Irreversible Glucuronyl C5-Epimerization in the Biosynthesis of Heparan Sulfate*

Åsa Hagner-McWhirter#, Jin-Ping Li**, Stefan Oscarson° and Ulf Lindahl#

#Department of Medical Biochemistry and Microbiology, Uppsala University, The Biomedical Center, Box 582, S-751 23 Uppsala, Sweden and °Department of Organic Chemistry, Arrhenius Laboratory, Stockholm University, S-106 91Stockholm, Sweden.

**To whom correspondence should be addressed

Address: Department of Medical Biochemistry and Microbiology, University of Uppsala, The Biomedical Center, Box 582, SE-751 23 Uppsala, Sweden

Tel: 46-18-4714241

Fax: 46-18-4714209

E-mail: jin-ping.li@imbim.uu.se

Running title: Glucuronyl C5-Epimerization in the Biosynthesis of Heparan Sulfate
ABSTRACT

Glucuronyl C5-epimerase catalyzes the conversion of D-glucuronic acid to L-iduronic acid units in heparan sulfate biosynthesis. Substrate recognition depends on the N-substituent pattern of the heparan sulfate precursor polysaccharide, and requires the adjacent glucosamine residue toward the non-reducing end to be N-sulfated. Epimerization of an appropriately N-sulfated substrate is freely reversible in a soluble system, equilibrium favoring retention of D-gluco configuration (Hagner-McWhirter, Å., Li, J.-P., and Lindahl, U. (2000) Biochem. J. 347, 69-75). We studied the reversibility of the epimerase reaction in a cellular system, by incubating human embryonic kidney 293 cells with D-[5-3H]galactose. The label was incorporated with glucuronic acid units into the heparan sulfate precursor polysaccharide, and was lost upon subsequent C5-epimerization to iduronic acid. However, analysis of oligosaccharides obtained by deaminative cleavage of the mature heparan sulfate chains indicated that all glucuronic acid units retained their C5-3H label, irrespective of whether they had occurred in sequences susceptible or resistant to the epimerase. All 3H label of the final products resisted incubation with epimerase in a soluble system, apparently due to blocking O-sulfate groups. These results indicate that glucuronic acid C5-epimerization is effectively irreversible in vivo, and argue for a stringent organization of the biosynthetic machinery.
INTRODUCTION

Heparan sulfate (HS)\(^1\) proteoglycans, on cell surfaces and in the extracellular matrix, have fundamental roles in development, homeostasis, and disease throughout the animal kingdom (1). Their biological activities are generally ascribed to binding of HS glycosaminoglycan chains to proteins (growth factors, receptors, enzymes and their inhibitors, matrix proteins, microbial proteins etc.) in more or less specific fashion. HS-protein interactions are largely ionic, selectivity of binding being dictated by the distribution of negatively charged carboxylate and sulfate groups along the HS chain (2). These features are largely established during HS biosynthesis, that appears to be regulated such that different types of cells, possibly also cells exposed to different stimuli, generate HS of different structures (3,4). Notably, however, further structural diversity may be introduced through endosulfatase action after completed biosynthesis (5).

HS biosynthesis, a Golgi process, is initiated by the generation of a linkage tetrasaccharide, GlcAß1,3Galß1,3Galß1,4Xyl-, onto a serine residue in the proteoglycan protein core (6,7). N-Acetylglucosamine (GlcNAc) and D-glucuronic acid (GlcA) residues are then transferred from the corresponding UDP-sugars to the non-reducing ends of nascent polysaccharide chains, concomitant with further modification of these polymers (3). N-Deacetylation and N-sulfation of GlcNAc units thus precedes C5-epimerization of GlcA to l-iduronic acid (IdoA) residues that in turn is followed by O-sulfation at different positions. Only a fraction of potential target units is attacked in each modification step, resulting in domain-type variability.
as well as more subtle structural diversity. The overall process is regulated in part by the substrate specificities of the various enzymes involved, all of which have now been cloned and expressed. However, additional regulatory mechanisms, still poorly understood, are required to explain the display of saccharide epitopes in HS chains. Our understanding of the regulation of HS biosynthesis has been hampered by the lack of information regarding the organization of the biosynthetic machinery. The enzymes involved are membrane-bound, with N-terminal transmembrane domains and C-terminal, lumenal catalytic domains, and are assumed to operate in closely concerted mode. Still, little is known regarding their topological and functional interaction, or regarding the kinetics of the various steps of HS chain formation and modification. The process has been described in terms ranging from kinetically controlled competition between different enzymes for common polymer substrates (8) to more strictly molded assembly lines (9).

The present work was undertaken to gain further insight into the biosynthetic process, by exploiting the properties of a unique step, i.e. the C5-epimerization of GlcA to IdoA residues. Solubilized (or recombinant) epimerase catalyzes a freely reversible reaction, involving abstraction of the C5-hydrogen of a target hexuronic acid (GlcA or IdoA) followed by reinsertion (presumably of a proton) to yield the alternative C5-configuration (Fig. 1). Incubation with epimerase of an appropriate polysaccharide substrate containing C5-\(^{3}H\)-labeled hexuronic acid residues will release the label from GlcA as well as IdoA units; conversely, incubation of unlabeled substrate in \(^{3}H_{2}O\) leads to incorporation of label into both epimers (10). Is epimerization reversible also during HS biosynthesis in an intact cell? The answer to this question bears not only on the kinetics of epimerization in the Golgi, but may also provide clues to the overall organization of the HS biosynthetic apparatus. We have
approached this problem by allowing cells to incorporate C5-3H-labeled GlcA residues into HS chains under formation, followed by analysis of the product with regard to residual label.

EXPERIMENTAL PROCEDURES

Materials - D-[U-14C]Gal (293 mCi/mm), D-[U-14C]GlcN hydrochloride (311 mCi/mm) and NaB\textsubscript{3}H\textsubscript{4} (55 Ci/mm) were purchased from Amersham Biosciences (Uppsala, Sweden). GlcA C5-epimerase was purified from bovine liver as described (11). Pre-packed PD-10 columns, DEAE-Sephacel and Sephadex G-50 were obtained from Amersham Biosciences. Bio-gel P-10 was purchased from Bio-Rad (Richmond, USA). Chondroitin ABC lyase (EC 4.2.2.4) and heparitinase I (EC 4.2.2.8) were obtained from Seikagaku (Japan). β-D-Glucuronidase from bovine liver (type B-10) was from Sigma.

Synthesis of [5-3H]Gal – A 5-ketohexofuranose 1 used as starting material was generated from 1,2-O-isopropylidene-[β]-d-galactofuranose (12) (75 mg, 0.34 mmol), following activation with bis(tri-n-butyltin) oxide and subsequent oxidation with Br\textsubscript{2} as described for the corresponding gluco-derivative (13) (Fig 2). The 5-oxo derivative 1 (52 mg, 70%) was obtained after silica gel chromatography (CHCl\textsubscript{3}:MeOH 19/1). NMR: $^{13}$C (D\textsubscript{2}O), d 25.14, 25.2, 66.2, 75.7, 85.0, 90.4, 106.7, 113.8, 209.4; $^1$H, d 1.29 (s, 3H), 1.37 (s, 3H), 4.43 (d, $J$=20 Hz, 1H, H-6a), 4.56 (s, 1H, H-4), 4.57 (d, $J$=3.66 Hz, 1H, H-2), 4.79 (s, 1H, H-3), 4.90 (d, $J$=20 Hz, 1H, H-6b), 6.03 (d, $J$= 3.66 Hz, 1H, H-1).
[5-3H]Gal was generated by reduction of 1 with NaB3H4 followed by mild acid hydrolysis of the isopropylidene acetal (Fig. 2). A sample of 1 (2 mg dissolved in 40 µl water) was added to 10 ml of methanol together with 50 mCi NaB3H4 and the mixture was incubated for 1 h at room temperature. After drop wise addition of 100 µl 1 M HCl to give pH 2-3 (in a fume hood), the mixture was incubated for another 30 min and was then heated to 55°C and evaporated to dryness. Addition of 20 ml methanol followed by evaporation to dryness as above was repeated 5 times. The reduction products were dissolved in 3 ml of water and were then analyzed by paper chromatography on Whatman no. 1 paper in butanol/ethanol/water (10:3:5 by volume), along with D-[U-14C]Gal as standard (running time 22 hours). A major component (65% of total radioactivity) appeared at the migration position of Gal, whereas 19 % migrated as altrose, another expected reaction product (L-enantiomer, Fig. 2). The yields of D-[5-3H] Gal in two separate preparations were 5.4 mCi and 4.6 mCi. The two batches were combined before use.

Metabolic Labeling of Cells – Human embryonic kidney HEK 293 cells were cultured in Dubecco’s MEM with Glutamax-II (Gibco, Life Technologies) using standard procedures at 37°C. The medium contained 4.5 g/L glucose and was supplemented with 10 % heat-inactivated fetal calf serum (Gibco), 60 µg/mL penicillin and 50 µg/mL streptomycin sulfate. When cells seeded in six 175 cm² flasks had reached ~80 % confluency, the medium was replaced with 15 mL medium supplemented as above, but containing only 1 g/L glucose and, in addition, 100 µCi/mL [5-3H]Gal and 5 µCi/mL [U-14C]GlcN (five 175 cm² flasks). To the sixth 175 cm² flask was instead added medium as above with 100 µCi/mL [5-3H]Gal but 10 µCi/mL [U-14C]Gal substituted for the labeled GlcN. The cells were metabolically labelled for 48 h.
Purification of Metabolically Radiolabeled HS – After labeling, the media were collected and the cell layers lysed by adding to each flask 10 ml of cold PBS containing 1% Triton X-100. All samples were kept on ice on a rocking plate for 1 h. Urea was added to a final concentration of 8 M to both the media and cell lysates and the mixtures were boiled for 15 min. The samples were then centrifuged at 11 000 x g at 4°C for 20 minutes. Supernatants from media and cell lysates were mixed and an equal volume of 0.1 M NaAc, pH 4 was added. The samples were applied on DEAE-Sephacel columns (10 ml) equilibrated with 50 mM NaAc buffer containing 2 M urea and 0.2 M NaCl, pH 4. After extensive washing (buffer as above), the columns were eluted with a linear gradient of 0.2 M - 1 M NaCl in the same buffer (total volume 100 ml). Fractions containing proteoglycans eluted between 0.5 and 0.75 M NaCl were pooled, dialysed against water and lyophilized.

The proteoglycans were further purified by gel chromatography on a Sephadex G-50 column (1 cm x 90 cm) in 0.2 M NH₄HCO₃. The proteoglycans, excluded from the gel, were pooled and desalted by lyophilization. The samples were treated with 1 U/ml of chondroitinase ABC. The HS chains were then released from proteoglycan core proteins by 8-elimination in 0.5 M NaOH for 16 hours at 4°C followed by neutralization with 4 M acetic acid.

The free HS chains were recovered by absorption to a 1-ml DEAE-Sephacel column equilibrated in 0.2 M NH₄HCO₃. The columns were washed with 0.2 M NH₄HCO₃, followed by 0.25 M NaCl, 0.2 M NH₄HCO₃ and were finally eluted with 2 M NH₄HCO₃. This purification procedure yielded pure HS as it was quantitatively degraded by treatment with heparitinase I (not shown).
**HS Structural Analysis** – Metabolically labeled HS chains (generally 400 x 10^3 dpm of ^3H) were cleaved at the sites of N-sulfated GlcN units by treatment with nitrous acid at pH 1.5 followed by reduction with NaBH₄ (14). Under these conditions GlcNSO₃ units are deaminated and converted to terminal 2,5-anhydromannitol residues, whereas GlcNAc units remain intact (15). Deamination mixtures were fractionated on a column (1.3 x 185 cm) of Bio-gel P-10 eluted with 0.5 M NH₄HCO₃. Effluent fractions were analyzed for ^3H and ^14C. Fractions corresponding to di- and tetrasaccharides were pooled, desalted by lyophilization and further analyzed separately. O-Sulfated disaccharides were separated by anion-exchange HPLC on a Partisil-10 SAX column (4.6 x 250 mm; Whatman), using a step gradient of KH₂PO₄ (0.012 M, 0.028 M and 0.168 M) at a flow rate of 1 ml/min. Fractions of 1 ml were collected and analyzed for radioactivity. O-sulfated disaccharides were first isolated by preparative high-voltage paper electrophoresis at pH 5.3 (0.083 M pyridine, 0.05 M acetic acid) and were then identified by paper chromatography (ethyl acetate/acetic acid/H₂O, 3/1/1 by volume) (10).

Samples of [^3H]Gal/^14C[GlcN]-labeled and [^3H]Gal/^14C[Gal]-labeled tetrasaccharides (150 x10^3 dpm and 30 x10^3 dpm ^3H, respectively) obtained by Bio-gel P-10 chromatography were digested with 10.4 U β-glucuronidase in 200 µl of 0.05 M NaAc pH 5 at 37°C overnight. Test incubations with appropriate disaccharide standards ascertained that the enzyme preparation contained β-glucuronidase but no α-iduronidase activity (not shown). The digestion products were analyzed by Bio-gel P-10 chromatography as described above.
RESULTS

The course of hexuronyl (HexA) – C5-epimerization in relation to subsequent polymer modification (O-sulfation) reactions in HS biosynthesis was investigated by monitoring the fate of C5-$^3$H atoms introduced with GlcA residues during the polymerization phase of the process. Incorporation of [5-$^3$H]GlcA units was achieved by incubating HEK 293 cells with [5-$^3$H]Gal (predicted to yield UDP-[5-$^3$H]GlcA via conversion to [5-$^3$H]Gal-1-phosphate and UDP-[5-$^3$H]glucose (16). Incubation of the resultant $^3$H-labeled HS with purified soluble epimerase (11) failed to release any significant label from the polysaccharide (data not shown), indicating that all residual [5-$^3$H]GlcA residues in the mature HS were inaccessible to the enzyme. Previous studies have shown that epimerase action is blocked by 2-O-sulfation of potential target HexA units, as well as by 6-O-sulfation of adjacent GlcN residues (17). The $^3$H label thus could reside either in GlcA units never recognized as substrate by the epimerase, or in substrate units blocked by O-sulfation. The latter alternative would apply only if such blocking is favored over reversible C5-epimerization, since a GlcA unit generated by “back-epimerization” of IdoA would be devoid of C5-$^3$H.

Conversely, the occurrence of unlabeled GlcA units in primary epimerase target positions would point to a relatively looser organization of the biosynthetic process, in which C5-epimerization would be allowed to revert to d-gluco configuration before onset of intervening O-sulfation (Fig. 3).

Primary susceptibility of GlcA units in the HS precursor polysaccharide to enzymatic C5-epimerization is dictated by the adjacent N-substituent pattern. The GlcA residues in -GlcNS-GlcA-GlcNS- and -GlcNS-GlcA-GlcNAc- sequences (reducing terminus to the right) thus are recognized as substrate by the epimerase
whereas those in -GlcNAc-GlcA-GlcNAc- and -GlcNAc-GlcA-GlcNS- sequences are not. These various structural constellations can be distinguished by deaminative cleavage of the polysaccharide with nitrous acid, that will attack N-sulfated GlcN units and break the corresponding glucosaminidic linkages, whereas N-acetylated GlcN residues are resistant (18). A HexA located between two GlcNS residues thus will be recovered in a disaccharide deamination product, whereas a -GlcNAc-GlcA-GlcNAc- structure will form part of a larger oligosaccharide. Tetrasaccharides derived from sequences of alternating N-acetylated and N-sulfated disaccharide units are particularly instructive, since the nonreducing-terminal HexA, but not the internal GlcA unit, represents a potential epimerase substrate in the intact parent polysaccharide chain (Fig. 4). We therefore attempted to determine the distribution of $^3$H label between the variously sized deamination products. To enable such comparison, a $^{14}$C reference label was introduced, through addition to the incubation medium of either [U-$^{14}$C]Gal or [U-$^{14}$C]GlcN along with the [5-$^3$H]Gal.

**Distribution of $^3$H Between HS Domains** – HEK 293 cells were metabolically labeled with [5-$^3$H]Gal, [U-$^{14}$C]GlcN or with [5-$^3$H]Gal, [U-$^{14}$C]Gal. Labeled HS was isolated and cleaved at GlcNS residues by treatment with HNO$_2$, and the resultant di- and oligosaccharides were separated by gel chromatography on a Bio-gel P-10 column. The internal GlcA residues of HexA-(GlcNAc-GlcA)$_n$-aMan$_r$ oligosaccharides correspond to units in the intact parent polymer that are not recognized as substrate by the epimerase whereas the nonreducing-terminal HexA residues represent potential target units (Fig. 4) (17). Consistent loss of $^3$H from such target units would result in progressively decreasing $^3$H/$^{14}$C ratios for oligosaccharides of decreasing size. The results shown in Fig. 5 conform to this prediction, irrespective of whether the $^{14}$C
The label was introduced through $[^{14}\text{C}]\text{GlcN}$ (Fig. 5A) or $[^{14}\text{C}]\text{Gal}$ (Fig. 5B). The largest (≥18-mer) fragments were expected to contain labeled Gal residues of the HS-protein linkage region, and were therefore excluded from further calculations. Instead, the $^3\text{H}/^{14}\text{C}$ ratio of the 16-mer was applied as a reference approximating complete retention of $^3\text{H}$, and used to calculate hypothetical ratios for the smaller oligosaccharides assuming complete loss of nonreducing-terminal $^3\text{H}$. Comparison with the experimentally found ratios indicated significant loss of $^3\text{H}$ from all oligosaccharide species (Table I). We therefore proceeded to analyze whether this lack of $^3\text{H}$ was restricted to IdoA units of the HS chain, or included also GlcA units formed by “back-epimerization” of IdoA residues.

**Analysis of Epimerase HexA Target Units** – Disaccharide deamination products are derived from HS sequences composed of consecutive N-sulfated disaccharide units, hence containing HexA units initially all susceptible to epimerase attack (Fig. 4). The disaccharide fractions were separated by anion-exchange HPLC (Fig. 6). As expected, all IdoA-containing species (*peaks 4, 5 and 6*) had lost their C5-$^3\text{H}$ label. By contrast, the $^3\text{H}/^{14}\text{C}$ ratios for the GlcA-containing disaccharides, GlcA2S-aMan$_R$ (*peak 2*), and GlcA-aMan$_R$6S (*peak 3*) were similar to that of the extended N-acetylated 16-mer reference structure, irrespective of whether the $^{14}\text{C}$ was introduced through $[^{14}\text{C}]\text{GlcN}$ (Fig. 6A) or $[^{14}\text{C}]\text{Gal}$ (Fig. 6B), thus demonstrating essentially quantitative retention of C5-$^3\text{H}$ (Table II). Non-O-sulfated disaccharides (6.4 % of total disaccharide units in N-sulfated domains) were isolated by paper electrophoresis and further separated into GlcA-aMan$_R$ and IdoA-aMan$_R$ species by paper chromatography (Fig. 7). Only the former component occurred in significant amounts, again with fully retained $^3\text{H}$ label (Table II). Principally similar results were obtained irrespective of whether the
\[^{14}\text{C}\] reference label had been introduced through \[^{14}\text{C}\]GlcN (Fig. 5A) or \[^{14}\text{C}\] Gal (Fig. 5B; Table II).

Tetrasaccharide deamination products, isolated by gel chromatography (Fig. 5), were digested with \(\beta\)-D-glucuronidase to yield GlcA monosaccharide and GlcNAc-GlcA-aMan\(_R\) trisaccharide, in addition to residual tetrasaccharide (Fig. 8). Most of the tetrasaccharides resisted \(\beta\)-glucuronidase digestion, indicating IdoA in nonreducing-terminal position. However, significant proportions were degraded to free GlcA and trisaccharide\(^3\), and analysis of these fractions showed that the total amounts of \(^3\text{H}\) released as \([^{3}\text{H}]\text{GlcA}\) by \(\beta\)-glucuronidase equalled those recovered in the trisaccharide digestion products (Fig. 8A, B). These findings point to essentially complete retention of \(^3\text{H}\) in GlcA residues that are potentially accessible to the epimerase in the intact polysaccharide substrate, yet retain \(\text{D-gluc}\) configuration and are recovered in nonreducing-terminal position of tetrasaccharide deamination products (Figs. 4, 9). This conclusion is further supported by the \(^3\text{H}/^{14}\text{C}\) ratios of products generated by \(\beta\)-glucuronidase digestion of tetrasaccharide from HS labeled with \([5^{3}\text{H};^{14}\text{C}]\text{Gal}\) (Figs 8B, 9B). The ratio for the released GlcA thus approached that of the trisaccharide product and was almost twice that of the enzyme-resistant, IdoA-containing tetrasaccharide\(^3\).

Susceptibility of GlcA units to the C5-epimerase during the initial phases of HS (and heparin) biosynthesis is dictated by positioning of these units in relation to N-acetyl and N-sulfate substituents. Taken together, the results presented here show that the potentially susceptible residues are either irreversibly converted to IdoA or escape altogether encounter with the enzyme.
DISCUSSION

A wealth of information points to strict control in HS biosynthesis, to yield polysaccharide chains of regulated composition and domain organization. HS preparations isolated from different mammalian organs thus differ in structure, whereas those obtained from the same organs of different individuals appear similar (19-22). The HS generated in a single tissue (human aortic wall) shows gradual structural change with increasing age of the individual (21,22). Immunohistochemical application of various anti-HS antibodies demonstrate cell-specific expression of HS epitopes of different structure (23,24). Regulation presumably applies also to the GlcA-IdoA conversion in HS biosynthesis, judging from the variable content and domain distribution of IdoA units in HS chains. The potential extent of IdoA formation is restricted by the N-substitution pattern that is established through the GlcNAc N-deacetylation/N-sulfation step before (or along with) GlcA C5-epimerization (3). However, structural analysis of HS chains after completed biosynthesis indicates that the potential target GlcA units (in -GlcNS-GlcA-GlcNS- and -GlcNS-GlcA-GlcNAc- sequences) are only partly converted to IdoA. Thus additional regulatory factors must come into play.

Reaction of the appropriate -GlcNS-HexA-GlcNS- substrate with C5-epimerase in a soluble system is freely reversible and yields an equilibrium product containing GlcA and IdoA in approximate 2:1 ratio (10). Yet heparin as well as N-sulfated domains in HS show extended sequences of consecutive IdoA-containing disaccharide units (25). It has been suggested that IdoA generation may be promoted by concomitant O-sulfation, coordinated to block “back-epimerization” to GlcA.
A model of “kinetically controlled” polymer modification predicts that regulation, hence the fine structure of the final HS product, be dictated by the relative amounts of the various enzymes involved (C5-epimerase, O-sulfotransferases) and their substrate specificities. In accord with this proposal, the HexA 2-O-sulfotransferase shows strong preference for IdoA over GlcA target units (28) and HexA2S units are not recognized as substrate by the epimerase (17). Moreover, incubation of microsomal enzymes from a heparin-producing mouse mastocytoma with exogenous polysaccharide substrate resulted in appreciable release of $^3$H from C5-$^3$H-labeled GlcA residues, along with some conversion of GlcA to IdoA. Tritium release exceeded the actual extent of C5-epimerization. Similar incubation in the presence of the sulfate donor, 3'-phosphoadenosyl-5'-phosphosulfate (PAPS) yielded O-sulfated products with significantly increased IdoA contents (29). On the other hand, generation of endogenous, microsomal polysaccharide from the appropriate UDP-sugar precursors and PAPS appeared not to entail $^3$H loss in excess of IdoA formation, suggesting more stringent control of interaction between the C5-epimerase and its substrate (29,30). It was therefore of interest to assess the extent of reversibility of the C5-epimerization reaction during HS biosynthesis in an intact cell.

Synthetic C5-$^3$H-labeled Gal was used as a vehicle for incorporation of [5-$^3$H]GlcA residues into HS precursor polysaccharide in 293 HEK cells, along with either $[^{14}\text{C}]$Gal or $[^{14}\text{C}]$GlcN as markers of the polysaccharide backbone. Analysis of oligosaccharides obtained by deaminative cleavage enabled assessment of $^3$H loss from potential epimerase target sequences (-GlcNS-GlcA-GlcNS-; -GlcNS-GlcA-GlcNAc-) as compared to non-target sequences (-GlcNAc-GlcA-GlcNS-; -GlcNAc-GlcA-GlcNAc-). The $^3$H/$[^{14}\text{C}]$ ratios of variously sized oligomers indicated retention of potentially labile C5-$^3$H atoms (Table I). Moreover, detailed examination of
disaccharide and tetrasaccharide fractions showed the same $^3$H/$^{14}$C ratios for potential target and non-target GlcA units. Thus all residual GlcA units fully retain the $^3$H label, irrespective of position in the HS chain. These findings demonstrate that the GlcA C5-epimerase reaction in HS biosynthesis is irreversible and does not approach equilibrium conditions. Similar conditions may apply to dermatan sulfate biosynthesis (31). The formation of IdoA units is the result of single encounters between susceptible GlcA residues and the epimerase, which are terminated once L-idO configuration has been attained. The (poorly understood) design of the biosynthetic “assembly line” rather than the amounts of the various enzymes is the key factor in control of polymer modification. Indeed, mouse embryos heterozygous with regard to the epimerase expressed about half the enzyme levels of wild-type littermates, yet produced HS of indistinguishable composition (14).

Incubation of the isolated, labeled HS with exogenous epimerase failed to release any significant amounts of $^3$H from the polysaccharide, suggesting that all potential target sites generated during the N-deacetylation/N-sulfation phase of the biosynthetic process had been blocked by subsequent O-sulfation. While this O-sulfation apparently occurred before any reversion of C5-epimerization, its regulatory role is unclear. Analysis of disaccharides derived from the N-sulfated domains indicated that most of the IdoA units were 2-O-sulfated, which could explain resistance toward back-epimerization (Fig. 6). The IdoA residues in domains composed of alternating N-acetylated and N-sulfated disaccharide units, that typically account for about half of the total IdoA in HS chains, are rarely 2-O-sulfated whereas adjacent 6-O-sulfation is common (20). Both GlcA- and IdoA-containing sequences are readily attacked by 6-O-sulfotransferases (32), and HexA C5-epimerization is precluded by adjacent 6-O-sulfate groups (17). O-Sulfotransferases, working in close
association with the epimerase, thus may restrict the extent of epimerization. In view of the virtually irreversible GlcA C5-epimerization, it seems more difficult to explain how O-sulfation may promote IdoA formation.

Previous generation of microsomal heparin-related polysaccharide yielded sulfated products that released $^3$H from [5-$^3$H]GlcA residues upon subsequent incubation with exogenous epimerase (29). While this phenomenon was presumably an artefact of the experimental system (incomplete O-sulfation), it nevertheless pointed to a sequestration of epimerase and substrate that apparently applies also to the intact biosynthetic apparatus. Following rapid interaction between the epimerase and its substrate, the polysaccharide chains are physically or functionally dislocated from the enzyme. The lack of reversibility in HexA C5-epimerization thus has bearing on general aspects of the HS biosynthetic apparatus and its organization.
REFERENCES

1. Bernfield, M., Gotte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Lincecum, J., and Zako, M. (1999) *Annu Rev Biochem* **68**, 729-777

2. Spillmann, D., and Lindahl, U. (1994) *Curr Opin Struct Biol* **4**, 677-682

3. Esko, J. D., and Lindahl, U. (2001) *J Clin Invest* **108**, 169-173

4. Kusche-Gullberg, M., and Kjellén, L. (2003) *Curr Opin Struct Biol* **13**, 605-611

5. Ai, X., Do, A. T., Lozynska, O., Kusche-Gullberg, M., Lindahl, U., and Emerson, C. P., Jr. (2003) *J Cell Biol* **162**, 341-351

6. Esko, J. D., and Zhang, L. (1996) *Curr Opin Struct Biol* **6**, 663-670

7. Sugahara, K., and Kitagawa, H. (2000) *Curr Opin Struct Biol* **10**, 518-527

8. Rosenberg, R. D., Shworak, N. W., Liu, J., Schwartz, J. J., and Zhang, L. (1997) *J. Clin. Invest.*** **99**, 2062-2070

9. Lidholt, K., Kjellén, L., and Lindahl, U. (1989) *Biochem J* **261**, 999-1007

10. Hagner-Mcwhirter, Å., Lindahl, U., and Li, J.-P. (2000) *Biochem J* **347**, 69-75

11. Campbell, P., Hannesson, H. H., Sandbäck, D., Rodén, L., Lindahl, U., and Li, J.-P. (1994) *J Biol Chem* **269**, 26953-26958

12. Morgenlie, S. (1973) *Acta. Chem. Scand* **27**, 3609-3610

13. Isuda, Y., Hanajima, M., Matsuhira, N., Okuno, Y., and Kanemitsu, K. (1989) *Chem. Pharm. Bull* **37**, 2344-2350

14. Li, J.-P., Gong, F., Hagner-McWhirter, Å., Forsberg, E., Åbrink, M., Kisilevsky, R., Zhang, X., and Lindahl, U. (2003) *J Biol Chem* **278**, 28363-28366
15. Shively, J. E., and Conrad, H. E. (1970) *Biochemistry* **9**, 33-43

16. Silbert, J. E., and Sugumaran, G. (2002) *IUBMB Life* **54**, 177-186

17. Jacobsson, I., Lindahl, U., Jensen, J. W., Rodén, L., Prihar, H., and Feingold, D. S. (1984) *J Biol Chem* **259**, 1056-1063

18. Shively, J. E., and Conrad, H. E. (1976) *Biochemistry* **15**, 3932-3942

19. Lindahl, B., Eriksson, L., and Lindahl, U. (1995) *Biochem J* **306**, 177-184

20. Maccarana, M., Sakura, Y., Tawada, A., Yoshida, K., and Lindahl, U. (1996) *J. Biol. Chem.* **271**, 17804-17810

21. Feyzi, E., Sallddeen, T., Larsson, E., Lindahl, U., and Salmivirta, M. (1998) *J. Biol. Chem.* **273**, 13395-13398

22. Kreuger, J., Prydz, K., Pettersson, R. F., Lindahl, U., and Salmivirta, M. (1999) *Glycobiology* **9**, 723-729

23. van den Born, J., Gunnarsson, K., Bakker, M. A. H., Kjellén, L., Kursche-Gullberg, M., Maccarana, M., Berden, J. H. M., and Lindahl, U. (1995) *J. Biol. Chem.* **270**, 31303-31309

24. Smits, N. C., Robbesom, A. A., Versteeg, E. M., Van De Westerlo, E. M., Dekhuijzen, P. N., and Van Kuppevelt, T. H. (2003) *Am J Respir Cell Mol Biol*

25. Casu, B., and Lindahl, U. (2001) in *Adv Carbohydr Chem Biochem* Vol. 57, pp. 159-206

26. Esko, J. D., and Selleck, S. B. (2002) *Annu Rev Biochem* **71**, 435-471

27. Conrad, H. E. (1998) *Heparin-binding proteins*, Academic press, San Diego

28. Rong, J., Habuchi, H., Kimata, K., Lindahl, U., and Kusche-Gullberg, M. (2001) *Biochemistry* **40**, 5548-5555
29. Jacobsson, I., Bäckström, G., Höök, M., Lindahl, U., Feingold, D. S.,
    Malmström, A., and Rodén, L. (1979) Journal of Biological Chemistry 254,
    2975-2982
30. Kusche, M., and Lindahl, U. (1990) J. Biol. Chem. 265, 15403-15409
31. Malmström, A. (1981) Biochem. J. 198, 669-675
32. Smeds, E., Habuchi, H., Do, A. T., Hjertson, E., Grundberg, H., Kimata, K.,
    Lindahl, U., and Kusche-Gullberg, M. (2003) Biochem J 372, 371-380
FOOTNOTES

*Supported by grants from Swedish Medical Research Council (2309), the Swedish Cancer Society (4708-B02-01XAA), the European Commission (QLK-CT-1999.00536) and Polysackaridforskning AB (Sweden).

1 Abbreviations: aManR, 2,5-anhydromannitol (generated by reduction of reducing-terminal anhydromannose units); GlcA, D-glucuronic acid; Gal, D-galactose; GlcN, D-glucosamine, GlcNAc, N-acetyl-D-glucosamine; GlcNS, N-sulfo-D-glucosamine; HexA, unspecified hexuronic acid; HS, heparan sulfate; IdoA, L-iduronic acid; 2S and 6S, 2-O-sulfate and 6-O-sulfate groups, respectively.

2 The heterogeneous appearance of the trisaccharide as well as the monosaccharide peaks is ascribed to variable 6-O-sulfation of GlcN units and to GlcA lactone/acid interconversion, respectively.

3 The slightly higher $^3$H/$^{14}$C ratio for the trisaccharide is probably due to some transfer of label from $[^3]$HGal also to the GlcN residues of HS (see Fig. 6, peaks 5 and 6). Degradation of the labeled HS with heparitinase I released $^3$H$_2$O, as predicted for eliminase action on $[5-^3]$H-GlcA residues. The released label accounted for ~95% of the total $^3$H incorporated, ~5% remaining associated with the resultant 4,5-unsaturated disaccharides, presumably in the GlcN residues (data not shown).
Table I. Ratios of $^3$H/$^{14}$C for oligomers generated by deaminative cleavage of metabolically radiolabeled ($[^3]$H[Gal/$[^{14}$C]GlcN) HS. Ratios were determined for the various peak fractions of the chromatogram shown in Fig. 5. Hypothetical ratios were calculated for the corresponding species devoid of $^3$H label at the non-reducing terminal HexA residues.

| Oligomer | $^3$H/$^{14}$C ratio found | Predicted $^3$H/$^{14}$C ratio assuming unlabeled terminal HexA unit$^a$ |
|----------|---------------------------|------------------------------------------------------------------|
| 16-mer   | 5.7                       | -                                                                |
| 14-mer   | 5.5                       | 4.9                                                              |
| 12-mer   | 5.2                       | 4.7                                                              |
| 10-mer   | 5.2                       | 4.6                                                              |
| 8-mer    | 4.8                       | 4.3                                                              |
| 6-mer    | 4.2                       | 3.8                                                              |
| 4-mer    | 4.1                       | 2.9                                                              |
| 2-mer    | 1.7                       | 0                                                                |

$^a$ Based on the assumption that the experimentally found ratio, 5.7, for the 16-mer fraction approximates the ratio for the epimerase-resistant -GlcNAc-GlcA-disaccharide unit. This reference ratio will be somewhat underestimated due to terminal IdoA formation also in the largest oligosaccharides.
Table II. Ratios of $^{3}\text{H}/^{14}\text{C}$ for disaccharides generated by deaminative cleavage of metabolically radiolabeled ($[^{3}\text{H}]\text{Gal}/[^{14}\text{C}]\text{GlcN}$ or $[^{3}\text{H}]\text{Gal}/[^{14}\text{C}]\text{Gal}$) HS. The various HexA-aMan$_{R}$ disaccharide species are derived from -GlcNS-HexA-GlcNS-sequences in the intact polysaccharide. Disaccharides were isolated by gel chromatography (Fig. 5) and were further separated by anion-exchange HPLC (Fig. 6), paper electrophoresis and paper chromatography (Fig. 7). The values represent means of two independent analytical runs. The 16-mer fraction from Fig. 5 is included to provide a reference ratio essentially reflecting a (GlcNAc-GlcA-)$_{n}$ sequence.

| Saccharide       | $^{3}\text{H}/^{14}\text{C}$ ratio |
|------------------|------------------------------------|
|                  | $[^{3}\text{H}]\text{Gal}/[^{14}\text{C}]\text{GlcN}$ | $[^{3}\text{H}]\text{Gal}/[^{14}\text{C}]\text{Gal}$ |
| GlcA-aMan$_{R}$  | 5.8                                 | 11.6                                 |
| GlcA2S-aMan$_{R}$| 5.7                                 | 11.9                                 |
| GlcA-aMan$_{R}6S$| 5.7                                 | 11.4                                 |
| 16-mer           | 5.7                                 | 11.3                                 |
FIGURE LEGENDS

Figure 1. Proposed reaction mechanism for GlcA C5-epimerase. C5-Epimerization involves abstraction of the C5 proton of GlcA followed by readdition of a proton from the medium to the resultant carbanion intermediate to generate IdoA. In a soluble system the reaction is freely reversible, as demonstrated by incubation of polysaccharide substrate, containing either GlcA or IdoA units, with epimerase in the presence of $^3$H$_2$O. The product contains C5-$^3$H-labeled GlcA as well as IdoA residues (reproduced with permission, from (10)).

Figure 2. Synthesis of tritium labeled galactose. Key: i) (Bu$_3$)$_2$SnO, Br$_2$, CHCl$_3$; ii) NaB$_3$H$_4$, MeOH; iii) HCl(aq)

Figure 3. Use of dual isotope labeling of cells to assess reversibility of GlcA C5-epimerization in HS biosynthesis. Cells are incubated with [5-$^3$H]Gal (open circle) and [$^{14}$C]GlcN (closed circle) to generate intracellular labeled UDG-GlcA and UDP-GlcNAc, respectively. Monosaccharide units are polymerized to N-acetylheparosan (catalyzed by the EXT1/EXT2 enzyme complex), that is further modified through N-deacetylase/N-sulfotransferase (NDST), GlcA C5-epimerase (Epi) and O-sulfotransferase (OST) reactions (3). The aim of the study is to clarify whether IdoA units can be “back-epimerized” to GlcA residues that will be detected by their lack of C5-$^3$H label. The scheme illustrates alternative pathways for a fully N-sulfated trisaccharide sequence that is arbitrarily 2-O- and 6-O-sulfated.
Figure 4. Relation between N-substituents and epimerase target units in HS precursor polysaccharide. Potential epimerase target units are indicated by arrows. Treatment with nitrous acid (HNO₂) generates di- and oligosaccharides, due to cleavage at GlcNS but not at GlcNAc units. Open circles, HexA; closed squares, GlcN (NAc, N-acetylated or NS, N-sulfated); closed arrowheads, anhydromannitol.

Figure 5. Gel chromatography of metabolically labeled HS following treatment with nitrous acid. 293 HEK cells were incubated with [5-³H]Gal/[U-¹⁴C]GlcN (A) or [5-³H]Gal/[U-¹⁴C]Gal (B). The metabolically labeled HS was cleaved by treatment with HNO₂, and the products were separated by gel chromatography on a Bio-Gel P-10 column. Effluent fractions were analyzed for radioactivity (open circles, ³H; closed circles, ¹⁴C). The ³H/¹⁴C ratios (closed triangles) were calculated (Table I) for oligosaccharides of different size (the numbers above each peak indicate number of monosaccharide units). Di- and tetrasaccharide fractions were pooled as indicated by the brackets. For further information see “Experimental Procedures”.

Figure 6. Anion-exchange HPLC of disaccharides obtained by deaminative cleavage of HS. Disaccharide fractions were isolated as shown in Fig. 5 from HS labeled with [5-³H]Gal/[U-¹⁴C]GlcN (A) or [5-³H]Gal/[U-¹⁴C]Gal (B). Samples were separated on a Partisil-10 SAX column, eluted with a step gradient of KH₂PO₄. Effluent fractions were analyzed for radioactivity (open circles, ³H; closed circles, ¹⁴C; dotted line, KH₂PO₄ concentrations). Identified peaks are indicated by numbers according to the elution positions of disaccharide standards: 1, GlcA-aMan₉/IdoA-aMan₉ (not separated); 2, GlcA2S-aManR; 3, GlcA-aMan₉6S; 4, IdoA-aMan₉6S; 5,
IdoA2S-aManR; and 6, IdoA2S-aManR6S. The $^3$H/$^{14}$C ratios for GlcA-containing
disaccharides are shown in Table II.

**Figure 7. Paper chromatography of non-**-sulfated disaccharides obtained by
deaminative cleavage of HS. Non-**-sulfated disaccharides, obtained by deamination
of HS labeled with [5-$^3$H]Gal/[U-$^{14}$C]GlcN, were separated from **-sulfated species
by high-voltage paper electrophoresis and further fractionated by paper
chromatography. The migration positions of GlcA-aManR (GM) and IdoA-aManR
(IM) standard disaccharides are indicated. Open circles, $^3$H; closed circles, $^{14}$C.
Similar experiments were conducted for [5-$^3$H]Gal/[U-$^{14}$C]Gal labeled HS (not
shown). The $^3$H/$^{14}$C ratios for the GM disaccharides are shown in Table II. For
additional information see “Experimental Procedures”.

**Figure 8. Digestion of tetrasaccharides with β-glucuronidase.** Tetrasaccharide
deamination products purified by gel chromatography (Fig. 5) from HS metabolically
labeled with [5-$^3$H]Gal/[U-$^{14}$C]GlcN (A) or [5-$^3$H]Gal/[U-$^{14}$C]Gal (B) were treated
with β-glucuronidase and the digests were separated on a Bio-Gel-P10 column. Open
circles, $^3$H; closed circles, $^{14}$C. The elution positions of tetrasaccharide (4),
trisaccharide (3) and monosaccharide (1) are indicated.

**Figure 9. Distribution of radiolabel in products obtained by digestion of
tetrasaccharides with β-glucuronidase.** Tetrasaccharides labeled with [5-
$^3$H]Gal/[U-$^{14}$C]GlcN (A) or [5-$^3$H]Gal/[U-$^{14}$C]Gal (B) were treated with β-
glucuronidase and the products were separated by gel chromatography (Fig. 8). The
scheme shows the predicted distribution of radiolabel in the various products, and the
$^{3}\text{H}/^{14}\text{C}$ ratios based on analysis of the [5-$^{3}\text{H}$]Gal/[U-$^{14}$C]Gal labeled components. The symbols used are as indicated.
Figure 1
Gal + GlcN
↓
Sugar metabolism
↓
UDP-GlcA + UDP-GlcN
↓
EXT 1/EXT 2
↓
-GlcNAc-GlcA-GlcNAc
↓
NDST
-GlcNS-GlcA-GlcNS
↓
OST
-GlcNS6S-GlcA-GlcNS
↓
Epi
-GlcNS-IdoA-GlcNS
Epi
-GlcNS-GlcA-GlcNS
↓
OST
-GlcNS6S-IdoA2S-GlcNS
↓
OST
-GlcNS6S-GlcA-GlcNS
Figure 4

\[
\text{NS} \rightarrow \text{NAc} \rightarrow \text{NS} \rightarrow \text{NAc} \rightarrow \text{NS} \rightarrow \text{NS}
\]

\[
\text{HNO}_2/\text{NaBH}_4
\]

\[
\text{NAc} \rightarrow \text{NAc} \rightarrow \text{NAc}
\]
Figure 6
Figure 7
Figure 8

A

Dpm (x 10^{-3})

Effluent volume (ml)

B

3H-Dpm (x 10^{-3})

14C-Dpm (x 10^{-3})

Effluent volume (ml)
Figure 9

**A**

\[ 	ext{GlcA} \rightarrow \text{IdoA} \rightarrow \text{GlcNAc} \rightarrow \text{ManR} \rightarrow \beta\text{-glucuronidase} \]

**B**

\[ 	ext{GlcA} \rightarrow \text{IdoA} \rightarrow \text{GlcNAc} \rightarrow \text{ManR} \rightarrow \beta\text{-glucuronidase} \]

\( ^3\text{H} / ^{14}\text{C} \) 8.9 11 5.4
