Biosynthesis of the Escherichia coli K4 Capsule Polysaccharide
A PARALLEL SYSTEM FOR STUDIES OF GLYCOSYLTRANSFERASES IN CHONDROITIN FORMATION

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Escherichia coli K4 bacteria synthesize a capsule polysaccharide (GalNAc-GlcA(fructose))ₙ with the carbohydrate backbone identical to chondroitin. GlcA- and GalNAc-transferase activities from the bacterial membrane were assayed with acceptors derived from the capsule polysaccharide and radiolabeled UDP-[¹⁴C]GlcA and UDP-[³H]GalNAc, respectively. It was shown that defructosylated oligosaccharides (chondroitin) could serve as substrates for both the GlcA- and the GalNAc-transferases. The radiolabeled products were completely degraded with chondroitinase AC; the [¹⁴C]GlcA unit could be removed by β-D-glucuronidase, and the [³H]GalNAc could be removed by β-N-acetylhexosaminidase. A fructosylated oligosaccharide acceptor tested for GlcA-transferase activity was found to be inactive. These results indicate that the chain elongation reaction of the K4 polysaccharide proceeds in the same way as the polymerization of the chondroitin chain, by the addition of the monosaccharide units one by one to the nonreducing end of the polymer. This makes the biosynthesis of the K4 polysaccharide an interesting parallel system for studies of chondroitin sulfate biosynthesis.

In the biosynthesis of capsule polysaccharides from E. coli, a similar mechanism has earlier been demonstrated for polysialic acid (NeuNAc)ₙ (Rohr, T. E., and Troy, F. A. (1980) J. Biol. Chem. 255, 2332–2342) and for the K5 polysaccharide (GlcAβ1–4GlcNAc1–4)ₙ (Lidholt, K., Fjelstad, M., Jann, K., and Lindahl, U. (1994) Carbohydr. Res. 255, 87–101). In contrast, chain elongation of hyaluronan (GlcAβ1–3GlcNAcβ1–4)ₙ is claimed to occur at the reducing end (Prehm, P. (1983) Biochem. J. 211, 181–189).

The biosynthesis of heparin/heparan sulfate and chondroitin sulfate polysaccharides has been intensively studied. These glycosaminoglycan chains are synthesized as proteoglycans. The chains are attached to serine units in the core protein via a tetrasaccharide linkage, and the polysaccharides are formed by the addition of monosaccharide units, one by one, from the corresponding UDP sugars to the nonreducing end of the growing chain (1). The glycosyl transferases involved in the polymerization reaction of heparin/heparan sulfate, GlcNAC- and GlcA-transferase, have been studied in different tissues (2–5) and purified from bovine serum (6). One very interesting finding from these studies is that the two glycosyl transferase activities seem to be catalyzed by one single protein with two catalytic sites. The glycosyltransferases involved in chain elongation of chondroitin sulfate, GalNAc- and GlcA-transferase, have so far not been purified, although their activities have been studied (7–11).

Two bacterial strains, Escherichia coli K5 and E. coli K4, synthesize polysaccharide capsules with the same backbone structures as heparin/heparan sulfate and chondroitin sulfate, respectively (Fig. 1). The K5 bacteria produce a (GlcA-GlcNAc)ₙ polymer (12) and the K4 synthesizes a (GalNAc-GlcA(Fru))ₙ sequence (13) having the same structure as chondroitin with the fructose units removed (Fig. 1B). We have previously studied the biosynthesis of the K5 polysaccharide and found that chain elongation occurs in the same way as heparin/heparan sulfate biosynthesis. We have also demonstrated that the bacterial enzymes have the same substrate specificity regarding N-sulfate groups on the oligosaccharide acceptor as the mammalian ones (14). Although the K5 bacteria produce a nonsulfated polymer, the bacterial GlcA-transferase, like its mammalian equivalent, preferred oligosaccharides with an N-sulfate group on the penultimate GlcNAc unit from the reducing end (14).

The structure of E. coli K4 capsule polysaccharide has been characterized by Rodriguez et al. (13), but the biosynthesis of the polymer has not been investigated. There are so far no reports of assays for the corresponding glycosyl transferases using endogenous primers or exogenous oligosaccharide acceptors. The aim of this study was to clarify whether polymerization of K4 polysaccharide occurred in the same way as that of the chondroitin polysaccharide. With bacterial K4 membranes as the enzyme source, we were able to assay the transfer of both [¹⁴C]GlcA and [³H]GalNAc to defructosylated K4 oligosaccharides (chondroitin oligosaccharides). The radiolabeled oligosaccharide products were identified by enzyme digestions. Both [¹⁴C]GlcA- and [³H]GalNAc-labeled products could be degraded by chondroitinase AC, the [¹⁴C]GlcA could be removed by β-D-glucuronidase, and the [³H]GalNAc could be removed by β-N-acetylhexosaminidase. These results show that the radiolabeled monosaccharide units are added to the nonreducing end of a chondroitin oligosaccharide. On the other hand, when fructosylated K4 oligosaccharides (with a GalNAc unit at the nonreducing end) were tested as acceptors for the GlcA-transferase, no transfer of [¹⁴C]GlcA could be detected. Taken together, these findings show that K4 polysaccharide polymerization is analogous to that of chondroitin and that information about biosynthesis of bacterial polysaccharide, both on protein and genetic levels, could be useful for understanding of chain elongation reaction in mammalian systems.

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1 The abbreviation used is: Fru, fructose.
EXPERIMENTAL PROCEDURES

Materials—Membranes from *E. coli* wild-type O5;K4;H4 (13) were obtained as described (15). In short, bacteria were grown to late logarithmic phase and centrifuged (10,000 × g, 10 min, 4°C). The sediment was suspended in 50 mM Hepes, pH 7.2, 30 mM magnesium acetate, and 1.5 mM dithioerythritol and centrifuged (10,000 × g, 10 min, 4°C). The sediment was resuspended in the same buffer, and the bacteria were disrupted by three passages of the suspension through a French pressure cell. The homogenate was centrifuged for 10 min at 10,000 × g to remove large bacterial fragments and then for 60 min at 100,000 × g at 4°C. The membrane pellet was resuspended in the same buffer. UDP-[14C]GlcA was prepared enzymatically from D-[14C]glucose (uniformly labeled, 321 mCi/mmol; The Radiochemical Center) as described (16), and UDP-[1-3H]GalNAc (6.3 Ci/mmol) was obtained from New England Nuclear. UDP-GlcA, UDP-GalNAc, and bovine liver β-D-glucuronidase (type B-10) were from Sigma, and chondroitinase ACI (Flavobacterium heparinum) and β-N-acetylhexosaminidase (Jack Bean) were from Seikagaku Corp., Tokyo, Japan.

Oligosaccharides of the type (GlcA-GalNAc)_n-GlcA-2,5-anhydro-D-talose (n ≥ 8) with alternating sugar residues as exist in chondroitin were derived from the *E. coli* K4 polysaccharide (13) (provided by Italfarmaco S.p.A., Milan, Italy). The K4 polysaccharide was first hydrolyzed by acid treatment at pH 1.5 at 80°C for 30 min to remove the fructose unit from the chondroitin backbone. For the cleavage of the polysaccharide and the separation of the oligosaccharide substrates, a similar method was used as for the K5 polysaccharide (5). In short, partial N-deacetylation (hydrazinolysis) of the K4 polysaccharide followed by deaminative cleavage at the resulting N-unsubstituted galactosamine units by treatment with nitrous acid at pH 3.9, gave a mixture of even numbered oligosaccharides. These were separated by gel chromatography on Sephadex G-50, and fractions larger than octasaccharides were recovered and desalted. These oligosaccharides have a GlcA unit at the nonreducing end and can serve as substrates for the GalNAc-transferase. Substrates for the GlcA-transferase were created by β-glucuronidase digestion of the oligosaccharides. An alternative method used to make substrates for the GlcA-transferase was also applied: K4 polysaccharide (10 mg) with fructose units still retained in the polymer were digested with chondroitinase AC I, 0.75 units in Tris-HAc buffer, pH 7.3, in a final volume of 2.5 ml for 20 h. The polysaccharide chains were cleaved at positions lacking fructose units, and oligosaccharides with an unsaturated uronic acid at the nonreducing end, β-D-gluco-4-enepyranosyluronic acid-GalNAc-(GlcA(Fru)-GalNAc)_n (n ≥ 0) were recovered. Unsaturated uronic acid was removed by treatment with 10 mM mercuric acetate for 30 min. These odd numbered oligosaccharides, still containing the fructose GalNAc-(GlcA(Fru)-GalNAc)_n, were desalted on a PD-10 column (Pharmacia Biotech Inc., Uppsala, Sweden) and tested as substrates for the GlcA-transferase. As a control, the Fru units were then removed by acid hydrolysis at pH 1.5 at 80°C for 30 min. The defructosylated oligosaccharides GalNAc-(GlcA-GalNAc)_n were then desalted on a PD 10 column and used as substrates for the GlcA-transferase.

Glycosyl Transfer to Oligosaccharides—Enzymatic transfer of GalNAc and GlcA units were studied by incubating acceptor oligosaccharides (0.5 mM corresponding to 90 μg of GlcA assay, as determined by the carbazole reaction), 2 μCi of UDP-[3H]GalNAc (0.5 mM UDP-GalNAc) or 1.35 μCi of UDP-[14C]GlcA (0.5 mM UDP-GlcA), and 400 μg of bacterial membrane protein in a total volume of 100 μl of 10 mM MnCl₂, 10 mM MgCl₂, 5 mM CaCl₂, 50 mM Hepes, pH 7.2, and 1% Triton X-100. After incubation at 37°C for 30 min, the reactions were stopped by the addition of 100 μl of 10% trichloroacetic acid, and the mixtures were
RESULTS

Preparation of Oligosaccharide Acceptors

Substrates for the GalNAc-transferase—The K4 polysaccharide was subjected to acidic hydrolysis, which removed the fructose units from the polymer. These polysaccharide products were then partially N-deacetylated with hydrazine and cleaved at the resulting free amino groups with nitrous acid, pH 3.9 (18). The resulting oligosaccharides, which have a GlcA unit at the nonreducing end, were separated by gel filtration. Oligosaccharides with ≥8 monosaccharide units were collected and used as acceptor oligosaccharides for the GalNAc-transferase.

Substrates for the GlcA-transferase—Oligosaccharide substrates for the GlcA-transferase were created in two different ways. 1) The oligosaccharides used for the assay of the GalNAc-transferase were treated with β-D-glucuronidase, which removes the GlcA unit at the nonreducing end of the oligosaccharide, converting the oligosaccharides to a GlcA acceptor substrate. 2) In an alternative approach, where the purpose was to create a substrate in which fructose units were retained, the fructosylated K4 polysaccharide was digested with chondroitinase AC. The polysaccharide was partially degraded (Fig. 2), probably due to random loss of fructose units. Chondroitinase AC degrades chondroitin, but it does not degrade the fructosylated K4 polysaccharide. The unsaturated hexuronic acid that appears at the nonreducing end of the oligosaccharides after the digestion was removed by mercuric acetate, and the remaining fructosylated oligosaccharides of GalNAc-(GlcA(Fru)-GalNAc), type could be tested as acceptors for the GlcA-transferase. These oligosaccharides were then defructosylated by acidic hydrolysis, and substrates with the structure GalNAc-GlcA-GalNAc were recovered (defructosylation of the polymer has been investigated in an earlier paper (13)).

Experimental Conditions

The experimental conditions were chosen to optimize the incorporation of radioactive sugar to oligosaccharide substrates in order to get a sufficient amount of radiolabeled product for further analysis and were based on earlier experiences of chain elongation reactions in E. coli K5 (14) membranes and microsomal membranes from mouse mastocytomas (5).

Because the incorporation of both [3H]GalNAc and [3H]GlcA to their specific substrates when incubated with E. coli K4 membranes solubilized in 1% Triton X-100 increased with incubation times of up to 30 min, this time was selected for the experiments described below.

The effect of enzyme concentration was tested. It was found that in 15-min incubations transfer of both [3H]GalNAc and [3H]GlcA was linear with protein concentrations up to 4 mg/ml (data not shown). The membrane concentration was thus set to 4 mg of membrane protein/ml of incubation volume.

The reactions above were carried out in a buffer containing 5 mM Ca2+, 10 mM Mn2+, and 10 mM Mg2+, as used for studies of chain elongation of the E. coli K5 polysaccharide. The requirement for metal ions in the E. coli K4 system was tested in both glycosyltransferase reactions (Fig. 3), and a mixture of all three metal ions showed the highest activity. These conditions were thus maintained in the following experiments.

Transfer of GalNAc

Oligosaccharides with ≥8 monosaccharide units with a GlcA at the nonreducing end were tested as substrates for the bacterial GalNAc-transferase. Incubations with UDP-[3H]GalNAc, oligosaccharides, and bacterial K4 membranes solubilized in 1% Triton X-100 resulted in a radiolabeled product that was not found in control incubations without the addition of oligosaccharides (Fig. 4A). Furthermore, the labeled product was completely degraded by chondroitinase AC, and after digestion, the label eluted as a monosaccharide on the gel filtration chromatogram (Fig. 4B). The released monosaccharide confirms that a single monosaccharide unit has been added to the nonreducing end of the chondroitin oligosaccharide (Fig. 4C). The main part of the incorporated [3H]GalNAc could also be removed by treatment with β-N-acetyhexosaminidase (Fig. 4B), an exo enzyme that removes β-linked GalNAc from the nonreducing end (Fig. 4C).

The addition of UDP-GlcA, the other nucleotide sugar, to those assays had no influence on the amount of incorporated radioactivity, and we could only detect the transfer of one GalNAc unit to the exogenous oligosaccharide acceptors (data not shown).

The incubations were also carried out with intact bacterial membranes, without the addition of Triton X-100. No polymerization was detected in any of these incubations in terms of higher incorporation of radioactivity or changed profiles in the
gel chromatograms, but transfer of one GalNAc unit could be assayed in the same way as for the solubilized system (data not shown). No differences could be seen between solubilized and intact membranes regarding GalNAc transfer.

Transfer of GlcA

GlcA-transferase activity was tested essentially as described for GalNAc-transferase, by the incubation of bacterial membrane with oligosaccharide acceptors with a terminal GalNAc (nonreducing end) and UDP-[14C]GlcA in 1% Triton X-100. Two different oligosaccharide substrates were tested (preparation method 2), one defructosylated chondroitin acceptor and one acceptor containing fructose units in the chain. A striking difference in the GlcA-transferase activity was noticed between the two different oligosaccharide substrates (Fig. 5A). The GlcA was readily transferred to the defructosylated acceptor, whereas the fructosylated acceptor did not incorporate GlcA at all (Fig. 5). From the elution profile of the product derived from the defructosylated substrate, it can also be shown that a heptasaccharide is large enough to serve as a substrate for the bacterial enzymes. The differences in the size of the substrate seen in Fig. 2 and in the product in Fig. 5 are mainly due to the detection method used. The gel filtration pattern of the substrate (Fig. 2) illustrates the content of hexuronic acid, whereas only the radiolabeled nonreducing ends are detected in the product. However, one cannot exclude the possibility that the difference might partly be due to GlcA-transferase having a preference for shorter oligosaccharide substrates. Digestion of the radiolabeled product by β-glucuronidase yielded labeled monosaccharides (Fig. 5B), demonstrating that the GlcA unit is added with a β-linkage to the nonreducing end of the oligosaccharide (Fig. 1D). Furthermore, when the oligosaccharide products were treated with chondroitinase AC, only labeled disaccharides could be detected (Fig. 5B), showing that the cleavage site of the enzyme is a disaccharide unit from the nonreducing end (Fig. 1D).

The GlcA-transferase activity was also tested in incubations with the addition of UDP-GalNAc. No change in incorporated radioactivity could be seen due to this addition, and no polymerization was detected in the gel chromatograms (data not shown). Intact membrane fractions, assayed in the absence of Triton X-100, showed the same GlcA-transferase activity as did solubilized systems (data not shown).

Degradation of 3H-Labeled Product

In order to test endogenous activity of polysaccharide degrading enzymes, oligosaccharides enzymatically labeled with [3H]GalNAc were recovered and incubated with fresh membrane enzymes. The radiolabeled product was recovered after separation from the UDP-[3H]GalNAc on a G-25 Sephadex gel filtration column (Fig. 6A). Four fractions, as indicated in the chromatogram, were pooled, concentrated, desalted on PD-10, evaporated, and added back to a membrane incubation with the same buffer used in the previous incubations. After 30 min at 37 °C, the sample was applied to the same gel filtration column (Fig. 6B). No degradation could be detected.
The mechanisms regarding polysaccharide biosynthesis has earlier been investigated for mammalian heparin/heparan sulfate, chondroitin sulfate, and hyaluronan synthesis. The hyaluronan polysaccharide, which does not form proteoglycans and is synthesized at the cell membrane (19), is claimed to elongate at the reducing end (20). For the hyaluronan producing bacterium *Streptococcus pyogenes* (21), the mechanism for chain elongation has so far not been studied. The biosynthesis of polysaccharic acid from capsule-producing *E. coli* bacteria has been intensively investigated, and Rohr and Troy (21) showed that the polymer, although its polymerization only involves one sugar unit, is elongated at the nonreducing end.

The biosynthesis of the *E. coli* K4 Capsule Polysaccharide is rather ambiguous, because the role of the fructose units in the polymer is obscure. The sugar donor for the fructose units is still unknown, and it is not clear how fructoses are added to the polymer during biosynthesis. The results of this study show that both the GlcA and the GalNAc units can be added to exogenous chondroitin oligosaccharides that lack fructose (Figs. 4 and 5). We have also used bacterial-derived chondroitin oligosaccharides in assays with mammalian enzymes, and we have found that these oligosaccharides work as well as chondroitin oligosaccharides derived from other sources (data not shown). Similar oligosaccharide substrates have previously been used in studies of chondroitin polymerization using enzymes from chick embryo chondrocytes (7) where these oligo-
saccharides were shown to be acceptors for the transfer GalNAc and GlcA units. Bacterial glycosyltransferases seem to have similar requirements for the acceptor substrates as for chondroitin sulfate-forming enzymes. The fact that fructosylated oligosaccharides were not acceptors for the GlcA-transferase (Fig. 5) strongly indicates that fructose units are added to the polymer either after the formation of the chondroitin backbone or as a second step after the addition of GlcA and of the GalNAc units further downstream in the polymer. Digestion of the radiolabeled products with β-D-glucuronidase and β-N-acetylhexosaminidase proves that the GlcA and the GalNAc mono-saccharides were added to the nonreducing end of the oligosaccharides, because radiolabeled monosaccharides were released from the enzymatically labeled product (Figs. 4B and 5B). The complete degradation of the [14C]GlcA- and [3H]GalNAc-labeled products with chondroitinase AC shows that the products are chondroitin (Figs. 4B and 5B).

These results suggest that bacterial biosynthesis of K4 polysaccharide proceeds in the same way as chondroitin biosynthesis and that the addition of fructose units is not critical for chain elongation. This makes the E. coli K4 bacterium a suitable parallel system for studies of chondroitin biosynthesis in the same way as E. coli K5 polysaccharide biosynthesis is a parallel system for studies of heparin biosynthesis.

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