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The mechanism of *Acetobacter xylinum* cellulose biosynthesis: direction of chain elongation and the role of lipid pyrophosphate intermediates in the cell membrane

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Abstract

The biosynthesis of *Acetobacter xylinum* ATCC 10821 cellulose has been studied with resting cells and a membrane preparation using ^{14}C-pulse and chase reactions, with D-glucose and UDPGlc, respectively. Cellulose was biosynthesized from UDPGlc, and it was found to be tightly associated with both the cells and the membrane. The cellulose chains could be released from the cells and the membrane preparation by treating at pH 2, 100 °C for 20 min. The cellulose chains that were released from the pulse and pulse-chase reactions were purified and separated from any low molecular weight substances by gel chromatography on Bio-Gel P4. They were then reduced with sodium borohydride and hydrolyzed with 4 M trifluoroacetic acid at 121 °C for 2 h. Labeled products from the acid hydrolyzates were separated by paper chromatography and found to be D-glucose and D-glucitol. The amount of radioactivity in the products was determined by liquid scintillation counting. It was found that the pulsed products from the resting cells gave a ratio of D-[^{14}C]glucitol to D-[^{14}C]glucose of 1:11, and after chasing, the ratio decreased to 1:36. The pulsed products from the membrane gave a ratio of D-[^{14}C]glucitol to D-[^{14}C]glucose of 1:12, and after chasing for 5 min the ratio decreased to 1:43, and after 10 min, the ratio decreased to 1:66. These results show that the labeled D-glucitol obtained from the reducing end of the cellulose chain is chased into the interior of the cellulose chain during synthesis, showing that the cellulose chain is elongated from the reducing end. An insertion mechanism for the synthesis of cellulose from UDPGlc is proposed that involves lipid pyrophosphate glycosyl intermediates and three membrane enzymes: lipid phosphate:UDPGlc phosphotransferase, cellulose synthase, and lipid pyrophosphate phosphohydrolase.

Keywords: *Acetobacter xylinum*; Cellulose synthase; Microbial cellulose synthesis; Pulse and chase reactions; Direction of cellulose elongation

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1. Introduction

Cellulose is one of the most abundant naturally occurring organic compounds on the Earth. It comprises the major component of plant cell-walls and is synthesized extracellularly by a few species of bacteria [1]. Cellulose is also a readily renewable source of carbohydrate that serves humans in a variety of ways such as in the manufacture of paper, films, explosives, and textiles. Efforts to study the biosynthesis of cellulose in plants have been limited by the inability to obtain the cellulose synthase and demonstrate in vitro synthesis by purified enzyme [2,3].

Several strains of *Acetobacter xylinum* elaborate relatively pure cellulose as an extracellular product that is extruded from the surface of the cell [4]. Cell-free preparations from *A. xylinum* that formed cellulose from UDP-D-glucose (UDPGlc) were reported by Glaser in 1958 [5]. The enzyme responsible for the synthesis is called cellulose synthase (EC 2.4.1.12). The synthesis was found to be stimulated by cyclo-3,6′:3′,6-diguanidine monophosphate (c-di-GMP) [3]. The synthase from *A. xylinum* has been purified and the catalytic subunit identified [6,7]. Sequence analysis of the catalytic subunit indicates that it is an anchored membrane protein [8].

The mechanism of cellulose chain-elongation has never been definitively determined and recently has become controversial. In 1995, Saxena et al. [9] proposed that the cellulose chain is elongated from its reducing end. This was based on deductions made from a comparative study of the sequence of several different polysaccharide synthesizing and hydrolyzing enzymes that synthesized or hydrolyzed α- and β-glycosidic linkages. Direct experimental evidence, however, was not presented. In 1997, Koyama et al. [10] inferred that the cellulose elaborated by *A. xylinum* and several other sources was synthesized by the addition of monomer units from UDPGlc to the nonreducing ends of the chains. This was based on the silver staining of the putative reducing ends of the cellulose chains and the microdiffraction-tilting electron crystallographic analysis of the cellulose fibers. The evidence here also was very indirect and arrived at by analogous reasoning.

To resolve these two opposite positions, we have studied the de novo synthesis of cellulose by *A. xylinum* cells and membranes using ^{14}C-pulse and chase techniques with D-glucose and UDPGlc, respectively. We find clear evidence that cellulose is biosynthesized by the addition of D-glucose to the reducing ends of the growing cellulose chains.

2. Materials and methods

Chemicals.—UDP-[^{14}C]glucose (73 mCi/mmol) was obtained from New England Nuclear (Boston, MA). [U-^{14}C]Glucose (240 mCi/mmol), unlabeled UDPGlc, and *Trichoderma viride* cellulase (10 IU/mg), were obtained from Sigma Chemical Co. (St. Louis, MO). Polyethylene glycol, PEG-4000, was from J. T. Baker Chemical Co. (Phillipsburg, NJ). Trifluoroacetic acid and $NaBH_4$ were from Fisher Scientific (Pittsburgh, PA). Bio-Gel P4 and P10 were from Bio-Rad (Hercules, CA). Materials for culture media were from Difco (Detroit, MI). All other inorganic chemicals and organic solvents were of reagent-grade quality or better.

Organism.—A. xylinum ATCC 10821 was used to study the synthesis of cellulose [5]. The organism was consecutively cultured at 30 °C under static conditions in 5 mL, 100 mL, and 1 L using the Hestrin and Schramm medium [11]. The cells were harvested from the cellulose pellicles that were formed at the air–liquid interface by filtering the pellicle through a 16-layer gauze that was washed three times with 50 mM Tris-HCl, pH 7.5, and the filtrate then centrifuged at 10,000×*g* for 10 min.

Preparation and assay of membrane-associated cellulose synthase.—A. xylinum membranes were prepared and assayed as described by Aloni et al. [12]: after suspension of the washed cells in 45 mL of buffer, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 1 mM EDTA (TME), containing 20% PEG-4000, the cells were sonicated using a Branson Sonifier 250 with a 12.7-mm tapped tip, giving two pulses with 100% power every second for 30 min. The sonicate was centrifuged at 12,000×*g* for 10 min and the pellet was suspended in 20 mL TME buffer. After removal of the unbroken cells by a short centrifugation at 1500×*g* for 3 min, the supernatant suspension was designated as *membrane preparation*. Centrifugation of this suspension at 12,000×*g* for 10 min at 4 °C gave the membrane preparation completely in the centrifuged pellet. The supernatant contained the cellulose synthase activator (c-di-GMP) and was designated as *activator preparation*.

Assay of cellulose synthesis.—The cellulose-synthesizing activity was assayed by measuring the

conversion of radioactivity from UDP-[^{14}C]Glc into 0.5 M NaOH-insoluble β-(1→4)-D-[^{14}C]glucan as described by Aloni et al. [13]. The method was modified by omitting the GTP activator precursor to decrease the rate of cellulose synthesis required by our pulse and chase studies. The enzyme assay was carried out by incubation of 80 μL of TME buffer containing 20% (w/v) PEG-4000, 20 μL of 0.5 M Tris-HCl (pH 9.6), 50 μM $MgCl_2$, 5 mM EDTA, 5 μL of UDP-[^{14}C]Glc (120 cpm/pmol, 270 μM), and 95 μL of membrane preparation at 30 °C for 10 min. The reaction was terminated by addition of 2 mL of 0.5 M NaOH containing 0.5 M $NaBH_4$. α-Cellulose (20 mg) was added as a carrier and the mixture was boiled for 15 min and filtered through a glass filter (Whatman GF/A) membrane, followed by six 4-mL washes with water, and one with 4 mL of MeOH. The radioactivity remaining on the membrane was measured by liquid scintillation counting (Packard 1600TR, Packard Instrument Co. Meriden, CT) using a toluene cocktail and was used to calculate the cellulose-synthesizing activity.

Pulsing and chasing of cells with D-*[^{14}C]glucose*.—The washed cell paste (200 mg) was pulsed for 1 h by incubating in 2 mL of 0.05 M sodium phosphate buffer (pH 6.0) containing 2.1 μM of D-[U-^{14}C]glucose (1 μCi), and 2 mM of KOAc at 30 °C [14]. At the end of the pulse, the cells were washed four times with 10 mL each of 0.05 M cold phosphate buffer by centrifuging each wash at 10,000×*g* for 10 min. Half of the centrifuged cells were removed and chased by incubating them for 1 h in the above buffer containing 100 mM unlabeled D-glucose. At the end of the chase, the cells were centrifuged and washed twice with 10 mL of water.

*Pulsing and chasing of membrane preparation by UDP-*D-*[^{14}C]glucose*.—The membrane preparation (3 mL), obtained from 200 mg of cell paste, was suspended in 6 mL of pH 8.6 buffer (70 mM Tris-HCl, 9 mM $MgCl_2$, 0.9 mM EDTA) and pulsed with 0.3 μM UDP-[^{14}C]Glc (0.2 μCi) for 10 min at 30 °C. The reaction digest was centrifuged at 30,000×*g* for 10 min and the pellet was washed four times with 10 mL each of cold 0.5 mM Tris-HCl buffer (pH 7.5) by centrifuging each wash at 30,000×*g* for 10 min. The radioactivity in the last wash was reduced to background. The pulsed enzyme was divided into three equal parts. To 3.0 mL of the pulsed enzyme, 0.5 mL of *activator preparation* (containing cellulose synthase activator, c-di-GMP) and unlabeled UDPGlc to give 10 μM were added. Chase was allowed to go 5 min in one part and 10 min in a second part. After chasing, the reaction was centrifuged and the resulting pellet was washed twice with 10 mL of 0.5 mM Tris-HCl buffer (pH 7.5).

Extraction and separation of glucan.—To release the ^{14}C label from the cells and membrane preparation, the pH of the mixtures was adjusted to 2.0 by addition of HCl and the suspensions were heated at 100 °C for 20 min. The cells and the membrane preparation were centrifuged and the supernatants were chromatographed on a column (1×50 cm) of Bio-Gel P4 and eluted with water. The fractions were monitored by liquid scintillation counting and the fractions containing labeled glucan were pooled and concentrated to 1 mL by rotoevaporation.

Characterization of reaction product.—Methylation analysis of the synthesized glucan was carried out according to the procedure of Hakomori [15] as modified by Mukerjea et al. [16]. The isolated glucans were dissolved in Me_2SO and mixed with Hakomori reagent followed by addition of MeI. Methylated samples were extracted with $CHCl_3$ and washed with water, and the $CHCl_3$ phase was evaporated to dryness. The samples were hydrolyzed with 4 M CF_3CO_2H at 121 °C for 2 h and analyzed by TLC with methylated D-glucose standards.

The reaction products were treated with *T. viride* cellulase. After reaction, the digests were filtered through Whatman GF/A glass membrane with α-cellulose as a carrier. The amount of radioactivity remaining on the membrane was measured by liquid scintillation counting, using a toluene cocktail.

Reduction and acid hydrolysis of labeled glucans.—The pulsed and chased glucans, that were released from the cells and the membrane preparation and purified by gel permeation chromatography on Bio-Gel P4, were reduced with $NaBH_4$ (10 mg/mL) by heating for 15 min at 100 °C. The glucans were then hydrolyzed with 4 M CF_3CO_2H in sealed vials for 2 h at 121 °C. The hydrolyzates were dried by rotary evaporation and borate was removed by evaporation with MeOH (3×4 mL). The samples were dissolved in 250 μL of water and 50-μL aliquots were loaded onto Whatman 3MM paper (20×50 cm) for descending chromatography for 16 h with 8:1:1:1 (v/v/v/v) $MeNO_2$–AcOH–EtOH–water saturated with boric acid at 25 °C

[17]. Labeled D-glucose and labeled D-glucitol, located with standards, were cut (2×2 cm) from the paper, and the radioactivity was determined by liquid scintillation counting using a toluene cocktail.

3. Results

A. xylinum was grown in 1 L of medium to produce cellulose pellicles at the liquid–air interface of the culture. *A. xylinum* cells were released from the cellulose pellicle to give 1.4 g of cellulose-free cell paste as described by Aloni et al. [12]. A membrane preparation was obtained by sonication of the cells, followed by centrifugation. The membrane preparation incorporated D-[^{14}C]glucose from UDP-[^{14}C]Glc into glucan at 30 °C over at least a 2-h period (see Fig. 1). Unreacted labeled substrates were removed by centrifugation at 30,000×*g* for 10 min followed by washing four times with 10 mL of Tris-HCl buffer. The labeled glucan was tightly bound to both cells and membrane preparation, but could be released by heating at 100 °C and pH 2 for 20 min. Significant amounts (70–80%) of ^{14}C were released into the aqueous phase. This mild acid treatment will hydrolyze glycosyl phosphate linkages from lipid pyrophosphate glycosyl complexes [18,19]. The treatment, however, does not hydrolyze the β-glycosidic bond of cellobiose or the bonds of cellulose, as judged by very sensitive TLC analysis [20] (data not shown). In addition, 33% of the label from pulsed cells could be extracted by 2:1, (v/v) $CHCl_3$–MeOH, indicating a lipid glycosyl intermediate is involved in the synthesis.

Greater than 90% of the labeled glucan obtained from the cells and the membranes was hydrolyzed by cellulase to give D-glucose and cellobiose as the exclusive products, and the methylation analysis of the glucan gave over 99% 2,3,6-tri- *O*-methyl-D-glucose as the major product (see Fig. 2), showing that the glucan has a β-(1→4) linked cellulose structure.

Since D-glucitol is produced by the reduction of the hemiacetal reducing end of glucans, the average chain-length of the labeled glucan synthesized by the membrane preparation as a function of time was determined from the ratio of (glucose + glucitol)/glucitol after reduction and hydrolysis of the labeled glucan. A plot of the average chain length of the synthesized cellulose over a 15-min pulse reaction is given in Fig. 3.

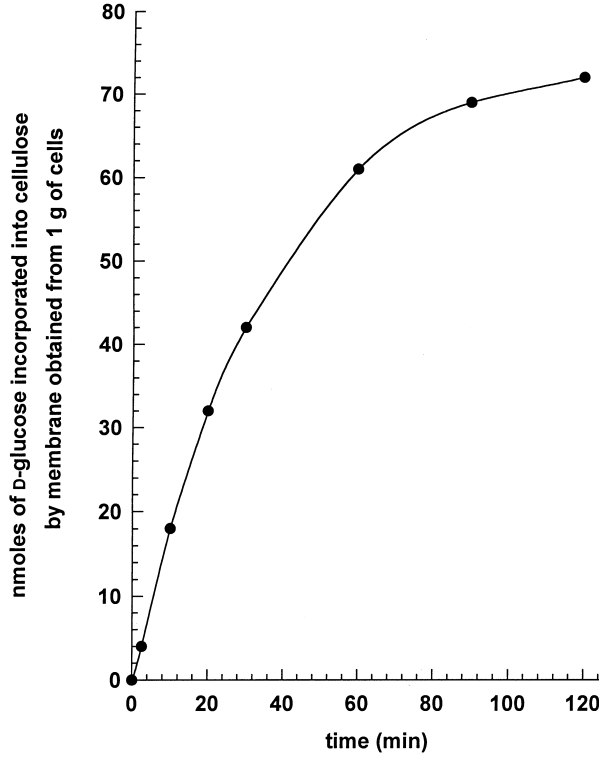

Fig. 1. Reaction time profile of the biosynthesis of cellulose by *A. xylinum* membranes. The reaction conditions were as described in Methods, except that samples were taken at various times.

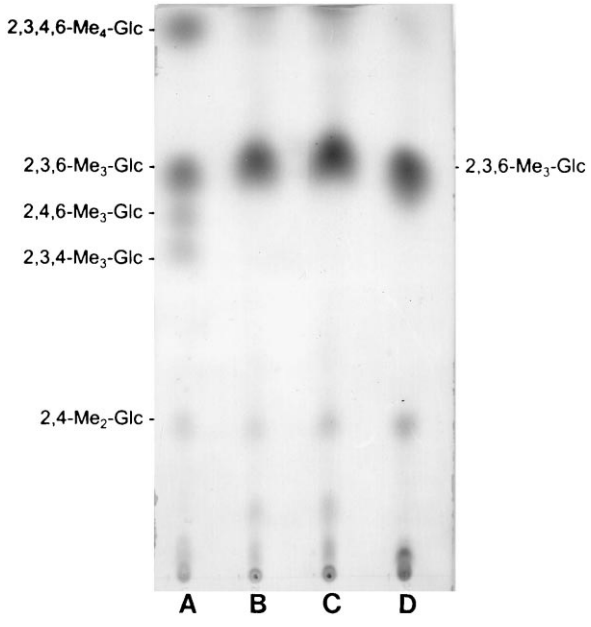

Fig. 2. TLC analysis of methylated and hydrolyzed products of *A. xylinum* synthesized glucan: A. *O*-methylated D-glucose standards; B. sigmacell cellulose; C. glucan produced by *A. xylinum* resting cells reaction with D-glucose; D. glucan produced by *A. xylinum* membranes reaction with UDPGlc.

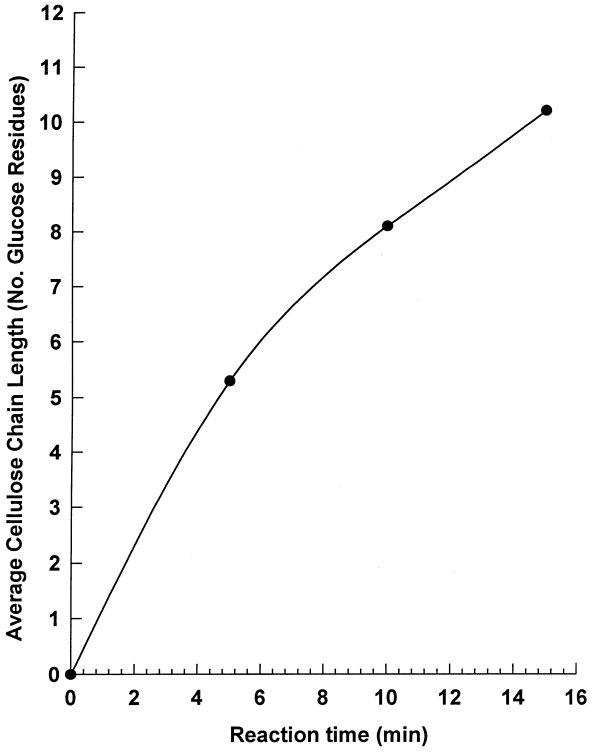

Fig. 3. The average chain-length of cellulose chains synthesized during pulse reaction of *A. xylinum* membrane with UDP-[^{14}C]Glc in 15 min. The membrane preparation was treated with UDP-[^{14}C]Glc, under pulse conditions as described in the Methods. Samples were obtained at 5, 10, and 15 min. After four cold washes, the labeled glucans were released by heating at 100 °C and pH 2.0 followed by Bio-Gel P4 gel filtration. The glucans were reduced, hydrolyzed and analyzed by paper chromatography. Average chain-lengths of glucans were determined from the ratio of (D-glucitol + D-glucose/D-glucitol).

The *A. xylinum* cells were pulsed with D-[^{14}C]-glucose, washed, and chased with nonlabeled D-glucose. The membrane preparation was pulsed with UDP-[^{14}C]Glc, washed, and chased with nonlabeled UDPGlc. [^{14}C]-Labeled products were released from both the pulsed and the pulse-chased cells and membranes by mild acid hydrolysis (pH 2, 100 °C for 20 min) as described in refs [18] and [19]. The products were separated from any low molecular weight substances by gel permeation chromatography on Bio-Gel P4.

The purified pulse and pulse-chase products, obtained from the Bio-Gel P4 column, were reduced with sodium borohydride, acid hydrolyzed, and the products separated and analyzed by descending paper chromatography. Labeled D-glucitol and D-glucose were obtained from both the pulse and pulse-chase cellulose products (Table 1). The pulsed products from the resting cells gave a ratio of D-glucitol to D-glucose of 1:11, and after chasing the ratio decreased to 1:36. The pulsed products from the membrane gave a ratio of D-glucitol to D-[^{14}C]glucose of 1:12, and after chasing for 5 min the ratio decreased to 1:43, and after 10 min, the ratio had decreased to 1:66. These results clearly show that the labeled D-glucitol obtained from the reducing end of the cellulose chain from a pulsed reaction is chased from the reducing end into the interior of the cellulose chain. The formation of D-[^{14}C]glucitol from the pulse experiments and its decrease after chasing shows that the cellulose is being synthesized by the transfer of D-glucose units to the reducing end of the growing cellulose chain.

4. Discussion

Pulsing with ^{14}C-labeled substrates and chasing with nonlabeled substrates of resting *A. xylinum* cells and membranes have shown that the reducing end of the cellulose chain is labeled in the pulse experiments and that this reducing end label is significantly decreased by chasing. These results provide direct experimental evidence that *A. xylinum* synthesizes cellulose by the addition of activated D-glucopyranosyl units to the reducing end of the growing cellulose chain. Previous studies of *Salmonella* O-antigen polysaccharide [21,22], bacterial cell wall murein [23,24], xanthan [25], and acetan [26] have shown that these β-linked polysaccharides are biosynthesized by the addition of a monomer or a repeating unit to the reducing end of the growing polysaccharide chain.

Cellulose biosynthesis by a particulate preparation from the Chlorophyta, *Protheotheca zopfi*, was shown to incorporate D-[^{14}C]glucose from UDP-[^{14}C]Glc into lipid pyrophosphate [18]. The lipid had the properties of a polyisoprenoid and was characterized as lipid pyrophosphate–glucose and lipid pyrophosphate–oligosaccharides ranging from cellobiose to cellodecaose. These could be released from the lipid pyrophosphate by mild acid hydrolysis at pH 2, 100 °C for 10 min. *Agrobacterium tumefaciens* also synthesizes cellulose and incorporates D-[^{14}C]glucose into cellulose from UDP-[^{14}C]Glc [19]. Some of the label could be extracted by 2:1, (v/v) chloroform–methanol to give a lipid fraction from which mild acid hydrolysis gave labeled D-glucose, cellobiose, cellotriose, and cellotetraose. When the [^{14}C]-labeled lipid

Table 1
Analysis of reduced and acid-hydrolyzed pulsed and chased cellulose synthesized by *A. xylinum* resting cells and membranes

Resting cells			
	D-Glucitol (cpm)	D-Glucose (cpm)	D-Glucitol/ D-Glucose
1-h pulse	1799	20,544	1:11
1-h chase	476	16,926	1:36
Membrane preparation			
	D-Glucitol (cpm)	D-Glucose (cpm)	D-Glucitol/ D-Glucose
10-min pulse	239	2906	1:12
5-min chase	46	1996	1:43
10-min chase	28	1840	1:66

fraction was added back to membrane preparations, the labeled carbohydrates were incorporated into cellulose. Evidence for the involvement of lipid pyrophosphate in the biosynthesis of cellulose by *A. xylinum* also has been found [14,27–29]. In the present study, we were also able to extract 33% of the ^{14}C pulsed-label with 2:1 (v/v) chloroform–methanol, indicating that lipid intermediates are involved. It is not surprising that only 33% of the pulsed label could be extracted with chloroform–methanol. Quantitative extraction of the lipid pyrophosphate carbohydrate intermediates are plagued with difficulties when the carbohydrate chain-lengths become greater than about 8 to 10 monomer residues because of the insolubility of the carbohydrate component in the lipid solvent. With lipid pyrophosphate–cellulose intermediates, this problem is further compounded due to the intermolecular association of the cellulose chains, making them insoluble in both lipid and aqueous solvents. Significant amounts (70–80%) of the pulse and the pulse-chase labeled material were released from both the resting cells and the membrane preparation by a mild acid hydrolysis at pH 2, 100 °C for 20 min, indicating that the labeled cellulose chains were attached to the lipid by a high-energy, pyrophosphate linkage. We, therefore, propose a mechanism (Fig. 4) for the elongation of the cellulose chain that involves lipid pryophosphate glycosyl intermediates and a so-called insertion reaction in which glucose from a lipid pyrophosphate intermediate is added to the reducing end of a growing cellulose chain also attached to a lipid pyrophosphate.

The mechanism proposed in Fig. 4 also involves three enzyme-catalyzed reactions. The first reaction is catalyzed by *lipid pyrophosphate:UDPGlc phosphotransferase (LP:UDPGlc-PT)*. In this reaction the phosphate group of a lipid monophosphate attacks the phosphate attached to the glucose in UDPGlc to give UMP and the lipid pyrophosphate–α-D-glucose (reaction 1 in Fig. 4A and B). The D-glucose residue attached to the phosphate is linked α and is retained from the original configuration of UDPGlc in that it is the pyrophosphate linkage of UDPGlc that is cleaved and not the α-glucopyranosyl phosphate linkage.

The second reaction is catalyzed by *cellulose synthase (CS)* and causes the polymerization of the glucose residues and their incorporation into cellulose. In this reaction, the 4-hydroxyl group of D-glucose of one of the lipid pyrophosphate glucopyranosyl units attacks C-1 of a D-glucose residue of another lipid pyrophosphate glucopyranosyl unit. This reaction gives the inversion of the configuration of the transferred glucose residue and the formation of a disaccharide with a β-(1→4) linkage (reaction 2 of Fig. 4B).

The third reaction is catalyzed by *lipid pyrophosphate phosphohydrolase (LPP)* and gives the hydrolysis of the phosphate from the lipid pyrophosphate that is formed in reaction 2 (reaction 3 in Fig. 4B). The phosphate of the lipid monophosphate then attacks another UDPGlc to give another lipid pyrophosphate glucopyranosyl unit (reaction 4 of Fig. 4B). The C-4 hydroxyl group of this glucose residue than attacks C-1 of the disaccharide (cellobiose) that is attached to the lipid pyrophosphate, giving the formation of a trisaccharide (cellotriose) with β-(1→4) linkages (reaction 5 in Fig. 4B). The synthesis of cellulose then continues in this fashion, using the three enzyme-catalyzed reactions, to add D-glucose residues to the reducing end of the growing cellulose chain, which is attached at the reducing end to a lipid pyrophosphate that is anchored in the cell membrane. The nonreducing end of the cellulose chain is thus extruded from the membrane away from the cell surface into the medium by the insertion mechanism. Cellulose synthase has been shown to be an integral membrane enzyme [8] and it is proposed that the three enzymes are in or near the lipid bilayer membrane. The three enzymes may be a part of a cellulose-synthesizing complex that is embedded in the membrane.

At first, it might be thought that lipid intermediates are not necessarily required and that the glucosyl unit and the growing cellulose chain could

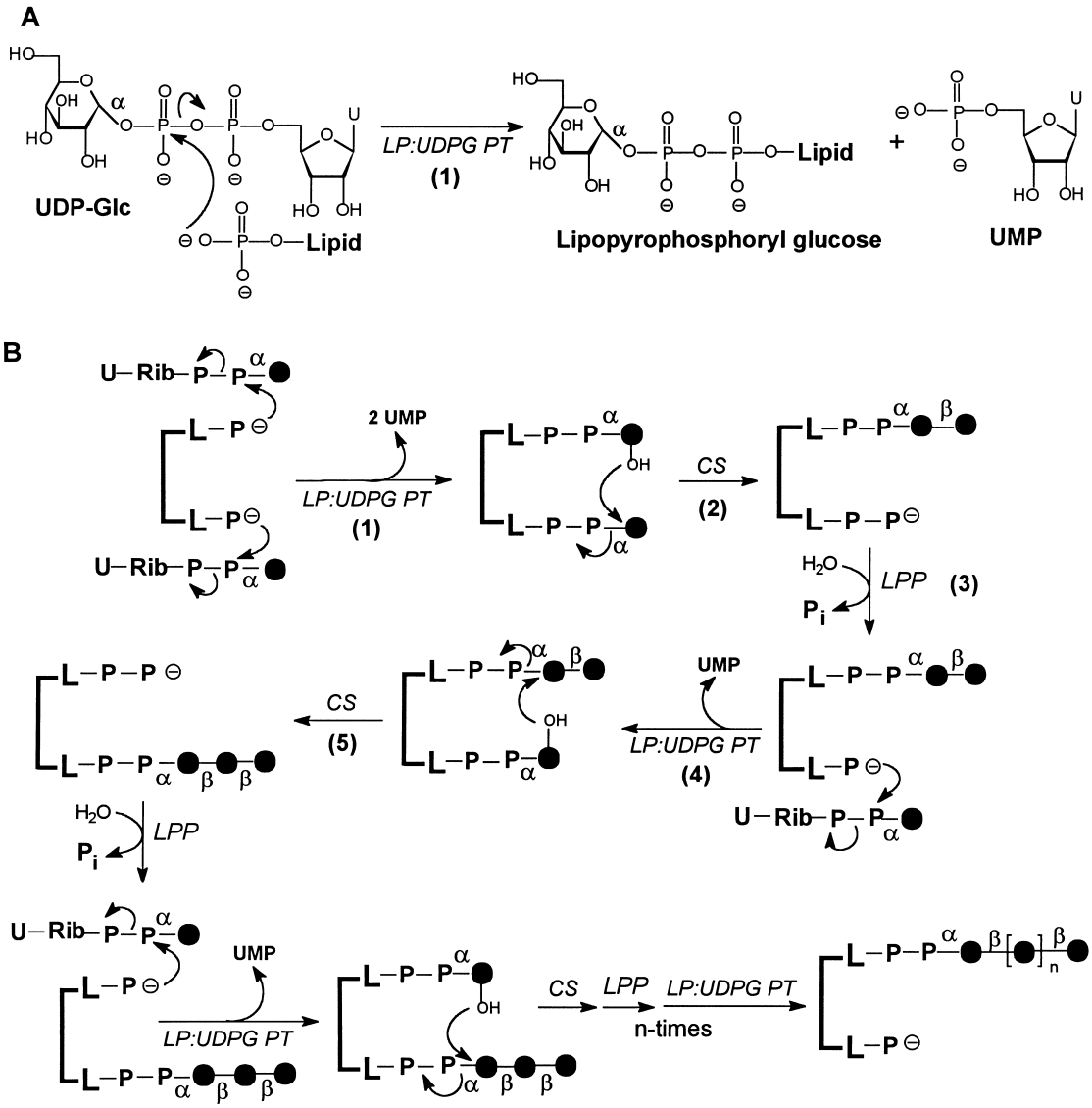

Fig. 4. Proposed mechanism for cellulose biosynthesis in a membrane involving three enzymes: A. reaction of UDPGlc with lipid monophosphate; B. biosynthesis of cellulose chain by reaction of lipopyrophosphoryl–glucose with a lipopyrophosphoryl glucosyl unit to give the formation of β-(1→4) linkage. (L = lipid, P = phosphoryl, Rib = D-ribose, U = uridine, ● = glucosyl residue, *LP:UDPG PT* = lipid phosphate:UDPGlc phosphotransferase, *CS* = cellulose synthase, and *LPP* = lipid pyrophosphate phosphohydrolase).

be directly attached to the cellulose synthase protein. The direct reaction of cellulose synthase with UDPGlc, however, would give inversion of the configuration of the glucose residue and the formation of a β-linked glucosyl–protein intermediate. The subsequent reaction of this glucosyl intermediate to give the incorporation of glucose into cellulose would also give inversion of the configuration and the formation of an α-linked glucan with a starch- or dextran-type structure. This type of reaction with the covalent attachment of glucose and polysaccharide to the enzyme has been shown for the biosynthesis of dextrans by dextransucrases [30–32]. In contrast, the involvement of lipid pyrophosphate intermediates would have the glucose residues attached by an α-configuration. On the nucleophilic addition of the glucose to the reducing end of a growing cellulose chain, also attached to a lipid pyrophosphate by an α-configuration, a β-(1→4) glycosidic linkage could be formed.

The proposed reducing-end, insertion mechanism has no need for a preformed oligosaccharide or polysaccharide primer [30] and is thus consistent with the finding that cellulose does not require the addition of an exogenous primer for its biosynthesis [33]. The requirements for polyisoprenyl phosphate and the three distinct enzymes for the biosynthesis of cellulose explains why the purification of a

single enzyme, synthesizing cellulose has met with failure.

Thus, the proposed mechanism for cellulose biosynthesis fulfills the requirements of (*a*) a lipid pyrophosphate intermediate, (*b*) membrane bound enzymes, (*c*) the addition of D-glucose to the reducing end of the cellulose chain, and (*d*) the inversion of the configuration of D-glucose in UDPGlc to give β-(1→4) glycosidic linkages. The pulse/chase results give direct experimental evidence for the speculations drawn from the comparative study of the structure and sequence analysis of cellulose synthase made by Saxena et al. [9]. It, however, does not support the conclusion drawn by Koyama et al. [10] that cellulose is elongated from the non-reducing end, which was obtained by silver staining of the putative reducing ends of cellulose made on a 5 to 7 day post-stationary phase cellulose-cell preparation.

The formation of cellulose by resting cells from glucose probably goes through the traditional enzymatic processes of reaction with glucokinase and ATP to give Glc-6-P, conversion into α-Glc-1-P by phosphoglucomutase, and then incorporation into UDPGlc by the reaction of UTP and pyrophosphorylase. The resulting UDPGlc then enters into the formation of cellulose by the above described synthetic pathway.

Cellulose biosynthesis by *A. xylinum* joins a growing list of polysaccharides (*Salmonella* capsular O-antigen polysaccharide [21,22], bacterial cell-wall peptidomurein [34], *Xanthanomonas campestris* xanthan [35], *Leuconostoc mesenteroides* B-512F dextran [30,31], *Streptococcus mutans* 6715 alternating comb dextran and mutan [30,32], that are biosynthesized by the addition of monosaccharide or repeating units to the reducing ends of their growing chains by the so-called insertion mechanism. *A. xylinum* cellulose biosynthesis also joins several polysaccharides (*Prot. zopfi* cellulose [18], *A. tumefaciens* cellulose [19], *Salmonella* O-antigen polysaccharide [21,22], bacterial cell wall peptidomurein [23,24] and *X. campestris* xanthan [25]) that are synthesized using polyisoprenyl pyrophosphate intermediates by enzymes embedded in a lipid bilayer membrane.

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