 70, 88, 117, 165, 197, and 202). Extensive data and classification can be found in the Carbohydrate-Binding Module Family Server (http://afmb.cnrs-mrs.fr/~pedro/CAZY/cbm.html).

The CBMs contain from 30 to about 200 amino acids and exist as a single, double, or triple domain in one protein. Their location within the parental protein can be both C- or N-terminal and is occasionally centrally positioned within the polypeptide chain. The three-dimensional (3D) structures of representative members of 23 CBM families have been deciphered so far, several in complex with their ligands. These data provide insight into the underlying mechanism of CBM-ligand recognition and interaction (for reviews, see references 26 and 81). Data from these structures indicate that CBMs from different families are structurally similar and that their carbohydrate binding capacity can be attributed, at least in part, to several aromatic amino acids that constitute the hydrophobic surface (for extended reviews on CBMs, see references 11, 12, 26, 70, 85, 196, and 197).

CBMs have been found in both hydrolytic and nonhydrolytic

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proteins. Proteins that possess hydrolytic activity (e.g., cellulases and xylanases) encompass a complex molecular architecture comprising discrete modules (typically, a catalytic module and one or more CBMs), which are normally joined by relatively unstructured linker sequences. The CBMs, by bringing the biocatalyst into intimate and prolonged association with its recalcitrant substrate, increase the rate of catalysis (70, 124, 192, 195–197). The CBMs present in proteins that do not possess hydrolytic activity comprise part of a scaffolding subunit that organizes the catalytic subunits into a cohesive multienzyme complex known as a cellulosome (11–13, 15, 47, 50, 53, 54, 123, 173, 212). The enzymatic complex was found to function more efficiently in substrate degradation, and removing the CBM from the enzyme or from the scaffolding in cellulosomes dramatically decreased its enzymatic activity (29, 42, 74, 84, 194, 201).

CBMs have also been found in several polysaccharide-degrading enzymes other than cellulases and xylanases. In *T. reesei*, CBMs have been identified in hemicellulase, endomannanase, and acetylxylanesterase (128, 188). CBMs have been recognized in esterase from *Penicillium funiculosum* (108), isomaltodextranase from *Arthrobacter globiformis* (82), arabinofuranosidases from *Aspergillus kawachii* and *Cellvibrio japonicus* (21, 136), pectate lyase from *Pseudomonas cellulose* (27), β-agarase from the marine bacterium JAMB-A94 (145), β-glucosidase from *Phanerochaete chrysosporium* (127), and dextranase from *Paenibacillus* sp. (59). An interesting observation was recently reported when a CBM was found in cytochrome (217). The presence of putative CBMs in plant endoglucanases has also been reported (30, 152, 199). Expansins, which are believed to play a role in nonhydrolytic cell wall expansion, are homologues to CBMs and possess cellulose binding capabilities in vitro (40). Most recently, a small olive pollen protein, Ole e 10 (10 kDa), was identified. Ole e 10 binds specifically to 1,3-β-glucans. The protein was described as an independent CBM and represents the first member of the new CBM family 43 (9).

In the phylum *Nematodea*, CBMs have been found in secretions of the root knot nematode *Meloidogyne incognita* (51) and in the soybean cyst nematode *Heterodera glycines* (65), and recently it was demonstrated that nematodes express expansin during plant feeding (110, 159).

CARBOHYDRATE BINDING, MODULATION OF POLYSACCHARIDE STRUCTURE, AND SOLVENT INTERFACE

Enzyme Concentration on the Surface of the Substrate—the Phase Transfer

In earlier studies of CBM-cellulose interactions, the presence of CBMs was shown to increase the effective concentration of the hydrolytic unit (or units, in the case of cellulosomes) on the surface of the carbohydrate substrate (14, 196). In fact, removing the CBM from the cellulase or from the scaffoldin in cellulosomes dramatically decreases enzymatic activity (2, 4, 20, 25, 29, 42, 74, 79, 194, 201, 218). However, a frequent observation in these studies is that the reduction in catalytic activity takes place only on insoluble substrates. Alternatively, CBMs were employed to improve the carbohydrate degrading activity. Interestingly, the addition of a CBM, derived from cellobiohydrolase II of *T. reesei*, to the *T. harzianum* chitinase resulted in increased hydrolytic activity of insoluble substrates (122). Moreover, replacing the CBM of endo-1,4-β-glucanase from *Bacillus subtilis* (Ben) with the CBM of exoglucanase I (Texl) from *T. viride* resulted in higher binding affinity and enhanced hydrolytic activity on the surface of the microcrystalline cellulose (105). Although the interaction of CBMs with cellulose is occasionally irreversible (29, 158), their contact with the cellulose surface is a dynamic process. Jervis et al. (96), using fluorescence recovery techniques, confirmed that CBM_{Cex} is mobile on the surface of crystalline cellulose. The mobility of CBMs may explain the function of CBMs other than to concentrate the catalytic activity on the substrate.

Substrate Binding and Selectivity

In recent years, the 3D structures of representative members from 22 CBM families have been resolved. Data from these structures indicate that CBMs from different families are structurally similar and that their binding to cellulose can be attributed, at least in part, to their hydrophobic surface, which is composed of several aromatic amino acids. CBMs are classified into seven "fold families," based on their 3D structures and functional similarities, into three types: (i) "surface binding" (type A), (ii) "glycan chain binding" (type B), and (iii) "small sugar binding" (type C). The structure-function relationship is discussed extensively in a review by Boraston et al. (26) and is not repeated here.

Nonhydrolytic Substrate Disruption

It has been proposed that some CBMs possess additional functions, such as disruption of substrate. The initial evidence supporting this notion was from CBM_{CenA}, from *C. fimi* endoglucanase A. More specifically, the CBM was able to disrupt the structure of cellulose fibers, resulting in the release of small particles without any detectable hydrolytic activity (49). In addition, CBM_{CenA} was able to prevent the flocculation of microcrystalline bacterial cellulose (72). Similar phenomena were also reported for other CBMs (7, 22, 66, 71, 109, 118, 148, 213). In addition, it was reported that structural disruption occurred when the starch binding domains (family 20 CBM) were bound to starch (68, 186). Another interesting discovery was reported when a CBM was applied to dental plaque polysaccharides (mainly fructan and glucan), which resulted in its dispersion, thereby removing and preventing plaque formation (64).

Lee et al. (113) provided the first physical evidence for the involvement of CBMs in altering of the fiber surface following cellulase treatment. In their study, two cellulases from *T. reesei*, exoglucanase (EGase) CBH I and EGase EG II, were applied separately and in combination to cotton fibers. Treatment with CBH I resulted in the appearance of distinct tracks along the longitudinal axis of the fiber, as visualized by atomic force microscopy, whereas EG II treatment appeared to cause peeling and smoothing of the fiber surface. When cellulase from *Thermotoga maritime*, which lacks the CBM, was used, no effect on the surface of the cotton fibers was discerned.

Surface/Interfacial Modifications

Recent studies have indicated that treatment of cellulose fibers with CBMs alters the interfacial properties of the fibers. This phenomenon was first reported by Cavaco-Paulo et al. (33), who demonstrated that treatment of cotton fibers with a CBM alters their affinity to dye. Suurnakki et al. (191) demonstrated that treatment of bleached chemical pulp with endoglucanases, cellobiohydrolases, and the catalytic domains from *T. reesei* could also change the interfacial properties. According to this study, the presence of the CBM in the intact enzyme had a beneficial effect on pulp properties such as viscosity and strength after refinement. Later, Pala et al. (149) demonstrated that treatment of fibers recycled from old paperboard containers composed of CBMs may improve both the tensile and burst indexes, as well as increase the pulp drainage rate. These observations inspired an in-depth research study into CBM-cellulose fiber interactions. It was shown that CBM treatment of cellulosic fibers results in an increased surface area as a consequence of cellulosic aggregate disruption, reduction in fiber acidity, and reduction of surface polarity (158). In general, the interaction between cellulose surfaces is dominated by double-layer repulsive forces attributed to the negative charge of cellulose surfaces. Nigmatullin et al. (140) have demonstrated, using atomic force microscopy, that despite an increase in surface charge following CBM binding, interfacial force profiles are less repulsive. This phenomenon may assist other molecules, such as xyloglucan and pectin, in interacting with cellulose surfaces.

UTILIZATION OF CBMs

In recent years the practical use of CBMs has been established in different fields of biotechnology, and the number of published articles and patents is constantly on the rise. Three basic properties have contributed to CBMs being perfect candidates for many applications: (i) CBMs are usually independently folding units and therefore can function autonomously in chimeric proteins; (ii) the attachment matrices are abundant and inexpensive and have excellent chemical and physical properties; and (iii) the binding specificities can be controlled, and therefore the right solution can be adapted to an existing problem. Utilization of CBMs has been extensively reported and reviewed in the literature (10, 75, 117, 146, 197, 202), and their use has been described in several patents (103, 104, 133, 134, 174–176). Therefore, this section summarizes only the basic principles of CBM application, along with recent developments.

Bioprocessing

Bioprocessing is the major application for CBMs, given that large-scale recovery and purification of biologically active molecules continue to be challenges for many biotechnological products. Biospecific affinity purification (affinity chromatography) has become one of the most rapidly developing divisions of immobilized affinity ligand technology. To date, several affinity tags, which vary in size from several amino acids to a complete protein, have been developed. Each individual affinity-based purification system embodies specific advantages (for reviews, see references 57, 80, 126, 167, 193, and 211). Many protein entities have been expressed when fused to CBMs, establishing CBMs as high-capacity purification tags for the isolation of biologically active target peptides at relatively low cost (24, 52, 102, 160, 164, 180, 183).

Production of recombinant proteins in plants has been recently recognized as one of the most cost-effective production systems. However, a major drawback of this system is that plants contain high levels of polysaccharides and phenolic components, which interfere with the purification process (55, 87, 90). The utilization of CBMs in the production of CBM fusion proteins in plants permits efficient production, taking advantage of the fact that the plant cell wall is composed of cellulose. In this system the plant manufactures both the target protein and its purification matrix (171).

Two-phase liquid separation systems for protein purification have been proposed in order to reduce the downstream processing of biological molecules (141, 205). Haynes et al. (83) proposed a novel two-phase separation system to purify proteins from aqueous solutions by utilizing family 4 CBMs, which bind to water-soluble cellulosic materials such as hydroxyethylcellulose. The system was composed of a phase-forming polysaccharide polymer to which a CBM can bind and a phase-inducing agent such as a polyethylene glycol. The solution containing a CBM-fused peptide or protein was mixed with the phase-forming oligosaccharide, followed by the addition of the phase-inducing agent. The two phases were then separated, and the target protein was purified. Shortly thereafter, Lam et al. (111) designed an advanced system based on a two-phase aqueous micellar system utilizing family 9 CBMs. Interestingly, the detergent *n*-decyl-β-D-glucopyranoside operates simultaneously as a phase former and as an affinity ligand. These systems may be useful for protein separation in large-scale, industrial fermentation plants.

Numerous reports have affirmed the feasibility of employing a CBM as an affinity tag for enzyme immobilization and processing. In these studies carbohydrates were used as an affinity support for enzyme immobilization, with high capacity, while retaining enzymatic activity; in some instances, increased enzymatic activity was reported (19, 77, 93, 98, 101, 102, 119, 130, 155, 162, 166). Recent studies have shown that a CBM serving as a fusion partner may have additional values. In the expression of CBM-lipase fusion protein in yeast, for example, it was shown that CBM also enhanced secretion (1).

Another area of increasing interest is bioethanol production from cellulosic material. Lignocellulose is the most abundant renewable natural resource for conversion to fuels. CBMs are the pivotal proteins able to target the catalytic modules of polysaccharidases that are needed for the breakdown of the cellulosic biomass to sugars, which can then be converted to liquid fuel (for extended reviews, see references 47, 54, and 139).

Matrix-assisted refolding of recombinant proteins is one of the approaches taken in order to prevent the aggregation of protein during the course of renaturation. At present, only histidine and arginine tags have been found to be suitable for this process, because they maintain matrix binding ability under denaturing conditions (73, 189). Recently, Berdichevsky et al. (17) demonstrated that a CBM (*C. thermocellum*) can be used as the attachment support for matrix-assisted refolding

of a single-chain antibody expressed in *Escherichia coli*. This CBM can bind cellulose in the presence of 6 M urea, and this method was shown to provide a threefold increase in protein yield compared with standard refolding procedures.

Phage display technology is a well-established tool for isolating biologically active molecules (38, 61, 67, 99, 163). One of the limitations preventing extensive implementation of this technology is the relatively high proportion of clones that lack insertions within the library. In a recent study, a CBM from *C. thermocellum* was fused to a single-chain antibody (scFv) and expressed as an scFv-CBM phage display library. The CBM tag allowed for rapid recovery of phages that displayed functional inserts, thus increasing the efficiency of the screening process for recombinant antibodies (16). Furthermore, a novel approach for high-throughput screening of shuffled recombinant scFvs was developed, based on their immobilization on cellulose-based supports (5).

Targeting

Cellulose is a major component of numerous commercial products, several of which are capable of being recycled. Therefore, CBMs can be used for the targeting of functional molecules to materials containing cellulose. The commercial potential of CBMs in this context was first realized for denim stonewashing, where cellulases were used as an alternative to the original abrasive stones (31, 32, 100). Another textile-associated CBM application used in numerous laundry powders is fabric targeting of recombinant enzymes that do not possess a native affinity for the cellulosic fibers (e.g., amylases, proteases, lipases, and oxidoreductases). This can be achieved by recombinant enzyme technology, where fusion to CBMs with a desired enzyme is achieved (203, 204). Additional substances can also be targeted to cellulosic fabrics. Fragrance-bearing particles conjugated to CBMs can be added to laundry powder, hence reducing the amount of fragrance needed in the product (18).

Cell Immobilization

Cell immobilization technology ranges from ethanol production and phenol degradation (137, 139) to mammalian cell attachment (107, 215) and whole-cell diagnostics (76, 169, 187). Surface-exposed CBMs can be an efficient means of whole-cell immobilization. Whole-cell immobilization by cellulosic material was first demonstrated when an *E. coli* surface-anchored CBM, derived from *C. fimi*, was attached to cellulose (62). The cells bound tightly to cellulose at a wide range of pHs, and the extent of immobilization was dependent on the amount of surface-exposed CBM (206). In a different study, *Staphylococcus carnosus* was chosen to display CBM_{Cel6A} from *T. reesei* on its cell surface, and the addition of the CBM predisposed the anchoring of bacterial cells to cotton fibers (115). Yeast was also shown to be cellulose immobilized via cell surface display of CBMs (138).

A different strategy for cell immobilization was demonstrated by the attachment of mammalian cells to a cellulosic surface coated with recombinant protein composed of the cell attachment peptide RGD fused to CBM_{CenA} from *C. fimi*. This approach is based on the preservation of the functional properties of the attachment ligand following its immobilization. In addition, it enabled cell immobilization without the need for expensive attachment factors (210). Furthermore, it was demonstrated that cellulose is an excellent inert matrix for presenting cytokines to target cells, where it demonstrated a more stimulating effect of proliferation (52), improved cellular adhesion (92), and stimulated receptor polarization in the cell membrane (97). This approach was used to improve the performance of vascular grafts (91) and tissue-engineered scaffolding for cartilage regeneration (92). In a study by Nordon et al. (142), a hollow-fiber device for analysis of ligand-mediated cell adhesion wherein cell adhesion strength can be measured under shear stress was established. This system permits evaluation of the interaction of molecular domains with cell surface receptors.

CBM Engineering for Different Applications

It is well established that expression of foreign proteins fused to CBMs results, for the most part, in high expression levels (24, 52, 101, 105, 147, 151, 160, 162, 166, 181–183). As a result, expression vectors (pET34 to pET38) incorporating CBMs as fusion tags were developed (143).

Several studies have shown the potential of CBMs for modifying the characteristics of several enzymes. The basic approach in CBM engineering was to replace or add a CBM in order to improve hydrolytic activity. Addition of a CBM derived from cellobiohydrolase II of *T. reesei* to *T. harzianum* chitinase resulted in increased hydrolytic activity of insoluble substrates (122). Replacement of the CBM of endo-1,4-β-glucanase from *B. subtilis* (Ben) with the CBM of exoglucanase I (Texl) from *T. viride* conferred higher binding, with enhanced hydrolytic activity on the microcrystalline cellulose. In addition, the hybrid enzyme was more resistant to tryptic digestion (105). Similar results were obtained with other endoglucanases (94, 112, 153).

Other studies involved actual modification of the CBM moiety to match a set of defined reaction conditions. Linder et al. (125), for example, rationally modified the small CBM from Cel7A cellobiohydrolase, derived from *T. reesei*, to be sensitive to changes in pH. By replacing the tyrosine residues in two different positions with histidine, they obtained a definite pH dependency. As a result of this manipulation, the binding efficiency of the mutant CBM, at an optimal pH value, was inferior to that of the wild type. In another study, a combinatorial library was created by introducing restricted variations in defined positions in CBM4-2, originating from *Rhodothermus marinus*. This library was then used to select CBM variants that would interact with different carbohydrates and with human immunoglobulin G, thus demonstrating that a CBM is a suitable scaffold for creating binding modules for different substrates (37). A similar approach was taken by Lehtio et al. (114) when screening for α-amylase inhibition in a combinatorial library of a CBM scaffold that was displayed on phage. Interestingly, they were able to recognize variants that selectively inhibit α-amylase and that were capable of competing with the binding of the amylase inhibitor acarbose. Using the same CBM library, Wernerus et al. (209) generated a metal binding protein that lost its original cellulose binding capacity. In another example, Smith et al. (184) utilized the flat hydro-

phobic face of the wedge-shaped CBM from *T. reesei* for introducing random mutations in seven side chains. The mutated CBM was then displayed on phage, and variants with high affinity to alkaline phosphatase were selected.

Fierobe et al. (58) employed a different strategy to design and produce active cellulosome. To construct the desired complex, they prepared a series of chimeric scaffolds. They obtained the molecular building blocks from the two *Clostridium* cellulosomes, *C. thermocellum* and *C. cellulolyticum*. The designed chimeric cellulosomes exhibited enhanced synergistic action on crystalline cellulose. Later, the same research group was able to show that active cellulosome can be assembled and secreted in bacteria (135). A similar approach was employed for the use of these proteins in affinity chromatography (44).

CBMs as Analytical Tools in Research and Diagnostics

The use of CBMs as analytical tools was first introduced when a bioassay was developed for characterizing the pulp fiber surface using cellulase (216). Unfortunately, for many years after this seminal work was published, there were no further research studies in that direction. However, in recent years this idea has been revived. McCartney et al. (129) developed novel molecular probes for detection of polysaccharides in plant cell walls using CBMs of different types. In their approach, recombinant CBMs fused to polyhistidine tags and anti-polyhistidine antibodies were used to detect polysaccharide-CBM interactions. Jamal-Talabani et al. (95) proposed that CBMs could be used for mapping the "glyco-architecture" of plant cells. Degani et al. (46) took a different approach when they fused the CBD to β-glucuronidase to determine the extent of wax removal from cotton fibers.

The latency of CBMs was also demonstrated in diagnostics. In order to optimize bioprocesses such as fermentation, it is very important to monitor the glucose levels. Phelps et al. (156) addressed this problem based on the reversible immobilization of chemically conjugated CBM-glucose oxidase (CBM_{Cex} from *C. fimi*), which can be repeatedly loaded onto a cellulose probe. Given that the binding is reversible, the sensor can be regenerated by replacing the originally bound enzyme with a fresh one (157, 200). Shoseyov et al. (177) developed a system based on CBMs that permits rapid detection of pathogenic microbes in food samples. In this method, a CBM is conjugated to a bacterium binding protein such as an epitope-specific monoclonal antibody and is loaded onto a cellulosic matrix (e.g., cotton gauze) that acts as a bacterial cell concentrator. The structure of the cotton gauze enables the passage of relatively large volumes of liquids, consequently permitting the isolation of sufficient bacteria, even from dilute samples. The eluted bacteria can then be utilized for enumeration and/or classification.

Recently, a simple and efficient strategy for the production of non-DNA microarrays was demonstrated, based on the affinity of a CBM for its 3D substrate. In this study, various microarray formats (conventional and single-chain antibody microarrays and peptide microarrays for serodiagnosis of human immunodeficiency virus patients), in which the binding determinant is fused to the CBM, were described. This CBM-based microarray technology overcomes many of the previous obstacles that have hindered fabrication of non-DNA microarrays and provides a technically simple alternative to conventional microarray technology (144).

Bioremediation

Wang et al. (207) genetically engineered bacteria to display simultaneously a CBM and an organophosphorus hydrolase. The CBM was used to immobilize the bacterial cells onto a cellulose support, and the organophosphorus hydrolase was used to hydrolyze nerve gas. The high degradation capacity and affinity for cellulose make this immobilized cell system an attractive alternative for nerve agent detoxification. Future applications may include protective cotton clothing against nerve gas and filters that may be charged and recharged by laundry cycles.

Heavy metals are major contributors to pollution; therefore, efficient removal systems are required. Recently, Xu et al. (214) reported the cloning and expression of a recombinant protein composed of a CBM fused to a synthetic phytochelatin. The immobilized sorbent was shown to be highly effective in removing cadmium at the level of parts per million.

Atrazine is a commonly used pesticide that is persistent in water, is mobile in soil, and is among the most frequently detected pesticides in groundwater. Therefore, its removal or detoxification from industrial wastewater is required before its disposal. Recently Kauffmann et al. (101) reported a novel method for enzymatic removal of atrazine from water. Atrazine chlorohydrolase (AtzA) was fused to a CBM and immobilized on cellulose. The active cellulose-AtzA resin was then used to dechlorinate atrazine. Hydroxyatrazine is an unregulated compound and is not leached from the soil. We recently constructed a fused protein consisting of a CBM and horseradish peroxidase for the oxidation of a model toxic phenol, 4-bromophenol. The oxidation reaction resulted in the formation of dimers to pentamers of phenols that adsorbed to the cellulosic matrix. These findings may have potential impact in treatment of wastewater contaminated with toxic phenols (119).

Modification of Fiber

The nonhydrolytic fiber disruption activity demonstrated by Din et al. (49) provided the first evidence that CBMs have potential in modification of fiber. It was shown that modification of the polysaccharide structure could be achieved with isolated CBMs. In this study the surface area of cellulosic materials (ramie cotton fibers) was roughened after treatment with a CBM (CBM_{CenA} from *C. fimi*). It was proposed that these treatments could be used to alter the dyeing characteristics of cellulose fibers (71). Cavaco-Paulo et al. (33) provided additional evidence when they demonstrated elevated levels of dye affinity following treatment with family II CBMs from *C. fimi*. This was especially notable with acid dyes.

We have used a genetic engineering approach in order to construct a novel reagent for cellulose cross-linking. Two cellulose binding modules from *Clostridium cellulovorans* were fused together to form a cellulose cross-linking protein (CCP). The recombinant bifunctional cellulose-binding protein was applied to Whatman filter paper and was found to enhance its mechanical properties, such as tensile strength and ability to

stretch, as well as Young's modulus and the energy to the breakpoint. In addition, it was shown that CCP treatment could transform filter paper into water-repellent material (116). Furthermore, a synergistic effect between CCP and cationic starch that resulted in higher mechanical performance of paper made in the presence of CCP was observed (120). Kitaoka and Tanaka (106) reported the production of a novel papermaking reagent by covalently binding activated anionic polyacrylamide (A-PAM) to a CBM originating from *T. viride* 1,4-β-glucan (CBM–A-PAM). In this manner they were able to produce a molecule containing more than one CBM copy that is capable of cellulose fiber cross-linking. Importantly, the dry and wet tensile strengths of paper prepared from CBM–A-PAM were increased. Recently, we constructed a bifunctional cross-linking molecule composed of starch and cellulose binding modules, termed CSCP (starch-cellulose cross-linking protein). This molecule was able to bind soluble and insoluble starch to cellulose. Additionally, this molecule was able to improve the mechanical properties of paper composed of cellulose fibers and starch (121).

Suurnakki et al. (191) tested the effects of EGases, cellobiohydrolases, and their core proteins (from *T. reesei*) on bleached chemical pulp. They reported that the presence of CBMs in the EGase had a beneficial effect on the pulp's properties. Similarly, Pala et al. (150) demonstrated that application of CBMs to secondary paper fibers improved drainability and resulted in paper with improved mechanical properties. They proposed that CBMs affect the interfacial properties of the fibers in both fiber-water and fiber-air interactions. A novel approach for the synthesis of cellulose synthetic polyester composite material was demonstrated, wherein a CBM fused to lipase, an enzyme capable of polymerizing monomers to polymer, was made. The proximity, enabled by the CBM, of the enzyme to the cellulose surface, facilitates a template-like synthesis of the polymer (78). At this stage, this novel approach takes advantage of only one property of the CBM, namely its binding to cellulose. In the future, however, it is likely that both the CBM's mobility on the fiber surface and its nonhydrolytic fiber disruption properties will enable the synthesis of interlaced composite materials with superior physical properties.

MODULATION OF PLANT CELL WALLS BY CBMs

Plant cell walls are important structures specifically designed for a variety of apparently opposing functions. On the one hand, cell walls are responsible for tensile strength, cell shape, and resistance to pathogen invasion. On the other hand, they must maintain reasonable flexibility against breaking forces and just enough permeability to allow building blocks and signaling molecules to enter the living cells. Therefore, modifications of living cell walls require a sensitive, highly synchronized system of signals, enzymes, and building blocks (185). Among plant enzymes, several families have been shown to bind to different carbohydrate components of the plant cell wall. The first and largest families identified were the expansin families (discussed extensively below). A novel E-type endo-β-1,4-glucanase with a putative CBM was isolated from ripening strawberry fruits (199), and, most recently, a small olive pollen protein, Ole e 10 (10 kDa) was identified. The ability of Ole e 10 to bind soluble polysaccharides has been demonstrated (9). Ole e 10 binds specifically to 1,3-β-glucans; in addition, this protein shows sequence identity with the noncatalytic C-terminal domains of several plant 1,3-β-glucanases (27 to 53% identity, 44 to 69% similarity). The protein can be described as an independent CBM and is the first member of the new CBM family 43 (9).

It is now well established that living microorganisms contain complex systems for the management of cellulose-containing materials. One of the pivotal players in these systems, which appear to be present primarily in systems devoted to cellulose degradation rather than to cellulose synthesis, is the cellulose binding module. This important, naturally occurring protein entity is part of a family of many endo-1,4-β-glucanase- and other polysaccharide-degrading enzymes. It plays an essential role in cellulose degradation and has the potential of modifying cellulose-containing materials. Significant progress has been made in recent years in better understanding and using these genes as tools for improving plants in modern agricultural and forest systems.

Expansins: the Main Family of Plant CBMs

A crude protein extract from the cell walls of growing cucumber seedlings was shown to possess the ability to induce the extension of isolated cell walls (131). A specific protein possessing that expansion activity was isolated and named expansin. It was further established that expansins induce nonhydrolytic activity on cell wall polymers, e.g., pectins and xyloglucans, which are tightly bound to the cellulose microfibrils (132). The in vitro effect of expansins on plant cell walls was found to be similar to that of bacterial and fungal CBMs, including swollenin, which is a family II CBM with a sequence similarity to plant expansins (168). This distinct CBM family, known today as the expansin family, was first identified as the grass allergen of group I, the major allergen from grasses (63). Some genes from this family have been characterized (3, 41, 56), but their function in plant pollen became clear only years later when the identity between these proteins and a second group of expansins, known as α-expansins, was established (39). Expansins are cell wall proteins that are involved in the loosening of the plant cell wall during plant growth as well as in the fruit softening process (40). The relatively high level of β-expansins in the pollen suggests its involvement in pollen germination and pollen penetration and in growth through the pistil. Expansins are composed of two segregated domains, a C-terminal CBM and an N-terminal domain that exhibits some sequence similarity with the family 45 endoglucanases (86). A 3D model of the CBM of the rye grass pollen allergen Lo1 pI (β-expansin) was constructed through homology modeling by Barre and Rouge (8), who found a groove and an extended strip of aromatic and polar residues that remarkably resemble the 3D structure of bacterial family III CBMs. Furthermore, the Barre and Rouge (8) model was developed according to the 3D structure of Phl p2 (48), a small (10.7-kDa) protein that belongs to group 2/3 grass allergens (expansin-like protein) and has a striking homology to the C-terminal (CBM) half of the β-expansins. Lol p3 (*Lolium perenne*) and its homologue, Phl p2 (both expansin-like proteins), possess significant cell wall loosening capability (L. C. Li, M. W. Shieh, and D. J. Cosgrove, Abstr. Plant Biol. Meet. Am. Soc. Plant Biol., abstr.

1259, 2003). These findings indicate that the minimal structural requirement for their activity is the CBM. A BLAST search of the protein databases against Phl p2 showed that almost all of the hits belong to proteins from the plant allergens and are classified as group I or group 2/3. On average, the sequence identity was found to be about 60%; the region of homology covers the whole sequence of group 2/3 allergens (expansin-like proteins) and the C-terminal (CBM) sequence of group I allergens (β-expansin) (48). A 10-kDa expansin-like allergen derived from maize pollen has already been implicated in the past as part of the group of proteins involved in the hydrolysis of style cell walls during pollen tube penetration (190). Although the specific carbohydrate binding determinant of expansins remains controversial, its ability to bind to cellulose was demonstrated. Microcrystalline cellulose, which was added to a plant extract containing α-expansin, was shown to deplete its creep activity (39).

A structural similarity search using the Dali, version 2.0, server (EMBL-EBI) (89) for CBM III (CBM of the cellulosome subunit S1 from *Clostridium thermocellum*, accession no. 1nbc) against the protein data bank revealed significant similarities with both the Phl p2 (expansin-like protein, 1who) and Phl p1 (β-expansin, 1n10) allergens from timothy grass (*Phleum pretense*). Cellulose binding sites typically contain aromatic/hydrophobic and polar amino acids. The amino acids of two cellulose binding sites of CBM III (Fig. 1A) were denoted according to Tormo et al. (198). The site shown at the bottom of Fig. 1A was proposed as a planar cleft, which binds crystalline cellulose. The site shown at the top of Fig. 1A was proposed as a groove that binds the amorphous single cellulose chain. Hypothetical cellulose binding clefts of the β-expansin and expansin-like proteins are presented in Fig. 1B and C, respectively. His16 and Glu48 in Ph1 p2 (expansin-like protein) and their analogs in β-expansin have already been proposed as part of their cellulose binding cleft (8). CBM III and Phl p2 were matched by pairwise protein structure alignment with the C-Alpha Matching Program (6, 60). The successful alignment revealed a close similarity along the protein backbone (Fig. 1D) and striking similarities of specific amino acids groups (Fig. 1E). The homology pattern, shown in Fig. 1D, reveals two potential parallel cellulose binding grooves in both CBM III and Phl p2. Cleft A is that reported by Tormo et al. (198), whereas cleft B was never reported for CBM III. It was previously suggested that Arg67, Val77, and Asp79 of Phl p2 cleft B are part of the binding site of this protein (8). The planar cellulose binding site of CBM III (bacterial CBD family III) is absent from the Phl p2 protein (Fig. 1F); this finding is in agreement with the results of McQueen-Mason and Cosgrove (132), which implicated the affinity of expansin to the *para*-crystalline (amorphous) part of the cellulose fibers. Furthermore, the two cellulose binding grooves (Fig. 1D) are parallel to each other, enabling the protein to slide between two cellulose chains and facilitate the disruption of the hydrogen bonding between adjacent chains in a wedge-like action. The similar 3D conformations of the two binding clefts of the two proteins further suggest the existence of a mutual potential substrate. Although this model is very attractive, more experimental work is necessary to validate it.

Effect of CBMs on Cellulose Biosynthesis

The recombinant bacterial family III CBM was shown to modulate cell elongation in vitro in peach (*Prunus persica* L.) pollen tubes and *Arabidopsis thaliana* seedlings. At low concentrations, the CBM enhanced the elongation of pollen tubes and roots, whereas at high concentrations, the CBM inhibited root and pollen elongation in a dose-dependent manner (181). The in vivo effect of the CBM on cellulose biosynthesis was also demonstrated. Recombinant CBM increased the rate of cellulose biosynthesis in *Acetobacter xylinum* by up to fivefold over the control. Electron microscopy of cellulose produced in the presence of the CBM revealed that the newly formed fibrils appear as splayed ribbons, instead of the uniform, thin, packed ribbons of the control fibers (181). The underlying mechanism by which the CBM affects cell wall metabolism is yet to be studied. The synthesis of cellulose can be divided into an initial polymerization step and a second step in which the individual glucan chains associate to form crystalline cellulose (28). A physicomechanical mechanism whereby the CBM slides between cellulose fibers and separates them in a wedge-like action has been postulated (118). This physicomechanical interference uncouples the cellulose-biosynthetic polymerization step from the crystallization step, resulting in an increased rate of cellulose biosynthesis (118, 170, 181). This model was further supported by additional in vitro experiments in which the application of recombinant CBM markedly reduced the wet tensile strength of cellulose paper when tested in an Instron Universal Testing Machine (118).

By introducing the *CBM* gene into plants under elongation conditions by a specific promoter and a cell wall-targeting signal peptide, we were able to express CBM proteins within the cell wall of plant tissue in vivo. Expression of a bacterial CBM (family III CBM) in transgenic plants resulted in accelerated growth, as demonstrated in tobacco (170, 178), poplar (118, 170, 179), and potato (L. Safra, Z. Shani, O. Shoseyov, and S. Wolf, Abstr. 6th Int. Cong. Plant Mol. Biol., abstr. S3-103, 2000). A similar effect was observed with a plant CBM (expansin) in transgenic *A. thaliana* (35), in rice (36) and in transgenic poplar plants (E. Mellerowicz, N. Nishikubo, M. Gray-Mitsumune, A. Siedlecka, and B. Sundberg, Abstr. 10th Cell Wall Meet., abstr. 61, 2004). Introduction of the *CBM* gene under the control of the elongation-specific *cel*1 promoter into transgenic poplar plants led to a marked increase in biomass production in selected clones compared to wild-type control plants (118). Analysis of the wood properties from transgenic poplar trees showed a marked increase in fiber cell length and in the average molecular weight of cellulose polymers and a significant decrease in the microfibril angle (179). These results coincided with increased burst, tear, and tensile indices of paper prepared from these transgenic wood fibers.

FUTURE ASPECTS

Numerous scientific publications describing the CBM structure's putative and well-proven modes of action and novel applications of CBMs are available today. It seems that nature selected this fascinating group of proteins to function in different life forms. The use of human CBMs for nonimmunogenic drug targeting and medical devices has much potential

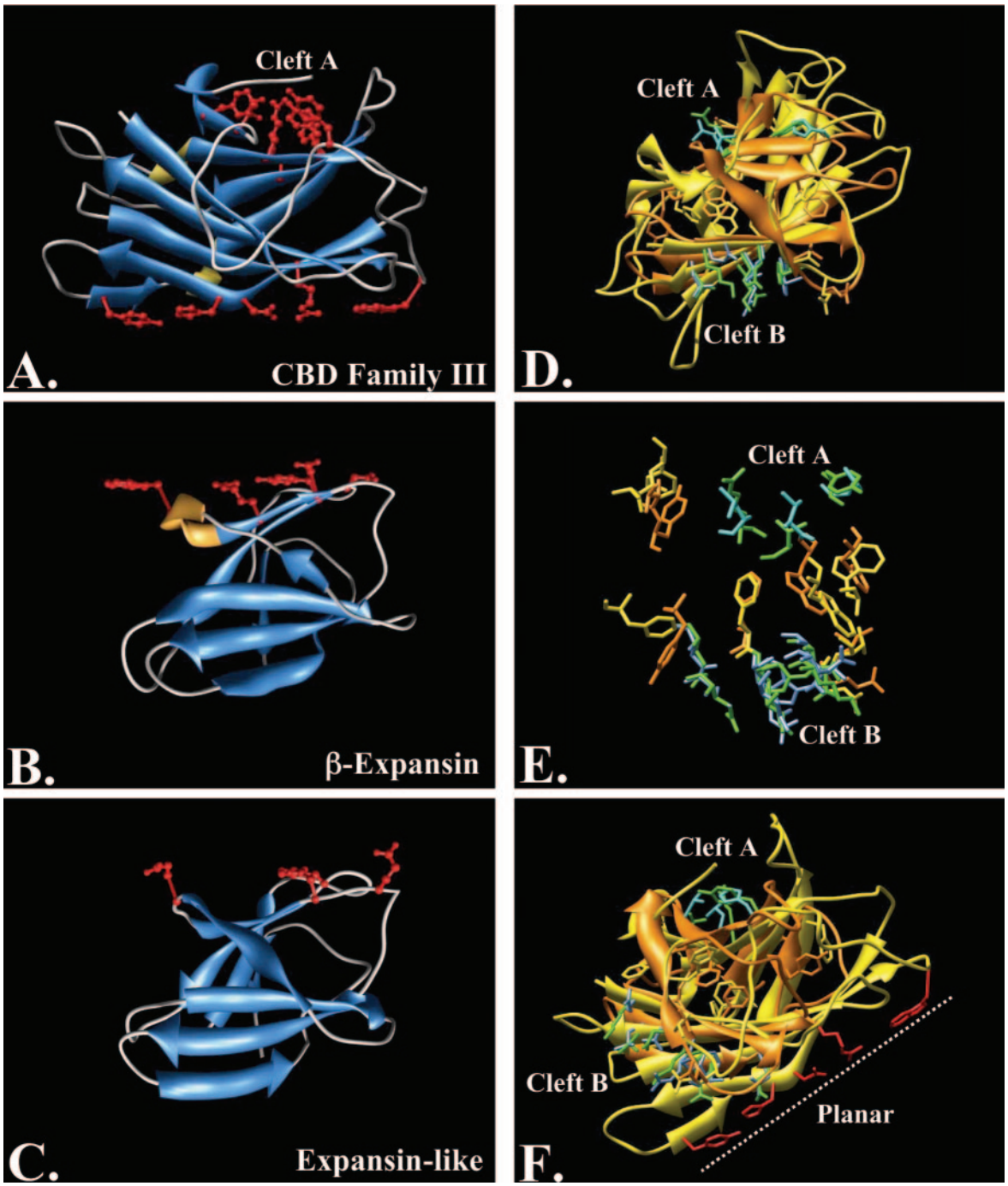

FIG. 1. 3D structures of plant and bacterial CBMs. (A to C) Structure of bacterial (A) and plant (B and C) CBMs (created with UCSF CHIMERA extensible molecular modeling system software, University of California, San Francisco) (154). β-Sheet fragments appear as blue strips; the amino acids at the binding sites are indicated in red (for CBD family III, they are Asp56, His57, Tyr67, Arg112, and Trp118 [planar] and Arg40, Tyr42, Tyr91, Glu93, Tyr127, Thr142, and Tyr144 [groove]; for β-expansin, they are Asn159, Tyr160, Trp194, Arg199, and Asp201; for the expansin-like protein, they are His16, Glu48, Asp55, and Glu57). (D to F) Alignment of the 3D backbone structures of the CBD family III (yellow) and expansin-like (orange) proteins. Selected homologous amino acids in panels D to F are indicated in blue (CBD family III) and green (expansin-like protein) (for groove I, they are Arg40, Tyr127, and Thr142 [CBD family III] and His16, Leu20, and Val51 [expansin-like protein]; for groove II, they are Lys3, Glu5, Lys23, Thr25, Gln108, Gln110, and Phe135 [CBD family III] and Glu30, Glu32, Trp41, Arg67, Leu69, Val77, and Asp79 [expansin-like protein]). Planar amino acids of CBD family III in F are indicated in red.

for application in the future. CBMs may bind cytokines, growth factors, and structural proteins to biocompatible polysaccharide scaffolds in order to selectively direct hard- and soft-tissue remodeling in reconstructive surgeries.

Another example of the important roles of CBMs in human metabolism is the unique CBM in the N-terminal region of the Laforin gene, a protein phosphatase involved in glycogen metabolism. A single mutation in the CBM, depleting its carbohydrate binding capability, is the cause of Lafora disease (34, 208). This CBM may be utilized in the future as a possibly nonimmunogenic CBM for drug targeting.

The self-assembly properties of different polysaccharides, together with novel nano-fabrication techniques, may enable the construction of 2D and 3D molecular crossroads through which different CBMs may be used to carry and transfer molecular cargo. These devices may be used to transfer drugs in one direction and simultaneously remove toxic molecules in the other direction, as well as to store and remove molecular information in computational devices. The realization of this concept will require a better understanding of the molecular mechanism by which CBMs bind and move on the respective polysaccharides. It will take time and effort to harness the full potential of these molecules. However, the potential of these molecules for improving life in many aspects cannot be overstated.

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