Expression of the *Streptococcus pneumoniae* Type 3 Synthase in *Escherichia coli*

ASSEMBLY OF TYPE 3 POLYSACCHARIDE ON A LIPID PRIMER*

Robert T. Cartee, W. Thomas Forsee, John W. Jensen, and Janet Yother†

From the Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294

Received for publication, July 11, 2001, and in revised form, October 4, 2001
Published, JBC Papers in Press, October 29, 2001, DOI 10.1074/jbc.M106481200

Synthesis of the type 3 capsular polysaccharide of *Streptococcus pneumoniae* is catalyzed by the membrane-localized type 3 synthase, which utilizes UDP-Glc and UDP-GlcUA to form high molecular mass \(3\beta-D-GlcUA-(1\rightarrow4)\beta-D-Glc-(1\rightarrow)n\). Expression of the synthase in *Escherichia coli* resulted in synthesis of a 40-kDa protein that was reactive with antibody directed against the C terminus of the synthase and was the same size as the native enzyme. Membranes isolated from *E. coli* contained active synthase, as demonstrated by the ability to incorporate Glc and GlcUA into a high molecular mass polymer that could be degraded by type 3 polysaccharide-specific depolymerase. As in *S. pneumoniae*, the membrane-bound synthase from *E. coli* catalyzed a rapid release of enzyme-bound polysaccharide when incubated with either UDP-Glc or UDP-GlcUA alone. The recombinant enzyme expressed in *E. coli* was capable of releasing all of the polysaccharide from the enzyme, although the chains remained associated with the membrane. The recombinant enzyme was also able to reinitiate polysaccharide synthesis following polymer release by utilizing a lipid primer present in the membranes. At low concentrations of UDP-Glc and UDP-GlcUA (1 \(\mu\)M in the presence of Mg\(^{2+}\) and 0.2 \(\mu\)M in Mn\(^{2+}\)), novel glycolipids composed of repeating disaccharides with linkages consistent with type 3 polysaccharide were synthesized. As the concentration of the UDP-sugars was increased, there was a marked transition from glycolipid to polymer formation. At UDP-sugar concentrations of either 5 \(\mu\)M (with Mg\(^{2+}\)) or 1.5 \(\mu\)M (with Mn\(^{2+}\)), 80% of the incorporated sugar was in polymer form, and the size of the polymer increased dramatically as the concentration of UDP-sugars was increased. These results suggest a cooperative interaction between the UDP-precursor-binding site(s) and the nascent polysaccharide-binding site, resulting in a non-processive addition of sugars at the lower UDP-sugar concentrations and a processive reaction as the substrate concentrations increase.

Type 3 polysaccharide, which is composed of the repeating subunit \(3\beta-D-GlcUA-(1\rightarrow4)\beta-D-Glc-(1\rightarrow)n\), is one of 90 different capsule types identified in the human pathogen *Streptococcus pneumoniae* (1–3). Synthesis of type 3 polysaccharide requires only a single glycosyltransferase, which utilizes UDP-Glc and UDP-GlcUA to form both glycosidic linkages of the repeating disaccharide (4–6). The type 3 synthase shares significant homology with a number of processive \(\beta\)-glycosyltransferases, including the hyaluronan synthases from prokaryotes and eukaryotes, the cellulose synthases from plants and bacteria, the chitin synthases from yeast, and the Nod factor synthases from *Rhizobium* (5, 7) It has recently been classified in family 2 of the glycosyltransferases (8). Elongation of type 3 chains in pneumococcal membranes has been demonstrated to occur at the nonreducing end of the polysaccharide (9), consistent with a previously suggested mechanism of growth for members of the processive \(\beta\)-glycosyltransferase family that involves the dual addition of sugars to the growing polymer (10, 11). An improved understanding of the three-dimensional structure of inverting glycosyltransferases has generated a more recent proposal that most family transferases utilize conserved structural domains and four conserved aspartates to form a single center where both the binding of the UDP-sugars and the glycosyl transfer reaction would take place (12). It was further speculated that a single active-site transferase could catalyze the formation of an alternating polysaccharide if the addition of each sugar served to fine-tune the affinity of the acceptor site for the succeeding nucleotide-sugar.

In a recent study of the polysaccharide release reaction mediated by the type 3 synthase in isolated *S. pneumoniae* membranes, the presence of either UDP-Glc or UDP-GlcUA was shown to dramatically affect the binding affinity of the enzyme for the conjugate UDP-sugar (13). In addition, polymer synthesis is significantly impaired following UDP-sugar-mediated release of the polysaccharide from the synthase in *S. pneumoniae* membranes, suggesting that some additional factor may be necessary for reinitiation of polymer synthesis (13). To further explore the chain initiation process as well as possible interactions between the nucleotide-sugar- and carbohydrate-binding sites, we have characterized the biosynthetic and polysaccharide release reactions of the recombinant type 3 synthase contained in *Escherichia coli* membranes.

EXPERIMENTAL PROCEDURES

Materials—UDP-[\(^{14}\)C]Glc (257 mCi/mmol) was obtained from Anotok; UDP-[\(^{3}H\)]GlcUA (338 mCi/mmol) was from ICN; and UDP-[\(^{1}H\)]Glc (1 Ci/mmol) from Amersham Biosciences, Inc. Econo-Safe scintillation mixture was from Research Products International Corp. Rabbit polyclonal antiserum specific for the C-terminal 14 amino acids of the type 3 synthase linked to keyhole limpet hemocyanin was obtained through Research Genetics, Inc. (Huntsville, AL). Biotin-conjugated goat anti-rabbit IgG and streptavidin-conjugated alkaline phosphatase were from Southern Biotechnology Associates, Inc. (Birmingham, AL). Mutanolysin, Sephacryl S-500HR, UDP-Glc, and UDP-GlcUA were obtained from Sigma. Nonidet P-40 was from Calbiochem.
chem, and Todd Hewitt broth, yeast extract, and Tryptone were from Difco. Bee venom phospholipase A$_2$ (1360 units/mg of protein), Bacillus cereus phospholipase C (1472 units/mg of protein), Clostridium perfringens phospholipase C (66 units/mg of protein), peanut phospholipase D (700 units/mg of protein), and Streptomyces chromofuscus phospholipase D (1000 units/mg of solid) were obtained from Sigma. The membrane layer plates and No. 3MM chromatography paper were from Whatman.

**Analytical Methods**—Chromatography on Sephacryl S-500 and S-300 was carried out on 1.4 × 57-cm columns eluted with a solution consisting of 0.1% Nonidet P-40, 0.02% sodium azide, either 200 mM ammonium acetate (pH 6.5) or 5 mM Tris (pH 7.5), and 200 mM NaCl as previously described (13). Preparation of type 3 polysaccharide-specific depolymerase and digestion with the depolymerase were carried out as described (13). The assay for polysaccharide release with E. coli membranes was as previously described for S. pneumoniae membranes, except that 100 mM imidazole (pH 7.5) and 10 mM MgCl$_2$, respectively (13). Protein concentrations were assayed by fluorosceamine (14) as previously described (15).

**Preparation of Glycolipid Products**—Glycolipids were synthesized in reaction mixtures containing 0.3 μCi of labeled nucleotide-sugar, other nucleotide-sugars as indicated, 100 mM Hepes (pH 7.5), 10 mM MgCl$_2$, and E. coli membranes (2 mg of protein) in a total volume of 0.6 ml. Following a 15-min incubation at 35 °C, the reaction was terminated by the addition of 2 ml of an ice-cold solution containing 100 mM Hepes (pH 7.5) and 10% glycerol, and the membranes were sedimented by centrifugation at 100,000 × g for 30 min. The membranes were washed two more times with 2.5 ml of wash solution as described above. The membranes were suspended in 0.2 ml of 0.1% NaCl and heated at 100 °C for 5 min. The labeled glycolipids were extracted with 0.25 ml of chloroform-methanol (2:1) containing 0.02% NaCl, 0.001% Nonidet P-40, and 2 mM EDTA (pH 7.5). The insoluble material was sedimented by centrifugation at 13,000 × g for 5 min; the supernatants were saved; and the pellets were extracted a second time. The supernatants containing the glycolipid fraction were combined for further analyses.

**Saponification, Acid Hydrolysis, and Enzymatic Digestions of Glycolipid Products**—Saponification was carried out at 35 °C for 20 min in 0.4 ml of 80% methanol containing 0.1 N NaOH. The mixture was neutralized with acetic acid, and the methanol was removed with a stream of nitrogen. Acid hydrolysis was carried out in 1.0 N HCl at 100 °C for the indicated times. The hydrolysates were neutralized with NaOH, and samples were analyzed by Sephacryl S-300 and paper chromatography. Phospholipase digestions were conducted in reaction mixtures containing 0.5 mg of protein and either 200 mM ammonium acetate (pH 6.5) or 5 mM Tris (pH 7.5) containing 0.1% Nonidet P-40, 0.02% sodium azide, either 200 mM ammonium acetate (pH 6.5) or 5 mM Tris (pH 7.5), and 200 mM NaCl as described (13). The assay for polysaccharide release with E. coli membranes was as previously described for S. pneumoniae membranes, except that 100 mM imidazole (pH 7.5) and 10 mM MgCl$_2$, respectively (13). Protein concentrations were assayed by fluorosceamine (14) as previously described (15).

**Type 3 Synthase Expression in E. coli**—We previously reported that overexpression of the type 3 synthase in E. coli results in death of the cells, with no detectable accumulation of protein, as determined by Coomassie Blue and silver staining (5). Arrecubieta et al. (6) observed similar toxic effects, but were able to observe low levels of recombinant protein. Using a rabbit polyclonal antiserum specific for the C-terminal 14 residues of the synthase, we have confirmed expression of the protein in our original strains and shown that it is of the same apparent molecular size as that expressed in S. pneumoniae (Fig. 1). The apparent molecular size (40 kDa) was smaller than the predicted size (48 kDa), consistent with observations for the streptococcal hyaluronan synthase (20). It was in contrast, however, to the apparent molecular size of 49 kDa reported by Arrecubieta et al. (6) for the type 3 synthase. In our system, cps3S expression was leaky, resulting in a constant low level production of the synthase that was increased only modestly by induction with isopropl-$\beta$-thiogalactopyranoside (Fig. 1). The presence of the protein was detectable only with the synthase-specific antiserum or using the assays described below.

**Type 3 synthase activity was determined by the incorporation of $^{14}$C from either UDP-$^{14}$C-glucose or UDP-$^{14}$C-glucuronic acid into a high molecular mass product. Membranes from the recombinant strain (JD424) actively synthesized polysaccharide when incubated with high concentrations (100 μCi/ml) of UDP-sugars, with the addition of 2 mg of membrane protein. Low levels of $^{3}$H occurred with membranes from the control strain containing the vector only (JD422). The activity of the recombinant enzyme was dependent on the presence of both UDP-glucose and UDP-glucuronic acid, was linear for 30 min, and was proportional to protein concentration. Activity was observed in the presence of both Mn$^{2+}$ and Mg$^{2+}$ and was optimal at pH 7.0—8.0, which
FIG. 1. Western immunoblot analysis of *S. pneumoniae* and *E. coli* expressing Cps3S. *E. coli* strains JD422 (vector control) and JD424 (Cps3S+) were grown to a cell density of 5 x 10^8 cfu/ml and induced for 2 h with isopropyl-β-D-thiogalactopyranoside. Samples of JD422 (1.2 x 10^8 cfu; V), uninduced JD424 (1.2 x 10^8 cfu; U), and induced JD424 (0.6 x 10^8 cfu; I) were separated along with membrane preparations (30 μg of total protein) of *S. pneumoniae* (S. pn) type 3 strain WU2 and JD908 (S-) on a 10% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose, and Western immunoblotting was performed as previously described (35) utilizing rabbit polyclonal antiserum directed to the C-terminal 14 amino acids of the type 3 synthase. The arrowhead indicates the position of Cps3S.

Polysaccharide Association with the Enzyme-Membrane Complex—To assess whether polysaccharide remained associated with the *E. coli* synthase-membrane complex during the course of the biosynthetic reaction, samples were taken from reaction mixtures and sedimented by centrifugation to separate released polysaccharide from membrane-associated polysaccharide. Virtually all of the polysaccharide product remained bound to the membranes even though most of the UDP-Glc was depleted by 30 min (data not shown). These results suggested that the polysaccharide was not released from the enzyme-membrane complex, which was in sharp contrast to the results observed with membranes from *S. pneumoniae* (9, 13). When polysaccharide release was assayed directly by incubating *E. coli* membranes in the presence of a single UDP-sugar, no significant release of polysaccharide from the membranes was observed (data not shown). Furthermore, there was no significant inhibition of polymer synthesis using *E. coli* membranes that had been pretreated with either UDP-Glc or UDP-GlcUA, which again was in contrast to the results observed in *S. pneumoniae* (13).

To explore the possibility that polymer was being released from the enzyme but not from the membrane, we examined polysaccharide synthesis in a dual isotope experiment. First, [14C]GlcUA-labeled polysaccharide-synthase-membrane complex was prepared (as described under “Experimental Procedures”) in a brief (30 s) reaction (Fig. 2A). Then, the labeled membranes were incubated with either UDP-Glc (Fig. 2B) or UDP-GlcUA (Fig. 2C) prior to a second round of polysaccharide synthesis in a reaction mixture containing UDP-[3H]Glc and UDP-GlcUA. In each instance, the formation of a high molecular mass 3H-labeled product was observed, but none of the 14C-labeled material was elongated. In contrast, when the membranes were preincubated in the absence of either UDP-precursor, approximately half of the 14C-labeled product was extended into a higher molecular mass form that coeluted with the 3H-labeled polysaccharide (Fig. 2D). Both the 14C- and 3H-labeled high molecular mass products were degraded by the type 3-specific depolymerase, indicating that both labels were incorporated into type 3 polysaccharide. These data indicate that incubation with a single substrate will actuate release of all the 14C-labeled polysaccharide from the enzyme and that the synthase is capable of reinitiating synthesis. These results contrast with those obtained in *S. pneumoniae*, where ~50% of the chains were released, and reinitiation did not occur (13).

Further experiments were carried out at low protein/substrate ratios to minimize changes in the substrate concentration during the course of the reaction. In a reaction containing 5 μM UDP-precursors and MgCl2, two products were present following a 30-min incubation (Fig. 3). Over 80% of the larger product was chased into a higher molecular mass polysaccharide by continuing the incubation with 200 μM concentrations of the UDP-sugars. These data indicated that <20% of the polysaccharide chains had been released from the synthase during the initial 30-min incubation. In contrast, a low molecular mass product, which was the primary product at low UDP-sugar precursor concentrations (2 μM or less), was further characterized.

Identification of the Low Molecular Mass Product as a Glycolipid—At low UDP-sugar concentrations, membranes from the synthase-containing strain (JD424) and from the vector control strain (JD422) incorporated radioactivity into products with very different properties. Membranes from JD424 incorporated Glc into a product that remained at the origin and another that migrated ~75% of the distance of the Glc standard (Fig. 4A). When analyzed by Sephacryl S-500, the origin product appeared to be a high molecular mass polymer, and the second product corresponded in size to a small oligosaccharide (data not shown). The JD422 membranes were unable to utilize UDP-GlcUA as a substrate, and the presence of UDP-GlcUA did not stimulate the formation of any additional Glc-labeled products (Fig. 4A). When incubated with low concentrations of UDP-sugars, membranes from the synthase-containing JD424 strain were greatly reduced in the above activities and instead incorporated isotope into products with different chromatographic properties. The GlcUA-labeled products (synthesized in reactions containing only UDP-GlcUA) migrated more rapidly than a monosaccharide when analyzed by paper chromatography (Fig. 4B). When incubated with UDP-Glc alone, JD424 membranes incorporated low levels of Glc into compounds with a chromatographic pattern similar to that of the GlcUA-labeled products. The presence of unlabeled UDP-GlcUA in the reaction mixture stimulated the incorporation of Glc 10-fold, suggesting copolymerization of these two sugars. Following saponification with 0.1 N NaOH for 15 min at 37°C (Fig. 5A) or acid hydrolysis for 7 min in 1 N HCl at 100°C (data not shown), both the GlcUA- and Glc-labeled products (synthesized in the presence of UDP-GlcUA) migrated more slowly than a monosaccharide when separated by paper chromatography and yielded a pattern that was suggestive of a series of oligosaccharides. Ninety percent of the products were liberated by acid hydrolysis for 7 min in 1 N HCl at 100°C, and 100% were liberated in 10 min. Fifty percent were liberated in 30 min in 0.1 N HCl at 100°C.

When separated by gel filtration on Sephacryl S-300, both the GlcUA- and Glc-labeled products eluted at a position sim-
ilar to that of Nonidet P-40 micelles (Fig. 5B). After saponification (Fig. 5B) or acid hydrolysis (data not shown), they eluted at the same position as small oligosaccharides. These properties are indicative of glycolipids, which might be expected to be soluble in organic solvents. Table I shows the distribution of the products when partitioned with chloroform, methanol, and water, similar to the procedure of Bligh and Dyer (21). At a neutral pH, 77% of the GlcUA-labeled products and 89% of the Glc-labeled products were found in the aqueous phase; but in an acidic solvent, 76 and 72% of these respective products partitioned at the interphase or in the organic phase. All of these properties are consistent with those expected for anionic glycolipids containing oligosaccharides of moderate length.

The glycolipid products were unaffected by heating with 50% phenol at 70°C for 2.5 h, which clearly distinguished them from undecaprenyl diphosphate and monophosphate sugars, which, under this condition, have respective half-lives of 4–10 and 60 min (22). The products were completely hydrolyzed by phospholipase D from S. chromofuscus as assessed by paper chromatography using a solvent of butanol/acetic acid/water (44:16:40). Most of the digested Glc-labeled product remained at the origin, suggesting a composition of somewhat larger oligomers than the GlcUA-labeled product (Fig. 6). The glycolipid products were unaffected by digestion with peanut phospholipase D or with phospholipase C from either B. cereus or C. perfringens. Phospholipase A2 from bee venom partially hydrolyzed the products, but only when added at 1000-fold over the level of S. chromofuscus phospholipase D, suggesting that a contaminating phospholipase may have been responsible for the partial liberation of the saccharide moiety.

The GlcUA-labeled product (labeled as described above in the absence of UDP-Glc) was resolved by thin-layer chromatography into approximately five bands, none of which were sensitive to digestion with the type 3-specific depolymerase. The Glc-labeled product was resolved by thin-layer chromatography into approximately eight bands and a component that remained at the origin. The origin material and the slowest migrating band were completely degraded by digestion with...
Formation of Polymer on a Lipid Primer—Because there were no oligomeric products smaller in size than that corresponding to detergent micelles, the data suggested that polysaccharide synthesis was initiated on a micellar component, the data suggested that growth was occurring by sugar addition at the nonreducing end. Approximately 15–25% of the Glc was released by β-glycosidase digestion of the Glc-labeled product, suggesting that a number of internal glucose residues are present in glycolipid products formed in the presence of both UDP-sugars.

The formation of polymer on a lipid primer was clearly established in scaled-up reactions containing 3 μM concentrations of both UDP-sugars. Only glycolipid was synthesized during the first 5 s of the reaction. It eluted at the position of a micelle on Sephacryl S-300 and, following saponification, appeared to have the size of a small oligosaccharide (Fig. 8A). By the incorporation of GlcUA into glycolipid during the first minute of the reaction (compare with UDP-GlcUA alone); however, this stimulation diminished at longer reaction times, presumably as more synthase was engaged in polymer synthesis. The 3 μM concentrations of the UDP-sugars present in the reaction are significantly below their K_M values and would be insufficient to engage all of the synthase in polymer synthesis.

The formation of polymer on a lipid primer was clearly established in scaled-up reactions containing 3 μM concentrations of both UDP-precursors. Only glycolipid was synthesized during the first 5 s of the reaction. It eluted at the position of a micelle on Sephacryl S-300 and, following saponification, appeared to have the size of a small oligosaccharide (Fig. 8A). By
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20 s, the intact product still consisted of a single micellar fraction; however, following saponification, a small quantity of intermediate-size polysaccharides was present in addition to the small oligosaccharides (Fig. 8B). By 60 s, some of the product had increased sufficiently in size so that it eluted from the glycolipid micellar component, and saponification resulted in a concomitant increase in the formation of polysaccharide-chains of a size comparable to those synthesized in MgCl₂. The transition from formation of primarily glycolipid to polymer occurred at UDP-sugar concentrations of 2 μM in MgCl₂ and 0.5 μM in MnCl₂ (Fig. 10). The 4-fold difference corresponds very closely with the relative Kₘ values of the type 3 synthase for the UDP-sugars in Mg²⁺ and Mn²⁺.

D I S C U S S I O N

S. pneumoniae membranes were previously shown to assemble type 3 polysaccharide by the addition of Glc and GlcUA to the nonreducing termini of pre-existing polysaccharide primers (9). When the concentration of either UDP-Glc or UDP-GlcUA drops below a critical concentration, which correlates with the apparent Kₘ values for the UDP-sugars in the biosynthetic reaction, the growing polysaccharide chain is released from the synthase by an abortive translocation process (13). Following release of the polymer from S. pneumoniae membranes, the type 3 synthase is greatly reduced in its ability to form additional polymer, suggesting that some unknown factor is involved in the initiation of polysaccharide synthesis. This investigation has shown that E. coli membranes contain a lipid that is capable of serving as a primer for type 3 synthase. The lipid nature of the primer was indicated by its 1) ready saponification, 2) co-migration with detergent micelles, 3) partitioning between aqueous and organic mixtures, 4) hydrolysis by phospholipase D, and 5) chromatographic mobility. The results are more consistent with a phosphoglycerol lipid than a polyprenol; however, the resistance of the primer to hydrolysis by phospholipase C cannot be explained.

Both type 3 polymer and glycolipid were synthesized by the synthase-containing E. coli strain JD424, and neither was synthesized by the vector control strain JD422. Formation of the glycolipid preceded the onset of polymer synthesis, and glycolipid synthesis diminished as the level of synthase engaged in polymer synthesis increased. A fraction of the glycolipid was cleaved by the type 3 polysaccharide-specific depolymerase from B. circulans, suggesting that the same repeating disaccharide sequence was present in both polymer and glycolipid. Finally, we could find no evidence of any other primer that was smaller than that corresponding to the detergent micelle elution volume when analyzed by gel filtration. All of these findings indicate that the type 3 synthase initiates polysaccharide synthesis on a lipid primer. We have recently identified a primer in S. pneumoniae with properties identical to those of the E. coli primer.

The formation of novel glycolipids due to the heterologous expression of a glycosyltransferase is not unexpected in view of several recent demonstrations that the expression of other glycosyltransferases in E. coli can give rise to novel glycolipid and phosphoglycolipid products (23, 24). One of the puzzling
features, as noted in those studies, is the ability of the glucosyltransferases to glycosylate hydrophobic acceptors embedded in the surface layer of the membrane and also hydrophilic acceptors. Of particular interest is the glucosylation of phosphatidylglycerol in recombinant *E. coli* expressing the *Staphylococcus aureus* diacylglycerol glucosyltransferase, although no such phosphoglycolipids have been found in *S. aureus* (24).

We do not yet know the extent of the modification of the endogenous lipid composition in the membranes of recombinant *E. coli* expressing the type 3 synthase; however, the composition of carbohydrate products synthesized from UDP-Glc was significantly altered. The membranes from wild-type *E. coli* synthesized an oligomeric product with properties similar to those of membrane-derived oligosaccharides, which are thought to utilize phosphatidylglycerol by an undefined pathway (25, 26). Membranes from *E. coli* containing the recombinant type 3 synthase synthesized almost none of this oligosaccharide product and also much less of a high molecular mass glucan, suggesting that these activities had been greatly reduced as a consequence of endogenous type 3 synthase activity.

**FIG. 8.** Gel filtration of products before and after saponification. Glycolipid and polysaccharide were synthesized in reactions of 5 s (A), 20 s (B), 60 s (C), and 120 s (D) containing 3 μM concentrations of both UDP-precursors as described in the legend to Fig. 7. The washed membranes were solubilized, and the products were analyzed by Sephacryl S-300 chromatography before (○) and after (○) saponification. The elution positions of Nonidet P-40 micelles (arrow 1) and Glc (arrow 2) are indicated.

**FIG. 9.** Effect of substrate concentration on polymer size. Products were synthesized in reaction mixtures containing MgCl₂ (A) or MnCl₂ (B). The reaction mixtures in A contained JID424 membranes (280 μg/ml protein), 100 mM Hepes (pH 7.5), 10 mM MgCl₂, UDP-[¹⁴C]Glc, and UDP-GlcUA, with both nucleotide-sugars present at a final concentration of 1 μM (○), 5 μM (■), 10 μM (□), or 20 μM (△). Following a 15-min incubation at 35 °C, the membranes were washed three times to remove any unincorporated nucleotide-sugars, and the products were solubilized and analyzed by chromatography on Sephacryl S-500. The reaction mixtures in B were as described for A, except that they contained 10 mM MnCl₂ and UDP-[¹⁴C]GlcUA, with both nucleotide-sugars present at a final concentration of 0.8 μM (○), 1.5 μM (□), and 4 μM (△). The elution positions of Nonidet P-40 micelles (arrow 1) and Glc (arrow 2) are indicated.

**FIG. 10.** Effect of substrate concentration on lipid-to-polymer transition. Aliquots from the reactions described in the legend to Fig. 9 containing MgCl₂ (squares) and MnCl₂ (circles) were analyzed by paper chromatography with a solvent of butanol/acetic acid/water (44:16:40). The chromatograms were cut into 2-cm strips, and the radioactivity present at the origin (open symbols) and in a component migrating more rapidly than standard Glc (closed symbols) was determined.
Possibly, the inability to express high levels of the synthase in *Escherichia coli* is a result of these alterations in lipid composition.

In contrast to *S. pneumoniae*, release of the polysaccharide from type 3 synthase in *E. coli* was not accompanied by release from the membranes. Presumably, this was due to the reten-

tion of a lipid anchor at the reducing end of the polymer. *E. coli* strains synthesize a variety of cell-surface polysaccharides, including group 2 and 3 capsular K antigens, the enterobacte-

rial common antigen, and colanic acid, which are attached to the outer membrane through a diacylglycerol phosphate moiety (27–29). Group 2 and 3 K antigens and colanic acid are exported across the inner membrane by an ABC transporter; and, at least in the case of colanic acid, the phosphatidic acid has been postulated to be the common recognition for the transporter (30). Little is known about the attachment of diacylglycerol phosphate to these polysaccharides during their biosynthesis (31).

At low concentrations of UDP-Glc and UDP-GlcUA, the type 3 synthase functioned largely in a non-processive manner, transferring only one to several sugar residues to the lipid primer. As the UDP-sugar concentrations were increased above 0.2 μM in the presence of MnCl₂ and above 1 μM in the presence of MgCl₂, the polysaccharide chains were dramatically length-

ened. Possibly, the growing nascent chain must attain a minimum length before it will become permanently engaged with the carbohydrate-binding site, thus allowing extensive proces-

sive elongation. We previously observed that heparin synthase exhibits a >100-fold decrease in *Kₘ* for the carbohydrate substrate as the size of the oligosaccharide increases from a tetra-

rasaccharide to an octasaccharide (32), suggesting that the affinity of the transferase for the growing polysaccharide increases markedly in the early stages of polymerization. A similar occurrence here would, in part, explain the difficulty in trapping oligosaccharides of intermediate size.

Type 3 synthase in *S. pneumoniae* is a bifunctional glycosyl-

transferase capable of adding Glc and GlcUA to the nonreduc-

tion terminus of the polysaccharide chain (9), which presumably remains bound to a carbohydrate-binding site specific for one to several repeating disaccharide sequences at the nonreducing end of the type 3 chain. The apparent *Kₘ* values for UDP-Glc and UDP-GlcUA in the reaction catalyzed by the synthase in *E.

coli* membranes were 26 and 20 μM in the presence of Mn²⁺ and 76 and 58 μM in the presence of Mg²⁺, respectively. The corre-

lation between these differences in these values in the presence of Mn²⁺ and Mg²⁺ and the approximate 4-fold difference in UDP-

sugar concentrations at which a glycolipid-to-polymer transition occurred in reactions in the presence of these two metal ions demonstrates the importance of the concentration of the UDP-precursors in modulating this reaction. The dramatic increase in polysaccharide chain length corresponding to a slight increase in UDP-sugar concentration is indicative of a highly cooperative binding mechanism, which allows the synthase to engage in non-processive addition of sugars at low UDP-precursor concen-

trations and processive polymer formation at higher sub-

strate levels.

When *S. pneumoniae* membranes are incubated in the com-

plete absence of the conjugate UDP-sugar, either UDP-Glc or UDP-GlcUA will actuate the release of the polysaccharide from the synthase with respective apparent *Kₘ* values of 880 and 0.004 μM (13). The presence of UDP-Glc decreases the affinity of the *S. pneumoniae* synthase for UDP-GlcUA by 3 orders of magnitude, and the presence of the latter increases the binding affinity of the former by 2 orders of magnitude. The release by either UDP-sugar is inhibited by the presence of the conjugate UDP-sugar, and all the results are consistent with the hypoth-

esis that both polymerization and release are catalyzed by interaction of the UDP-sugars with the same set of binding sites on the synthase. These data are consistent with a possible allosteric interaction between two nucleotide-binding sites. However, in view of the recent proposal that the processive glycosyltransferase family² synthases contains only a single nucleotide-binding site (12), a variety of mechanisms need to be considered for the type 3 synthase that would allow for both a processive and non-processive reaction mode. In *E. coli*, 100% of the polymer was released from the membranes, whereas only 50% release occurred with *S. pneumoniae* membranes (15). These results suggest that other factors may affect release in *S. pneumoniae* or that there are differences in the enzyme expressed in the two systems (modification in *S. pneumoniae*, for example). Reductions in surface capsule levels are important for colonization of the nasopharynx, whereas enhanced levels are necessary in systemic infections (33). In the type 3 strain WU2, ~60% of the polysaccharide is released from the cell during laboratory culture in enriched medium (34), but different environmental conditions may alter this ratio.³ Neither the mechanisms that regulate expression of the type 3 capsule in *S. pneumoniae* nor the mechanisms that control the percentage of polymer that remains cell-associated are known. Thus, an understanding of the processive/non-processive synthetic mechanism of the type 3 synthase and the modulation of polysaccharide release by UDP-sugar concentrations may provide insights into how *S. pneumoniae* regulates the amount of surface-localized polysaccharide.

**Acknowledgment**—We thank Katherine Scheier for obtaining the synthase-specific antiserum.

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Robert T. Cartee, W. Thomas Forsee, John W. Jensen and Janet Yother

*J. Biol. Chem.* 2001, 276:48831-48839.
doi: 10.1074/jbc.M106481200 originally published online October 29, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M106481200

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