Overexpression of UDP-glucose dehydrogenase in *Escherichia coli* results in decreased biosynthesis of K5 polysaccharide

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The *Escherichia coli* K5 capsular polysaccharide (glycosaminoglycan) chains are composed of the repeated disaccharide structure: -GlcA β 1,4-GlcNAc α 1,4- (where GlcA is glucuronic acid and GlcNAc is *N*-acetyl-D-glucosamine). The GlcA, present in most glycosaminoglycans, is donated from UDP-GlcA, which, in turn, is generated from UDP-glucose by the enzyme UDP-glucose dehydrogenase (UDPGDH). The formation of UDP-GlcA is critical for the biosynthesis of glycosaminoglycans. To investigate the role of UDPGDH in glycosaminoglycan biosynthesis, we used K5 polysaccharide biosynthesis as a model. *E. coli* was transformed with the complete gene cluster for K5 polysaccharide production. Additional transformation with an extra copy of UDPGDH resulted in an approx. 15-fold increase in the *in vitro* UDPGDH enzyme activity compared with the strain lacking extra UDPGDH. UDP-GlcA levels were increased

3-fold in overexpressing strains. However, metabolic labelling with [¹⁴C]glucose showed, unexpectedly, that overexpression of UDPGDH lead to decreased formation of K5 polysaccharide. No significant difference in the K5 polysaccharide chain length was observed between control and overexpressing strains, indicating that the decrease in K5-polysaccharide production most probably was due to synthesis of fewer chains. Our results suggest that K5-polysaccharide biosynthesis is strictly regulated such that increasing the amount of available UDP-GlcA results in diminished K5-polysaccharide production.

Key words: bacterial capsule, *Escherichia coli*, glycosaminoglycan, K5 polysaccharide, UDP-glucose dehydrogenase, UDP-glucuronic acid.

INTRODUCTION

Bacterial capsule polysaccharides play important roles in the invasiveness and pathogenicity of bacteria [1]. The expression of *Escherichia coli* capsular polysaccharides (K-antigens) is believed to enable the bacteria to evade host defences [2]. Although most *E. coli* strains are harmless commensal organisms, certain encapsulated strains are considered invasive pathogens. Infection results in a diversity of clinical symptoms, including septicaemia, urinary-tract infections and neonatal meningitis. The bacterial strain *E. coli* K5 produces a capsule polysaccharide, composed of a D-glucuronic acid-*N*-acetyl-D-glucosamine (GlcA β -GlcNAc)_n polymer, identical with the unmodified mammalian glycosaminoglycan precursor in heparin/heparan sulphate biosynthesis [3] also called *N*-acetylheparosan. The *E. coli* K5 capsule gene cluster contains genes encoding KfiA, KfiB, KfiC and KfiD proteins. The assembly of *E. coli* K5 capsule polysaccharide requires the concerted action of the KfiA and KfiC proteins [4]. The genes *kfiA* and *kfiC* encode glycosyltransferases that add alternating GlcA and GlcNAc to the non-reducing end of the K5 polysaccharide [4] (Scheme 1). The *Pasteurella multocida* Type D strain, which recently has been shown to synthesize *N*-acetylheparosan, utilizes, in contrast with *E. coli* K5, only a single enzyme in the polymerization reaction [5]. The role of KfiB is not known, but mutations in *kfiB*, as in any of the other genes in the cluster, abolish K5-polysaccharide synthesis [6]. The *kfiD* gene encodes UDP-glucose dehydrogenase (UDPGDH), which oxidizes UDP-glucose to UDP-GlcA (Scheme 1).

The UDPGDH enzyme cDNA has been cloned and characterized from several animal, plant and bacterial sources. The eukaryotic enzyme is a homohexamer composed of a trimer of dimers of 52 kDa subunits [7], whereas the bacterial UDPGDH is

reported to be active as a homodimer of 47 kDa subunits in *E. coli* [8] and as a monomer in group A streptococci [9]. The conserved primary sequences of the prokaryotic and eukaryotic UDPGDH enzymes suggest that they are likely to use a common catalytic mechanism [10,11]. In many bacterial strains, UDPGDH provides UDP-GlcA necessary for the synthesis of capsular polysaccharide [1]. In mammalian cells UDP-GlcA has a major biological role as an essential building block of glycosaminoglycans (heparan sulphate, chondroitin sulphate and hyaluronan). It has been proposed that glycosaminoglycan biosynthesis is regulated through the availability of UDP-GlcA, which, in turn, depends on UDPGDH activity [12,13]. Since the mammalian and bacterial glycosyltransferases have similar substrate specificities *in vitro*, the chain-elongation processes of heparan sulphate and K5 polysaccharide are believed to be analogous [14]. Using *E. coli* as a model system, we evaluated how K5-capsular-polysaccharide biosynthesis is influenced by the availability of UDP-GlcA. In the present paper we demonstrate that overexpression of UDPGDH results in an increased formation of UDP-GlcA, but, paradoxically, also to a decreased synthesis of the K5 polysaccharide. Our results provide novel information as to the influence of the UDPGDH activity levels on glycosaminoglycan biosynthesis in bacteria.

MATERIALS AND METHODS

Materials, bacterial strains and plasmids

D-[U-¹⁴C]Glucose was from Amersham Biosciences and New England Nuclear (NEN) (300 mCi/mmol). Mono Q (HR 5/5), DEAE-Sephacel, Sepharose CL-2B, PD10 (Sephadex G-25)

Abbreviations used: BCA, bicinechonic acid; GlcA, D-glucuronic acid; GlcNAc, *N*-acetyl-D-glucosamine (2-deoxy-2-acetoamido-D-glucose); IPTG, isopropyl β -D-thiogalactoside; p[NH]ppA, adenosine 5'-[β , γ -imido]triphosphate; UDPGDH, UDP-glucose dehydrogenase.

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Scheme 1 K5-polysaccharide biosynthesis

UDPGDH (KfiD) irreversibly oxidizes UDP-Glc to UDP-GlcA. KfiA and KfiC extend the polysaccharide chain by the alternating additions of GlcA and GlcNAc from the corresponding UDP-sugars to the non-reducing end of the growing chain.

and Superose 6 (HR 10/30) were from Amersham Biosciences. D-Glucurono- γ -lactone was from Merck. Heparitinase I (EC 4.2.2.8) and heparinase (EC 4.2.2.7) were purchased from Seikagaku Corporation (Tokyo, Japan). All other reagents used were from Sigma unless stated otherwise.

E. coli strain JM 109 (DE3) (Promega), carrying pPC6, a plasmid that constitutively expresses the K5 capsule gene cluster [15], was transformed either with the plasmid pGPR 105 containing the *kfiD* gene, encoding the UDPGDH enzyme, subcloned in the plasmid vector pGEM [15] (overexpressing strain) or with the pGEM vector alone (control strain). All transformations were carried out using the CaCl_2 method. The expression of the *kfiD* gene, under control of the *lac* operon, was induced with 0.5 mM IPTG (isopropyl β -D-thiogalactoside). The bacteria were grown at 37 °C in Luria-Bertani medium (Qbiogene, Carlsbad, CA, U.S.A.) supplemented with 35 $\mu\text{g}/\text{ml}$ chloramphenicol and 100 $\mu\text{g}/\text{ml}$ ampicillin.

Assay for UDPGDH activity

Bacterial cultures of overexpressing and control strains were grown at 37 °C to an attenuation (D_{600}) of 0.6. Protein expression was induced with 0.5 mM IPTG for 30 min and then 1 mM Glc was added. After a further 15 min growth, bacteria were harvested by centrifugation (2000 g , 10 min, 4 °C). The resulting pellet was washed with sodium phosphate buffer and the bacteria were lysed using the French press. Insoluble debris was removed by centrifugation at 100 000 g for 1 h. The protein content of the supernatant was measured with the BCA (bicinchoninic acid) protein determination kit (Pierce).

The determinations for UDPGDH activity were essentially as in [16]. Standard determinations for UDPGDH activity were carried out in 25 μl of 10 mM Tris/HCl, pH 8.7, containing 0.16 μCi

of UDP-D- $[^{14}\text{C}]$ glucose (Amersham Biosciences), 0.5 mM UDP-glucose, 0.5 mM NAD^+ , 1 mM p[NH]ppA (adenosine 5'- $[\beta,\gamma\text{-imido}]$ triphosphate) and 1 $\mu\text{g}/\text{ml}$ pepstatin. To initiate enzyme reaction approx. 3 μg of supernatant protein was added to the mixture, which was then incubated for 20 min at 30 °C. The reaction was terminated by freezing. The resultant UDP- $[^{14}\text{C}]$ GlcA was separated from unreacted UDP- $[^{14}\text{C}]$ glucose by anion-exchange chromatography on a MonoQ column eluted with a linear gradient extending from 50 to 240 mM NaCl. Activity was expressed as c.p.m./mg of protein and was calculated from the radioactivity of the measured product area after subtraction of the background radioactivity of an incubation mixture lacking added protein.

Isolation of metabolically labelled UDP-GlcA

To determine the influence of UDPGDH overexpression on UDP-GlcA production, bacterial strains were metabolically labelled with $[^{14}\text{C}]$ glucose (Amersham Biosciences). At 30 min after IPTG induction, 1 ml bacterial cultures were given glucose (final concentrations 0.5 or 1 mM) mixed with 10 μCi of $[^{14}\text{C}]$ glucose and incubated for 15 min at 37 °C. The incubation was stopped by cooling on ice, and the bacteria were pelleted by centrifugation at 4 °C at 9500 g for 10 min. The cell pellet was washed with 50 mM Tris/HCl, pH 7.2, centrifuged again, and, after removal of the supernatant, the cell pellet was collected and frozen at -20 °C. Before lysis of the cell pellet, 1 mM p[NH]ppA, 0.5 mM UDP-GlcA and 0.5 mM UDP-GlcNAc were added as carriers, together with UDP- $[^3\text{H}]$ GlcA (120 $\times 10^3$ c.p.m.) as an internal reference standard. The cell pellet was lysed by addition of 200 μl of B-PER[®] Bacterial Protein Extraction Reagent (Pierce) according to the manufacturer's protocol and the membranes removed by centrifugation at room temperature (16000 g for

5 min). The membrane-free supernatant was applied to a 1 ml DEAE-Sephacel ion-exchange column equilibrated in 50 mM acetate buffer, pH 4.0. The column was washed twice with 1 ml portions of the same acetate buffer, followed by three successive 1 ml washes with deionized water. The nucleotide sugars were eluted with 0.2 M NH_4HCO_3 , evaporated, and further purified by descending paper chromatography in 95% ethanol/1 M sodium acetate (7:3, v/v), pH 5.0, for 20 h. Material which co-migrated with the authentic UDP-GlcA was eluted with water and subjected to anion-exchange chromatography on MonoQ. Material eluted in the position of the authentic UDP-GlcA was quantified by liquid-scintillation counting, and the amount of cellular UDP-GlcA determined after correction for losses calculated from the difference in reference UDP-[^3H]GlcA radioactivity at the beginning and at the end of the purification.

Isolation of [^{14}C]K5 polysaccharide

K5 polysaccharide was metabolically labelled by incubating the bacterial strains with [^{14}C]glucose essentially as described above, with a 1 mM glucose concentration. After incubation for 15 min, duplicate samples (200 μl) were taken from each incubation, immediately put on ice and then centrifuged (5500 g for 10 min at 4 °C). The membrane pellet was dissolved in ethanol and sonicated until homogeneity was achieved. After addition of carrier K5 polysaccharide (0.2 mg), the mixture was dried, washed twice with ethanol, followed by one acetone wash, and, finally, incubated in 1 M MgCl_2 at 37 °C for at least 3 h. After centrifugation (5500 g, 10 min), the supernatant was applied to an ion-exchange DEAE-Sephacel column in 50 mM sodium acetate, pH 4.0, containing 50 mM NaCl. Labelled K5 polysaccharide was eluted with 0.5 M NaCl in the same buffer. The DEAE-Sephacel eluates were desalted on PD10 columns eluted with 10% (v/v) ethanol, dried, and resuspended in water. [^{14}C]-labelled K5 polysaccharide was further separated from unrelated labelled material by gel filtration on a Superose 6 HPLC column eluted in 1 M NaCl/0.1% Triton X-100/10 mM phosphate buffer, pH 8.0, at a flow rate of 0.5 ml/min. Fractions containing K5 polysaccharide were identified by sensitivity to digestion with the K5 bacteriophage (see below).

Enzymic digestions

Digestion with the K5 bacteriophage, which contains a K5-polysaccharide-specific lyase [17], was carried out in 50 mM Tris (pH 8.0)/3 mM sodium acetate/0.1 mg/ml BSA, containing saturating amounts of the bacteriophage [18]. Heparitinase I digestion was carried out according to the instructions of the manufacturer, with the following exceptions: acetate buffer was exchanged for 0.1 M Tris, pH 7.2, and BSA was omitted from the incubation mixture. Both changes were performed to reduce background absorbance at 232 nm. The enzyme mixtures were incubated for 2 h or overnight at 37 °C.

Isolation and quantification of unlabelled K5 polysaccharide

To isolate the total cellular K5 polysaccharide pool, bacterial cultures of three separate colonies were grown as described above. Protein expression was induced with 0.5 mM IPTG for 30 min and then 1 mM glucose was added. Aliquots of 100 ml were collected from each sample 30 min after the addition of IPTG (first time point) and 30 min after glucose addition (second time point). The collected samples were treated essentially as the metabolically labelled K5 polysaccharide, except that the addition of carrier K5 polysaccharide and the gel-chromatography step

were omitted. The eluate from the DEAE-Sephacel column was desalted on a PD10 column eluted with 10% (v/v) ethanol. The amounts of K5 polysaccharide in the purified samples were determined both colorimetrically with the carbazole assay [19] and enzymically by digestion with heparitinase I. Enzyme digestion was performed in microcuvettes sealed with Parafilm M[®] in a total volume of 200 μl containing 2 munits of heparitinase I. The linearity of the measurements was determined using standard D-glucurono- γ -lactone for the carbazole reaction and a purified K5 preparation for the heparitinase digestion. Experiments with non-transformed *E. coli* JM 109 (DE3), which does not synthesize K5 polysaccharide, showed negligible background levels in both assays. The amount of K5 polysaccharide was determined as the difference in the amount of polysaccharide at the first and second time points, divided by the protein concentration of the latter time point. Protein concentrations, determined by the BCA method, varied between 0.2 and 0.3 mg/ml in the different incubations.

Size analysis of [^{14}C]-labelled K5 polysaccharide

The size distribution of purified [^{14}C]-labelled K5 polysaccharide from control and UDPGDH-overexpressing strains was analysed by gel chromatography on a Sepharose CL-2B column (1 cm $\times$ 87 cm). Separations were performed in 50 mM Tris/HCl (pH 8.0)/1 M NaCl at a flow rate of 20 ml/h. Fractions (1 ml each) were collected and analysed by liquid-scintillation counting. Size-defined hyaluronan standards of 430 and 120 kDa (from Dr P. Heldin, Department of Medical Biochemistry and Microbiology, Biomedical Center, Uppsala, Sweden) were used to calibrate the column.

RESULTS

Effect of UDPGDH transformation on enzyme activity and product formation

E. coli strain JM 109 (DE3) (pPC6), which constitutively expresses all the enzymes required for K5 biosynthesis, was transformed with either plasmid pGPR105 containing an extra IPTG-inducible copy of the *E. coli* K5 *kfiD* gene [15] or the corresponding pGEM vector plasmid. The UDPGDH activity was assessed *in vitro* by measuring the formation of UDP-[^{14}C]GlcA from UDP-[^{14}C]glucose (see the Materials and methods section). Protein extracts from UDPGDH-overexpressing cultures displayed an approx. 15-fold increase in UDPGDH activity compared with that of the control (Figure 1). UDPGDH activity levels varied between 0.7 and 1.3 nmol/min per mg of protein for the control strain and 14 and 17 nmol/min per mg of protein for the overexpressor.

To determine the effect of overexpression on the production of UDP-GlcA in living cells we metabolically labelled bacterial cultures with [^{14}C]glucose (see the Materials and methods section). The synthesized [^{14}C]-labelled UDP-GlcA was purified by DEAE-Sephacel chromatography followed by descending paper chromatography and finally quantified by anion-exchange chromatography. The yields of UDP-[^{14}C]GlcA were approximately three times higher with UDPGDH-overexpressing clones (54×10^3 and 55×10^3 d.p.m./mg of protein for two different clones) than with corresponding control clones (18×10^3 and 13×10^3 d.p.m./mg of protein for two different clones). The lower increase in UDP-GlcA formation, about three-fold as compared with the 15-fold increase in enzyme activity, could have several causes. The situation could, for example, arise from limiting substrate amounts or reflect *in vivo* inhibition of the enzyme which is known to be feedback-inhibited by its own product [20].

Figure 1 UDPGDH-activity assay

Cell lysates from UDPGDH-overexpressing (○, ●) and control (□, ■) strains were assayed for UDPGDH activity. Cultures from separate colonies were harvested, solubilized and centrifuged. The resulting protein supernatant was incubated with UDP- ^{14}C glucose. The newly formed UDP- ^{14}C GlcA was separated from non-utilized UDP- ^{14}C glucose by anion-exchange chromatography on a MonoQ column (see the Materials and methods section). An incubation without protein addition (x) was used to determine the background level of radioactivity eluted as UDP- ^{14}C GlcA.

Effect of UDPGDH overexpression on K5-polysaccharide production

The analysis described above showed that overexpression of UDPGDH resulted in increased formation of UDP-GlcA. We next examined if the increased UDP-GlcA production influenced the biosynthesis of the K5 polysaccharide by metabolically labelling overexpressing and control clones with ^{14}C glucose. The resultant labelled macromolecules were fractionated by Superose 6 gel chromatography. Three distinct peaks were observed in the elution pattern from both the control strain and the overexpressing strain (Figures 2A and 2B). The high-molecular-weight material eluting in peak I was completely degraded to smaller fragments by digestion with the K5 bacteriophage, which specifically cleaves K5 polysaccharide, producing mainly deca- to hexa-mers (Figures 2A and 2B). Also, heparitinase digestion completely degraded the peak I material, but to disaccharides. Peak II material from control and overexpressing strains were purified and digested with heparitinase. Analysis of the digest showed no significant formation of smaller digestion products (results not shown). Gel chromatography using a column separating low-molecular-mass compounds revealed that the size of peak III material corresponded to that of a disaccharide. The possibility that peaks II and III represented K5-polysaccharide precursor molecules was investigated by pulse-chase experiments. No successive transfer of material from peaks III and II to peak I was found. Furthermore, non-transformed bacterial cultures deficient in K5-polysaccharide synthesis also yielded peaks II and III, but not the high-molecular-mass peak I (Figure 2C). Taken together, these results indicate that only peak I represents K5 polysaccharide, whereas peaks II and III represent unidentified labelled products, recovered in the purification. Unexpectedly, the yields of ^{14}C -labelled K5 polysaccharide, i.e. material susceptible to digestion with the K5 bacteriophage, decreased about 3-fold in the UDPGDH-overexpressing strain. Incorporation of ^{14}C into K5 polysaccharide, determined as mean values of duplicate determinations from three separate cultures, was 36 ± 5 c.p.m./ μg of protein and 114 ± 15 c.p.m./ μg of protein for the overexpressing and control strains respectively (Table 1).

Figure 2 Analysis of K5 polysaccharide on a Superose 6 column

Metabolically labelled K5 polysaccharide was purified from bacterial cultures as described in the Materials and methods section. The resulting labelled polysaccharides from the control (A) and the overexpressing (B) strains were subjected to chromatography on a Superose 6 column, before (open symbols) and after (closed symbols) digestion with the K5 phage lyase. The retarded components corresponding to peaks II and III have not been identified. They were also found in the parent non-transformed bacterial strain that is unable to produce K5 polysaccharide (C) and represent low-molecular-mass components, unrelated to K5 polysaccharide synthesis (see the Results section).

We also assayed the *in vitro* transferase activities of the K5 elongation enzymes [4]. No significant change in transferase activity was observed, suggesting that overexpression of UDPGDH did not affect the polymerization capacity (results not shown).

In metabolically radiolabelled cultures, overexpression of UDPGDH could possibly cause changes in the specific radioactivity of ^{14}C glucose, and thus lead to erroneous conclusions about the amount of K5 polysaccharide synthesized. Therefore we determined the amount of cellular K5 polysaccharide both chemically and enzymically. The K5 content in the overexpressing

Table 1 Effect of UDPGDH overexpression on K5-polysaccharide formation

Standard incubation mixtures (see the Materials and methods section) from control and UDPGDH-overexpressing strains were analysed for K5-polysaccharide content. The values indicated after metabolic labelling are given as means from three different cultures $\pm$ sample S.D. and represent radioactivity incorporated into peak I in Figure 2. The data obtained by carbazole analysis and heparitinase I digestion (A_{232}) are means $\pm$ sample S.D. from three separate experiments.

Clone	Amount of K5 polysaccharide		
	Metabolic labelling (c.p.m./ μ g of protein)	Carbazole (ng/mg of protein)	Heparitinase (ng/mg of protein)
Control	114 $\pm$ 15	1050 $\pm$ 265	1335 $\pm$ 295
Overexpressor	36 $\pm$ 5	320 $\pm$ 80	405 $\pm$ 185

and control strain was calculated by comparing the results from uronic acid determination by the carbazole method and the change in absorbance due to formation of an unsaturated uronic acid residue after heparitinase I digestion as described in the Materials and methods section. The results from both measurements were highly similar and agreed well with the results obtained after metabolic labelling of K5 polysaccharide. Thus overexpression of UDPGDH indeed resulted in an approx. 3-fold reduction in K5 production (Table 1).

K5 polysaccharide chain length

Our results showed that the amounts of K5 polysaccharide formed were reduced when UDPGDH activity was raised. This reduction could result from initiation of fewer chains, from premature termination of chain polymerization or from decreased polymerization rate. The size of the purified 14 C-labelled K5 polysaccharide (peak I material in Figure 2) was investigated by gel chromatography on a Sepharose CL-2B column. The K5 polysaccharide isolated from control and overexpressing clones showed virtually identical elution patterns, suggesting that overexpression had resulted in synthesis of fewer, but equally sized, chains (Figure 3).

DISCUSSION

UDPGDH forms UDP-GlcA in plants, bacteria and animals, thereby furnishing an essential structural component for polysaccharide synthesis. The kinetic properties of the enzyme have been thoroughly studied [9,20,21] and the crystal structures of UDPGDH from *S. pyogenes* in ternary complexes with UDP-xylose/ NAD^+ and UDP-GlcA/ NAD^+ have been determined [22]. However, despite the key role of UDPGDH in generating the UDP-GlcA substrate for the synthesis of bacterial polysaccharides and mammalian glycosaminoglycans, little is known about the influence of the enzyme activity on polysaccharide synthesis. Unexpectedly, our results in this report show that increased levels of UDP-GlcA, generated by overexpression of the *E. coli* UDPGDH in a K5-polysaccharide-producing *E. coli* strain, resulted in decreased production of the bacterial capsular polysaccharide. Furthermore, our results showed that the chain length was largely unaffected, such that the reduction would mainly be due to synthesis of fewer chains. Chemical and enzymic analysis of the cellular K5-polysaccharide pool gave similar results to metabolic radiolabelling, showing that our data were not influenced by changes in the specific radioactivity of the labelled UDP-sugar precursor.

Figure 3 Analysis of purified K5 polysaccharide by gel chromatography

14 C-labelled K5 polysaccharide obtained from metabolically labelled UDPGDH-overexpressing (●) or control (□) bacterial cell cultures was purified on Superose 6. Fractions corresponding to peak I in Figure 2 were pooled and analysed by chromatography on a Sepharose CL-2B column. Arrows indicate the elution positions of size-defined hyaluronan standards. *Serratia marcescens* and [^3H]water were used as markers for the void volume (V_0) and total volume (V_t) respectively.

The catalytic efficiency of the GlcA and GlcNAc transferases, assayed *in vitro* using exogenous substrates, was not significantly affected by overexpression of UDPGDH. This finding suggests that the reduction in the amount of K5 polysaccharide synthesized was not due to loss of either GlcA or GlcNAc transferase activity *per se*. However, the possibility cannot be completely excluded that the overexpression of UDPGDH causes a disturbance in the complex of K5-assembling proteins at the inner membrane, thus disturbing the polymerization activity. The decrease in the amount of K5 polysaccharide synthesized could reflect a reduction in the rate of initiation of new chains and/or in the rate of chain elongation. UDPGDH produces UDP-GlcA from UDP-Glc, which, in turn, is derived from glucose 6-phosphate. Overexpression of UDPGDH and the resultant increase in UDP-GlcA may have the net effect of decreasing the pool of glucose 6-phosphate, which is in equilibrium with fructose 6-phosphate, a precursor of UDP-GlcNAc. Therefore, paradoxically, one effect of increasing the synthesis of UDP-GlcA may be to reduce the levels of UDP-GlcNAc. This could have two effects on the synthesis of the K5 polysaccharide. First, it is known that the initiation of K5 biosynthesis involves the addition of GlcNAc to a membrane acceptor, which is subsequently extended by the processive transferase KfiA and KfiC enzymes (M. Pourhossein and I. Roberts, unpublished work). As such, a decrease in the intracellular pools of UDP-GlcNAc could reduce the rate of initiation of K5 biosynthesis and the number of K5 chains made. Secondly, a reduction in the intracellular pools of UDP-GlcNAc could result in the availability of UDP-GlcNAc becoming rate-limiting for the KfiA transferase, such that the rate of chain elongation is reduced in the presence of excess UDP-GlcA.

In addition to any effects as a consequence of UDP-GlcNAc availability, it is also possible that the increased levels of UDP-GlcA may directly interfere with the chain-elongation process. Previous studies with a heparin-producing mouse mastocytoma

microsomal fraction have demonstrated that the relative proportions of UDP-GlcNAc and UDP-GlcA affect the rate of polymerization and the chain length [23]. When the two sugar nucleotides were added at widely different concentrations, a curtailed polymerization was observed. The length of the heparin precursor chains was directly proportional to the rate of polymerization. The inhibition was believed to be due to competition between the two UDP-sugars for nucleotide binding sites on the glycosyltransferases. We have previously found that the mammalian and bacterial polymerization enzymes show similar kinetics and substrate specificities [11]. Therefore a consequence of an increase in the levels of UDP-GlcA within the cell may be that the excess UDP-GlcA is able to transiently occupy the binding site for UDP-GlcNAc in KfiA and thus function as a competitive inhibitor and thereby lower the K5 production.

The activity of UDPGDH has been proposed to be rate-limiting in glycosaminoglycan synthesis in mammalian cells [12,13]. Our results show that, in *E. coli*, this is not the case, and that increasing UDP-GlcA concentrations does not increase K5-polysaccharide biosynthesis. The production of an extracellular polysaccharide capsule is a complex process, involving sugar metabolism, sugar nucleotide supply, enzyme kinetics and membrane transport proteins. Regulated increase in K5 capsular polysaccharide production is likely to require co-ordinate changes in a number of these steps.

We thank Dr Pasqua Oreste (Ricerche Sperimentali Montale, Montale, Italy) for providing standard K5 polysaccharide and Dr Paraskevi Heldin (Department of Medical Biochemistry and Microbiology, Biomedical Center, Uppsala, Sweden) for kindly providing hyaluronan standards. We are indebted to Professor Ulf Lindahl (Department of Medical Biochemistry and Microbiology, Biomedical Center, Uppsala, Sweden) for valuable support and discussions. This work was supported by grant no.13401 from the Swedish Research Council, The Swedish Foundation for Strategic Research (the programme 'Glycoconjugates in Biological Systems') Polysackaridforskning AB (Uppsala, Sweden) and Konung Gustav V 80-års fond.

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Received 7 March 2003/20 May 2003; accepted 30 May 2003

Published as BJ Immediate Publication 30 May 2003, DOI 10.1042/BJ20030365