Alteration of In Vivo Cellulose Ribbon Assembly by Carboxymethylcellulose and Other Cellulose Derivatives

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ABSTRACT In vivo cellulose ribbon assembly by the Gram-negative bacterium Acetobacter xylinum can be altered by incubation in carboxymethylcellulose (CMC), a negatively charged water-soluble cellulose derivative, and also by incubation in a variety of neutral, water-soluble cellulose derivatives. In the presence of all of these substituted celluloses, normal fasciation of microfibril bundles to form the typical twisting ribbon is prevented. Alteration of ribbon assembly is most extensive in the presence of CMC, which often induces synthesis of separate, intertwining bundles of microfibrils. Freeze-etch preparations of the bacterial outer membrane suggest that particles that are thought to be associated with cellulose synthesis or extrusion may be specifically organized to mediate synthesis of microfibril bundles. These data support the previous hypothesis that the cellulose ribbon of A. xylinum is formed by a hierarchical, cell-directed, self-assembly process. The relationship of these results to the regulation of cellulose microfibril size and wall extensibility in plant cell walls is discussed.

Most cellulose-synthesizing organisms produce cellulose fibrils with characteristic dimensions and crystallinity. The cellulose fibrils produced by the green alga Chaetomorpha melagonium, the bacterium Acetobacter xylinum, and cotton have average crystallite sizes of 14, 7.5, and 5.7 nm, respectively (1). The supramolecular organization of cellulose may also change during cell development. For example, small cellulose fibrils are present in the primary walls of certain green algae, whereas their secondary walls are composed of larger, more crystalline fibrils (2, 3). The biological processes that regulate synthesis of fibrils with specific size and crystallinity are just beginning to be understood. Significant progress in elucidating the mechanism and control of cellular fibril biogenesis has been made by studying normal and altered cellulose ribbon assembly in the Gram-negative bacterium Acetobacter xylinum (4–8).

Previously, fluorescent brightening agents have been shown to prevent normal ribbon assembly by disrupting microfibril crystallization in vivo. It has been proposed that the brighteners bind with small aggregates of glucan chains that are extruded through pores in the outer membrane (6), thereby separating the glucan chain polymerization from microfibril crystallization (7, 8). In this paper we report that carboxymethylcellulose (CMC) and other cellulose derivatives also alter ribbon assembly. These substituted celluloses disrupt ribbon assembly at a higher level of organization than do the fluorescent brighteners; they prevent the normal fasciation of bundles of microfibrils but do not prevent microfibril crystallization. This report extends preliminary observations previously reported (9) and augments macroscopic and kinetic evidence reported by Ben-Hayim and Ohad (10) that CMC alters cellulose synthesis in A. xylinum.

The disruption of ribbon assembly at two levels of organization by fluorescent brighteners and cellulose derivatives can be correlated with (a) the substructure of the ribbon as described in other reports (4, 5, 11), and (b) the organization of particles and pores thought to be associated with cellulose synthesis in the bacterial outer membrane. These data support the hypothesis that cellulose ribbon biogenesis occurs by a hierarchical, cell-directed, self-assembly process in A. xylinum (7–9, 12). This mechanism may be applicable, in general, to cellulose synthesis in other organisms. Furthermore, the demonstration that cellulose derivatives can alter ribbon assembly during synthesis supports the hypothesis that hemicellulosic polymers have a role in regulation of cellulose fibril size (13–15) and cell wall extensibility (16, 17) during plant development.

MATERIALS AND METHODS

Culture Conditions

A. xylinum, strain ATCC 23769, from the American Type Culture Collection (Rockville, MD) was grown on Hestrin and Schramm's medium (18) for 24 h at 28°C in still Roux bottles (Corning Glass Works, Science Products Div., Corning, NY). The cellulose pellets produced at the air/liquid interface of the medium...
were soaked in cold 0.05 M KH2PO4 buffer, pH 6. Cells were released by twisting a pellicle around a wooden applicator stick. The cell suspension obtained was kept on ice and used without dilution.

Chemicals

Samples of CMC (cellulose gum 4M6F, 7LF, 7HF, 7MF, 12M8P) hydroxypropylcellulose (KhecE), and hydroxyethylcellulose (Natrosol LR) were gifts from Hercules Inc. (Wilmington, DE). Methylcellulose (Methocel A15-LV) and hydroxypropylmethylcellulose (Methocel K4M) were gifts from the Dow Chemical Co. (Indianapolis, IN). Xanthan gum (a bacterial polysaccharide that has a β-(1,4) glucose backbone and a three-sugar sidechain on alternate glucose residues [19]) was a gift from Kelco, Div. Merck & Co., Inc. (Clark, NJ). Laminarin and cellulose were obtained from the Sigma Chemical Co. (St. Louis, MO).

Experimental Incubation Conditions

All incubations were performed by floating Formvar/carbon-coated copper grids with attached cells side down on a solution of 0.05 M KH2PO4 buffer, pH 6, containing 1% glucose (control) or 1% glucose plus 0.1–1.0% (wt/vol) of a cellulose derivative for 3–10 min. (A. xylinum cells adhere to the coated grid when it is touched to the surface of the cell suspension.) Cells were also incubated in 1% cellulose, 1% starch, 0.2% laminarin, 0.2% agar, 0.2–1.0% xanthan gum, and 5% glycerol to test for alterations induced by the repeating unit of cellulose, by nonspecific interactions with polymers in solution, by solution viscosity, or through binding of water by the polymers.

Specimen Preparation and Electron Microscopy

Grids were washed in water after incubation and negatively stained with 1% aqueous uranyl acetate containing 0.02% (wt/vol) bacitracin as a spreading agent. Cells actively synthesizing cellulose were freeze-etched in a Balzers 360M freeze-etch apparatus (Balzers, Hudson, NH) using platinum-carbon resistance electron micrographs were taken at 75 kV.

X-ray Diffraction

Samples of altered cellulose induced by 0.1% CMC were collected by centrifugation and air-dried into thin films. The x-ray diffraction pattern was recorded using Cu Ka radiation (λ = 1.54 Å) in an evacuated Searle (Searle Radiographics Inc., Des Plaines, IL) focusing toroid.

RESULTS

A. xylinum cells incubated in glucose, as described, synthesize a twisting ribbon of cellulose (Fig. 1). Subunits of the ribbon begin at specific points in a longitudinal line along the cell surface, become thicker, and add to the composite ribbon (Fig. 3). Complete ribbons vary in width between 40 and 60 nm. High resolution micrographs reveal that the ribbon has internal striations and substructure (Fig. 2). Occasionally, microfibrils measuring 3–4 nm wide and bundles of microfibrils are pulled away from the ribbon (Fig. 2, arrow). Freeze-etch micrographs of actively synthesizing cells show that the particles in the outer membrane thought to be associated with cellulose synthesis are organized into discontinuous and sometimes overlapping segments (Fig. 4).

When 0.1% CMC is added to the incubation medium, separate bundles of cellulose microfibrils are synthesized along the longitudinal axis of the cell (Fig. 5). The bundles twist, revealing a narrow and a wide axis. Typical bundles are 6–12 nm wide, but occasionally fibrils measuring 3–4 nm or less are present (Fig. 6). The bundles of microfibrils often form a loose coil (Fig. 5). Other ribbons synthesized in the presence of CMC are more coherent but lack the tight organization and characteristic twist of normal ribbons (Fig. 7). Both types of CMC-altered ribbons can be observed on the same grid. Reversals in the direction of ribbon synthesis can be observed in cells incubated in cellulose derivatives (Fig. 8) but not in cells incubated in control medium. The reversals are accompanied by transient, extreme disorganization of the cellulose (Fig. 9).

When A. xylinum is grown in medium containing 0.1% CMC for 24 h (shaking at 50 rpm to keep the CMC dispersed), a slime of bacterial cellulose combined with CMC forms throughout the medium. Cells incubated, with shaking, in glucose alone produce tough balls of intertwining normal ribbons. X-ray diffraction shows that the CMC-altered cellulose has cellulose I crystalinity (Fig. 10).

Similar degrees of alteration in ribbon assembly are induced by (a) various concentrations of CMC (0.1–1.0%), (b) CMCs with various degrees of substitution (DS = 0.4, 0.7, and 1.2) when molecular weight is constant (250,000) and (c) CMCs with varying molecular weights (90,000, 250,000, and 700,000) when degree of substitution is constant (DS = 0.7). Carboxymethylcellulose with a DS of 0.7 is slightly more effective (as judged by induction of highly splayed ribbons) in altering ribbon assembly than CMC with DS = 0.4 or 1.2.

Incubation in neutral cellulose derivatives and in cellulobiose results in altered ribbons similar to the more coherent CMC-altered ribbon shown in Fig. 7. Occasionally cells producing loosely coiling bundles similar to those in Fig. 5 are observed after incubation in methylcellulose. In order of increasing effectiveness in altering cellulose assembly, the neutral derivatives tested are: hydroxypropylcellulose (DS = 3), hydroxyethylcellulose (DS = 1.5), hydroxypropylmethylcellulose (DS = 1.1–1.6), and methylcellulose (DS = 1.6–1.9).

No alteration in ribbon assembly was observed when cells were incubated in starch, laminarin, agar, xanthan gum, or glycerol.

DISCUSSION

A cellulose derivative that effectively alters cellulose assembly must be able to associate closely with native cellulose. Close association would require that enough of the glucan backbone of the derivative be exposed to allow at least partial hydrogen bonding between the native cellulose and the derivative. Therefore, the low DS of CMC is probably one reason that it alters ribbon assembly more effectively than the other derivatives tested. A DS of 0.4, 0.7, or 1.2 indicates that there are 4, 7, or 12 carboxymethyl substituents per 10 glucose residues. A completely substituted glucan chain would have a DS = 3 since there are three free hydroxyls on each glucose. Substitution of 4, 7, or 12 carboxyls per 10 glucose molecules would not shield the glucan backbone of CMC, allowing CMCs in this range of DS to associate closely with the bacterial cellulose. In close agreement with the data of Ben-Hayim and Ohad (10), our results indicate that CMC with DS = 7 is most effective in altering ribbon assembly.

An increased number of substituents or large substituent groups may shield the cellulose backbone. Such shielding probably explains why hydroxypropylcellulose (DS = 3) and hydroxyethylcellulose (DS = 1.5), or the CMC of DS = 2.2 tested by Ben-Hayim and Ohad (10), are less effective in altering cellulose assembly. The DS of hydroxyethylcellulose is not much higher than that of one of the CMCs tested, but its ethylene oxide substituents undergo extensive secondary substitution (20). The resulting long side chains may effectively shield the glucan backbone. Similarly, xanthan gum does not alter cellulose assembly, even though it has DS = 1, because its...
FIGURE 1  *Acetobacter xylinum* (left) produces an extracellular, twisting ribbon of cellulose. Bar, 1 μm × 30,000.

FIGURE 2  High magnification view of a normal ribbon in which surface striations are visible. A 3.5-nm microfibril is pulled away from the ribbon surface (arrowhead). Bar, 0.1 μm × 300,000.

FIGURE 3  A normal *A. xylinum* cell in which subunits of the ribbon, probably corresponding to microfibril bundles, begin at separate points (arrowheads) along the cell surface. The microfibril bundles aggregate to form the composite ribbon (right). Bar, 1 μm × 30,000.

FIGURE 4  Freeze-etch micrograph of the PF face of the bacterial lipopolysaccharide layer showing the linear row of particles that are thought to be associated with cellulose synthesis. Overlapping rows and discontinuous arrays of particles are visible. Bar, 0.1 μm × 100,000.

FIGURE 5  Cellulose synthesized in the presence of CMC. Microfibril bundles have not fasciated to form the normal composite ribbon. Bar, 1 μm × 30,000.

FIGURE 6  A high magnification view of separated bundles of cellulose produced during incubation with CMC. Bundles are typically 6–12 nm wide, but fibrils as small as 3 nm are occasionally observed (arrowheads). Ferritin (120 Å diameter) is present for internal calibration. Bar, 0.1 μm × 140,000.
three-sugar side chains wrap completely around the glucan backbone (19).

After a derivative coats the bundles of microfibrils, its neutral or charged substituents would prevent further hydrogen bonding between subunits of the native cellulose through steric hindrance or electrostatic repulsion. Alteration of cellulose ribbon assembly by celllobiose is probably minimal because celllobiose is the unsubstituted repeating unit of cellulose. By comparing kinetic experiments performed at different pH, Ben-Hayim and Ohad (10) demonstrated that electrostatic repulsion between charged carboxyl groups increases the effectiveness of CMC in altering cellulose synthesis. A negatively charged substituent is not a requirement for induction of altered ribbon assembly, however, since the neutral derivative, methylcellulose, is almost as effective as CMC.

It is unlikely that altered cellulose assembly is a nonspecific result of the presence of polymers in solution because (a) variable degrees of alteration can be obtained with similar cellulose derivatives, and (b) normal ribbons are synthesized in α-(1,4)-starch, β-(1,3)-laminarin, and agar. High viscosity of the incubation medium or binding of water by the polymers are also unlikely explanations for the observed alterations since normal ribbons are produced in high viscosity xanthan gum and in 5% glycerol.

FIGURE 7 A more coherent altered ribbon synthesized in the presence of CMC that lacks the tight organization and characteristic twist of normal ribbons. Bar, 1 μm. X 30,000.

FIGURE 8 Reversal in direction of ribbon synthesis frequently occurs in CMC when the normal assembly mechanism is disrupted. Reversal is accompanied by transient and extreme disorganization of the cellulose product. Bar, 1 μm. X 15,000.

FIGURE 9 Higher magnification of region of transient disorganization. The coiling cellulose in this disorganized zone is probably completely noncrystalline. Bar, 0.5 μm. X 50,000.

FIGURE 10 An x-ray diffractogram of cellulose synthesized in 0.3% CMC. Cellulose I crystallinity is unaltered by incubation in CMC.

FIGURE 11 Microfibril crystallization is disrupted in the presence of fluorescent brighteners. The cellulose synthesized forms a broad band of small fibrils (minimum width, 1.5 nm) instead of the normal twisting ribbon. Bar, 1 μm. X 15,000.
Disruption of cellulose ribbon assembly at two different levels of structural organization by fluorescent brighteners (Fig. 11) and cellulose derivatives suggests that the ribbon is assembled hierarchically from 3–4-nm microfibrils and bundles of microfibrils. Microfibrils measuring 3–4 nm wide have been described repeatedly as a component of A. xylinum ribbons (4, 5, 11, 21, 22). A variety of evidence suggests that bundles of microfibrils are also structural subunits of ribbons: (a) separate bundles are synthesized during incubation in CMC; (b) high resolution negative staining reveals striations within normal ribbons and, occasionally, bundles of microfibrils are pulled away from the ribbon (Fig. 2, and reference 22); and (c) the first step in the degradation of A. xylinum cellulose by purified cellulases is the spaying of ribbons into bundles (11, 22). Other evidence suggests that the cellulose-synthesizing apparatus is specifically organized to mediate synthesis of microfibril bundles: (a) negative staining of the bacterial cell surface shows that subunits of the ribbon begin at separate points, become thicker, and combine into the composite ribbon (Fig. 3); and (b) freeze-etch micrographs show that particles in the outer membrane are arranged into discontinuous and sometimes overlapping segments (Fig. 4). These separated segments of particles may correspond to sites of synthesis of separate bundles of microfibrils. (The extrusion pores associated with these particles are not often seen because the outermost layer of the bacterial outer membrane is usually split away by the fracture plane.)

Therefore, cellulose ribbon biogenesis can be described as a hierarchical, cell-directed self-assembly process in which glucan chain aggregates crystallize into 3–4-nm microfibrils, microfibrils combine into bundles, and bundles of microfibrils fasciate to form the composite ribbon. The cellulose-synthesizing apparatus is organized (or cell-directed) to promote the ordered self-assembly of parallel glucan chains at the cell surface, so that the composite ribbon with its hierarchical substructure of microfibrils and microfibril bundles is produced (12). The concept of a hierarchical assembly mechanism of microfibrils and the ribbon was first suggested by Zaar (5) and is strongly supported by our observations of altered microfibril and ribbon assembly. Fluorescent brighteners can penetrate between glucan aggregates synthesized and extruded at closely spaced sites, before they interact and crystallize into microfibrils (7, 8). Polymeric cellulose derivatives can interfere only with the fasciation of bundles of microfibrils that are synthesized along slightly separated arrays of sites.

Kinetic data also support the hypothesis that cellulose assembly occurs by a hierarchical process. In the presence of fluorescent brighteners that disrupt microfibril crystallization, the rate of glucan chain polymerization increases 200–400%. Therefore, microfibril crystallization, and perhaps the assembly of microfibrils into bundles, are major rate-limiting steps in cellulose ribbon biogenesis. Ben-Hayim and Ohad (10) present data that demonstrate that the rate of cellulose synthesis increases ~30% in the presence of CMC. Thus the final step in ribbon assembly, the fasciation and hydrogen bonding of bundles of microfibrils, is also slightly rate limiting.

The normal assembly mechanism must be highly coordinated to produce a ribbon from the interaction of glucan chain aggregates arising from an array of synthesis sites along the length of the cell. Reversals in the direction of ribbon assembly are infrequently observed under normal incubation conditions but are frequently observed in the presence of CMC when the assembly process is disrupted. The transient extreme disorganization of the cellulose product during reversal emphasizes the precise coordination of the synthesizing machinery that normally results in production of microfibrils, bundles of microfibrils, and the composite ribbon.

The principles of the assembly mechanism described for A. xylinum may be applicable to cellulose synthesis in eucaryotic organisms. The 7.5 nm average crystallite size (1), typical of A. xylinum cellulose, is probably determined by the arrangement of the synthetic machinery that mediates the formation of bundles of microfibrils. Other organisms could produce cellulose with larger or smaller crystallites, depending on the specific arrangement of their cellulose-polymerizing enzymes. Microfibril-terminal particle arrays that correspond to the size and substructure of cellulose microfibrils have been observed in algae (2, 3) and higher plants (23). It has been frequently suggested that hemicelluloses in plant cell walls interact with cellulose to regulate fibril size (13–15) and wall extensibility (16, 17). The fibril size of A. xylinum cellulose is reduced when CMC is present, and the altered cellulose forms a slime in shaking solutions rather than a tough, cohesive ball. Hemicelluloses present during synthesis of new cell walls could interact with cellulose in a similar way and thereby regulate the physical properties of the wall. Testing the effects of purified plant cell wall components on cellulose assembly in A. xylinum could be a useful bioassay method for investigating the relationship of wall matrix components to cellulose.

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