Native folded-chain cellulose II

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A mutant strain of *Acetobacter xylinum* was isolated, which produces cellulose of anomalous band-like morphology in contrast to straight, crystalline microfibrils of cellulose I normally produced by the wild-type strain. The band material extruded perpendicularly from the cell surface constitutes strand-like structures with lateral dimensions of 10 nm. Electron diffraction analysis revealed that these strands are composed of cellulose II crystallites within which the molecular chains are oriented perpendicular to the strand axis. Because the average length of glucan chains in this material was determined to be about 10 times the width of the 10 nm wide strand, the elucidated features make it highly likely that this form of cellulose II involves chain folding which gives rise to the antiparallel structure.

(Keywords: microbial cellulose; cellulose II; chain folding)

INTRODUCTION

Cellulose, a major constituent of the plant cell wall, is known to have a variety of allomorphs, of which the predominant native form is cellulose I[1]. Extensive attempts have been made[2,3] to determine the crystalline form and arrangement of β-1,4 glucan chains out of several possible structures: (i) extended, parallel; (ii) extended, antiparallel; or (iii) folded, antiparallel. While structure (i) is the most likely based on biological and physicochemical considerations, its confirmation has not been easy owing to insufficient diffraction data resulting from the small crystallite sizes. During the past 10 years, however, new approaches showing asymmetry in labelling of reducing ends[4,5] and in the mode of enzymatic degradation[6], lattice images showing extensive crystalline order[7-10], and the discovery of a single chain unit cell[11] collectively have established the 'extended and parallel' structure of cellulose I beyond doubt.

On the other hand, the structure of cellulose II, which is typically formed from cellulose I via artificial treatments such as alkali swelling or regeneration from the dissolved state, has remained unsolved because of unavailability of specimens suited for the above-cited techniques. Without resolution of this problem for cellulose II, we cannot understand why cellulose I occurs as a predominant allomorph in nature. In this contribution we report that a mutant strain of *Acetobacter xylinum*, a cellulose-synthesizing bacterium, produces *in vivo* an anomalous form of cellulose ('band' material; see Results and discussion), and we present evidence that the product is cellulose II existing in the form of a folded-chain antiparallel structure.

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EXPERIMENTAL

Synthesis of band cellulose by a mutant strain

The parent strain, *Acetobacter xylinum* (ATCC 23769), which is one of the few strains that occasionally produces band material, was cultured in Schramm-Hestrin (SH) medium[12] for 3–4 weeks without replenishment. A milky-white layer formed at the top of the translucent pellicle. While this pellicle consisted mostly of normal ribbon cellulose, the milky layer was found to have a high content of band material. When the cells in this layer were plated on solidified SH medium, three types of colonies were formed: (i) rough and cohesive; (ii) large, flat and non-cohesive; and (iii) small and non-cohesive. The strain derived from a small and non-cohesive colony (type (iii)) was found to be responsible for extensive band production.

When inoculated into fresh SH medium, this mutant strain produced a weak and translucent film at the surface of the medium, and this material contained almost exclusively band material. The strain could be maintained through successive inoculations retaining the band-producing activity. The efficiency of cellulose production was estimated to be approximately one-fiftieth of the parent strain. All experiments below were performed with the band material obtained from this strain.

Preparation of specimens and electron microscopy

The native band-rich cellulose sample, with or without hot alkali treatment (boiling for 30 min in 2% sodium hydroxide followed by washing with water), was mounted on a Formvar coated grid and negatively stained with 2% uranyl acetate. For the purposes of high-resolution observation and electron diffraction, the specimen was mounted on an ultrathin carbon film deposited on a mica

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plate. The electron diffraction patterns were recorded under carefully controlled conditions to minimize the beam damage to the material. Negative staining with methylamine tungstate[13] also was utilized for high magnification observations. Both the morphology and electron diffraction patterns of the material were not adversely affected by the alkali treatment.

Degree of polymerization

The band-rich cellulose sample purified by hot alkali, as described above, was dried through exchange of the medium to acetone. The sample was then converted to cellulose nitrate by treatment with a nitric acid–phosphorus pentoxide mixture[14]. The cellulose trinitrate was dissolved in tetrahydrofuran and analysed by a size exclusion chromatograph using a set of polystyrene gel columns (TSK-GEL, GMH-xl 300 mm + G2500H6 300 mm, Tosoh, Inc., Tokyo).

RESULTS AND DISCUSSION

The normal structure of cellulose produced by the wild type of *A. xylinum* (strain ATCC 23769) is a ribbon composed of crystalline microfibrils[15]. Careful examination of the material reveals that it contains small quantities of anomalous material having a dispersed, band-like morphology (denoted herein as 'band' material, in contrast to normal 'ribbon' material)[16]. This band material occasionally forms when cellulose is disturbed by shear forces upon separating cells from the native product[17]. In other words, the production of the band material is likely to be the result of an unstable or altered condition in which the normal process of cell-directed self-assembly of cellulose[18] fails to operate. The structure of the band material has not been understood because of the rarity of its occurrence with the normal strains. To overcome this difficulty, we examined various culture conditions and found that a nutritional restriction could induce a mutation leading to abundant production of the band material (see Experimental section).

Electron microscopic examination revealed that the product of the mutant contained abundant 'band' material, with relatively small amounts of normal ribbon cellulose (less than 5% by visual assessment) (*Figures 1A, B*). These two materials were occasionally observed to be sequentially attached to each other,

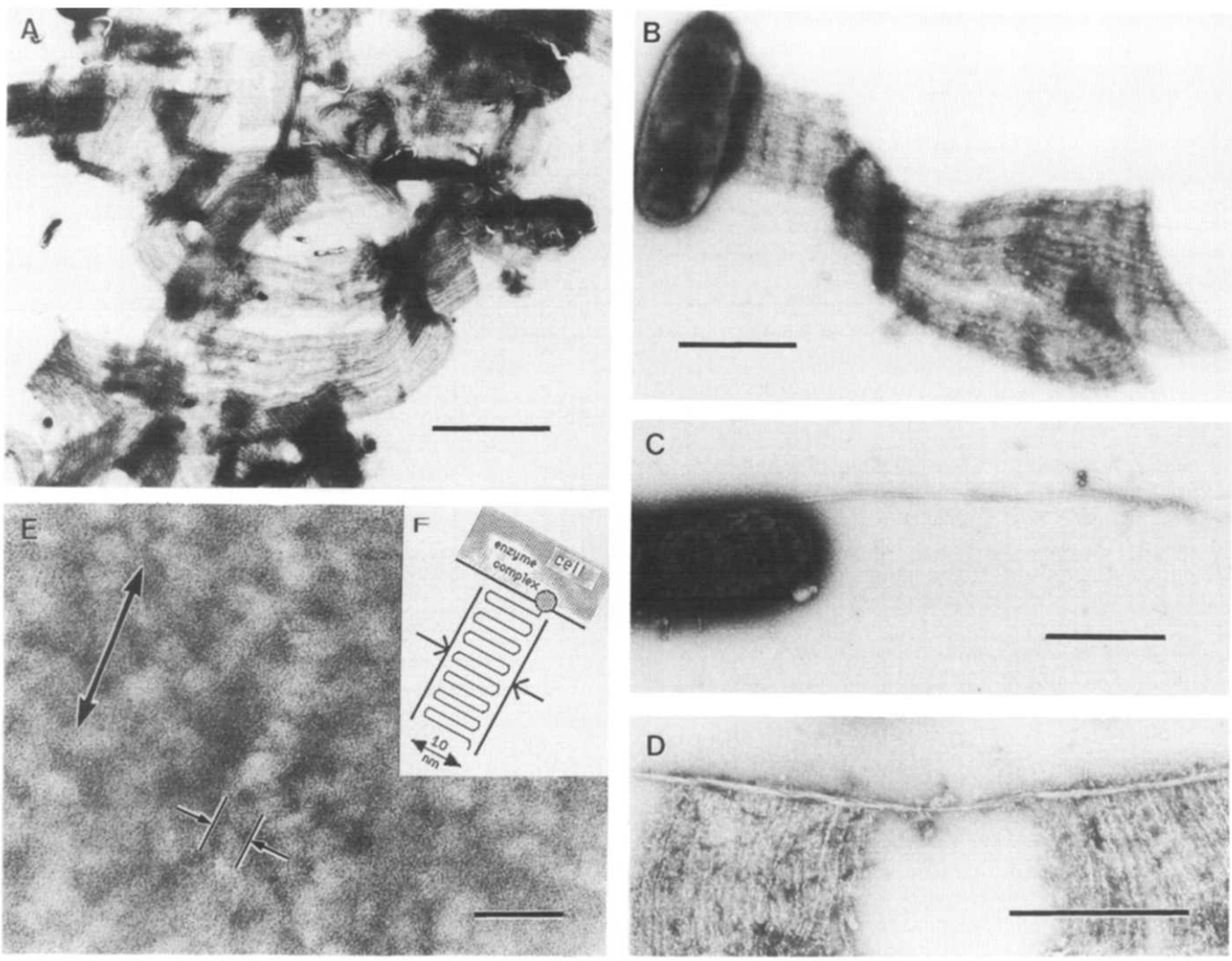

Figure 1 Electron micrographs of negatively stained *Acetobacter* cellulose. (A) 'Band' material taken from the mutant culture (scale bar, 2 μm). (B) Mutant cell and newly produced 'band' material taken from the mutant culture (scale bar, 1 μm). (C) Wild-type cell and normal ribbon-shaped cellulose (scale bar, 1 μm). (D) Area showing transition between ribbon formation and band formation (scale bar, 1 μm). (E) Strand-like structure at high magnification. Typical width of the strand as shown by the opposing arrows is 10 nm. Double-headed arrow shows the direction of band extrusion (scale bar, 20 nm). (F) A schematic drawing of the possible arrangement of the folded glucan chain within the strand. The specimens were negatively stained with 2% uranyl acetate (A–D) or 2% methylamine tungstate (E)

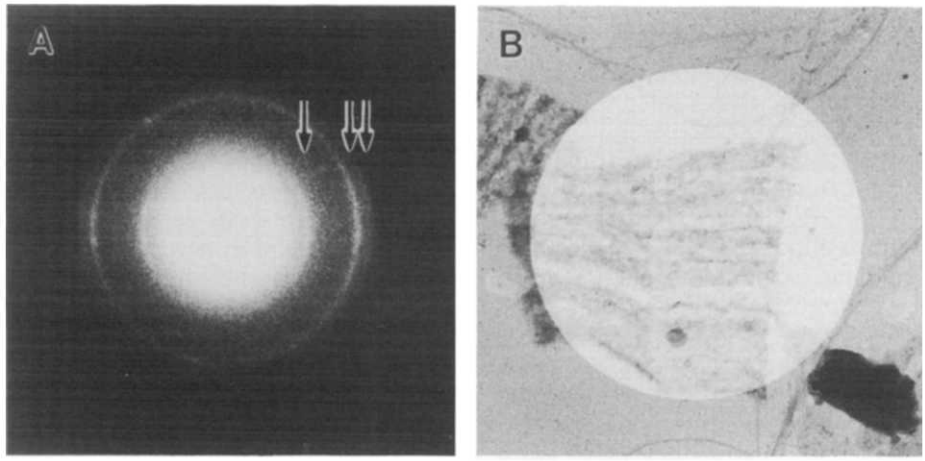

Figure 2 (A) Electron diffraction pattern of band material; (B) defocus contrast image of corresponding area. The diameter of the area irradiated for diffraction (bright circle in B) is 1.6 μm. The pattern is properly aligned relative to the image. Arrowheads show three major equatorial reflections, (1 $\bar{1}$ 0), (1 1 0) and (2 0 0), of cellulose II, with determined *d*-spacings of 0.71 nm, 0.44 nm and 0.40 nm, respectively. The strong anisotropy in intensity shows the orientation of the molecular axis perpendicular to the direction of band extrusion

demonstrating transitions between the formation processes thereof (*Figure 1D*)[19]. Observations at higher magnification (*Figure 1E*) show that the band material consists of small irregular granules loosely linked to form strand-like structures lying in the direction of band extrusion. The typical number of strands in the band formed by a single cell was about 50, which agreed with the number of 1.5 nm wide subfibrils constituting the normal ribbon cellulose[15]. This number also corresponds to the number of pores on the cell surface from which cellulose subfibrils are extruded[20].

The overall appearance of this 'native' band material is similar to that of the 'altered band' material formed by the wild-type cell in the presence of certain cellulose-binding dyes[18]; however, the structures of these two band materials are significantly different in that the latter consists of short, disrupted subfibrils of a width of 1.5 nm in contrast to the 10 nm wide strands of the former. Additionally, the 'altered band' is known to give cellulose I on removal of the dye, while the 'native band' has been found to be cellulose II, as described below. Therefore, the difference in morphology is considered to reflect a basic difference in the molecular arrangements within these cellulose materials.

A knowledge of the molecular arrangement of the glucan chains is needed for understanding the mechanism(s) of band formation. The electron diffraction pattern of a selected area of the band (*Figure 2A*) shows three main equatorial reflections [(1 $\bar{1}$ 0), (1 1 0) and (2 0 0)] of cellulose II*. All these diffractions form fairly long arcs, but still show remarkable orientation of the molecular axis perpendicular to the direction of band extrusion. The absence of the meridional (0 0 4) reflection indicates that the crystalline order in the direction of the molecular axis is limited.

To reconcile this orientation of glucan chains with the strand-like morphology, we must assume either: (a) that the cellulose molecules are regularly folded into a strand with the folded parts forming the edge of the strand; or (b) that they are disrupted and stacked as short fragments with an average degree of polymerization (DP) of 20, corresponding to the observed 10 nm width of the strands.

* Lattice planes are indexed according to ref. 3

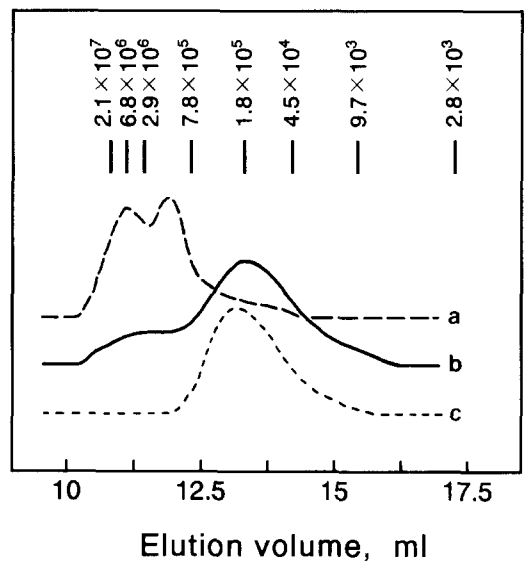

Figure 3 Size exclusion chromatograms of trinitrates of (a) normal *Acetobacter* cellulose, (b) 'band' material and (c) commercial microcrystalline cellulose of DP 200. Vertical bars show positions of monodisperse polystyrenes of indicated molecular weights

For determining which is the case, the DP distribution of the band cellulose was determined by size-exclusion chromatography of the nitrated sample.

The major peak of the sample corresponded with the position of a polystyrene standard with a molecular weight of 100 000, which corresponds to an average DP of 200 for cellulose nitrate (see *Figure 3*). This DP is significantly greater than 20. Thus, the present results strongly point to the occurrence of chain folding in the band material. (The average DP of 200 is significantly lower than that of the normal ribbon material (> 1000), and the cause of this difference should be studied in relation to the mechanisms of the polymerization and ribbon/band formation.)

There have been several reports[21,22] indicating the possibility of chain folding in the lamellae of cellulose II. Preparation of these samples involved delicate controls of recrystallization, and reproducibility of the results has not been established[23]. The epitaxial growth of cellulose

II crystallites in mercerization of *Valonia* cellulose has also indicated possible chain foldings[24], but conclusive evidence has been lacking. Therefore the present result is regarded as the most plausible evidence for the occurrence of chain folding in cellulose II.

Logically, chain folding in a crystal of a linear polymer having polarity does not necessitate inclusion of an equal number of oppositely oriented chains in the unit cell, i.e. a parallel-chain structure may still arise as aggregated domains in a folded-chain crystal. Such a structure, however, would involve complicated arrangements at the folding sites and seems highly unlikely. Therefore, it is reasonable to assume that the individual glucan chain foldings lead to the proposed 'antiparallel' arrangement in the unit cell of the native band form of cellulose II.

As stated earlier, we already have convincing evidence for the 'extended and parallel' structure of cellulose I. Based on these facts and the present finding of the likeliness of antiparallel structure for cellulose II, we can now construct a conceptual model for the molecular arrangement in the two major allomorphs of cellulose and interpret our model in relation to the biological and chemical processes leading to the formation of cellulose I and cellulose II.

Cellulose molecules are probably synthesized by organisms with the same molecular polarity*, and this leads to the formation of parallel-chain cellulose I when the crystallization is properly controlled to keep the original molecular arrangement[27]. On the other hand, cellulose forms the thermodynamically more stable crystal of cellulose II having antiparallel structure, either when no dominant chain polarity is present or when molecular chains can be folded upon crystallization. In other words, the formation of the thermodynamically unfavourable form of cellulose I is dictated by the unidirectionality of synthesis combined with some spatial conditions within the enzyme complex; this allows the cellulose molecules to become fasciated, retaining extended chain conformation[27]. The importance of the glucan chain population at the site of synthesis in determining the morphology, and the possible crystal structure of cellulose synthesized *in vitro*, have recently been shown[28].

That cellulose I is converted ('mercerized') to cellulose II via treatment with concentrated alkali without dissolution of cellulose has been a major obstacle to acceptance of the hypothesis of 'parallel cellulose I and antiparallel cellulose II'. The present finding strongly supporting chain folding, in this instance of cellulose II, will serve as a basis for verifying the proposed mechanism of intermingling of neighbouring microfibrils having opposite polarities[29].

A similar situation of possible chain folding in the form of ribbons or lamellae has been known for glucomannan[30,31]. This form of glucomannan, however, was obtained only under carefully controlled artificial conditions. In contrast, the present band form of cellulose is synthesized in an aqueous environment as an underivatized native product as a result of an anomalous biosynthetic mechanism. Similar conditions may be involved in other systems that are known to produce cellulose II *in vivo*[32].

While the early models based on diffraction and packing analyses of cellulose I[2,3] postulated the presence of intramolecular hydrogen bonds, suggesting remarkably rigid and straight conformations, a recent modelling study demonstrated that the cellulose molecule can form compact folds without significant disadvantage in conformational energy[33]. Still, it is obvious that the occurrence of such foldings needs certain anomalous condition(s) in the synthetic mechanism, which must be elucidated in terms of the structure and nature of the enzyme complex synthesizing cellulose. (The latest conformation energy analysis supporting our folded-chain cellulose has been provided by French[34]. According to this analysis, a cellulose chain can be folded into a hairpin within the nominal constraint limits.)

* The polarity of cellulose synthesis, i.e. whether the reducing end is directed away from the synthesizing site, the opposite condition, or possibly both conditions, is to be further clarified. There is, however, a general belief that β-1,4 glucans are synthesized with their reducing ends directed away from the synthesizing sites, as reported for α-1,4 glucan[25,26]

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