Biosynthesis of dermatan sulphate

Assay and properties of the uronosyl C-5 epimerase

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During biosynthesis of dermatan sulphate D-glucuronate (GlcA) residues are converted to L-iduronate (IdoA) residues via the reaction

The reaction occurs on the polymer level and is catalysed by a C-5 uronosyl epimerase. The reversible release of the C-5 hydrogen was utilized as a measure of the enzyme activity with 5-³H-labelled chondroitin as a substrate. ³H released during incubation was distilled and quantified by liquid-scintillation counting. The epimerase has a low pH optimum (5.6) and requires divalent cations, Mn²⁺ being the most efficient for activity. The *K_m* for chondroitin is 1.2×10^{-4} M. The epimerase is largely associated with the microsomal fractions (90%). Two-thirds of the activity can be solubilized by detergents. Microsomes from cultured fibroblasts contain two different uronosyl epimerases, one for the biosynthesis of heparan sulphate and one for that of dermatan sulphate. The two epimerases have different cofactor and pH requirements.

Both heparin/heparan sulphate and dermatan sulphate contain the rather unusual sugar residue L-iduronate. The formation of this sugar occurs by C-5 epimerization of D-glucuronate residues in polysaccharide precursors (Höök *et al.*, 1974; Malmström *et al.*, 1975). The process is enhanced by concomitant sulphation of the polysaccharide precursors. The initial step in the epimerization process is an abstraction of the C-5 hydrogen of the target uronosyl residues (Jacobsson *et al.*, 1979; Malmström, 1981). This property has been utilized to construct an assay for the heparosan epimerase with a 5-³H-labelled polymer as a substrate. Enzymatic activity is measured by the release of ³H. The same approach has now been used to assay the dermatan sulphate epimerase. Properties of the epimerase are described, and evidence is presented to show that the two epimerases are different.

Experimental

Materials

Chondroitin 4-sulphate from beef nasal septum was prepared as described (Fransson & Malm-

Abbreviations used: IdoA, iduronic acid; Mes, 4-morpholine-ethanesulphonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

ström, 1971). Dermatan sulphate was a gift from Dr. L. Rodén, Birmingham, AL, U.S.A., and it was isolated from heparan side fractions by alkaline copper precipitation (Lindahl *et al.*, 1965). The remaining heparan sulphate was removed after treatment with HNO₂ at pH 1.5 (Shively & Conrad, 1976) and chromatography on a column of Sephadex G-50. In order to decrease the amount of glucuronate in dermatan sulphate, 100 mg of dermatan sulphate was dissolved in 5 ml of 0.1 M-Tris/HCl buffer, pH 7.3, and 0.1 units of chondroitinase AC-II were added three times over a period of 16 h. The digest was subsequently chromatographed on a column (2.2 cm × 145 cm) of Sephadex G-100, which was equilibrated in 0.2 M-acetic acid/pyridine buffer, pH 5.0. The major part (90%) of the remaining polysaccharide that chromatographed in the void volume was pooled. Chondroitin sulphate and chondroitinase AC-treated dermatan sulphate was desulphated three times with acid methanol as described by Kantor & Schubert (1957). After this treatment the galactosaminoglycans contained less than 0.1% of sulphate. As this procedure partly depolymerizes the polysaccharides the desulphated de-esterified product was chromatographed on a Sephadex G-100 column. Material containing uronic acid and eluting between *K_{av}* 0.15 and 0.35 was pooled.

Heparin (155 units/mg) was purchased from Inolex Pharmaceutical Division, Park Forest South, IL, U.S.A. Dextran sulphate (M_r 60000) and Sephadex G-100 were obtained from Pharmacia. Ion-exchange resin (DE-52) was a product of Whatman. Materials for cell culture work were purchased from Flow Laboratories. [$5\text{-}^3\text{H}$]Heparan (specific radioactivity 1245 d.p.m./ μg of uronic acid) was a gift from Dr. L. Rodén and was prepared enzymically (Prihar *et al.*, 1980) by incubating desulphated and re-*N*-sulphated heparin with partially purified epimerase and $^3\text{H}_2\text{O}$. [$5\text{-}^3\text{H}$]Chondroitin was prepared from epiphyses of 12-day-old chicken, which were incubated with 1 mCi of [$5\text{-}^3\text{H}$]glucose in 5 ml of Krebs–Ringer solution for 6 h. The epiphyses were digested with papain and the chondroitin sulphate was recovered by cetylpyridinium chloride precipitation (Fransson & Malmström, 1971). The [$5\text{-}^3\text{H}$]chondroitin sulphate was desulphated as described by Kantor & Schubert (1957). The final product [$5\text{-}^3\text{H}$]chondroitin had a specific activity of 18200 d.p.m./ μg of uronic acid. Chondroitinase ABC digestion followed by distillation showed that 40% of the radioactivity was located at C-5 of the uronosyl residues (chondroitinase ABC acts as an ‘eliminase’ and digestion of a substrate containing $5\text{-}^3\text{H}$ -labelled glucuronosyl or iduronosyl residues liberates ^3H and creates 4,5-unsaturated glycuronosyl residues.) Adenosine 3'-phosphate 5'-phosphosulphate was prepared by using enzymes from a rat liver high speed supernatant as described by Jacobsson *et al.* (1979).

Epimerase enzyme was prepared from cultured human skin fibroblasts. The cells were cultured as described earlier (Malmström *et al.*, 1975). Cells in monolayer between 5 and 12 passages were rinsed twice with phosphate-buffered saline (137 mM-NaCl/3 mM-KCl/8 mM- Na_2HPO_4 /1 mM- KH_2PO_4 , pH 7.4) detached by shaking with 0.5 mM-disodium EDTA in phosphate-buffered saline and collected by centrifugation at 400 *g* for 2 min. The cells were rinsed with phosphate-buffered saline and then dispersed in 10 vol. of 0.25 M-sucrose, pH 7.4, and homogenized with ten strokes at 2000 rev./min in a Potter–Elvehjem homogenizer. The homogenate was centrifuged at 600 *g* for 10 min. The supernatant was subsequently centrifuged at 10000 *g* for 20 min. The 600 *g* pellet was rehomogenized and recentrifuged as above. This procedure was repeated four times. The 10000 *g* supernatants were combined and centrifuged at 105000 *g* for 1 h. The 600 *g*, the 10000 *g* and the 105000 *g* pellets were suspended by gentle homogenization in 0.05 M-Hepes, pH 6.5, in a Potter–Elvehjem homogenizer and stored at -60°C . Solubilization of microsomal enzyme was carried out as described by Schwartz & Rodén (1975). Briefly, microsomal preparations were sonicated in 0.1 M-Hepes, pH 6.2, containing 0.5% Nonidet P-40.

After sonication solid KCl was added to a final concentration of 0.5 M and the mixture was incubated for 30 min. Finally the solubilized enzyme was separated from the remaining membranes by centrifugation at 105000 *g* for 1 h.

Enzyme assays

The dermatan sulphate epimerase was assayed in a final volume of 100 μl of 25 mM-Mes, pH 5.6, containing 10 mM- MnCl_2 , 0.5% Nonidet NP 40, 68000 d.p.m. (4 μg of uronic acid) of [$5\text{-}^3\text{H}$]chondroitin and 50–150 μg of microsomal protein. In the standard procedure the samples were incubated for 4 h and then boiled for 3 min. After addition of 200 μl of water the samples were distilled (Jacobsson *et al.*, 1979) and 200 μl was counted in a Packard 2650 liquid-scintillation counter.

In order to study the pH-dependence of the enzyme the reaction was carried out either in 25 mM-sodium acetate (pH 4.5–5.6), 25 mM-Mes (pH 5.3–6.5) or 25 mM-Hepes (pH 7.0–7.5).

Heparan sulphate epimerase was assayed in a final volume of 100 μl of 50 mM-Hepes, pH 7.4, containing 50 mM-KCl, 15 mM-EDTA, 0.5% Nonidet NP 40, 29900 d.p.m. (24 μg of uronic acid) of [$5\text{-}^3\text{H}$]heparin and 50–150 μg of microsomal protein. The procedure was then as above.

Analytical methods

Protein was quantified by the method of Lowry *et al.* (1951). Before analysis, interfering salts were removed by trichloroacetic acid precipitation of the protein followed by resolubilizing it in alkali as described by Malmström (1981). Uronic acid was measured with the carbazole reaction (Bitter & Muir, 1962). Chondroitinase-ABC digestions were carried out as described by Yamagata *et al.* (1968).

Results

Incubation of [$5\text{-}^3\text{H}$]chondroitin with a particulate enzyme preparation from cultured fibroblasts resulted in a linear release of ^3H with time. The ^3H was recovered by distillation after it had equilibrated with the water of the incubation medium (Scheme 1). The pH optimum of the reaction was approx. 5.5. The same optimum was obtained both with sodium acetate and Mes buffers, although the enzyme was 1.6-fold more active in the latter buffer. The enzyme is, however, active over a pH range from 5 to 7. The epimerase has an absolute requirement for divalent cations, Mn^{2+} being the most efficient (Fig. 1), while Ca^{2+} and Mg^{2+} are poorer activators. The optimal concentration of Mn^{2+} is 10 mM, but at higher concentrations there is inhibition. The enzyme is sensitive to ionic strength, as increasing concentrations of KCl or NaCl (Fig. 1)

Fig. 1. *Effect of different divalent cations and ionic strength on the epimerase activity*

Microsomal enzyme preparations from fibroblasts were incubated with [5-³H]chondroitin in the presence of various concentrations of Mg²⁺ (Δ), Ca²⁺ (○), Mn²⁺ (●) or KCl (▲) under otherwise standard incubation conditions. The experiment with KCl was performed with 10mM-MnCl₂ in the incubation mixture.

in the presence of optimal divalent-ion concentrations decreased the activity. Detergents considerably enhanced the reaction rate and Nonidet at a concentration of 0.5% increased the activity 1.5-fold. The K_m for [5-³H]chondroitin was 1.2×10^{-4} M, expressed as molarity of glucuronate residues. The concentration of substrate used in the subsequent assays was, to economize on the substrate, 2.3×10^{-4} M. Under these conditions the release of ³H was linear with time for at least 8 h and linear with concentration of microsomal protein up to 250 μg.

In order to elucidate the substrate specificity of the epimerase various polysaccharides were added to the incubation mixture. Thus, if the added derivative is a substrate, a decrease of specific activity occurs which would result in a decrease in the release of ³H. As shown in Fig. 2, addition of chondroitin diminished the release of ³H, as did dermatan, which should be the product in the reaction. Sulphated polysaccharides were, however, much better inhibitors of ³H release than were non-sulphated polysaccharides. Again, dermatan sulphate was more inhibitory than was chondroitin sulphate. Heparin was the best inhibitor. Dextran sulphate

Fig. 2. *Effect of various polysaccharides on the epimerase activity*

Microsomal enzyme was incubated under standard conditions with different concentrations of chondroitin (▼), dermatan (□), chondroitin sulphate (■), dermatan sulphate (○), or heparin (●).

Fig. 3. *Effect of adenosine 3'-phosphate 5'-phosphosulphate on epimerase activity*

Microsomal enzyme was incubated under standard conditions with different concentrations of adenosine 3'-phosphate 5'-phosphosulphate.

(results not shown) was as inhibitory as chondroitin sulphate, which suggests that the inhibition of ³H release was not only caused by substrate competition by the added polysaccharide. Adenosine 3'-phosphate 5'-phosphosulphate also inhibited the release of ³H from [5-³H]chondroitin above a concentration of 1mM (Fig. 3). No other experiments have yet been performed to elucidate the mechanism of the inhibition.

The distribution of the epimerase in different subcellular fractions was also investigated. Table 1

Table 1. *Distribution of epimerase activity in various subcellular fractions*

Fibroblast were cultured, harvested, homogenized and fractionated as described in the Experimental section. The various fractions were assayed for epimerase activity and protein.

Fraction	Total protein (mg)	Total activity (%)	Specific activity (d.p.m. of ^3H released/h per mg of protein)
600 g pellet	29.8	4	130
10 000 g pellet	27.9	36	1340
105 000 g pellet	19.6	53	2860
105 000 g supernatant	52.5	8	150

shows that the enzyme is largely (more than 90%) associated with the microsomal membranes. The highest specific activity was found in the 105 000 g pellet. The distribution of the activity between the 10 000 g pellet and 105 000 g pellet fractions varies between preparations, but the highest specific activity was always obtained in the 105 000 g fraction. The epimerase is firmly bound to the microsomes, as freezing followed by centrifugation released less than 20% of the enzyme activity. Sonication in detergent and subsequent addition of KCl to 0.5 M, however, solubilized 60% of the activity. The remainder was still found within the membranes.

As the dermatan sulphate epimerase is similar to the heparan sulphate epimerase, experiments were designed to investigate if the two enzymes were identical. Experiments were therefore carried out with a biosynthetically labelled heparin derivative and fibroblast microsomes at pH 7.4 with EDTA (optimal conditions for heparosan epimerase) as described by Malmström *et al.* (1980) and indeed heparan sulphate epimerase activity was noted (Fig. 4a). The reaction was linear for at least 2 h. When [$5\text{-}^3\text{H}$]chondroitin was incubated under the same conditions, no release of ^3H could be seen (Fig. 4a). The two substrates were also incubated under conditions optimal for the dermatan sulphate epimerase. As shown in Fig. 4(b) incubation with [$5\text{-}^3\text{H}$]chondroitin resulted in a linear release of ^3H with time, whereas no activity with the ^3H -labelled heparin derivative was detected (Fig. 4b). It can therefore be concluded that there are two different epimerases or active sites, one involved in dermatan sulphate biosynthesis and one in heparan sulphate biosynthesis.

Discussion

As shown earlier (Malmström, 1981) incubation of fibroblast microsomes with UDP[$5\text{-}^3\text{H}$]glucuronate, UDP[U- ^{14}C]glucuronate, UDP-*N*-acetylgalactosamine and adenosine 3'-phosphate 5'-phosphosulphate yields a dermatan sulphate polymer with $5\text{-}^3\text{H}$ - and U- ^{14}C -labelled glucuronate residues but only U- ^{14}C -labelled iduronate residues. Thus, release of ^3H appears to accompany the poly-

Scheme 1. *Epimerase action on [$5\text{-}^3\text{H}$]chondroitin*

mer-level C-5 epimerization of glucuronate to iduronate (see also Malmström *et al.*, 1975). In the present study, incubation of an identical microsomal preparation with [$5\text{-}^3\text{H}$]chondroitin as a substrate resulted in release of ^3H (Scheme 1). The ^3H which may be recovered from the medium as $^3\text{H}_2\text{O}$ must be derived from the glucuronate residues of chondroitin, because exhaustive digestion of this substrate with chondroitinase-ABC liberated the same amounts of ^3H . Hence, ^3H release may be used as a measure for epimerase activity.

Little is known about the regulation of the epimerase activity and also how the co-polymeric structure of the final product is attained. It has been shown earlier (Malmström *et al.*, 1975) that epimerization and sulphation are tightly coupled during biosynthesis of dermatan sulphate. Incubation of UDP-glucuronic acid and UDP-*N*-acetylgalactosamine with microsomes yielded chondroitin with less than 10% of iduronic acid, while after addition of adenosine 3'-phosphate 5'-phosphosulphate synthesis of dermatan sulphate with approx. 60% iduronic acid was observed. At least three different explanations for the increase of iduronic acid residues by concomitant sulphation can be postulated, namely: (i) the optimal substrate for the epimerase is a sulphated structure, (ii) sulphation of the newly formed iduronosyl-containing disaccharide removes it from an equilibrium which favours glucuronosyl residues, or finally (iii) epimerase and sulphotransferase may be located together in the endoplasmic reticulum so that epimerization is directly following by sulphation.

The first possibility can be ruled out, as chondroitin is a much better substrate for the epimerase than is chondroitin sulphate (A. Malmström, unpublished work). The present results do not permit a distinction between the latter two possibilities.

Fig. 4. Comparison of the activity of heparan sulphate and dermatan sulphate epimerases at (a) optimal conditions for the former enzyme and (b) optimal conditions for the latter enzyme

Microsomal enzyme preparations from fibroblasts were incubated with a 5-³H-labelled heparin derivative (●—●) or with [5-³H]chondroitin (●---●) as described in the Experimental section.

Sulphated polymers, however, efficiently inhibit the epimerase. This may indicate that sulphation is important as a regulator of the epimerase and that sulphation of the substrate prevents the action of the

enzyme. Therefore adenosine 3'-phosphate 5'-phosphosulphate may be an important regulator of epimerization, since in high concentrations it was inhibitory to the process. This effect is most likely not operative *in vivo*, as the concentration of adenosine 3'-phosphate 5'-phosphosulphate in the fibroblast is much lower than 2 mM (B. Särnstrand, G. Ericksson, & A. Malmström, unpublished work).

The dermatan sulphate and the heparan sulphate epimerases are most likely different enzymes, as pH optima, cation requirements and sensitivity to ionic strength are different. Furthermore, as shown in Fig. 4, the dermatan sulphate epimerase is inactive when incubated under optimal conditions for the heparan sulphate epimerase, and *vice versa*. Further research involving purification and separation of the enzyme molecules are required to establish definitely this concept.

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