Biosynthesis of heparin/heparan sulphate: mechanism of epimerization of glucuronyl C-5

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In the biosynthesis of heparin and heparan sulphate, D-glucuronic acid residues are converted into L-iduronic acid (IdoA) units by C-5 epimerization, at the polymer level. The reaction catalysed by the epimerase occurs by reversible abstraction and readdition of a proton at C-5 of target hexuronic acid residues, through a carbanion intermediate, with or without an inversion of configuration at C-5 [Prihar, Campbell, Feingold, Jacobsson, Jensen, Lindahl and Rodén (1980) *Biochemistry* **19**, 495–500]. Incubation of chemically N-sulphated capsular polysaccharide from *Escherichia coli* K5 ([4GlcA β 1-4GlcNSO₃ α 1-]_n), or of O-desulphated heparin (predominantly [4IdoA α 1-4GlcNSO₃ α 1-]_n) with purified C-5 epimerase from bovine liver, resulted in the interconversion of glucuronic acid and IdoA residues, which reached equilibrium (30–40% IdoA/total hexuronic acid) after approx. 1 h of incubation. Similar incubations performed in the presence of ³H₂O resulted in progressive labelling at C-5 of the target hexuronic acid units of either substrate polysaccharide. Contrary to chemical D-gluco/L-ido equilibrium, established within 1 h of incubation, the accumulation of ³H label continued for at least 6 h.

This isotope effect suggests that the second stage of the reaction, i.e. the re-addition of a proton to the carbanion intermediate, is the rate-limiting step of the overall process. Analysis of the 5-³H-labelled polysaccharide products showed that the ³H was approximately equally distributed between glucuronic acid and IdoA units, irrespective of incubation time (from 15 min to 72 h) and of the relative proportions of the two epimers in the substrate. This finding points to a catalytic mechanism in which the abstraction and re-addition of C-5 protons are effected by two polyprotic bases, presumably lysine residues. Previous experiments relating to the biosynthesis of dermatan sulphate were similarly interpreted in terms of a two-base epimerization mechanism but differed from the present findings by implicating one monoprotic and one polyprotic base function [Hannesson, Hagner-McWhirter, Tiedemann, Lindahl and Malmström (1996) *Biochem. J.* **313**, 589–596].

Key words: C-5 epimerase, glucuronic acid, iduronic acid.

INTRODUCTION

Heparin and heparan sulphate (HS) are structurally related glycosaminoglycans. Owing to their polyanionic character they interact with many different proteins, including enzymes and enzyme inhibitors, cytokines, growth factors and extracellular matrix proteins, and therefore influence a variety of biological processes ([1,2], and references therein). Both polysaccharides are synthesized as proteoglycans, in which they are linked to serine residues in defined core proteins through a glucuronyl-galactosyl-galactosyl-xylosyl tetrasaccharide sequence. Heparin is produced by connective-tissue-type mast cells and is stored in cytoplasmic granules in these cells [3,4]. HS proteoglycans, in contrast, are generated by most mammalian and many non-mammalian cells and are found primarily on cell surfaces and in extracellular matrices [5,6].

Polysaccharide formation, which is believed to take place in the Golgi system, is initiated by the sequential addition of alternating D-glucuronic acid (GlcA) and N-acetyl-D-glucosamine (GlcNAc) units to the non-reducing termini of nascent acceptor structures. The resultant polymers are subsequently modified, presumably in close association with chain elongation [7], through a series of reactions involving N-deacetylation/N-sulphation of the GlcNAc residues, C-5 epimerization of GlcA to L-iduronic acid (IdoA) units, and finally O-sulphation at various positions (reviewed in [1,2]). These reactions occur consecutively,

such that the products of early events provide the substrates for later steps. Polymer modification is regulated, at least in part, by the substrate specificities of the enzymes involved in the process. For instance, N-sulphate groups are prerequisite to the GlcA C-5 epimerization as well as to the O-sulphation reactions. Owing to restriction of the initial polymer-modification reaction, the N-deacetylation/N-sulphation of GlcNAc units, HS therefore contains not only less N-sulphate, but also less IdoA and O-sulphate, than heparin.

It has been proposed that IdoA units in glycosaminoglycans promote interactions with proteins owing to their conformational flexibility [8]. The generation of such residues, by the C-5 epimerization of GlcA units, is of particular interest because it is the only reaction in the polymer-modification process that is strictly dependent on enzymic catalysis; the N-deacetylation, N-sulphation and O-sulphation reactions can all be achieved, albeit in a less selective fashion, by a purely chemical approach [9]. The C-5 epimerase involved in the biosynthesis of heparin/HS has been purified from bovine liver [10] and cloned from a bovine lung cDNA library [11]. The epimerase reaction occurs by the abstraction and re-addition of the C-5 proton via a carbanion intermediate with an inversion of configuration such that the carboxy group is shifted across the plane of the pyranose ring [12–14]. In a solubilized enzyme system the reaction is freely reversible, and incubations performed in the presence of ³H₂O result in the incorporation of ³H into both IdoA and GlcA units

Abbreviations used: aMan₆, 2,5-anhydromannitol (formed by reduction of the terminal 2,5-anhydromannose residues with NaBH₄); HexA, hexuronic acid (unspecified); HS, heparan sulphate; IdoA, L-iduronic acid.

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Scheme 1 Basic mechanism for the heparin/HS hexuronic acids (HexA) C-5 epimerization reaction

The reaction involves the reversible abstraction of a proton at C-5 of target HexA residues, leading to the formation of a carbanion intermediate. The C-5 proton is exchangeable with protons of the medium. Incubations of polysaccharides containing 5- ^3H -labelled HexA (GlcA or IdoA) units with the epimerase result in the formation of $^3\text{H}_2\text{O}$, as exploited in a convenient enzyme assay [16]. Conversely, incubations performed in the presence of $^3\text{H}_2\text{O}$ yield polysaccharide products with 5- ^3H -labelled GlcA as well as IdoA units. The upper sequence illustrates the repeating disaccharide unit of N-sulphated K5 polysaccharide; the lower sequence represents the major disaccharide unit of O-desulphated heparin.

(see Scheme 1). In the present study the course of such labelling was studied to gain further insight into the mechanism of the epimerase reaction.

EXPERIMENTAL

Materials

A derivative of the *Escherichia coli* K5 capsular polysaccharide, in which the GlcNAc residues had been N-deacetylated essentially quantitatively by hydrazinolysis, and then N-sulphated by extensive treatment with trimethylamine- SO_3 complex [15], was a gift from Benito Casu (Ronconi Institute, Milan, Italy). O-Desulphated pig mucosal heparin was given by Lennart Rodén (University of Alabama at Birmingham, Birmingham, AL, U.S.A.). This material had been prepared through N/O-desulphation of the polysaccharide by treatment with DMSO followed by re-N-sulphation [16]. Treatment of the modified heparin with HNO_2 at pH 1.5 followed by reduction of the products with NaB^3H_4 yielded disaccharides in which GlcA accounted for approx. 17% of total unspecified hexuronic acids (HexA) (results not shown).

Glucuronyl C-5 epimerase was purified from bovine liver by a series of chromatographic steps [10], yielding one major band on SDS/PAGE. The specific activity of the enzyme preparation was 4.4×10^8 c.p.m. of ^3H released/30 min per mg of protein with 5- ^3H -labelled O-desulphated heparin as substrate (specific radioactivity 32.8×10^6 c.p.m./mg of HexA). The purified enzyme was stored in 10% (v/v) glycerol at 4°C. Before use, the enzyme preparation was passed through a PD-10 column equilibrated in assay buffer. $^3\text{H}_2\text{O}$ (90 Ci/mol, 1 Ci/200 μl) and NaB^3H_4

(28 Ci/mmol) were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Sephadex G-15 and prepacked PD-10 columns (Sephadex G-25) were purchased from Pharmacia Biotech (Uppsala, Sweden).

Incubations with C-5 epimerase

For standard incubations, polysaccharide substrate (50 μg) was incubated with approx. 150 ng of enzyme protein (determined by the method of Bradford [17]) at 37°C in 100 μl of 0.05 M HEPES, pH 7.4, containing 0.015 M EDTA, 0.015% (v/v) Triton X-100 and 0.1 M KCl. The incubations were performed either in unlabelled medium or in the presence of 50 mCi of $^3\text{H}_2\text{O}$. The reactions were stopped at selected time points by adding 500 μl of 0.05 M sodium acetate/1.5 M LiCl (pH 4.0). To the incubations extended for 72 h were added fresh aliquots of enzyme (approx. 75 ng) after 24 and 48 h. Samples were passed through PD-10 columns equilibrated with 0.2 M NH_4HCO_3 to separate the polysaccharide constituent from free $^3\text{H}_2\text{O}$. The isolated polysaccharide was repeatedly freeze-dried to eliminate NH_4HCO_3 and any residual free $^3\text{H}_2\text{O}$.

Analysis of polysaccharides

For the analysis of polysaccharide composition, samples were treated with HNO_2 at pH 1.5 [18] and the resultant hexuronyl-anhydromannose disaccharides were reduced with 0.5 mCi of NaB^3H_4 [19]. The labelled HexA-[1- ^3H]aMan_R disaccharide products (in which aMan_R stands for 2,5-anhydromannitol) were isolated by gel chromatography on a column (1 cm $\times$ 200 cm) of Sephadex G-15 in 0.2 M NH_4HCO_3 , then desalted by repeated freeze-drying. The disaccharides were further purified by high-voltage paper electrophoresis on Whatman no. 3MM paper, in 0.083 M pyridine/0.05 M acetic acid (pH 5.3) at 80 V/cm for 40 min. Portions of the paper strips corresponding to HexA-aMan_R disaccharides were cut out and the labelled material was extracted with water. These samples were analysed by paper chromatography on Whatman no. 1 paper in ethyl acetate/acetic acid/water (3:1:1, by vol.), along with GlcA-[1- ^3H]aMan_R and IdoA-[1- ^3H]aMan_R standards [20]. After the separation, the paper strips were dried and cut into 1 cm pieces which were transferred to 6 ml scintillation vials. The samples were extracted in the vials with 1 ml of water for at least 15 min at room temperature; they were then analysed for radioactivity by scintillation counting after the addition of 4 ml of scintillation fluid. The relative proportions of GlcA and IdoA were calculated from the corresponding disaccharide peak areas. Polysaccharide samples that had been incubated with epimerase in the presence of $^3\text{H}_2\text{O}$ were analysed in a similar fashion, except that the disaccharides formed on deaminative cleavage were reduced with 50 μmol of unlabelled NaBH_4 instead of with NaB^3H_4 [21]. Polysaccharides were quantified by the carbazole reaction for HexA [22]. Radioactivity was measured in a Beckman LS 6000 liquid-scintillation counter with Optiphase HiSafe scintillation fluid.

RESULTS

The course of the HexA C-5 epimerization reaction was followed during incubations of purified epimerase with two distinct polysaccharide substrates, one (N-sulphated K5 polysaccharide) containing GlcA as the only HexA component, the other (O-desulphated heparin) predominantly IdoA. The effects of incubation were analysed by determining the change in the molar proportions of GlcA and IdoA. To define the rate-limiting step of the reaction, the time course of HexA epimerization was

Figure 1 HexA composition and C-5 ^3H labelling of N-sulphated K5 polysaccharide and O-desulphated heparin incubated with HexA C-5 epimerase from bovine liver

Polysaccharide samples were incubated with bovine liver C-5 epimerase for different periods in the absence (left panels) or presence (right panels) of $^3\text{H}_2\text{O}$, as described in the Experimental section. The products were deaminated and the resultant disaccharides were reduced with NaB^3H_4 (left panels for each substrate) or with unlabelled NaBH_4 (panels to the right for each substrate), to yield HexA-[1- ^3H]aMan_R and [5- ^3H]HexA-aMan_R species. The GlcA- and IdoA-containing isomers were separated by paper chromatography. Paper chromatograms are shown of disaccharides derived from N-sulphated K5 polysaccharide and O-desulphated heparin that had been incubated with epimerase for the indicated periods. The distribution of label seen after 72 h of incubation did not differ appreciably from that obtained after 1 h (results not shown). The migration distances of GlcA-aMan_R (GM) and IdoA-aMan_R (IM) disaccharide standards are indicated by arrows. For additional information, see the Experimental section.

related to the kinetics of ^3H incorporation during incubations performed in the presence of $^3\text{H}_2\text{O}$. Finally, the mechanism of epimerization was assessed by measuring the distribution of the incorporated ^3H label between the resultant GlcA and IdoA residues.

Compositional analysis of polysaccharides

After incubation with the C-5 epimerase for different periods, the polysaccharides were recovered and subjected to deaminative cleavage with nitrous acid (see the Experimental section). The

products (approx. 95% disaccharides; results not shown) were reduced with NaB^3H_4 and the resultant HexA-[1- ^3H]aMan_R disaccharides were purified by high-voltage paper electrophoresis and finally separated into GlcA-[^3H]aMan_R and IdoA-[^3H]aMan_R species by paper chromatography (see Figure 1, left panels for each type of substrate). The proportions of the two HexA C-5 epimers were calculated from peak areas (Table 1).

A plot of HexA composition against incubation time shows that the proportion of IdoA reached its maximal level, approximately one-third of the total HexA, after approx. 1 h of incubation of N-sulphated K5 polysaccharide with the epimerase

Table 1 C-5 epimerization and $5\text{-}^3\text{H}$ labelling of HexA units during incubation of N-sulphated K5 polysaccharide and O-desulphated heparin with epimerase

N-sulphated K5 polysaccharide and O-desulphated heparin were incubated with purified bovine liver GlcA C-5 epimerase in buffer containing unlabelled water or $^3\text{H}_2\text{O}$, as described in the Experimental section. The polysaccharide products recovered after the indicated periods were cleaved with HNO_2 , pH 1.5, and the resultant disaccharides were reduced with NaB^3H_4 (for incubations performed in unlabelled water) or with NaBH_4 (for incubations performed in the presence of $^3\text{H}_2\text{O}$). The generated HexA-[$1\text{-}^3\text{H}$]aMan_R and [$5\text{-}^3\text{H}$]HexA-aMan_R disaccharides were isolated and the isomers containing GlcA and IdoA were separated by paper chromatography (Figure 1). The relative quantities of 5-epimeric species were calculated from peak areas. For additional information see the Experimental section.

Substrate	Incubation time	GlcA/(GlcA + IdoA) (%)	[$5\text{-}^3\text{H}$]GlcA/ ([$5\text{-}^3\text{H}$]GlcA + [$5\text{-}^3\text{H}$]IdoA) (%)
N-sulphated K5 PS	0 min	100	—
	5 min	88	56
	15 min	80	44
	30 min	72	47
	1 h	67	60
	72 h	67	60
O-desulphated heparin	0 min	17	—
	5 min	29	40
	15 min	36	60
	30 min	50	60
	1 h	56	62
	72 h	59	65

(Figure 2A). This ratio was not changed further during extensively prolonged incubation with the addition of fresh enzyme. With the heparin-derived substrate, in contrast, the reaction started from a large excess of IdoA that subsequently decreased owing to epimerase action (Figure 2B). As with the K5 incubations, equilibrium was reached after approx. 1 h, albeit at a somewhat higher proportion of IdoA (approx. 40% of total HexA). The overall time course of HexA C-5 epimerization was therefore the same for the two substrates.

Enzymic incorporation of ^3H

Previous experiments demonstrated that incubations of HexA C-5 epimerase with O-desulphated heparin in the presence of $^3\text{H}_2\text{O}$ result in the labelling of both GlcA and IdoA residues on C-5 [1,2]. A reaction mechanism was proposed that involved a freely reversible inversion of configuration at C-5, around a planar carbanion intermediate (Scheme 1). The $^3\text{H}/^1\text{H}$ exchange could be exploited to provide a convenient epimerase assay [16]. In this procedure, epimerase activity is defined by the rate of $^3\text{H}_2\text{O}$ release from a substrate enzymically prelabelled at the 5-position of the HexA units.

In the present study we compared the rates of ^3H incorporation into the two polysaccharide substrates (N-sulphated K5 polysaccharide and O-desulphated heparin) during incubations with epimerase in the presence of $^3\text{H}_2\text{O}$. The two incorporation curves were highly similar and increased continuously for several hours (Figure 3A). Prolonged incubation indicated that radiochemical equilibrium, i.e. a plateau of ^3H incorporation, was not attained within 6 h (Figure 3A, inset). This finding thus stands in marked contrast with the chemical equilibrium, which was established within 1 h of incubation under identical conditions (Figure 2). An isotope effect of approx. 20 was estimated on the basis of initial rates of ^1H and ^3H incorporation, deduced from Figures 2 and 3(A) respectively.

Figure 2 Change in HexA composition of polysaccharide substrates during incubation with C-5 epimerase

N-sulphated K5 polysaccharide (A) and O-desulphated heparin (B) were incubated with bovine liver C-5 epimerase for different periods. The recovered polysaccharides were processed to generate HexA-[$1\text{-}^3\text{H}$]aMan_R disaccharides, which were further separated by paper chromatography (see Figure 1). The relative amounts of disaccharide units containing GlcA (○) and IdoA (●) were calculated from peak areas.

Polysaccharide samples recovered after incubation with epimerase for different periods of time in the presence of $^3\text{H}_2\text{O}$ were deaminated with HNO_2 ; the disaccharides formed were reduced with (unlabelled) NaBH_4 (see the Experimental section). The resultant [$5\text{-}^3\text{H}$]GlcA-aMan_R and [$5\text{-}^3\text{H}$]IdoA-aMan_R species were separated by paper chromatography and quantified, as illustrated in Figure 1. The ^3H was approximately equally distributed between GlcA and IdoA, also after short incubation times (Figure 3B) and regardless of whether the substrate contained exclusively GlcA or predominantly IdoA units (Figure 4; Table 1).

DISCUSSION

Epimerization at C-5 of HexA units, located at internal positions of polysaccharides, is not unique to heparin/HS biosynthesis. A similar inversion of configuration also generates IdoA from GlcA residues in dermatan sulphate [23] and L-guluronic acid from D-mannuronic acid units in alginate biosynthesis [24]. Although distinct epimerase enzymes catalyse these reactions, a common basic mechanism has been implicated, as illustrated for the epimerase reaction in heparin/HS biosynthesis in Scheme 1.

Figure 3 Incorporation of ^3H into substrate polysaccharides during incubation with C-5 epimerase in the presence of $^3\text{H}_2\text{O}$

N-Sulphated K5 polysaccharide ($\circ$) and O-desulphated heparin ($\bullet$) were incubated with C-5 epimerase in the presence of $^3\text{H}_2\text{O}$, as described in the Experimental section. (A) Accumulated incorporation of ^3H into substrate polysaccharides; (B) distribution of label between GlcA and IdoA units after incubation for the indicated periods. The results in (B) were calculated from peak areas related to the experiment illustrated in Figure 1.

The present study was undertaken to gain a deeper insight into the latter reaction.

The freely reversible nature of the reaction was apparent from the course of HexA C-5 epimerization during incubations of substrate polysaccharides containing excess amounts of either GlcA or IdoA units. An equilibrium level of epimerization, corresponding to the conversion of approximately one-third of the GlcA residues into IdoA units was achieved within 1 h of incubation of N-sulphated K5 polysaccharide. Conversely, approximately half of the IdoA in O-desulphated heparin reverted to GlcA during a similar incubation. The somewhat higher equilibrium level of IdoA in the latter case presumably reflects the occurrence of some residual O-sulphate groups in the substrate polysaccharide. Previous work showed that HexA units adjacent (on either side) to 6-O-sulphated glucosamine residues are not accessible to the epimerase [25]. Incubations of either substrate in the presence of $^3\text{H}_2\text{O}$ revealed an isotope effect, which resulted in the continued incorporation of label into HexA units long after chemical equilibrium between GlcA and IdoA had been reached (Figure 3A). The ^3H incorporation

Figure 4 Relationship between overall HexA composition of epimerase substrates and C-5 configuration of enzymically $^5\text{-}^3\text{H}$ -labelled HexA residues

The Figure relates the change in HexA composition of substrate polysaccharides ($\circ$, N-sulphated K5 polysaccharide; $\bullet$, O-desulphated heparin) during incubation with C-5 epimerase, to the distribution of $^5\text{-}^3\text{H}$ -label, incorporated from $^3\text{H}_2\text{O}$ between GlcA and IdoA residues. For further information see Table 1.

reflects the second phase of the epimerization reaction, i.e. the re-addition of a proton to the carbanion intermediate (Scheme 1). The isotope effect therefore suggests that this step is rate-limiting in the overall process.

A further analysis of ^3H incorporation, assessing the ratios of $^5\text{-}^3\text{H}$ -labelled GlcA and IdoA, provided more detailed insight into this mechanism. In a previous study of the HexA C-5 epimerization reaction in dermatan sulphate biosynthesis, the relative extent of labelling of the two HexA species was found to vary with the overall HexA composition of the polysaccharide substrate [26]. Although the incubation of chondroitin, which contains exclusively GlcA units, with epimerase in the presence of $^3\text{H}_2\text{O}$ resulted in the formation of $^5\text{-}^3\text{H}$ -labelled GlcA as well as IdoA units, a similar incubation of dermatan, which contains predominantly IdoA residues, labelled GlcA units preferentially. This finding was interpreted in terms of a two-base mechanism, initially proposed for the conversion of (*R*)-mandelate into (*S*)-mandelate catalysed by mandelate racemase [27]. The selective labelling pattern was explained through the combined action of a monoprotic (histidine) and a polyprotic (lysine) proton donor [26]. The present results show that the epimerase involved in heparin/HS biosynthesis promotes the $^5\text{-}^3\text{H}$ labelling of GlcA and IdoA units equally, regardless of the composition of the substrate polysaccharide, and thus suggest that the catalytic mechanism of this enzyme differs from that implicated in the formation of dermatan sulphate. This difference could be explained by a reaction mechanism by which the abstraction and re-addition of C-5 protons by the heparin/HS-associated epimerase is effected by two polyprotic bases (Scheme 2). Scheme 2(A) shows the transfer of a C-5 proton from an IdoA residue to one of these bases to create the carbanion intermediate and a positively charged ammonium function. Three alternative events might now occur. (1) The original unlabelled proton might be added back to the C-5 carbon, thus generating an unlabelled IdoA unit. (2) The ammonium group of the same lysine residue

Scheme 2 Tentative mechanism for the heparin/HS epimerization reaction of HexA at C-5, based on ${}^3\text{H}$ labelling patterns in the presence of ${}^3\text{H}_2\text{O}$

The epimerase reaction is shown as predicted for an IdoA (predominant in O-desulphated heparin) (**A**) or GlcA (N-sulphated K5 polysaccharide) (**B**) substrate residue. The postulated involvement of two polyprotic bases ($-\text{BH}_2$, i.e. lysine amino groups), ${}^3\text{H}$ -labelled through proton exchange with ${}^3\text{H}_2\text{O}$ in the medium, invariably results in the 5- ${}^3\text{H}$ labelling of both GlcA and IdoA residues. R and R' represent the unspecified portions of the HexA pyranose ring. For additional information see the text.

will rotate during the lifetime of the intermediate, such that a ${}^3\text{H}$ proton, introduced through the exchange of ammonium protons with solvent, is added to the intermediate; the product will be a $[5\text{-}^3\text{H}]\text{IdoA}$ residue. (3) A ${}^3\text{H}$ proton will be added from the base at the opposite side of the intermediate, yielding a 5- ${}^3\text{H}$ -labelled GlcA unit. The attack of a GlcA target unit in the polysaccharide substrate involves a similar sequence of events, except that the two base functions have switched roles (Scheme 2B). According to this model, GlcA and IdoA residues will both be labelled, irrespective of the HexA composition of the substrate polymer. If one of the two base functions is monoprotic, as postulated for the dermatan epimerase, it will preferentially abstract and re-add the same proton and will therefore contribute to unequal labelling of GlcA and IdoA residues [26]. The reaction mechanisms will, it is hoped, be defined in detail through studies of the physical interactions between enzyme and substrate, once recombinant proteins are available.

The catalytic mechanism based on the combined action of monoprotic and polyprotic bases, implicated for both dermatan sulphate epimerase and mandelate racemase [26,27], seems to be associated with an absolute requirement for bivalent ions (Mg^{2+} and Mn^{2+} respectively). A similar requirement (for Ca^{2+}) has been demonstrated for the mannuronan C-5 epimerase in alginate biosynthesis [28]. In contrast, the heparin/HS epimerase shows no such dependence, in accord with the presumably different reaction mechanism.

How do the equilibrium characteristics of the heparin/HS HexA C-5 epimerase reaction relate to the actual process of polysaccharide biosynthesis in the intact cell? It is recalled that IdoA can account for as much as 90% of the total HexA in heparin chains, which therefore contain extended sequences of consecutive IdoA-containing disaccharide units [29–31]. HS has

a more complex structure, with more equal proportions of GlcA and IdoA residues, both of which occur in extended N-sulphated domains as well as in sequences composed of intermixed N-acetylated and N-sulphated disaccharide units [19]. In both polysaccharides, and in heparin in particular, a significant proportion of the IdoA residues are 2-O-sulphated; it has been argued that 2-O-sulphation will withdraw the newly formed IdoA units from the *D-gluc*/*L-ido* equilibrium and thereby promote their accumulation [32,33]. Although studies of heparin biosynthesis in a mastocytoma microsomal preparation have indeed shown that the formation and 2-O-sulphation of IdoA units are tightly coupled processes [1,34], there was no indication of any equilibrium being established between the two C-5 epimers. Available results suggest that the formation [35] and modification [34] of extended heparin chains occur on a time scale of less than 1 min, possibly in a consecutive order. Moreover, the introduction of 5- ${}^3\text{H}$ -labelled GlcA units (from the corresponding UDP-sugar nucleotide) in the microsomal heparin precursor polysaccharide, followed by enzymic polymer modification (initiated by the addition of the sulphate donor, 3'-phosphoadenosine-5'-phosphosulphate) yielded a product in which the radiolabel, as expected, had been eliminated from the generated IdoA residues but was retained by GlcA units, including those residues that satisfied the substrate requirements of the C-5 epimerase [1,36]. These residues had therefore not been touched by the enzyme. It should be emphasized that these findings apply to heparin biosynthesis in mast cells and that we still do not know whether the same or different GlcA C-5 epimerases are involved in the biosynthesis of heparin and HS. The epimerase purified from bovine liver [10] and cloned from a bovine lung cDNA library [11] is presumably related to the biosynthesis of HS; no distinct epimerase has yet been associated

with mast cells. Notably, enzyme preparations from bovine liver and mouse mastocytoma showed no apparent functional differences [37]. To improve our understanding of the GlcA C-5 epimerization reaction, including the regulatory aspects, as part of the biosynthesis of polysaccharides in intact cells, we need to characterize the functions of the appropriate epimerase enzymes in the context of the biosynthetic machineries, with emphasis on interactions with other enzymes, auxiliary proteins and the membranes of the Golgi system.

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