Biosynthesis of dermatan sulphate

Defructosylated *Escherichia coli* K4 capsular polysaccharide as a substrate for the D-glucuronyl C-5 epimerase, and an indication of a two-base reaction mechanism

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The capsular polysaccharide from *Escherichia coli* K4 consists of a chondroitin {[GlcA(β1→3)GalNAc(β1→4)]$_n$} backbone, to which β-fructofuranose units are linked to C-3 of D-glucuronic acid (GlcA) residues. Removal of the fructose units by mild acid hydrolysis provided a substrate for the GlcA C-5 epimerase, which is involved in the generation of L-iduronic acid (IdoA) units during dermatan sulphate biosynthesis. Incubation of this substrate with solubilized fibroblast microsomal enzyme in the presence of ^3H_2O resulted in the incorporation of tritium at C-5 of hexuronyl units. A K_m of 67×10^{-6} M hexuronic acid (equivalent to disaccharide units) was determined, which is similar to that (80×10^{-6} M) obtained for dermatan (desulphated dermatan sulphate). $V_{max.}$ was about 4 times higher with dermatan than with the K4 substrate. A defructosylated K4 polysaccharide isolated after incubation of bacteria with D-[5-^{3}H]glucose released ^3H_2O on reaction with the epimerase, and thus could be used to assay the enzyme. Incubation of a K4 substrate with solubilized microsomal epimerase for 6 h in the presence of ^3H_2O resulted in the formation of about 5% IdoA and approximately equal amounts of ^{3}H in GlcA and IdoA. A corresponding incubation of dermatan yielded approx. 22% GlcA, which contained virtually all the ^{3}H label. These results are tentatively explained in terms of a two-base reaction mechanism, involving a monoprotic L-*ido*-specific base and a polyprotic D-*gluco*-specific base. Most of the IdoA residues generated by the enzyme occurred singly, although some formation of two or three consecutive IdoA-containing disaccharide units was observed.

INTRODUCTION

Proteoglycans bearing galactosaminoglyan chains occur in most extracellular matrices [1]. Large proteoglycans of the aggrecan/versican type are mainly substituted with chondroitin 4- and 6-sulphate (CS) chains, i.e. polysaccharides containing D-glucuronic acid (GlcA) as the only hexuronic acid constituent, in alternating sequence with *N*-acetylgalactosamine (GalNAc) units. A galactosaminoglycan molecule containing one or more L-iduronic acid (IdoA) units is referred to as dermatan sulphate (DS). Certain proteoglycans may carry either CS or DS chains, depending on tissue source. Thus aggrecan proteoglycans in sclera bear DS chains with a rather low proportion of IdoA residues [2]. In fibrous connective tissue, the small proteoglycans, decorin and biglycan, bear DS with a high proportion of IdoA [about 70% of total hexuronyl (HexA) residues] [2,3]. In other tissues, such as bone, decorin is substituted with CS chains only [4]. The detailed structure of DS is generally complex. Disaccharide units containing IdoA or GlcA residues tend to be arranged in block structures, i.e. in a non-random fashion, along the polysaccharide chain [5]. The co-polymeric structure is presumably a prerequisite to the biological properties of DS. Proposed functions include control of cell proliferation, which apparently requires DS of high IdoA content [6], control of collagen deposition in tendons [7], effects on cell differentiation [8] and self-association of DS chains [9]. DS also has properties of pharmacological and clinical interest, such as the ability to inhibit thrombin through heparin cofactor II [10]; CS shows no such activity.

The biosynthesis of the polysaccharide chains in DS proteoglycans involves three major steps: formation of the linkage region that joins the polysaccharide chain to the protein core, assembly of repeating [GlcA(β1→3)GalNAc(β1→4)]$_n$ disaccharide units, and modification of the resultant polysaccharide backbone. The modifications include formation of IdoA units through C-5 epimerization of GlcA residues and O-sulphation at various positions of the disaccharide units [11]. The most abundant O-sulphation positions are C-4 and C-6 of the GalNAc and at C-2 of the IdoA units [12]. Although the mechanisms that determine the extent of C-5 epimerization are unknown, it is noteworthy that treatment of fibroblasts with transforming growth factor β results in the production of DS with a lower proportion of IdoA [13].

The HexA epimerization reaction involves the reversible abstraction of a proton from the C-5 position of the target uronic acid residue, which is then replaced by a proton from the medium [14]. The enzyme can thus be assayed by measuring the release of ^{3}H (recovered as ^3H_2O) from either chondroitin {[GlcA(β1→3)GalNAc(β1→4)]$_n$} or dermatan {[IdoA(α1→3)GalNAc(β→4)]$_n$} containing 5-^{3}H-labelled HexA units. Such an assay, using labelled chondroitin as substrate, was employed to establish the basic kinetic properties of DS epimerase from cultured human fibroblasts [15]. The 5-^{3}H-labelled chondroitin was prepared from epiphyses of 12-day-old chicken,

Abbreviations used: $aTal_R$, 2,5-anhydro-D-talitol (formed by the reduction of anhydrotalose units, obtained by deamination of galactosamine residues); CS, chondroitin sulphate; DS, dermatan sulphate; GalNAc, 2-acetamido-2-deoxy-D-galactose (*N*-acetyl-D-galactosamine); GlcA, D-glucuronic acid; HexA, hexuronic acid (unspecified); IdoA, L-iduronic acid.

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which had been incubated with [5-^{3}H]glucose. Alternatively, labelled substrates were obtained by incubating either chondroitin or dermatan (provided by chemical desulphation of CS or DS respectively) with crude epimerase in the presence of ^3H_2O [16]. After extended incubation with either substrate, the ratio of [5-^{3}H]GlcA to [5-^{3}H]IdoA reached a value of approx. 85:15 which appeared to represent equilibrium. The sulphated polysaccharides, DS and CS, were both inactive as substrates, indicating that HexA C-5 epimerization precedes sulphation [16]. However, epimerization and sulphation seem to be tightly coupled, as the formation of IdoA in a microsomal biosynthetic system is strongly promoted by concomitant sulphation of the polysaccharide [11,14].

The capsular K4 antigen of *Escherichia coli* O5:K4:H4 is a polysaccharide that has the same structure as chondroitin, except that β-fructofuranose is attached to C-3 of the GlcA residues [17]. The present report demonstrates that the polysaccharide backbone obtained after hydrolytic removal of the fructose residues can be used as a model substrate for the HexA C-5 epimerase involved in DS biosynthesis. Moreover, this substrate, along with the fully epimerized isomer, dermatan, was used to gain more detailed insight into the mode of action of the epimerase.

EXPERIMENTAL

Materials

Defructosylated *E. coli* K4 polysaccharide (subsequently called K4 polysaccharide) was a gift from Benito Casu (G. Ronzoni Institute, Milan, Italy). DS from pig skin [16] was digested with chondroitin AC lyase, separated from degradation products by gel chromatography on Sephadex G-100, and desulphated by treatment with methanolic HCl [18]. GlcA-[1-^{3}H]aTal$_R$ and IdoA-[1-^{3}H]aTal$_R$ disaccharide standards were prepared from K4 polysaccharide and dermatan respectively, by N-deacetylation (treatment with 30% aqueous hydrazine/1% hydrazine sulphate, at 100 °C for 4 h), followed by deaminative cleavage with HNO_2 at pH 3.9, and reduction of the products with NaB^3H_4 [19]. *E. coli* U1-41 (O5:K4:H4; Freiburg collection number 2616), which generates the K4 capsular polysaccharide [17], was given by K. Jann, Max Planck Institut für Immunbiologie, Freiburg, Germany. Chondroitin AC lyase I and chondroitin ABC lyase were obtained from Seikagaku.

D-[1-^{14}C]Glucose (56.8 mCi/mmol) and ^3H_2O (90 Ci/mol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. and D-[5-^{3}H]glucose (15.7 Ci/mmol) from Dupont/NEN. Sephadex G-15, G-50, G-100 and DEAE-Sephacel, as well as prepacked PD-10 (Sephadex G-25), Superose 6 HR 10/30 and Superose 12 HR 10/30 columns and Fast Desalting Column HR 10/10, were purchased from Pharmacia. DEAE-cellulose (DE-52) was from Whatman.

Metabolically labelled K4 polysaccharide

E. coli U1-41 (O5:K4:H4) stock (20 μl) was precultured in 2 ml of Luria–Bertoni medium [10 g/l tryptone (Difco), 5 g/l yeast extract (Difco), 10 g/l NaCl] containing 0.5% glucose at 37 °C overnight. Half of the preculture was added to 45 ml of this medium lacking glucose and was incubated at 37 °C for 2 h. After the addition of 200 μCi of D-[1-^{14}C]glucose or, in a separate incubation, 200 μCi of D-[5-^{3}H]glucose, the cultures were incubated for an additional 24 h period. After centrifugation at 3500 ***g*** for 30 min, the supernatant was applied to a 2 ml column of DEAE-Sephacel, equilibrated with 50 mM sodium acetate/50 mM NaCl, pH 4.0. The column was washed with 20 ml of the same buffer and then eluted using a linear NaCl gradient (0.05–1.0 M in 50 mM sodium acetate, pH 4.0; total gradient volume, 80 ml). A distinct peak of radioactivity and HexA-containing material appeared near the middle of the gradient (Figure 1a). The fractions containing the labelled polysaccharide were pooled, desalted by chromatography on a short Sephadex G-15 column equilibrated with 10% ethanol and lyophilized. Analysis of the products by a colorimetric procedure using the ketohexose-specific modified cysteine/H_2SO_4 reagent [20] indicated approx. 312 μg of fructose/mg of polysaccharide. Samples were defructosylated by mild acid hydrolysis (HCl to pH 2.8) at 23 °C for 12 h [17] followed by passage through a PD-10 column. Approx. 40% of the label, presumably representing the released fructose residues, appeared in the low-molecular-mass region of the chromatogram. Colorimetric analysis of the macromolecular portion showed no detectable residual fructose (< 5 μg/mg). The final yield of the ^{14}C-labelled polysaccharide was about 1.2 mg (specific radioactivity 1.6×10^6 d.p.m./μmol of HexA, determined by the carbazole reaction [21]), whereas that of the

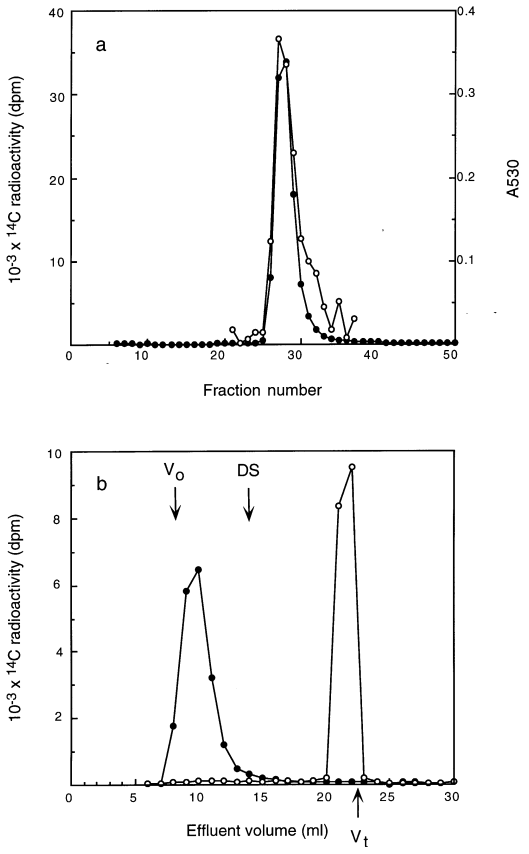

Figure 1 Isolation and characterization of ^{14}C-labelled polysaccharide from *E. coli* strain K4

Bacteria were cultured in the presence of D-[1-^{14}C]glucose as described in the Experimental section, and the culture was centrifuged. (**a**) The supernatant was fractionated by chromatography on DEAE-Sephacel, eluted by use of a linear salt gradient (0.05–1.0 M NaCl in 50 mM sodium acetate, pH 4.0), starting with fraction 11 and ending with fraction 50. Effluent fractions were analysed for radioactivity (●) or HexA (○; carbazole reaction). Fractions containing the labelled polysaccharide were pooled and dialysed; fructose residues were eliminated by mild acid hydrolysis. (**b**) The product was analysed by gel chromatography on a column of Superose 6 HR 10/30, equilibrated with 4 M guanidinium chloride/0.05 M sodium acetate, pH 5.8, before (●) or after (○) digestion with chondroitin AC lyase I. For comparison, the peak elution position of a sample of pig skin DS is shown (arrow). For additional information see the Experimental section.

^{3}H-labelled polysaccharide was about 0.7 mg (1.4×10^6 d.p.m./μmol of HexA). Digestion with chondroitin AC lyase resulted in virtually quantitative conversion of the defructosylated ^{14}C-labelled polysaccharide into labelled disaccharides (Figure 1b). Similar digestion of the 5-^{3}H-labelled polysaccharide resulted in the formation of ^3H_2O, released by elimination from [5-^{3}H]GlaA residues and quantified by the distillation procedure described [22].

Incubations with microsomal epimerase

Human embryonic skin fibroblasts were cultured as described previously [11]. Confluent cultures were washed with PBS and the cells were detached with a 'rubber policeman'. The cells were suspended in 0.25 M sucrose/0.05 M Hepes, pH 6.5, and homogenized in a Potter–Elvehjem homogenizer. The resultant homogenate was differentially centrifuged, and the 600 ***g***, 10000 ***g*** and 105000 ***g*** pellets were suspended in 0.05 M Hepes, pH 6.5 [15]. The 10000 ***g*** pellet, which contained the highest epimerase activity, was used as enzyme source in the incubations.

Incubations were performed at 37 °C, in a volume of 100 μl, containing 0.05 M Hepes, pH 6.5, substrate polysaccharide (various amounts for K_m determinations; 25 μg in other incubations), 10 mM $MnCl_2$, 0.25 % Nonidet P40 and 10 μg of microsomal protein. After completed incubations of epimerase with defructosylated K4 polysaccharide containing 5-^{3}H-labelled GlcA units, the ^3H_2O formed was recovered by distillation and quantified by liquid-scintillation counting [22]. Other experiments involved incorporation into the polysaccharide substrate of ^{3}H from ^3H_2O (50 mCi) in the incubation medium. To recover the labelled polysaccharide, each sample was mixed with 1.5 ml of 6 M urea/0.05 M sodium acetate, pH 5.8, and then applied to a small (1 cm × 2 cm) DEAE-cellulose column (DE-52; Whatman), which was washed with successive 5 ml portions of urea buffer until the radioactivity in 200 μl of the effluent was less than 500 d.p.m. The column was then washed with 3 ml of 0.1 M NH_4Cl and the polysaccharides were eluted from the anion-exchange resin with 2 × 1.5 ml of 1 M NH_4Cl. The eluate fractions were combined, repeatedly freeze-dried with intermittent addition of water, and finally subjected to gel chromatography on a Superose 12 HR 10/30 column (Pharmacia), equilibrated with 4 M guanidinium chloride/0.05 M sodium acetate, pH 5.8. The polysaccharide fractions were pooled, dialysed against water/0.5 M NaCl, three additional changes of water, and finally lyophilized. In some experiments (described under 'Mode of action of the epimerase' in the Results section), a simplified isolation procedure was used with satisfactory results. Each sample was mixed with 500 μl of 0.05 M sodium acetate/1.5 M LiCl, pH 4.0, and passed through a PD-10 column (Sephadex G-25; Pharmacia) equilibrated with 0.2 M NH_4HCO_3; most of the ^3H_2O remained in the column, simplifying disposal. Final purification was achieved by binding the polysaccharides to approx. 1 ml columns of DEAE-Sephacel (Pharmacia), followed by elution with 2 M NH_4HCO_3. The fractions containing the samples were pooled and lyophilized.

Analysis of polysaccharides

Polysaccharide samples were either exhaustively digested with chondroitin lyases (ABC or AC I) or cleaved with HNO_2 at pH 3.9 after N-deacetylation. Digestion with chondroitin lyase (ABC and AC I) was performed with 25 m-units of enzyme in 300 μl of 0.1 M Tris/HCl/0.1 M sodium acetate, pH 7.3, at 37 °C overnight. After digestion of ^{3}H-containing samples with chondroitin ABC lyase, the released ^{3}H, in the form of ^3H_2O, was recovered after distillation and measured by liquid-scintillation counting [22]. The products of chondroitin AC lyase digestion were applied (along with 1 mg of chondroitin sulphate added as a 'carrier') to a long column (1 cm × 190 cm) of Sephadex G-50, equilibrated with 1 M NaCl. Effluent fractions of volume approx. 2 ml were collected at a rate of about 6 ml/h and analysed for radioactivity (either ^{3}H alone or ^{3}H along with ^{14}C when ^{14}C-labelled K4 polysaccharide was used as a substrate).

The overall composition of galactosaminoglycans was determined by analysis of HexA-[1-^{3}H]aTal$_R$ disaccharides, generated by N-deacetylation followed by deaminative cleavage and reduction of the products with NaB^3H_4 [19]. Briefly, samples were subjected to hydrazinolysis (70 % hydrazine/30 % H_2O/1 % hydrazine sulphate) at 96 °C for 4 h, and then treated with HNO_2 at pH 3.9 (deamination of N-unsubstituted hexosamine units). The resultant disaccharides were reduced with NaB^3H_4, recovered by gel chromatography on Sephadex G-15 in 0.2 M NH_4HCO_3, and then desalted by lyophilization. The labelled disaccharides were separated by paper chromatography on Whatman no. 1 paper in ethyl acetate/acetic acid/water (3:1:1, by vol.), along with GlcA-[1-^{3}H]aTal$_R$ and IdoA-[1-^{3}H]aTal$_R$ standards. Paper strips were cut into 1 cm pieces that were analysed by liquid-scintillation counting, and the GlcA/IdoA ratios were calculated.

RESULTS

Defructosylated K4 polysaccharide as an epimerase substrate

Defructosylated K4 polysaccharide and dermatan were both incubated with fibroblast microsomal enzyme in the presence of ^3H_2O, recovered and exhaustively digested with chondroitin ABC lyase, as described in the Experimental section. Chondroitin ABC lyase will release ^{3}H from C-5 of both GlcA and IdoA units as ^3H_2O. The time course of ^{3}H labelling of the polysaccharide was determined (Figure 2). Tritium was incorporated into the K4 polysaccharide, albeit at a lower rate than into dermatan, in agreement with previous results for chondroitin [16]. A series of incubations was conducted for 4 h at different substrate concentrations to establish the basic kinetic parameters. The two polysaccharide substrates showed similar K_m values [67×10^{-6} M HexA (equivalent to disaccharide units) for the K4 polysaccharide and 80×10^{-6} M HexA for dermatan] in reasonable

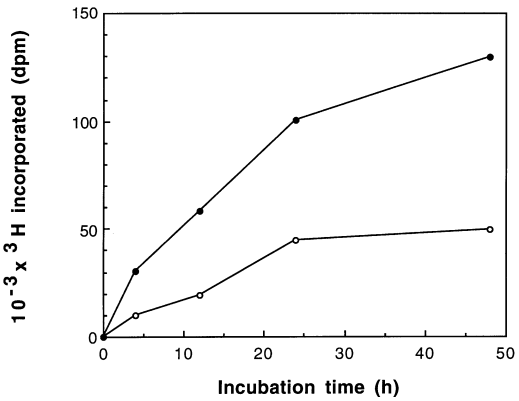

Figure 2 Incorporation of ^{3}H into K4 polysaccharide and dermatan

Solubilized microsomal epimerase was incubated with K4 polysaccharide (○) or with dermatan (●) in the presence of ^3H_2O. After incubation for the indicated periods of time, the samples were recovered and digested with chondroitin ABC lyase, as described in the Experimental section, to release the ^{3}H incorporated at C-5 of GlcA and IdoA units. The ^3H_2O formed was recovered by distillation, and quantified by liquid-scintillation counting. For additional information see the Experimental section.

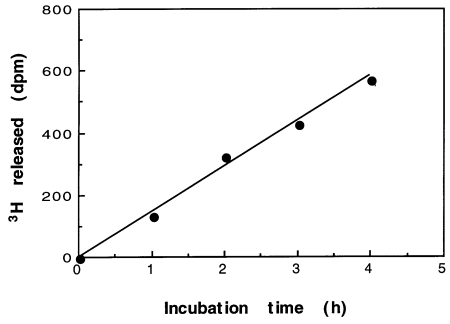

Figure 3 Release of tritium from metabolically 5-^{3}H-labelled K4 polysaccharide

Labelled K4 polysaccharide was prepared (including defructosylation) after the addition of D-[5-^{3}H]glucose to a culture of *E. coli* K4. Samples of about 20000 d.p.m. of ^{3}H were incubated with solubilized microsomal epimerase for the indicated periods of time, and the released ^3H_2O was recovered by distillation and quantified. For additional information see the Experimental section.

agreement with the value (120×10^{-6} M) previously determined for chondroitin [15]. Under the conditions of the assay, $V_{max.}$ was about 4 times higher for dermatan (190 d.p.m. of ^{3}H/h per μg of microsomal protein) than for K4 polysaccharide (46 d.p.m. of ^{3}H/h per μg of protein). This difference is probably not due to factors such as residual fructose residues in the K4 polysaccharide or differences in molecular mass, since previous experiments using dermatan and chondroitin of the same molecular mass gave similar results (^{3}H was incorporated about 5 times faster into dermatan than into chondroitin during short incubations of 1 h or less [16]).

Table 1 Analysis of polysaccharides incubated with C-5 epimerase in the presence or absence of ^3H_2O

Metabolically ^{14}C-labelled K4 polysaccharide and unlabelled dermatan were incubated with solubilized microsomal enzymes in the presence or absence of ^3H_2O, as described in the Experimental section. The polysaccharides recovered after incubation with ^3H_2O were digested with chondroitin AC lyase I, and the products were separated by gel chromatography on Sephadex G-50 (Figure 5). The relative amounts of [5-^{3}H]GlcA and [5-^{3}H]IdoA (column A) were calculated from the peak areas relating to ^3H_2O (at the V_t of the column) and to oligosaccharides repectively. Samples recovered from incubations in unlabelled water were processed to generate HexA-[1-^{3}H]aTal$_R$ disaccharides, which were then separated into the GlcA- and IdoA-containing isomers by paper chromatography (Figure 4). The relative amounts of GlcA and IdoA (column B) were calculated from the peak areas. The composition of samples derived from metabolically ^{14}C-labelled K4 polysaccharide (column B, values in parentheses) was calculated as described in the text, from the proportions of labelled oligosaccharides separated after digestion with chondroitin AC lyase I (Figures 5a and 5b). For further information, see the Experimental section.

Substrate	Incubation time (h)	A [5-^{3}H]GlcA / [5-^{3}H]GlcA + [5-^{3}H]IdoA (%)	B GlcA / GlcA + IdoA (%)
K4 polysaccharide	6	51	95 (97)
	72	63	85 (90)
Dermatan	6	> 98	22
	72	87	58

Capsular K4 polysaccharide containing 5-^{3}H-labelled GlcA residues was prepared by incubating *E. coli* K4 bacteria with D-[5-^{3}H]glucose, as described in the Experimental section. A sample of the product reisolated after hydrolytic release of the fructose

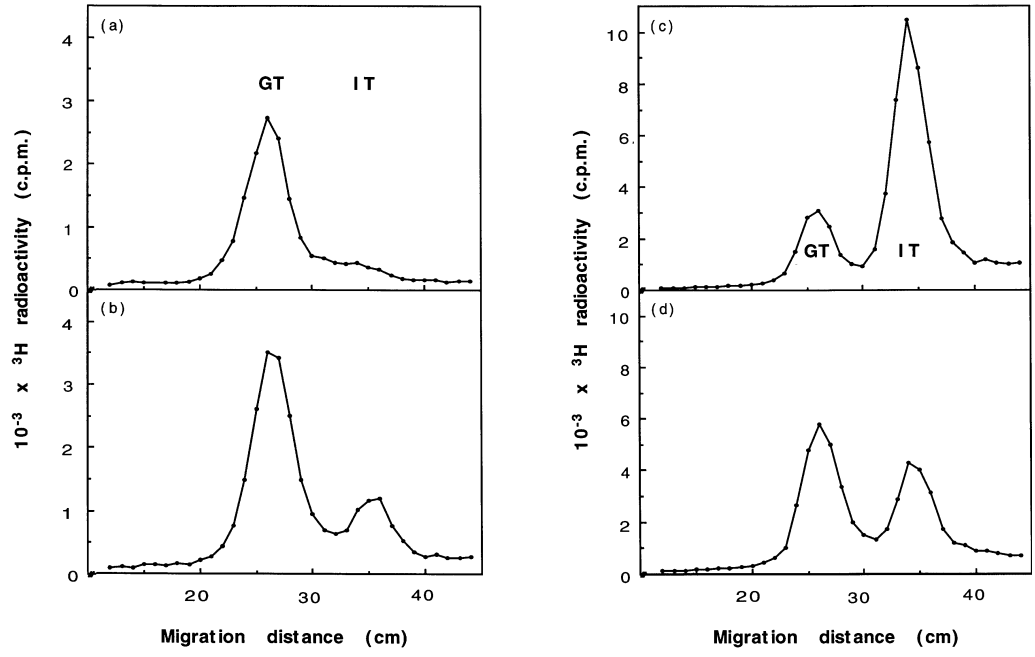

Figure 4 Disaccharide composition of polysaccharides incubated with microsomal epimperase

Polysaccharide samples (25 μg) were incubated with solubilized microsomal epimerase, recovered and processed to generate HexA-[1-^{3}H]aTal$_R$ disaccharides which were then separated by paper chromatography. Paper chromatograms are shown of disaccharides derived from K4 polysaccharide which had been incubated for (**a**) 6 h and (**b**) 72 h, and from dermatan which had been incubated for (**c**) 6 h and (**d**) 72 h with the epimerase. The two peaks were identified as GlcA-aTal$_R$ (GT) and IdoA-aTal$_R$ (IT), by comparison with standard disaccharides (not shown). For additional information see the Experimental section.

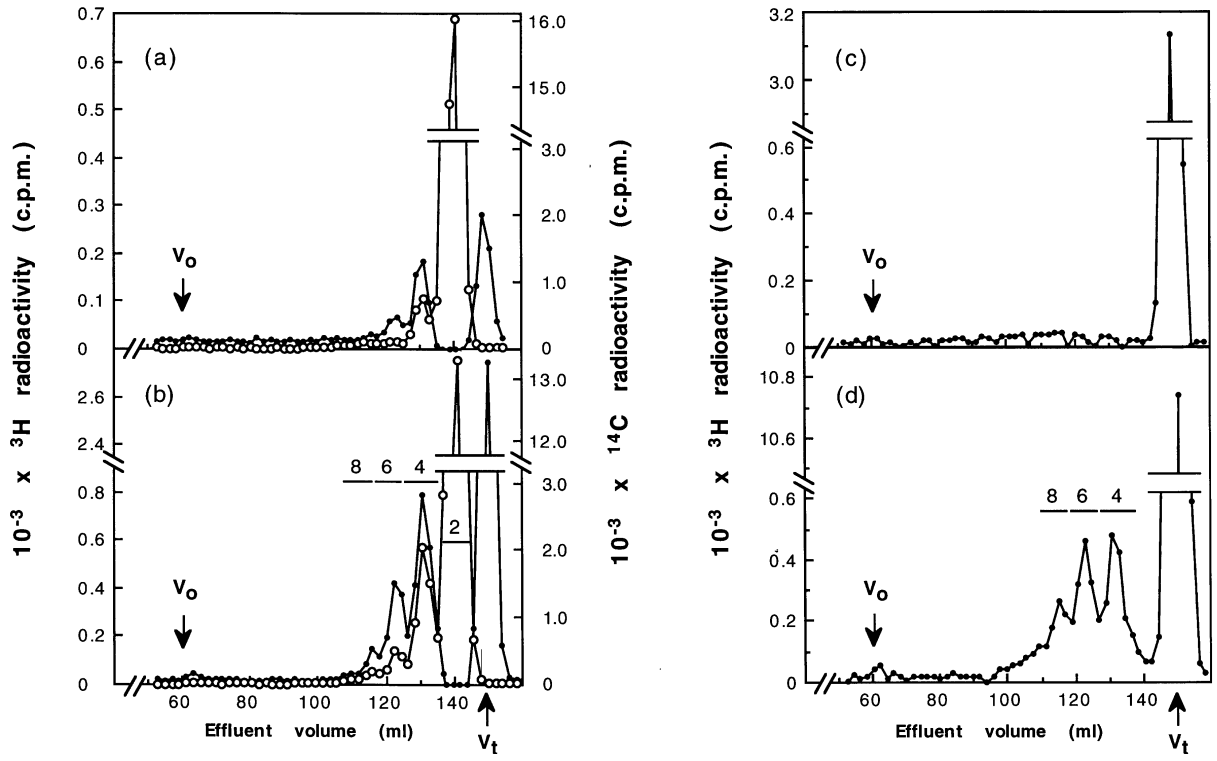

Figure 5 Digestion of radiolabelled polysaccharides with chondroitin AC lyase I

^{14}C-labelled K4 polysaccharide and unlabelled dermatan were incubated with solubilized microsomal epimerase and ^3H_2O, recovered and digested with chondroitin AC lyase I, and the products were separated by gel chromatography on Sephadex G-50. Effluent fractions were analysed for ^{3}H (●) and for ^{14}C (○). The chromatograms were derived from K4 polysaccharide that had been incubated for (**a**) 6 h and (**b**) 72 h, and from dermatan that had been incubated for (**c**) 6 h and (**d**) 72 h. The approximate elution positions of di-, tetra-, hexa- and octa-saccharides are indicated by horizontal lines in (**b**). The peak of ^{3}H at the V_t of the column represents ^3H_2O.

substituents was exhaustively digested with chondroitin AC lyase I, resulting in quantitative conversion of the labelled polymer into low-molecular-mass components as demonstrated by gel chromatography on a Fast Desalting Column HR 10/10 (results not shown). About 40% of the radiolabelled digestion products was recovered as ^3H_2O by distillation, and thus represented 5-^{3}H atoms of GlcA residues in the intact polysaccharide; the remaining 60% of the label would be located at C-5 of GalNAc units. Incubation of the defructosylated 5-^{3}H-labelled K4 polysaccharide with microsomal epimerase resulted in the release of ^3H_2O which was quantified after distillation [22]. The formation of ^3H_2O was linear with time for at least 4 h (Figure 3), after which period about 8% of the ^{3}H label in the GlcA residues, or about 3% of the total ^{3}H of the polysaccharide, had been released. Varying the amounts of microsomal protein from 0.5 to 50 μg likewise led to a linear increase in ^3H_2O release during a 2 h incubation (results not shown). A K_m value of 52×10^{-6} M HexA was determined from a series of 2 h incubations at different substrate concentrations.

Mode of action of the epimerase

Polysaccharide substrates, either dermatan or defructosylated K4 (^{14}C-labelled), were incubated with microsomal epimerase in the presence or absence of ^3H_2O. The recovered products were analysed with regard to the D-*gluco*/L-*ido* ratios of ^{3}H-labelled HexA residues, as well as total HexA units of the polysaccharides. To determine the total HexA composition, the polysaccharides incubated in the absence of ^3H_2O were N-deacetylated by hydrazinolysis, followed by deaminative cleavage to disaccharides which were subsequently radiolabelled by reduction with NaB^3H_4 (see the Experimental section). The resultant products, containing either a GlcA or an IdoA unit linked to an ^{3}H-labelled $aTal_R$ residue, were separated by paper chromatography and quantified by liquid-scintillation counting. The K4 substrate, initially composed of GlcA–GalNAc disaccharide units only (results not shown), contained a minor proportion of IdoA units, estimated to be about 5% of the total HexA, after incubation for 6 h with the epimerase (Figure 4a). After 72 h of incubation, the IdoA content had increased to about 15% of the total HexA (Figure 4b; Table 1), which is the same as the equilibrium ratio previously estimated after extensive incubations of chondroitin, or dermatan, in ^3H_2O [16]. The IdoA units of dermatan were relatively rapidly converted into GlcA residues, which amounted to 22 and 58% of the total HexA units after 6 h (Figure 4c) and 72 h (Figure 4d) respectively of incubation with microsomal epimerase. Together with the results of the K4 substrate incubations, these values cover a broad range of products with different GlcA/IdoA ratios.

The ^{14}C label of the K4 substrate enabled an alternative approach to the determination of the GlcA/IdoA ratio, involving digestion of the epimerase incubation products with chondroitin AC lyase I. The resultant oligosaccharides were separated by gel chromatography. Since the lyase cleaves *N*-acetylgalactosaminidic linkages to GlcA but not to IdoA units [23], the total GlcA contents would be represented by the sum of the ^{14}C radioactivity in the disaccharide, plus half of that in the tetrasaccharide, one-third of that in the hexasaccharide peak, etc.

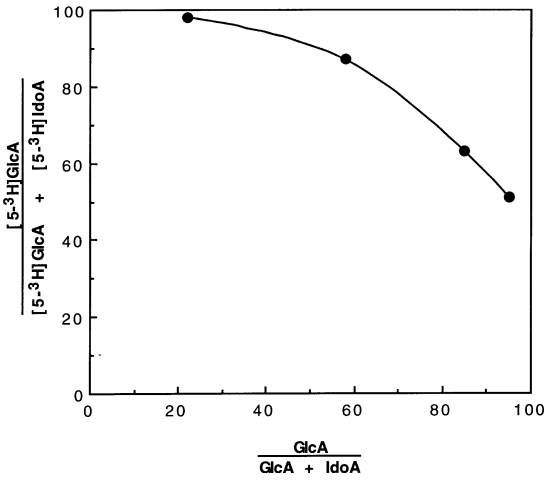

Figure 6 Relationship between overall HexA composition of epimerase substrates and C-5 configuration of enzymically ^{3}H-labelled HexA residues

The proportion (%) of 5-^{3}H-labelled GlcA present in the K4 polysaccharide and dermatan samples incubated with microsomal epimerase in the presence of ^3H_2O was plotted against the proportion (%) of total GlcA present. For further information see Table 1 and the text.

Assessment of the [^{14}C]oligosaccharide patterns thus derived from the K4 samples incubated with epimerase for 6 h (Figure 5a) or for 72 h (Figure 5b) gave values for the HexA composition that were in good agreement with those calculated from the chemical-degradation procedure (Table 1).

The distribution of ^{3}H in the polysaccharide samples incubated with epimerase in the presence of ^3H_2O was investigated by exhaustive digestion with chondroitin AC lyase I followed by gel chromatography (see the Experimental section). Owing to the substrate specificity and the mechanism of action of this enzyme [23], the C-5 ^{3}H of GlcA residues will be recovered as ^3H_2O, whereas the C-5 ^{3}H of IdoA units will remain unaffected, and thus be isolated with IdoA-containing oligosaccharides (tetrasaccharides or larger; no ^{3}H-labelled disaccharides can be formed). The gel chromatograms of ^{3}H-labelled degradation products derived from the dermatan and K4 substrates were strikingly different. After incubation of the K4 polysaccharide with epimerase and ^3H_2O for 6 h, the amount of ^3H_2O generated on digestion of the product with chondroitin AC lyase I approximately equalled the total ^{3}H label in the tetrasaccharide (single [5-^{3}H]IdoA unit) and hexasaccharide (two consecutive [5-^{3}H]IdoA units) fractions (Figure 5a, Table 1). With dermatan, on the other hand, virtually all of the ^{3}H label appeared in the peak of ^3H_2O derived from [5-^{3}H]GlcA (note the lack of ^{3}H-labelled oligosaccharides in Figure 5c). Prolonged incubation of the K4 substrate, leading to an increased proportion of IdoA units (Table 1), was accompanied by a shift in the ^{3}H-incorporation pattern, such that a larger proportion of the label (63% of the total ^{3}H, as calculated from the chromatogram in Figure 5b) occurred in D-*gluco* configuration. Extended incubation of dermatan, increasing the proportion of GlcA units (Table 1), also resulted in some ^{3}H-labelled IdoA residues (13% of the total ^{3}H; Figure 5d). When the proportion of [5-^{3}H]GlcA is plotted against the proportion of total GlcA, the combined results of the dermatan and K4 substrate incubations form a continuous pattern (Figure 6). Epimerase action on an [IdoA-GlcNAc]$_n$ substrate thus appears to result in virtually exclusive 5-^{3}H-labelling of GlcA units, whereas a [GlcA-GalNAc]$_n$ substrate affords approximately equal labelling of GlcA and IdoA units.

The IdoA formed during incubation of the K4 polysaccharide with the microsomal epimerase occurred largely as isolated units, surrounded by GlcA-containing disaccharide residues, as tetrasaccharides were the major oligosaccharide products generated by chondroitin AC lyase digestion (Figures 5a and 5b). However, smaller proportions of hexasaccharides and even octasaccharides were noted, indicating the formation of consecutive IdoA-containing disaccharide units. This tendency was more clearly seen after extended incubation of the dermatan substrate (Figure 5d; however, see the Discussion section).

DISCUSSION

The C-5 epimerization of GlcA to IdoA units is a key step in the biosynthesis of DS proteoglyans. DS chains attached to different core proteins, or produced by different types of cell, can vary greatly in IdoA/GlcA ratio [24–26]. Such variations in IdoA content are undoubtedly of functional significance, as IdoA imparts additional conformational flexibility to the polysaccharide chain, which may result in tighter binding to various proteins [27]. The mechanisms controlling the extent of GlcA epimerization are poorly understood, and will probably be fully elucidated only when the epimerase has been cloned and characterized in detail. However, pending the isolation of the enzyme, valuable information can be gained by studying the epimerization reaction using crude epimerase in appropriate model systems.

The results of the present report demonstrate that the (defructosylated) K4 polysaccharide provides a useful artificial substrate for such studies. Incubation of the K4 substrate with ^3H_2O in the presence of solubilized microsomal epimerase yields a product that contains 5-^{3}H-labelled HexA units. Owing to the reversibility of the epimerase reaction, this product can be used for assay purposes, as required in enzyme purification. An alternative approach to the generation of a similar substrate, as demonstrated in the present study, is to utilize defructosylated K4 polysaccharide produced by metabolic labelling of *E. coli* K4 bacteria with [5-^{3}H]glucose. This procedure avoids handling of large amounts of ^3H_2O and does not require access to crude epimerase. The K_m determined for release of ^3H_2O from the metabolically labelled substrate was similar to that observed for ^{3}H-labelling of defructosylated K4 polysaccharide on incubation with epimerase in the presence of ^3H_2O.

The K4 substrate was further used, along with the isomeric polymer, dermatan, to study the course of the epimerization process, as catalysed by crude solubilized microsomal epimerase. Incubations performed in ^3H_2O confirmed previous findings [16] that the reaction is freely reversible, and thus leads to ^{3}H-labelling of both GlcA and IdoA residues. However, the introduction of label into the two alternative configurations was found to vary with the overall HexA composition of the substrate. Whereas limited epimerization of the K4 polysaccharide, resulting in the formation of about 5% IdoA units, was accompanied by approximately equal distribution of ^{3}H between GlcA and IdoA isomers, the reverse experiment with dermatan as a substrate yielded virtually exclusive labelling of GlcA units, which constituted approx. 22% of the HexA residues (Table 1). This finding would be compatible with a two-base mechanism, involving a monoprotic and a polyprotic base, such as recently proposed for the bacterial enzyme mandelate racemase [28]. The HexA C-5 epimerase and the mandelate racemace catalyse the same general type of reaction, i.e. the abstraction of a proton from one side of a chiral carbon adjacent to a carboxyl group followed by the addition of a proton to the other side, resulting in inversion of configuration. Both enzymes also require bivalent cations for activity. In the racemase, a monoprotic base (histidine)

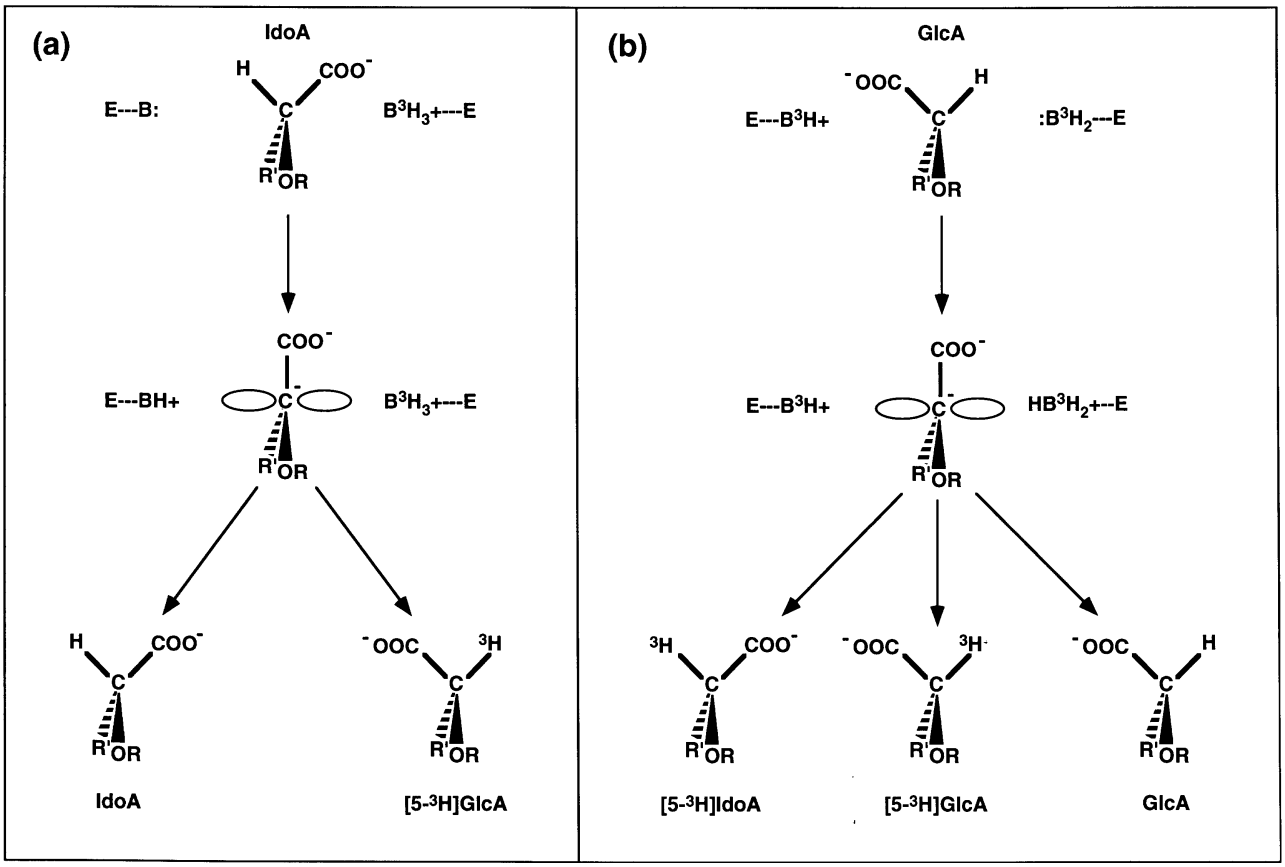

Figure 7 Schematic diagram of tentative two-base reaction mechanism for the HexA C-5 epimerase in ^3H_2O

A monoprotic L-*ido*-specific base (-B:) and a polyprotic D-*gluco*-specific base (:B^3H_2-) result in preferential ^3H-labelling of GlcA when the enzyme (E) acts on IdoA (**a**), but in ^3H-labelling of both GlcA and IdoA when it acts on GlcA (**b**). R and R′ represent the remaining portions of the pyranose ring. For additional information, see the text.

abstracts the (*R*)-proton and a polyprotic base (lysine) abstracts the (*S*)-proton of the respective mandelate enantiomers. If, in the epimerase, a monoprotic base (such as histidine) abstracts the C-5 proton of IdoA to produce a carbanionic intermediate, and if there is little or no exchange of that proton with solvent during the lifetime of the intermediate, then re-addition of the proton to the intermediate will give back an unlabelled IdoA residue, whereas addition of ^3H from the (polyprotic) base on the opposite face of the intermediate will result in a labelled GlcA residue (Figure 7a). On the other hand, if a polyprotic base such as lysine abstracts the C-5 proton of GlcA to produce the carbanionic intermediate, and if the ammonium group rotates rapidly during the lifetime of the intermediate, then solvent-derived ^3H from the ammonium group could be added back to the intermediate to produce labelled GlcA, in addition to the unlabelled GlcA that would result from re-addition of the original proton. Moreover, addition of ^3H from the (monoprotic) base on the opposite face of the intermediate will result in labelled IdoA (Figure 7b). Such a mechanism would predict preferential labelling of GlcA units when the enzyme is acting on a substrate containing exclusively IdoA (dermatan), but labelling of both IdoA and GlcA residues when the enzyme is acting on a GlcA-containing substrate (chondroitin/K4). The results obtained after 6 h of incubation (Table 1) are in accord with this prediction. The ^3H-labelling of IdoA units observed after prolonged incubation of dermatan presumably reflects 'back-epimerization' of GlcA residues formed during the earlier phase of the reaction. It should be emphasized that this interpretation, although conceptually appealing, remains tentative, pending the availability of recombinant enzyme for more thorough mechanistic studies.

The distribution of IdoA units in polysaccharides incubated with the epimerase could be assessed from the elution patterns on gel chromatography, after digestion with chondroitin AC lyase I. After 72 h of epimerization, approaching equilibrium conditions, about 15% of the GlcA units of the K4 polysaccharide had been converted into IdoA (Table 1). Although these residues occurred largely as isolated units (recovered in tetrasaccharides after chondroitin AC lyase I digestion), an appreciable proportion (about 35% of the total IdoA) formed sequences of two or three consecutive IdoA-containing disaccharide units (recovered in hexa- or octa-saccharides) (Figure 5b). This proportion is higher than that (approx. 11%) calculated on the basis of an entirely random distribution of the IdoA units, pointing to a tendency towards processivity in the mode of action of the enzyme. An even higher proportion (about 60%) of consecutive IdoA-containing disaccharide units was observed after extended incubation of dermatan (Figure 5d); however, the significance of this finding is unclear, as it seems likely that some of the ^3H-labelled, thus *de novo*-epimerized, IdoA units had been introduced adjacent to pre-existing unlabelled IdoA residues which would confer resistance toward chondroitin AC lyase I cleavage. Although these results taken together readily demonstrate the ability of the epimerase to catalyse the formation of IdoA units adjacent to either GclA- or IdoA-containing disaccharide units,

there was no indication for the generation of the extended IdoA-containing block structures that are typically seen in many DS preparations. Likewise, the synthesis of IdoA *de novo* never approached the levels seen in native DS. These discrepancies apparently reflect the characteristics of the incubation system used (with solubilized enzyme and substrate) as compared with the intact biosynthetic system (with membrane-bound components). The organization of the intact system conducive to the generation of extended IdoA-containing sequences is poorly understood, but presumably involves single encounters between the epimerase and target GlcA units, thus avoiding 'back-epimerization' and the unfavourable equilibrium attained in the solubilized system. Coupling of epimerization in the intact system with 4-O-sulphation of adjacent GalNAc residues would further bias the process work toward IdoA formation.

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