Biosynthesis of Type 3 Capsular Polysaccharide in *Streptococcus pneumoniae*

ENZYMATIC CHAIN RELEASE BY AN ABORTIVE TRANSLOCATION PROCESS*

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The type 3 polysaccharide synthase from *Streptococcus pneumoniae* catalyzes sugar transfer from UDP-Glc and UDP-glucuronic acid (GlcUA) to a polymer with the repeating disaccharide unit of (3)-β-D-GlcUA-(1→4)-β-D-Glc-(1→4). Evidence is presented that release of the polysaccharide chains from *S. pneumoniae* membranes is time-, temperature-, and pH-dependent and saturable with respect to specific catalytic metabolites. In these studies, the membrane-bound synthase was shown to catalyze a rapid release of enzyme-bound polysaccharide when either UDP-Glc or UDP-GlcUA alone was present in the reaction. Only a slow release of polysaccharide occurred when both UDP sugars were present or when both UDP sugars were absent. Chain size was not a specific determinant in polymer release. The release reaction was saturable with increasing concentrations of UDP-Glc or UDP-GlcUA, with respective apparent $K_m$ values of 880 and 0.004 μM. The apparent $V_{max}$ was 48-fold greater with UDP-Glc compared with UDP-GlcUA. The UDP-Glc-actuated reaction was inhibited by UDP-GlcUA with an approximate $K_i$ of 2 μM, and UDP-GlcUA-actuated release was inhibited by UDP-Glc with an approximate $K_i$ of 5 μM. In conjunction with kinetic data regarding the polymerization reaction, these data indicate that UDP-Glc and UDP-GlcUA bind to the same synthase sites in both the biosynthetic reaction and the chain release reaction and that polymer release is catalyzed when one binding site is filled and the concentration of the conjugate UDP-precursor is insufficient to fill the other binding site. The approximate energy of activation values of the biosynthetic and release reactions indicate that release of the polysaccharide occurs by an abortive translocation process. These results are the first to demonstrate a specific enzymatic mechanism for the termination and release of a polysaccharide.

The type 3 capsular polysaccharide is composed of the repeating cellobioseic acid disaccharide unit (3)-β-D-GlcUA-(1→4)-β-D-Glc-(1→4), and the antiphagocytic property of this polymer is essential in the virulence of *Streptococcus pneumoniae*. The glucan backbone of type 3 polysaccharide is identical to that for hyaluronic acid; however, the overall structure differs in having the carboxyl groups on the 1–4-linked rather than the 1–3-linked glucosyl units, and there are no acetamido constituents. *In vitro* studies have shown that the biosynthesis of type 3 polysaccharide occurs by the alternate addition of Glc and GlcUA† from UDP-Glc and UDP-GlcUA (2), and work in our laboratory has recently demonstrated that growth of the polymer occurs at the nonreducing end (3). Genetic analysis has established that a single open reading frame (cps3S) encodes the protein that catalyzes the formation of the glycosidic linkages in the type 3 polysaccharide and that the predicted Cps3S protein has significant homology to a number of polysaccharide synthases which form polymers composed of β (1–4)linked repeating disaccharide units (4). On the basis of hydrophobic cluster analysis (5), Cps3S has been included as a member of a family of closely related, processive, β-glycosyltransferases (6). By analogy to hyaluronate synthase from *Streptococcus pyogenes* (7), Cps3S is presumed to have two separate binding sites for the two UDP sugars, with each site catalyzing a distinct glycosidic linkage. Because no export system has been found to exist for type 3 polysaccharide or for most of the other polysaccharide members synthesized by this family of enzymes, it has been suggested that these enzymes catalyze the vectorial extrusion of the growing polysaccharide chain, possibly through a pore or channel (6, 8). Although some evidence has been presented for a role for phospholipids in creating a pore in the hyaluronate synthase complex (9, 10), very little is known about the binding of the polysaccharide to the enzyme complex or about the mechanism of translocation during polymer formation.

Models of polysaccharide biosynthesis that can explain the mechanism of chain termination and release have not been extensively investigated. Genetic analyses in Gram-negative bacteria have indicated the existence of molecular determinants for the length of the chains attached to the lipid A-core component of the lipopolysaccharide molecule (11). Recent evidence has suggested that the size of mammalian hyaluronate chains may be isozyme-dependent (12). In the synthesis of hyaluronic acid in *S. pyogenes*, Sugahara et al. (13) observed that a trichloroacetic acid-insoluble fraction was a precursor to a soluble fraction. The selective inhibition by p-chloromercuri-ribenzoate of the soluble fraction prompted the suggestion that its release might be an enzymic process. However, there was no detectable difference in the molecular size of these two fractions. Prehm (14) noted that the omission of one obligatory assay component, such as MgCl$_2$, UDP-GlcNAc, or UDP-GlcCUA, resulted in a low but distinct shedding of pulse-labeled, mammalian hyaluronate chains. We previously showed that the release of type 3 polysaccharide chains from the membrane-

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† The abbreviations used are: GlcUA, glucuronic acid; Mes, 2-(N-morpholino)ethanesulfonic acid; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine.
enzyme complex was markedly stimulated when an excess of one UDP substrate was added at the point of depletion of the corresponding UDP sugar (3). Here, we describe the catalytic nature of this polysaccharide release reaction.

**EXPERIMENTAL PROCEDURES**

**Materials and Analytical Methods—**UDP-[14C]Glc (257 mCi/mmol) was obtained from Andotek, and UDP-[14C]GlcUA (338 mCi/mmol) was from ICN. Econo-Safe scintillation mixture was from Research Products International, Corp. Mutanolysin, Sephacryl S-500-HR, UDP-Glc, and UDP-GlcUA were purchased from Sigma, Nonidet P-40 was from Calbiochem, and Todd Hewitt Broth and yeast extract were from Difco. Protein was determined with the micro protein determination kit from Biochem, and Todd Hewitt Broth and yeast extract were from Difco. International, Corp. Mutanolysin, Sephacryl S-500-HR, UDP-Glc, and the time of incubation was 3 min.

**Preparation and Assay of Type 3 Synthase—**Membranes containing type 3 synthase were isolated from the encapsulated WU2 S. pneumoniae strain (16) as described previously (3). Membranes were stored at –80 °C in a solution containing 100 mM Hepes (pH 8.0), 10% glycerol, and 10 mM sodium thioglycollate, at a protein concentration of 3 mg/ml. Type 3 synthase activity was determined as described previously (3). Reaction mixtures containing 100 mM Hepes (pH 8.0), 10 mM MnCl₂, and either UDP-Glc or UDP-GlcUA (0.02 mM) were incubated for 10 min at 35 °C with membranes containing 15 μg of protein. The products were separated by paper chromatography with a solvent containing ethanol (95%)/1 N ammonium acetate (pH 5.5), 65:35 (v/v), and quantified by liquid scintillation counting.

**Preparation of [14C]Polysaccharide-Synthase Complex—**Membranes (1.2 mg of protein) were labeled with [14C]Glc in a reaction mixture consisting of 100 mM Hepes (pH 6.0), 10 mM MnCl₂, 25 μM UDP-[14C]Glc (0.8 μCi), and 25 μM UDP-Glc-UA in a total volume of 0.8 ml. The mixture was incubated for 5 min at 35 °C, and the reaction was terminated by the addition of 6.0 ml of ice-cold buffer containing 100 mM Hepes (pH 7.5) and 10% glycerol. The membranes were sedimented by centrifugation at 100,000 × g for 30 min. The pellets were gently suspended with a loosely fitting teflon pestle in 1.0 ml of wash buffer for 2–3 s on a vortex mixer. The suspension was diluted to 6 ml with wash buffer, and the membranes were collected by centrifugation at 100,000 × g for 30 min. The wash step was repeated a total of five times. Prior to the final centrifugation, the membranes were suspended in 1.0 ml of wash buffer, frozen, thawed, and then diluted to 6.0 ml with wash buffer. The final pellet was suspended in 100 mM Hepes (pH 7.5) and 10% glycerol protein concentration of 0.1 mg/ml. The washing was required to remove all of the UDP-Glc-UA, which apparently binds with high affinity to the membranes. After washing the membrane pellet five times, the recoveries of synthase activity and enzyme-bound labeled polysaccharide were 85–90% of the yields obtained prior to these washes. In addition, the chain length distribution of polysaccharides was not changed by the washing procedure. The labeled polysaccharide synthase complex could be maintained in an active state for up to 2 months by storage at –80 °C.

**Polysaccharide substrate of different size chains was synthesized by controlling the nucleotide sugar concentration and the time of incorporation of [3H]Glc or [14C]Glc in the biosynthetic reaction. A lower molecular weight polysaccharide substrate complex was prepared as described above, except that the reaction mixture contained 1.0 μM UDP-[14C]Glc-UA and 25 μM UDP-Glc, and the time of incubation was 3 min. A higher molecular weight polysaccharide substrate complex was prepared as above except the reaction mixture contained 250 μM UDP-[14C]Glc-UA and 250 μM UDP-Glc and the time of incubation was 25 min.

**Assay of Polysaccharide Release—**[14C]Polysaccharide-synthase complex (20,000 cpm) was incubated at 35 °C in a 100-μl reaction mixture consisting of 100 mM Hepes (pH 8.0), 10 mM MnCl₂, and either UDP-Glc or UDP-GlcUA as indicated. The reaction was terminated by placing the reaction tubes on ice and adding 2 volumes of ice-cold buffer containing 100 mM Hepes (pH 7.5) and 10% glycerol. The membranes were sedimented by centrifugation at 100,000 × g for 30 min at 4 °C. The supernatants were saved, and the pellets were solubilized in 300 μl of a solution containing 0.1 N NaCl and 0.5% Nonidet P-40. The radioactivity in both the supernatant and pellet fractions was determined by scintillation counting, and the activity was expressed as the percentage of the total radioactivity found in the supernatant.

**Type 3 Polysaccharide-specific Depolymerase—**Depolymerase was partially purified by ammonium sulfate precipitation from the culture medium of Bacillus cereus (American Type Culture Collection 14175), as described previously (17). The ammonium sulfate precipitate was dissolved in 0.053 M sodium phosphate (pH 6.0) at a protein concentration of 1.9 mg/ml. Labeled polysaccharide (10,000 cpm) was digested for 1 h at 34 °C with depolymerase (2 μg of protein) in a 200-μl reaction mixture containing 40 mM Mes (pH 6.0) and 0.1% Nonidet P-40. The digest was then heated at 95 °C for 3 min, and the precipitate was removed by centrifugation at 10,000 × g for 5 min. The products were analyzed by chromatography on Sephacryl S-500 as described below and by paper chromatography in solvents of butanol/pyridine/H₂O (44:16:40) v/v/v, and ethanol (95%)/1 N ammonium acetate (pH 7.0) (73 v/v). The radioactivity present in 1-cm strips was determined by liquid scintillation counting.

**Depolymerase Digestion of Intact Polysaccharide-Synthase Com-plex—**Both unlabeled and [14C]-labeled polysaccharide membranes (600 μg) were suspended in 200 μl of 0.1 N Hepes (pH 6.0) and incubated with 2 μg of depolymerase at 34 °C for 10 min. The reaction was terminated by the addition of 3.4 ml of an ice-cold solution containing 0.1 N Hepes (pH 7.5) and 10% glycerol, and the membranes were collected by sedimentation at 100,000 × g for 30 min. The membranes were washed two more times in the same manner. The labeled membranes were suspended in 100 μl of the wash buffer and stored at –80 °C. The unlabeled membranes were suspended in a standard reaction mixture, and the polysaccharides were then labeled as described under “Preparation of [14C]Polysaccharide-Synthase Complex.”

**Sephacryl S-500 Column Chromatography—**Gel filtration chromatography was carried out on a 1.4 × 37-cm column of Sephacryl S-500 eluted with a solution consisting of 5 mM Tris (pH 7.5), 200 mM NaCl, 0.1% Nonidet P-40, and 0.02% sodium azide at a flow rate of 20 ml/h. Prior to application to the column, polysaccharide samples were solubilized with 0.2% Nonidet P-40, heated to 100 °C for 3 min, and clarified by centrifugation at 10,000 × g for 5 min. Column fractions of 1.0 ml were collected, and 0.33-ml aliquots were mixed with 3.0 ml of scintillation fluid for determination of radioactivity.

**RESULTS**

**Characterization of Polysaccharide-Synthase Complex—**To explore the mechanism of chain termination and release, we first prepared a membrane fraction containing labeled polysaccharide-synthase complex by incorporating [14C]Glc from UDP-[14C]Glc in the presence of UDP-GlcUA, as described under “Experimental Procedures.” As shown in Fig. 1, the labeled polysaccharide-synthase complex was incubated at 35 °C in a 100-μl reaction mixture containing 100 mM Hepes (pH 8.0), 10 mM MnCl₂, and either UDP-Glc or UDP-GlcUA as indicated. The reaction was terminated by placing the reaction tubes on ice and adding 2 volumes of ice-cold buffer containing 100 mM Hepes (pH 7.5) and 10% glycerol. The membranes were sedimented by centrifugation at 100,000 × g for 30 min at 4 °C. The supernatants were saved, and the pellets were solubilized in 300 μl of a solution containing 0.1 N NaCl and 0.5% Nonidet P-40. The radioactivity in both the supernatant and pellet fractions was determined by scintillation counting, and the activity was expressed as the percentage of the total radioactivity found in the supernatant.
membranes were free of contaminating radioactive nucleotide, and the product was degraded by digestion with type 3 polysaccharide-specific depolymerase from B. circulans, thus confirming the identity of the polysaccharide (17). Incubation of the labeled polysaccharide-synthesizing membranes in a biosynthetic reaction mixture containing 250 \( \mu \text{M UDP-Glc} \) and 250 \( \mu \text{M UDP-GlcUA} \) chased the radioactivity into a higher molecular weight product that eluted near the void volume of a Sephacryl S-500 column (Fig. 1). Elongation of the polysaccharide demonstrated that the \(^{14}\text{C}-\)labeled chain was still engaged with the active site of the enzyme and that the synthase was still active and able to extend the polymer.

**Enzymatic Characteristics of Polysaccharide Release—** Incubation of the \(^{14}\text{C}-\)polysaccharide-synthesizing membranes in the presence of either UDP-Glc or UDP-GlcUA alone resulted in rapid release of the polysaccharide from the membranes into the supernatant (Fig. 2). The rate of release was more rapid in the presence of UDP-Glc alone and was linear for 5 min. Release actuated by UDP-GlcUA was linear for up to 10 min. Only a very slow release occurred in the absence of both nucleotide sugars. A slow rate of polymer release was also observed when both nucleotide sugars were present, a condition that supports rapid synthesis of the polysaccharide (3).

Release of polysaccharide in the presence of UDP-Glc was dependent on \( \text{MnCl}_2 \) or \( \text{MgCl}_2 \), whereas release by UDP-GlcUA was not (shown in Table I for \( \text{MnCl}_2 \)). In the absence of divalent cation, similar levels of release were observed when both UDP sugars were present or with UDP-GlcUA alone, again indicating a metal ion requirement for UDP-Glc (Table I). UDP-xyllose also acteduated polysaccharide release in the presence of divalent cation. UDP-xyllose did not, however, support synthase activity in combination with UDP-GlcUA, and it inhibited the biosynthetic reaction when both UDP-Glc and UDP-GlcUA were present (data not shown). The presence of UDP did not result in significant release, and the addition of 50 \( \mu \text{M UDP-GlcNAc} \), UDP-Gal, or ADP-Glc resulted in no more release than with UDP (data not shown).

The effect of pH on the release reaction is shown in Fig. 3. Polysaccharide release actuated by UDP-Glc was optimal at pH 7.0 in Hepes buffer. UDP-GlcUA-actuated release was inhibited in Hepes buffer when the pH was less than 8.0, and the optimal pH for this reaction was 7.5 in imidazole buffer as shown. The pH profile for the UDP-GlcUA reaction in Tricine buffer was similar to that for imidazole (data not shown). Both imidazole and Tricine buffers inhibited the UDP-Glc-actuated reaction when the pH was above 6.0. A number of different buffers were tested, and none provided optimum conditions for both reactions at their optimal pH. The determinations of all catalytic constants for a direct comparison of both the release reaction and the polymerization reaction were therefore carried out at pH 8.0 in Hepes buffer.

**Effect of Polysaccharide Length and Other Factors on Release—** To assess the effect of chain size on polysaccharide release, synthase complex consisting of either high or low molecular weight polysaccharide was synthesized as described under “Experimental Procedures.” The size of the chains was analyzed by chromatography on Sephacryl S-500, following solubilization of the synthase complex with detergent. The low molecular weight chains were included in the column bed and gave a broad profile (Fig. 4A), whereas the high molecular weight sample eluted near the excluded column volume (Fig. 4B). The gel filtration profiles of the polymer released by UDP-Glc or UDP-GlcUA were similar to the initial polysaccharide.
profiles, indicating that the release reaction shows no specificity for polysaccharide size. Although both low and high molecular weight chains were readily released, the initial release of the smaller size polymer was more rapid (Fig. 5). To further assess the significance of the chain size in the release reaction, the activation energies of release were determined with low and high molecular weight polysaccharide chains. The Arrhenius plots of the release activity with UDP-GlcUA were linear between 15 and 35 °C for the low and between 20 and 40 °C for the high molecular weight chains. The essentially identical approximate activation energies of 29 and 28 kcal/mol, respectively, indicate that the synthase is nonspecific with regard to polysaccharide chain size. However, at any given temperature, the rate of release was more rapid with smaller polymer, demonstrating that the more rapid release with the smaller chains was a thermal property of the reaction.

In the above experiments, complete release of the polysaccharide was not observed, even at higher concentrations of nucleotide sugar (data not shown). Therefore, a possible additive release by the sequential addition of UDP sugars was investigated. Polysaccharide-enzyme complex was first incubated with either UDP-Glc or UDP-GlcUA for 20 min as in Fig. 2, resulting in 54 and 40% release, respectively, of the polysaccharide into the supernatant. After washing to remove the first nucleotide sugar, incubation of the membranes with the same or the alternative UDP sugar resulted in no more than 8% additional release over that observed with the control membranes (data not shown).

Depolymerase digestion of the synthase complex was employed to explore the possibility that some of the polysaccharide chains might be entangled at their reducing ends or anchored to the membranes, thereby preventing their free release. Labeled polysaccharide membranes were prepared by: (a) the standard protocol; (b) the standard protocol followed by depolymerase digestion; or (c) depolymerase digestion followed by the standard labeling protocol. The depolymerase digestion removed 67% of the radioactivity from the labeled polysaccharide chains. Longer digestions did not significantly reduce the amount of membrane-bound radioactivity, indicating that all the accessible length of the chains had been cleaved. Incubation of the membrane preparations for 20 min with 100 μM UDP-Glc resulted in 34, 56, and 34% release, respectively, for preparations a, b, and c. Thus, depolymerase treatment still did not allow complete release of the polysaccharide.

Reaction Constants of Polysaccharide Release—The effect of the concentration of UDP-Glc on the initial rate of polysaccharide release is shown in Fig. 6A. The plot exhibited typical hyperbolic kinetics, the double reciprocal plot was linear, and the apparent K_m value was 880 μM. Release of polysaccharide as a function of the concentration of UDP-GlcUA also exhibited hyperbolic kinetics (Fig. 6B), and the apparent K_m value was 0.004 μM. The apparent V_max value for UDP-Glc release was 48-fold greater than that for UDP-GlcUA, indicating that the release mechanism is much more readily facilitated by saturating levels of UDP-Glc.

Polysaccharide release was inhibited by increasing the concentration of one UDP sugar in the presence of a fixed level of the conjugate UDP sugar (Fig. 7). UDP-Glc inhibited release...
actuated by UDP-GlcUA, with an approximate $K_i$ of 5 mM. UDP-GlcUA inhibited release actuated by UDP-Glc, with an approximate $K_i$ of 2 mM. It is noteworthy that these values are very similar to the respective apparent $K_m$ values of 12 and 9 mM that we previously reported for UDP-Glc and UDP-GlcUA in the biosynthesis of type 3 polysaccharide (3).

The polysaccharide release reaction was markedly temperature-dependent. Below 15 °C there was no significant activity, but above this temperature the activity increased rapidly up to 40 °C. Arrhenius plots of the release activity were linear between 20 and 40 °C and approximate activation energies of 32 and 28 kcal/mol were calculated for the release reactions actuated by UDP-Glc and UDP-GlcUA, respectively (Fig. 8). The Arrhenius plot for the biosynthetic reaction was linear from 15 to 40 °C, and the approximate activation energy was 17 kcal/mol.

**Nascent Chain Release**—Release of low molecular weight polysaccharide chains by either UDP-Glc or UDP-GlcUA (Fig. 5), suggested that release of endogenous, nascent polysaccharide chains might also be possible. Incubation of the membranes containing type 3 synthase with either 50 μM UDP-Glc or UDP-GlcUA prior to initiating chain elongation, resulted in a rapid time-dependent inhibition of the synthase activity (Fig. 9). When both UDP sugars were added or when neither was added, the time-dependent inhibition was much slower. The maximum reduction in synthase activity was 66% when the membranes were incubated with UDP-Glc and 63% with UDP-GlcUA, consistent with the comparable maximum release of polysaccharide observed in Fig. 5 and in other similar reactions. These data suggested that endogenous nascent polysaccharide chains are released by the same mechanism. Further, release of nascent polysaccharide chains was concentration-dependent, and plots of the inhibition of the polymerization reaction as a function of the concentration of UDP-Glc or UDP-GlcUA were hyperbolic, and the double reciprocal plots were linear (data not shown), yielding respective apparent $K_m$ values of 230 μM for UDP-Glc and 0.015 μM for UDP-GlcUA (Table II). Nascent chain release, as determined by synthase inhibition, also demonstrated the same specificity for other UDP nucleotides as reported above for polysaccharide release (data not shown). These results further confirm the similarity of the release reaction for polysaccharide product and nascent polysaccharide chains.

**DISCUSSION**

There has been considerable progress in the understanding of the biosynthesis of a variety of carbohydrate polymers; however, most investigations have provided relatively little information with regard to possible mechanisms of termination and release of completed polysaccharide chains. The results obtained here indicate that chain release can occur by an enzymatic mechanism that may provide insight into the translocation process of the polymerization reaction. Indeed, the release reaction appears to be a futile attempt to translocate the growing polysaccharide chain when only a single substrate binding site is occupied.
Conjugate UDP-nucleotide is increased above its concentration sufficient to bind to its active site, and the concentration of the conjugate UDP-precursor is insufficient to bind to the other substrate binding site. As the concentration of the polysaccharide release occurs when one UDP sugar is present at a concentration that both polymerization and release are catalyzed by polysaccharide chains to the synthase or that the synthase contains additional sites on the synthase. These results indicate that polysaccharide release is rapidly reduced, apparently as the chain may be unable to reattach if it either now lacked the appropriate recognition sequence or had been shifted too far out of alignment with the enzyme binding site. It has been suggested that the driving force for polymer translocation could be generated by the hydrolysis of the pyrophosphoryl linkage of the nucleotide sugar. However, no evidence has been presented for this hypothesis, and it is possible that the energy could be derived from constrainment of the enzyme upon binding of the nucleotide sugar. The experimental activation energy for the polymerization reaction was about half that observed for the release reaction, suggesting that the rate-limiting process is not known in any detail, but a polymer translocation step, as has been postulated in the formation of other β-glycans, is a likely candidate as the common mechanistic step that would incrementally move the polymer between disaccharide additions during the biosynthetic reaction or, alternatively, eject the polymer in the release reaction. If detachment of the polymer from the carbohydrate binding site were to occur following the binding of a single UDP-precursor or alternatively as a consequence of the addition of a single monosaccharide, the chain may be unable to realign itself. A low level of polysaccharide release was determined as described in the legend to Fig. 7. The approximate activation energies (ΔE) were determined as described in the legend to Fig. 8. Labeled UDP-GlcUA was incorporated in the synthase assay when the concentration of UDP-Glc was varied to actuate release. Conversely, labeled UDP-Glc was incorporated when UDP-GlcUA was varied. The apparent Km values for polysaccharide release were determined as described in the legend to Fig. 6. The approximate Ki values for polysaccharide release were determined as described in the legend to Fig. 7. 

**TABLE II**

**Reaction constants**

| Reaction | Nucleotide sugar | Km | Ki | ΔE (kcal/mol) |
|----------|------------------|----|----|--------------|
| Polysaccharide release | UDP-Glc | 880 | 5 | 32 |
| Polysaccharide release | UDP-GlcUA | 0.004 | 2 | 28 |
| Nascent chain release | UDP-Glc | 230 | | |
| Nascent chain release | UDP-GlcUA | 0.015 | | |
| Polymerization | UDP-Glc + UDP-GlcUA | 12* | 17 | |
| Polymerization | UDP-Glc | 9* | | |

*Reported in Ref. 3.

The mechanism of polymerization for type 3 polysaccharide is not known in any detail, but a polymer translocation step, as has been postulated in the formation of other β-glycans, is a likely candidate as the common mechanistic step that would incrementally move the polymer between disaccharide additions during the biosynthetic reaction or, alternatively, eject the polymer in the release reaction. If detachment of the polymer from the carbohydrate binding site were to occur following the binding of a single UDP-precursor or alternatively as a consequence of the addition of a single monosaccharide, the chain may be unable to realign itself. A low level of polysaccharide release was determined as described in the legend to Fig. 7. The approximate activation energies (ΔE) were determined as described in the legend to Fig. 8. Labeled UDP-GlcUA was incorporated in the synthase assay when the concentration of UDP-Glc was varied to actuate release. Conversely, labeled UDP-Glc was incorporated when UDP-GlcUA was varied. The apparent Km values for polysaccharide release were determined as described in the legend to Fig. 6. The approximate Ki values for polysaccharide release were determined as described in the legend to Fig. 7. Labeled UDP-GlcUA was incorporated in the synthase assay when the concentration of UDP-Glc was varied to actuate release. Conversely, labeled UDP-Glc was incorporated when UDP-GlcUA was varied.

**FIG. 8.** Arrhenius plots of the type 3 synthase polymer formation and polysaccharide release activities. Polysaccharide release in the presence of 50 μM UDP-Glc for 3 min (●), 50 μM UDP-GlcUA for 8 min (○), or type 3 synthase activity (■) was determined by the standard protocols at the indicated temperatures. A control release in the absence of UDP sugar was conducted at each temperature and subtracted from the experimental release values. The lines were fitted by linear regression analysis.

**FIG. 9.** Nascent polysaccharide chain release as determined by the inhibition of polymer formation. Membranes were incubated at 35 °C in a 100-μl reaction mixture consisting of 100 mM Hepes (pH 8.0), 10 mM MgCl2, and 50 μM UDP-Glc (●), 50 μM UDP-GlcUA (○), both UDP sugars at 50 μM of each (■), or no addition (□). At the indicated times, 10-μl samples were removed and assayed for synthase activity as described under "Experimental Procedures.”

Inhibition of the release reaction occurred at Ki values similar to the biosynthetic Km values, consistent with the hypothesis that both polymerization and release are catalyzed by interaction of the UDP sugars with the same set of binding sites on the synthase. These results indicate that polysaccharide release occurs when one UDP sugar is present at a concentration sufficient to bind to its active site, and the concentration of the conjugate UDP-precursor is insufficient to bind to the other substrate binding site. As the concentration of the conjugate UDP-nucleotide is increased above its Km, the rate of polysaccharide release is rapidly reduced, apparently as the normal polymerization cycle is re-established. Because the type 3 synthase has not been purified, it is conceivable either that the release reaction involves an additional protein interacting with the synthase or that the synthase contains additional UDP-precursor binding sites. However, all of the data are readily explained by a single peptide with a single set of binding sites for UDP-Glc and UDP-GlcUA, whereby binding of either UDP sugar would trigger release of the polysaccharide chain by: (a) constrainment of the enzyme upon binding of the nucleotide sugar, (b) hydrolysis of the nucleotide sugar, or (c) transfer of the sugar to the polysaccharide.

We have so far been unable to demonstrate that the abortive translocation of polysaccharide chains is accompanied by the addition of either Glc or GlcUA. However, considering the low level of isotope that would be incorporated by the addition of a single monosaccharide and the increased background incorporation that occurs when the level of protein is necessarily scaled up, we cannot exclude the possibility that a low level of transfer to the released chain does take place. Nevertheless, release of the polysaccharide accompanied by the transfer of a single sugar seems unlikely for two reasons. First, the triggering of translocation due to the formation of either a single glucosidic or a single glucuronidic linkage, although not inconceivable, does not readily fit the current model of growth of β-glycans by repetitive disaccharide addition (5, 18). Second, the observed release of polysaccharide by UDP-xylose does not
support this mechanism, because xylose is not incorporated during polymer formation.

In contrast to the $K_v$ values, the apparent $K_m$ values for UDP-Glc and UDP-GlcUA were dramatically different in the abortive translocation reaction and the polymerization reaction. It would appear to be significant that the $K_m$ value for UDP-Glc is 2 orders of magnitude higher and that for UDP-GlcUA is 3 orders of magnitude lower. To the extent that the apparent $K_m$ values reflect binding affinities, these data suggest that the binding of UDP-Glc greatly decreases the affinity of the synthase for UDP-GlcUA and that the binding of the latter greatly enhances the affinity of the former. Although the chemistry of the release reaction is still undefined, the translocation step would presumably be slow in comparison with the association and dissociation of the nucleotide sugars. Hence, the $K_m$ values would be predicted to be similar to the dissociation constants and should reflect the affinities of the synthase for the nucleotide sugars.

Previous investigations of $S$. pyogenes hyaluronate biosynthesis have noted a sigmoidal response to increasing concentrations of UDP-GlcNAc (19, 20), and the latter have interpreted these results as evidence of cooperativity and “cross-talk.” The possibility of a cooperative interaction of the two UDP sugar precursors has also been suggested in view of the enhanced photoaffinity labeling of $S$. pyogenes hyaluronate synthase with $^{32}$P5-azido-UDP-GlcNAc in the presence of UDP-GlcUA (21). We have also observed sigmoidal kinetics in our system when the concentration of one UDP-precursor is varied in the presence of the conjugate UDP sugar. However, under some conditions this effect could readily be the consequence of a decrease in abortive translocation as the concentration of the nucleotide sugar is increased. This added complication precludes any simple interpretation of these data.

Nascent polysaccharide chains, which are present in our membrane preparations and which serve as primer in the polysaccharide elongation assay, were also released under the identical conditions and by the same apparent mechanism as the elongated $[^{14}$C]$polymer$. We have no evidence to indicate that $S$. pneumoniae membrane preparations are able to self-prime under the conditions of our assay, and following the release of some of the chains, all of the remaining polymerization activity appeared to occur by the elongation of pre-existing chains, without any evidence of the initiation of new low molecular weight chains. Taken together, the similarity of the conditions that actuate release of the nascent polysaccharide and also of the elongated $[^{14}$C]$polymer$ strengthen the hypothesis that both polymerization and release are catalyzed by the interaction of the UDP sugars with the same set of binding sites on the synthase. The failure to achieve complete release of polysaccharide from the membranes, even after depolymerase treatment to free potentially tethered chains, is at present an unexplained observation. It is perhaps significant that clinical type 3 isolates produce both cell-bound and released forms of the polysaccharide and the fraction of each varies with the strain under study (22). An understanding of the physiological relevance of the in vitro release will thus be an important step in understanding type 3 polysaccharide biosynthesis.

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