PROBING THE RELATIONSHIP OF POLYMERIZATION AND CRYSTALLIZATION IN THE BIOGENESIS OF CELLULOSE I

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Summary: Direct dyes and fluorescent brightening agents (which hydrogen bond with cellulose) and carboxymethylcellulose (which closely associates with native cellulose) have been used in vivo to alter cellulose assembly by the gram-negative bacterium Acetobacter xylanum. The dyes and brighteners alter cellulose biogenesis at a different level of fibril organization than carboxymethylcellulose. Light and electron microscopy, X-ray diffraction, and biochemical kinetics have been used to examine the nature of these alterations. From these data, we have proposed (1,2,3) that polymerization and crystallization are coupled processes which can be experimentally separated in vivo, and that biogenesis of cellulose I fibrils occurs by a cell-directed, self-assembly process in A. xylanum. The relationship of such a mechanism to control of fibril substructure and width, and to control of crystallite size in A. xylanum, algae, and higher plants is discussed.

Keywords: Cellulose, fibril biogenesis, Calcofluor White ST, carboxymethylcellulose, Acetobacter xylanum

Introduction

Recent work in our laboratory has shown that direct dyes and fluorescent brightening agents dramatically alter the in vivo assembly of cellulose ribbons by the gram-negative bacterium Acetobacter xylanum (1,2,3). A. xylanum normally produces a highly crystalline, extracellular ribbon of cellulose (Figure 1). A variety of compounds used as industrial dyes and brighteners for cellulose products and as biological stains for cellulose and chitin (4) bind to the subunits of the ribbon as they are synthesized in vivo, prevent their normal aggregation, and radically alter the morphology and crystallinity of the cellulose (Figure 2). Most of the dyes and brighteners found so far to alter cellulose assembly are planar derivatives of trans-stilbene with substituents capable of hydrogen bonding with the hydroxyl groups of linear β(1,4) polysaccharides (5). The alteration of cellulose assembly by the fluorescent brightener Calcofluor White ST will be discussed here.

Similarly, carboxymethylcelluloses (CMC) of varying degrees of polymerization and substitution have been shown to alter cellulose assembly by A. xylanum, but at a higher level of organization than the dyes and brighteners (3) (Figure 3). It is likely that the high molecular weight CMC associates closely with the larger subunits of the cellulose ribbon (6), and prevents the degree of association and hydrogen bonding necessary for formation of the typical twisting ribbon. Experimental use of these probes which interrupt cellulose assembly at different levels has allowed, for the first time, direct investigation of the relationships between glucan chain polymerization, microfibril crystallization, and fibril assembly in the biogenesis of cellulose I.

Cellulose biogenesis in Acetobacter xylanum

Acetobacter xylanum is an excellent experimental organism with which to study the assembly of cellulose. It is easily maintained in culture;

Figure 1: Negatively stained A. xylanum synthesizing a twisting ribbon of cellulose under normal culture conditions (bar 1 μm); Figure 2: A. xylanum synthesizing cellulose with altered morphology and crystallinity in the presence of 0.25 mM Calcofluor White ST (bar 1 μm); Figure 3: A. xylanum synthesizing separate bundles of cellulose in the presence of 0.1% carboxymethylcellulose (bar 0.5 μm).
experimental conditions can be readily manipulated; the cellulose ribbon is composed of pure native parallel chain cellulose I (7); and ribbon production can be monitored by light and electron microscopy (8). Using *A. xylinum* it is possible to examine and alter cellulose synthesis without the complications which arise in studies of multicellular organisms which produce cellulose along with many other polymers.

Further, the substructure of the cellulose ribbon synthesized by *A. xylinum* is very similar to the substructure of large cellulose fibrils in other organisms. The 3.5 nm microfibrils which may be characteristic of many cellulososes (9) are occasionally observed fractured away from the ribbons. Larger bundles of microfibrils are also evident, appearing as striations within the ribbon (9,10). The microfibrils are formed by end synthesis in association with a stationary, longitudinal row of presumed enzyme complexes and extrusion pores in the bacterial envelope (8,11). Microfibril terminal globules, presumed to contain cellulose polymerizing enzymes and to move within the fluid membrane, have been observed in the membranes of certain algae and plants (12,13,14). Therefore, comparisons are possible between the structure and biosynthesis of ribbons in *A. xylinum* and the cellulose fibrils in other cellulose synthesizing organisms.

The reader is referred to the pertinent references cited above for elaboration and documentation of our work with alteration of cellulose assembly in *A. xylinum*, but the most important data and conclusions are summarized below.

**Alteration of cellulose assembly by Calcofluor White ST**

When Calcofluor White ST (4,4′-bis(4-amino-6-bis(2-hydroxyethyl)amino-triazin-2-ylamino)-2,2′-stilbenesulfonic acid) is added to cultures of *A. xylinum*, the bacteria synthesize broad bands of fine fibrils with smallest dimensions of about 1.5 nm. Larger fibrils appear to arise by fasciation of these small fibrils. Experiments with cells attached to Formvar-carbon-coated grids indicate that the synthesis of altered cellulose is inducible or reversible within seconds depending on the presence or absence of Calcofluor in the medium. The observed rapid reversibility and other data suggest that Calcofluor exerts its effect by hydrogen bonding with the cellulose product and not by interfering with cell metabolism or the synthesizing enzymes themselves (1).

**Incorporation of 14C-glucose into an alkali-insoluble, cellulase-digestible product indicates that rate of polymerization increases up to four hundred percent compared to controls incubated in Calcofluor-free media. Rate of glucose oxidation to CO2 increases only 10-15%. Free Calcofluor is depleted from the medium as it binds to newly synthesized cellulose. When the concentration of free Calcofluor falls below 0.1 μM, the rate of polymerization returns to that of the control. As verified by electron microscopy, production of normal ribbons also occurs at concentrations of Calcofluor below this apparent threshold concentration (2).**

The increase in rate of polymerization of cellulose in the presence of Calcofluor can be related to the lack of microfibril crystallization and ribbon assembly. Polarization microscopy of wet Calcofluor-induced cellulose shows that the altered product is ordered, while X-ray diffraction of the altered cellulose in the wet state reveals no detectable crystallinity. *A. xylinum* cellulose has approximately equal crystallinity in the wet and dry states (1). The kinetic and diffraction data together suggest that: (1) polymerization and crystallization are coupled processes; (2) the two processes can be experimentally separated in vivo by addition of hydrogen bonding dyes and fluorescent brightening agents; and (3) crystallization is the rate limiting step in cellulose biogenesis by *A. xylinum*.

Cellulose I crystallinity is regained in the altered cellulose after drying; and crystallite size depends on the concentration of Calcofluor used to induce the altered product. It is unusual that cellulose I forms after drying when the wet altered cellulose shows no detectable crystallinity. Anti-parallel chain cellulose II, the most thermodynamically favored polymorph of cellulose, usually forms upon regeneration of crystalline cellulose from a solution of random glucan chains. Therefore, in the Calcofluor-induced cellulose the glucan chains must be tactoidal, that is they must be oriented (in parallel) even though they are not crystalline in the wet state (1).

Since Calcofluor is able to interrupt cellulose assembly before the point of microfibril crystallization, we have proposed that a 1.5 nm tactoidal aggregate of glucan chains is extruded by each pore in the bacterial envelope. In the absence of interfering agents, several of these small glucan aggregates fasciate and cocrystallize by self-assembly into a 3.5 nm microfibril, which may be the minimum fibril size able to have true cellulose I crystallinity (15). The organization of extrusion pores seen in freeze etch micrographs is consistent with the proposal that crystalline microfibrils are assembled from the tactoidal product of several pores. Segregated triplets of pores, each of which may cooperatively produce one crystalline 3.5 nm microfibril, have been observed along the longitudinal row of pores in the bacterial envelope (3). Through this specific organization of the synthetic machinery, cellulose assembly is cell-directed.

**Alteration of cellulose assembly by carboxymethyl-cellulose**

The hypothesis that microfibrils crystallize from adjacent tactoidal glucan aggregates suggests a hierarchical assembly mechanism for the cellulose ribbon which is substantiated by the observed CNC alteration of ribbon assembly. Instead of the band of fine fibrils produced in the presence of Calcofluor, a loose intertwining group of 8-16 individually twisting bundles is synthesized in the presence of CNC. These bundles originate at defined points along the cell surface and grow thicker as glucan chains are added from successive pores. CNC interrupts cellulose ribbon assembly at the level of fasciation of these bundles to form the twisting ribbon, while it apparently cannot interfere with the crystallization of microfibrils or their aggregation into bundles. Freeze etch micrographs again suggest that the extrusion sites may be specifically organized to mediate synthesis of the different ribbon subunits. Discontinuous rows of extrusion pole triplets, each of which may form one separate bundle, have been observed in the cell envelope after freeze etching (3).

**Conclusions**

On the basis of the data summarized here, we have proposed that ribbon biogenesis occurs by a hierarchical cell-directed, self-assembly process which is critically mediated by the arrangement of synthesizing and extrusion sites in the bacterial cell surface (1,2,3). Mechanisms of cellulose fibril
biogenesis consistent with the mechanism of hierarchical assembly proposed for A. xylinum have been advanced for the algae Oocystis apiculata and Micrasterias denticulata (12,13). These algae synthesize broad cellulose fibrils in association with differentiated membranes. Morphological evidence obtained by freeze etching suggests that fibril size and substructure may be determined by the number and arrangement of globular membrane bound enzyme complexes and/or associated membrane structures such as the rosettes in the membranes of Micrasterias. In contrast, the cellulose fibrils in corn root and in pinto and mung bean seedling are small and without visible substructure (14). Correspondingly, the microfibril terminal globules (which are presumed to contain synthesizing enzymes) occur singly, without the kind of secondary organization seen in the algae. Therefore, cellulose fibril size may be determined by the number of associated enzyme complexes. It is also possible that crystallite size is regulated, at least in part, by the proximity of enzyme complexes producing tactoidal glucan aggregates capable of interaction before final crystallization (1).

Along with the distribution of synthesizing enzymes, the presence of polymers capable of hydrogen bonding with cellulose may have a role in regulation of crystallite size in cell walls. In primary cell walls, the matrix of hemicelluloses and pectins may prevent the extensive inter-cellulose hydrogen bonding required for formation of larger fibrils and crystallites. In some secondary cell walls the absence of these polymers may allow large fibril and crystallite formation by organized enzyme complexes. Preliminary experiments show that pectin, xylan, and mannan interfere with normal ribbon production by A. xylinum in much the same way as CMC prevents aggregation of bundles into a typical ribbon. Further experiments with such high polymers may help elucidate the role of the different cell wall polymers in the structure and function of plant cell walls.

Historically, cellulose researchers have realized that the mechanism of cellulose biosynthesis and our understanding of its fundamental physical character and properties are inextricably related. It is evident that the more fully the biosynthesis of cellulose and its interaction with other wall polymers is understood, the better we will be able to exploit the potential of cellulose as a natural fiber and a renewable resource. The use of probes which alter cellulose assembly in A. xylinum has allowed significant advances in our understanding of cellulose fibril biogenesis. Further work, particularly with electron diffraction of these altered cellulosics, promises to be equally rewarding in elucidating the crystalline character and mechanism of biosynthesis of cellulose.

Literature Cited: