A method for the sequence analysis of dermatan sulphate

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We are attempting to develop methods for the sequencing of glycosaminoglycans from their reducing end. Here we describe a procedure for the analysis of dermatan sulphate from pig skin. The glycosaminoglycan is released from its parent proteoglycan by exhaustive proteolysis by using both endo- and exo-peptidases. The amino group of the residual serine residue is conjugated with a *p*-hydroxyphenyl group, which in turn is iodinated with ¹²⁵I (the Bolton–Hunter reagent, BHR). The ion-exchange-purified end-labelled dermatan sulphate is then degraded partially or completely by various enzymic or chemical means to yield fragments extending from the labelled serine residue to the point of cleavage. The various products are separated by gradient PAGE, detected by autoradiography and quantified by videodensitometry. Complete digestion with chondroitin ABC lyase affords the labelled fragment ΔHexA-GalNAc(SO₃)-GlcA-Gal-Gal-Xyl-Ser(BHR). The structure was confirmed by sequential degradation from the non-reducing end by chondroitin AC lyase, HgCl₂, and β-galactosidase. Periodate oxidation cleaves most of the Xyl even without treatment with alkaline phosphatase, showing that Xyl is not substituted with phosphate. Results from partial and selective periodate oxidation indicate that most of the non-sulphated IdoA residues are located towards the non-reducing end. Partial or complete digestions with testicular hyaluronidase (in the presence of an excess of β-glucuronidase) or chondroitin AC lyase identify the positions of GlcA residues. The results confirm that HexA next to Gal is always GlcA. Moreover, GlcA is common in the first three disaccharide repeats. Results with testicular hyaluronidase indicate that the distribution of clustered GlcA-GalNAc repeats is periodic and peaks at positions 1–3, 8–9 and around 25. Although there must be chains that contain IdoA in nearly all of the available positions, regions that have not been fully processed during biosynthesis are markedly non-random.

INTRODUCTION

Proteoglycans, which consist of a central protein core substituted with sulphated polysaccharide side chains, occur in the intercellular matrix, at the cell surface and in intracellular storage granules [for reviews see Fransson (1987), Heinegård & Sommarin (1987) and Ruoslahti (1988)]. The proteoglycans are diverse in structure as well as function. Their core proteins may vary in *M_r* from 11 000 to 220 000 (Ruoslahti, 1988), and the number of side chains from one to 100 (Heinegård & Sommarin, 1987). The proteoglycans participate in extracellular-matrix assembly, cell adhesion and growth control. Both the protein and the glycan side chains are involved in these processes.

The glycan side chains, termed glycosaminoglycans, are generally linked via their reducing ends to hydroxy amino acid residues of the core protein. A common linkage sequence is -GlcA-Gal-Gal-Xyl-Ser- (for reviews see Fransson, 1985, 1987). The Xyl residue may be 2-phosphorylated, which has been shown for chondroitin sulphate (Oegema *et al.*, 1984), heparan sulphate (Fransson *et al.*, 1985), dermatan sulphate (Glössl *et al.*, 1986) and heparin (Rosenfeld & Danishefsky, 1988). Biosynthesis of the glycan chains proceeds by repeated alternating addition of hexosamine and uronic acid residues at the non-reducing end. Because there is no precise termination mechanism, the chains display considerable length polydispersity. After polymerization the chains are modified into their final structure by uronosyl epimerase and various sulphotransferases. Dermatan sulphate is formed when the D-GlcA residues of the chondroitin precursor are extensively epimerized to L-IdoA (L-iduronic acid) before sulphation (Malmström & Åberg, 1982).

The structural variability of the glycosaminoglycan side chains of proteoglycans must be controlled in some manner, as

structures with specific properties can be reproducibly formed, notably the antithrombin-binding sequence in heparin (Lindahl *et al.*, 1986), the contact zones for dermatan sulphate or heparan sulphate self-association (Fransson, 1987) and the features that confer antiproliferative activity on to heparin (Wright *et al.*, 1989). However, it is not known whether the majority of the different disaccharide repeats in glycosaminoglycans are distributed randomly or in some ordered fashion. The disaccharide sequence of a glycan can be determined either from the non-reducing or from the reducing end. We are attempting to develop methods that would permit sequencing from the reducing end. In the present paper we describe a method for the sequencing of dermatan sulphates obtained after exhaustive proteolysis. The results indicate that clustered D-GlcA-GalNAc repeats are distributed periodically in a non-random fashion.

EXPERIMENTAL

Materials and equipment

Pig skin dermatan sulphate was obtained after solubilization of the tissue by papain and further fractionated by ethanol precipitation into preparations DS-18 and DS-36 as described elsewhere (Fransson, 1968; Fransson *et al.*, 1979). A linkage region fragment GlcA-GalNAc-GlcA-Gal-Gal-Xyl-Ser(peptide) was the same preparation as described previously (Fransson, 1968). The proteolytic enzymes used were papain (EC 3.4.22.2; Sigma type III), Pronase (EC 3.4.24.4; Sigma type VI), proteinase K (EC 3.4.24.4; Bethesda Research Laboratories), carboxypeptidase A (EC 3.4.17.1; Sigma type I) and leucine aminopeptidases (EC 3.4.11.1; Sigma type III-CP). The carbohydrate-splitting enzymes used were chondroitin ABC lyase

Abbreviation used: BHR, Bolton–Hunter reagent.

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(EC 4.2.2.4), chondroitin AC-I lyase and AC-II lyase (EC 4.2.2.5; all from ICN Biomedicals), testicular hyaluronidase (EC 3.2.1.35; 15000 units/mg, from Leo, Helsingborg, Sweden), β -D-glucuronidase (EC 3.2.1.31; Sigma grade B-10) and β -D-galactosidase (EC 3.2.1.23; Sigma grade IX). Another enzyme was alkaline phosphatase (EC 3.1.3.1; Sigma type III).

The following special chemicals were used: sulphosuccinimidyl 3-(4-hydroxyphenyl)propionate (sulpho-SHPP from Pierce Chemical Co.), acrylamide and *NN'*-methylenebisacrylamide (Serva), *NNN'*-tetramethylethylenediamine (Sigma Chemical Co), Na^{125}I (specific radioactivity 1.7×10^4 Ci/g, > 98 % pure; Cintichem, Tuxedo, NY, U.S.A.) and chloramine-T (BDH Chemicals); other chemicals were of AnalaR grade.

The column media used were: fast-desalting (FD) Sephadex G-25 and Mono Q HR (Pharmacia-LKB) and PartiSil 10 SAX and DE-53 DEAE-cellulose (Whatman). For autoradiography we used Hyperfilm (Amersham) or X-omat AR5 (Kodak) and a Gevamat 60 developing machine (Agfa-Gaevert). For scanning of gels a videodensitometric system developed by Mr. Lars Kopp (Makab, Göteborg, Sweden) was employed.

Proteolytic procedures

Digestions with papain were carried out in 50 mM-sodium phosphate buffer, pH 7.0, containing 10 mM-EDTA and 10 mM-cysteine hydrochloride at 65 °C and with proteinase K in 150 mM-NaCl/5 mM- CaCl_2 /10 mM-Tris/HCl buffer pH 7.4, at 37 °C, both for 24 h and with a substrate/enzyme mass ratio of 100:1. For Pronase (substrate/enzyme mass ratio 50:1), carboxypeptidase A and leucine aminopeptidase (1 unit per 50 mg of substrate) we used 50 mM-Tris/HCl buffer, pH 8.0, containing 1 mM- MgCl_2 at 37 °C overnight.

The various preparations of proteolytic enzymes were assayed for the presence of phosphatase activity by using *p*-nitrophenyl phosphate in 50 mM-Tris/HCl buffer pH 8.0, as a substrate. Incubations were carried out for 1 h at 37 °C, the pH was raised to 10 by the addition of 1 M-NaOH and the A_{410} was recorded. The procedure was standardized with alkaline phosphatase.

Preparation of linkage-region fragments

Dermatan sulphate preparation DS-36, which was obtained after proteolysis with papain, was digested exhaustively with chondroitin ABC lyase; 500 mg in 25 ml of 10 mM-EDTA/0.1 M-Tris-acetate buffer, pH 7.3, and dialysed against the same buffer, was treated with 500 munits of enzyme at 37 °C for 24 h. Samples were checked for u.v. absorption at 232 nm. The solution was further digested overnight by the addition of 50 munit portions of enzyme until the u.v. absorption remained constant (four additions). The digest was chromatographed in 25 ml portions on a fast-desalting column (FD-G-25) connected to a Pharmacia-LKB f.p.l.c. system (Lindblom *et al.*, 1989) and eluted with MilliQ water at a rate of 1 ml/min. The effluent was analysed for u.v.-absorbing material (A_{232}) with a variable-wavelength detector (LKB 2151) connected to an LKB 2210 recorder. Material that appeared in the disaccharide position and material of larger size were pooled separately and freeze-dried. The latter material (12 mg) was subjected to ion-exchange h.p.l.c. on a 3 ml PartiSil 10 SAX column connected to a Varian Vista 5500 liquid chromatograph containing a UV-200 detector. The column was eluted with a gradient of 0–0.6 M-potassium phosphate buffer, pH 4.3, at rate of 1 ml/min. The effluent was also analysed for orcinol-positive material (Fransson, 1968).

Radioiodination of dermatan sulphate preparations with Bolton–Hunter reagent (BHR)

The pig skin dermatan sulphate preparations DS-18 and DS-36 have been analysed elsewhere (Fransson *et al.*, 1979). DS-18

has an M_n of 17 500 and a very low GlcA content, probably only one to three residues per chain, whereas DS-36 has an M_n of 14 500 and approx. seven or eight GlcA residues per chain. Both preparations were subjected to exhaustive proteolytic procedures (see above), and 3-(4-hydroxyphenyl)propionylated at the *N*-terminus of the residual Ser residue followed by radioiodination as outlined below:

Step 1. Dermatan sulphate (0.5–5 mg) was dissolved in 0.1 M-sodium borate buffer, pH 8.5 (400 $\mu\text{g}/\mu\text{l}$), and shaken at room temperature overnight with sulphosuccinimidyl 3-(4-hydroxyphenyl)propionate (100 $\mu\text{g}/\mu\text{l}$). After addition of 2 μl of 0.2 M-glycine in borate buffer per μl of reaction mixture, dialysis against water and freeze-drying, samples were dissolved in 50 mM-Tris/HCl buffer, pH 7.5, to a final concentration of approx. 10–20 mg/ml. The preparations contained 0.8–1 mol of hydroxyphenyl group/mol of dermatan sulphate. This was assessed from the A_{275} by using either tyrosine or hydroxyphenyl-succinimide (treated with water) as a standard and unmodified dermatan sulphate as a blank.

Step 2. Portions (10 μg –2 mg) of 3-(4-hydroxyphenyl)-propionylated dermatan sulphate in 100–200 μl of 50 mM-Tris/HCl buffer, pH 7.5, were mixed with 0.2–0.4 mCi of Na^{125}I and 20 μl of chloramine-T solution (0.6 mg/ml of 50 mM-Tris/HCl buffer, pH 7.5). After 2 min at room temperature the reaction was terminated by the addition of 200 μl of $\text{Na}_2\text{S}_2\text{O}_5$ solution (1.2 mg/ml of Tris buffer) followed by 400 μl of KI solution (10 mg/ml). The reaction mixtures were applied to columns (1 ml) of DE-53 DEAE-cellulose equilibrated in 6 M-urea/0.2 M-sodium acetate buffer, pH 5.8, which were then washed with 5 bed-volumes of the equilibrating buffer, followed by the same buffer containing 0.5 M-sodium acetate and by 50 mM-Tris/HCl buffer, pH 7.5, to remove non-glycosaminoglycan material. The radiolabelled dermatan sulphate was displaced with five 1 ml portions of 4 M-guanidinium chloride/50 mM-sodium acetate buffer, pH 5.8. The effluent was analysed for ^{125}I with an LKB 1271 RiaGamma counter. Radiolabelled dermatan sulphate was recovered by precipitation with 3 vol. of 95 % (v/v) ethanol at –20 °C, centrifuged, redissolved in 100 μl of 25 % (w/v) sodium acetate, reprecipitated with 1 ml of ethanol, centrifuged, washed with absolute ethanol, re-centrifuged and freeze-dried. The final material was dissolved in water to a concentration of 50 $\mu\text{g}/\mu\text{l}$ or 2×10^7 – 5×10^7 c.p.m./ μl . The purity of the radiolabelled dermatan sulphate was checked by ion-exchange h.p.l.c. on Mono Q as described previously (Lindblom *et al.*, 1989). The effluent was monitored for ^{125}I radioactivity, for u.v. absorption at 275 nm and for glycosaminoglycan by the dimethylmethylene Blue method (Farndale *et al.*, 1986) with a Multiscan photometer. The mass and all of the radiolabel appeared in the same position of the chromatogram (results not shown). Dermatan sulphate prepared by alkaline-borohydride treatment (0.5 M-NaOH/0.05 M- NaBH_4 at room temperature overnight) was also subjected to radioiodination. Serine was 3-(4-hydroxyphenyl)propionylated and radiolabelled by the same procedure; the product was isolated by gel filtration (in water) on Sephadex G-25 packed in a disposable 1 cm $\times$ 10 cm column.

Degradation procedures

Cleavage of all hexosaminidic bonds in the main dermatan sulphate chain was performed with chondroitin ABC lyase in

10 mM-EDTA/0.1 M-Tris-acetate buffer, pH 7.3, at 37 °C. The same conditions were used for the cleavage of hexosaminidic bonds to D-GlcA by chondroitin AC-I lyase. The chondroitin AC-II lyase was used in a 50 mM-sodium acetate buffer, pH 6.0. Clustered GlcA-GalNAc units were cleaved by testicular hyaluronidase in 0.15 M-NaCl/50 mM-sodium acetate buffer, pH 5.5. Non-sulphated L-IdoA residues were selectively oxidized and cleaved as described previously (Fransson & Carlstedt, 1974). Non-reducing terminal unsaturated uronic acids were removed by the mercuric salt procedure developed by Ludwigs *et al.* (1987). Treatments with alkaline phosphatase and β -galactosidase (exoglycosidase) were as described previously (Fransson *et al.*, 1985). For further details see the legends to the appropriate Figures.

Gradient PAGE

The procedure used is based on the methods described by Rice *et al.* (1987) and Turnbull & Gallagher (1988). We cast gels between glass plates that were 18 cm wide, 40 cm high and 0.75 mm apart using a vertical LKB stand and gradient mixer and a pump (Alitea, Sweden). The mixing chamber was filled with a weighed 22 ml solution of acrylamide/bisacrylamide (T 30%; C 3.6%) in 10 mM-EDTA/0.1 M-Tris/0.1 M-borate buffer, pH 8.3 (resolving gel buffer), containing 15% (w/v) sucrose, as well as 10 μ l of *NNN'*-tetramethylethylenediamine and 100 μ l of 10% (w/v) ammonium persulphate as catalysts for the polymerization. The reservoir was filled with the same weight of acrylamide/bisacrylamide (T 20%; C 0.5%) in the same buffer containing 1% (w/v) sucrose and the same amount of catalysts. The solutions were pumped at a rate of 5 ml/min to form a linear gradient with a high concentration at the bottom and a low one at the top. The gel was allowed to form under 2-methylpropan-1-ol for approx. 1 h and was then rinsed with resolving gel buffer. On top of this gel we cast a 5 ml stacking gel consisting of acrylamide/bisacrylamide (T 5%; C 0.25%) in 10 mM-

EDTA/0.1 M-Tris/0.1 M-borate buffer, pH 6.3 (stacking gel buffer), containing the same amount of catalysts as above and with a ten-place comb to form the wells. The glass plates with the gels were then placed in a vertical electrophoresis unit (made in a local workshop) containing an upper and a lower reservoir. Before use the wells were rinsed and filled with 0.2 M-Tris/1.25 M-glycine buffer, pH 8.3 (upper reservoir buffer). The various samples were freeze-dried, dissolved in 10 μ l of 25% (w/v) sucrose in stacking gel buffer with or without 0.1% Bromophenol Blue and layered into the bottom of the wells with a Hamilton syringe (801 RNE). The lower reservoir contained resolving gel buffer. Electrophoresis was carried out for approx. 20 h with 600 V (constant voltage) and 8–10 mA. Standards were detected by using 0.08% Azure A in water followed by destaining in water. Radioiodinated compounds were located by placing the wet gel against X-ray film for 12–48 h at room temperature followed by development of the film. Tracks were scanned by videodensitometry in the transmittance mode.

RESULTS AND DISCUSSION

Specificity of 125 I-radiolabelling of the Ser residue in dermatan sulphate preparations

The dermatan sulphates, exhaustively digested with proteinases, were radiolabelled with the use of Bolton–Hunter reagent (BHR) in a two-step procedure with the aim of introducing a radioactive marker exclusively at the reducing end of the chain. Control experiments showed that dermatan sulphate treated with alkaline borohydride incorporated less than 1% of the amount of 125 I recovered in the 3-(4-hydroxyphenyl)-propionylated material.

To isolate and identify radiolabelled linkage-region fragments, i.e.

Fig. 1. Ion-exchange h.p.l.c. of oligosaccharides and linkage-region fragments from dermatan sulphate

(a) Dermatan sulphate preparation DS-36 was obtained after extensive proteolysis and then exhaustively treated with chondroitin ABC lyase followed by separation of disaccharides from larger fragments by fast-desalting f.p.l.c. on FD-G-25. Saccharides larger than disaccharide were separated by ion-exchange h.p.l.c. on PartiSil 10 SAX. For details see the Experimental section. The effluent was monitored for unsaturated uronic acids by u.v. absorption (A_{232} , —) and for orcinol-positive material (A_{670} , -----). The arrow is the elution position of an unsaturated sulphated disaccharide and the bar indicates the pooled linkage-region fragment, which was desalted and freeze-dried. (b) and (c) The same dermatan sulphate preparation was 3-(4-hydroxyphenyl)propionylated and labelled with radioiodine as described in the Experimental section. Samples (400 μ g) containing 20×10^6 c.p.m. of 125 I in 50 μ l of digestion buffer were digested with (b) 50 munits of chondroitin ABC lyase alone at 37 °C for 5 h or (c) with chondroitin ABC lyase followed by 5 munits of chondroitin AC-I lyase overnight. The digests were chromatographed on PartiSil 10 SAX and the effluents were monitored by γ -radiation spectrometry. The bar in panel (c) indicates pooled material, which was desalted and freeze-dried.

Fig. 2. Electrophoresis of oligosaccharides and linkage-region fragments from dermatan sulphate

Lane a, 100 μ g of disaccharide obtained from dermatan sulphate DS-36 after digestion with chondroitin ABC lyase followed by fast-desalting f.p.l.c. Lane b, the same amount of linkage-region fragment Δ HexA-GalNAc(-SO₄)-GlcA-Gal-Gal-Xyl-Ser isolated from the same digest after ion-exchange h.p.l.c. (Fig. 1a). Lane c, 200 μ g of oligosaccharides derived from the same dermatan sulphate after exhaustive digestion with testicular hyaluronidase (20 mg of preparation DS-36/ml of digestion buffer was treated with 4 munits at 37 °C for 24 h). Lanes a–c were stained with Azure A. Lanes d and e, the same preparation of dermatan sulphate as described in the Experimental section. Samples (80 μ g) containing 4×10^6 – 5×10^6 c.p.m. of ¹²⁵I in 10 μ l of digestion buffer were treated with (lane d) 10 munits of chondroitin ABC lyase at 37 °C for 5 h or (lane e) with chondroitin ABC lyase followed by 5 munits of chondroitin AC-I lyase overnight. Lanes f–i, the same dermatan sulphate preparation was 3-(4-hydroxyphenyl)propionylated and labelled with radioiodine as described in the Experimental section. Samples (80 μ g) containing 4×10^6 – 5×10^6 c.p.m. of ¹²⁵I in 10 μ l of digestion buffer were treated with (lane f) 10 munits of chondroitin ABC lyase at 37 °C for 5 h or (lane g) with chondroitin ABC lyase followed by 5 munits of chondroitin AC-I lyase overnight. Lanes h and i, the same dermatan sulphate preparation was 3-(4-hydroxyphenyl)propionylated and labelled with radioiodine as described in the Experimental section. Samples (80 μ g) containing 4×10^6 – 5×10^6 c.p.m. of ¹²⁵I in 10 μ l of digestion buffer were treated with (lane h) 10 munits of chondroitin ABC lyase at 37 °C for 5 h or (lane i) with chondroitin ABC lyase followed by 5 munits of chondroitin AC-I lyase overnight. Lanes d–i were subjected to autoradiography. For further details see the Experimental section. The positions of standard oligosaccharides (i.e. lane c) are indicated on the left. Abbreviation: BPB, Bromophenol Blue.

radioiodinated dermatan sulphate DS-36 was exhaustively digested with chondroitin lyases followed by gel and ion-exchange chromatography as described in the Experimental section. For comparison an unmodified sample of dermatan sulphate was

also degraded. The latter preparation was exhaustively digested with chondroitin ABC lyase to yield the expected products (1) disaccharide Δ HexA-GalNAc(-SO₄) and (2) the linkage-region fragment Δ HexA-GalNAc(-SO₄)-GlcA-Gal-Gal-Xyl-Ser (see also Fransson, 1985). The linkage-region fragment was purified by fast-desalting f.p.l.c. and ion-exchange h.p.l.c. In the latter procedure the expected linkage-region fragment was eluted after 47 min (Fig. 1a); remaining disaccharide emerged after 40 min (see the arrow). The radioiodinated and chondroitin ABC lyase-treated dermatan sulphate yielded a major labelled component that was also eluted after 47 min on ion-exchange h.p.l.c. (Fig. 1b). This fragment should have the structure Δ HexA-GalNAc(-SO₄)-GlcA-Gal-Gal-Xyl-Ser(-BHR). When the same dermatan sulphate was digested with both chondroitin ABC lyase and chondroitin AC-I lyase the major radiolabelled component emerged after 34 min (Fig. 1c). The latter fragment should have the structure Δ HexA-Gal-Gal-Xyl-Ser(-BHR), as chondroitin AC lyase removes the residual repeating disaccharide (see Fransson, 1985). There were also minor components eluted after 47 min and 60 min; these probably represent linkage-region fragments with additional disaccharide repeats, arising from incomplete degradation by the enzyme (see also Figs. 2 and 3 below).

These linkage-region fragments were further examined by gradient PAGE before and after various degradations. The fragment isolated from unmodified dermatan sulphate after exhaustive digestion with chondroitin ABC lyase (see bar in Fig. 1a) migrated to a position between dermatan sulphate hexasaccharide and octasaccharide (Fig. 2, lane b). Although a linkage-region fragment Δ HexA-GalNAc(-SO₄)-GlcA-Gal-Gal-Xyl-Ser(-BHR) is approximately of hexasaccharide size, its charge density is lower than that of a regular hexasaccharide, [HexA-GalNAc(-SO₄)]₃. Chondroitin ABC lyase as well as combined chondroitin ABC lyase and chondroitin AC-I lyase digests of radioiodinated dermatan sulphate were run in lanes d and e respectively (Fig. 2). The major component obtained after treatment with chondroitin ABC lyase only (lane d) migrated to the same position as the corresponding linkage-region fragment from unmodified dermatan sulphate (lane b). No radiolabelled material appeared in the disaccharide position (see lane d), where most of the Azure A-positive material was located (result not shown here). After treatment with both enzymes the major component had a slightly higher mobility (lane e), in accordance with its presumed structure, Δ HexA-Gal-Gal-Xyl-Ser(-BHR). [Removal of the last disaccharide repeat causes a greater decrease in net negative charge density than removal of the preceding ones, which results in a relatively small increase in electrophoretic mobility (see lanes d and e).] There were also (lanes d and e) minor bands corresponding to linkage-region fragments with additional disaccharide repeats. The results also suggest that the major chondroitin AC-I lyase-released fragment is formed to a minor extent by chondroitin ABC lyase and, conversely, that chondroitin AC-I lyase does not completely release the last repeating disaccharide unit.

The major linkage-region fragment obtained from radiolabelled dermatan sulphate after combined chondroitin ABC lyase and chondroitin AC-I lyase treatments, and isolated as described in Fig. 1(c), was homogeneous on electrophoresis (lane f in Fig. 2). This component should have a non-reducing terminal unsaturated uronic acid (Δ HexA), which was removed by treatment with HgCl₂ followed by passage through Dowex 50 (Ludwigs *et al.*, 1987). The product, which had lost a charged residue, migrated to a more retarded position (lane g in Fig. 2). There was also another, even more retarded, component, which disappeared upon incubation in an aqueous buffer (lane h). The component in lane h should have the structure Gal-Gal-Xyl-

Fig. 3. Electrophoresis of partial chondroitin lyase digests of end-labelled dermatan sulphate

Dermatan sulphate preparation DS-18 was 3-(4-hydroxyphenyl)-propionylated and labelled with radioiodine as described in the Experimental section. Samples ($15 \mu\text{g}$) containing 5×10^5 c.p.m. of ^{125}I in $50 \mu\text{l}$ of digestion buffer were treated with 1 munit of (a) chondroitin ABC lyase (Ch-ABC-ase) or (b) chondroitin AC-I lyase (Ch-AC-I-ase) at 37°C for the indicated periods of time. Incubations were stopped by boiling for 2 min. The positions of oligosaccharides obtained by treatment of unmodified dermatan sulphate DS-36 with testicular hyaluronidase are indicated on the left. They have the general structure $[\text{HexA-GalNAc}(\text{SO}_3)_n]$ with n ranging from 2 to approx. 50 (see Fig. 2, lane c). The HexA positions (disaccharide repeat number) as represented by the various radiolabelled components are indicated on the right (panel c). Abbreviation: BPB, Bromophenol Blue.

Ser(-BHR), i.e. a blocked *N*-terminus and a negatively charged *C*-terminus. To demonstrate that it contained non-reducing terminal Gal, it was digested with β -galactosidase. As shown in lane i in Fig. 2, the major product was a fast-moving component, which should be Xyl-Ser(-BHR). The component in lane f, $\Delta\text{HexA-Gal-Gal-Xyl-Ser}(-\text{BHR})$, was insensitive to β -galactosidase (results not shown).

Dermatan sulphate preparations that had been obtained simply with papain proteolysis before end-labelling yielded electrophoretic patterns that were indistinguishable from those obtained with samples pretreated also with Pronase, proteinase K, carboxypeptidase A or leucine aminopeptidase (results not shown). This indicates that heterogeneity in the peptide portion of dermatan sulphate may not be a serious problem. It should be added that the Pronase preparation, but not the other proteinases

Fig. 4. Electrophoresis of end-labelled dermatan sulphate preparation DS-36 after (lane a) periodate oxidation-alkaline elimination or (lane b) digestion with chondroitin ABC lyase

Lane a, dermatan sulphate preparations DS-18 and DS-36 (papain proteolysis) were treated with alkaline phosphatase, 3-(4-hydroxyphenyl)propionylated and labelled with radioiodine or directly 3-(4-hydroxyphenyl)propionylated and radiolabelled as described in the Experimental section. Samples ($15 \mu\text{g}$) (treated or untreated with phosphatase) containing 4×10^5 – 9×10^5 c.p.m. of ^{125}I were oxidized with periodate (final volume $50 \mu\text{l}$) for 5 min, 30 min, 1 h and 6 h. Oxidations were stopped by the addition of mannitol and oxidized sugar rings were cleaved in alkali. Only the 1 h oxidation of dermatan sulphate DS-36 is shown here. Lane b, dermatan sulphate DS-36 was treated with chondroitin ABC lyase for 30 min (see also Fig. 3). The gel was exposed for 72 h to enhance minor components. The positions of standard oligosaccharides (same as in Fig. 2 lane c) are shown to the left. GGXS indicates the position of the linkage-region fragment Gal-Gal-Xyl-Ser (see also Fig. 2); Ser indicates the position of 3-(4-hydroxyphenyl)-propionylated and radiolabelled serine.

or peptidases, contained detectable phosphatase activity; 1 mg of the Pronase preparation could release $1 \mu\text{mol}$ of phosphate in 20 h.

Partial degradations of end-labelled dermatan sulphate by chondroitin lyases

Partial degradation of reducing end-labelled dermatan sulphate with chondroitin ABC lyase or chondroitin AC-I lyase followed by electrophoresis should enable us to identify fragments extending from the Ser residue to a particular point of cleavage (a GalNAc-HexA bond or a GalNAc-GlcA bond). Brief digestion of dermatan sulphate DS-18 with chondroitin ABC lyase, which cleaves all GalNAc-HexA bonds, produced a series of fragments (see Fig. 3a) that were distributed uniformly into approx. 30 bands from a position just above the hexasaccharide position (indicated on the far left) up to the size distribution of the intact chains. The shortest fragment should have the structure $\Delta\text{HexA-GalNAc}(\text{SO}_3)_n\text{-GlcA-Gal-Gal-Xyl-Ser}(-\text{BHR})$, and correspond to the HexA position of the first repeating disaccharide

Fig. 5. Densitometric scans of electrophoretograms of partially degraded end-labelled dermatan sulphate

Dermatan sulphate preparation DS-36 (papain-treated) was 3-(4-hydroxyphenyl)propionylated and labelled with radioiodine as described in the Experimental section. Samples were partially digested with chondroitin ABC lyase or chondroitin AC-I lyase as described in the legend to Fig. 3 or partially oxidized with periodate and cleaved by alkali as described in the legend to Fig. 4. After electrophoresis and autoradiography for 24 h the tracks were scanned by videodensitometry. The scans are (a) digestion with chondroitin ABC lyase for 15 min, (b) digestion with the same enzyme for 30 min, (c) digestion with chondroitin AC-I lyase for 5 min and (d) periodate oxidation for 1 h. The positions of standard oligosaccharides (same as in Fig. 2, lane c) are indicated at the bottom.

(see Fig. 3c; for references see Fransson, 1985). Hence the longest discernible fragment contained 30 repeating units, corresponding to an M_n of at least 15000, which is in good agreement with the estimated average size of the intact chains (M_n 17500). When the same dermatan sulphate was treated with corresponding amounts of chondroitin AC-I lyase for the same periods of time, the major product was a linkage-region fragment with the expected structure $\Delta\text{HexA-Gal-Gal-Xyl-Ser}(-\text{BHR})$ (Fig. 3b; for references see Fransson, 1985). As this component was the major one even after very short periods of incubation, we conclude that the sequence nearest to the peptide is always $-\text{GalNAc-GlcA-Gal-Gal-Xyl-Ser}$. In many chains of this preparation this may be the only GlcA residue.

Partial degradation of end-labelled dermatan sulphate by oxidation-elimination

The major uronic acid in dermatan sulphate is IdoA, which can be 2-*O*-sulphated to a minor extent (Fransson, 1985). Non-sulphated IdoA can be oxidized with periodate under conditions (low pH and temperature) that preclude oxidation of GlcA.

Fig. 6. Electrophoresis of partial testicular-hyaluronidase digests of end-labelled dermatan sulphates

Dermatan sulphate preparation DS-36 (papain proteolysis) was treated with alkaline phosphatase (AP), 3-(4-hydroxyphenyl)propionylated and labelled with radioiodine or directly 3-(4-hydroxyphenyl)propionylated and radiolabelled as described in the Experimental section. Samples (80 μg) (untreated or treated with phosphatase) containing 2×10^6 c.p.m. of ^{125}I in 50 μl of digestion buffer were treated with 0.4 munit of testicular hyaluronidase at 37 $^\circ\text{C}$ for the indicated periods of time. Incubations were stopped by boiling for 2 min. A densitometric scan of track DS-36-AP (2 min) is shown to the right (panel c) and the positions of standard oligosaccharides (same as in Fig. 2, lane c) to the left. LR indicates the position of linkage-region fragment $\text{GlcA-GalNAc}(-\text{SO}_4)\text{-GlcA-Gal-Gal-Xyl-Ser}$ (Fransson, 1968).

IdoA- SO_4 is, of course, always inaccessible to oxidation. Moreover, non-substituted Xyl in the linkage region (see above) has a periodate-sensitive glycol group; a phosphorylated Xyl residue would be periodate-resistant. Hence periodate oxidation of end-labelled dermatan sulphate followed by base-catalysed elimination and electrophoresis can be used to assess the degree of substitution of Xyl as well as to provide information about the distribution of non-sulphated IdoA. Both phosphatase-treated and untreated dermatan sulphates DS-18 and DS-36 (obtained via papain proteolysis only) were subjected to this analysis. All preparations afforded major bands ranging from intact chains down to approximately the decasaccharide standard, indicating that most of the non-sulphated IdoA was located distally. Only the result of a 1 h oxidation of preparation DS-36 is shown here (Fig. 4, lane a). Direct comparison of the banding pattern obtained after brief oxidation (Fig. 4, lane a) with that obtained after partial chondroitin ABC lyase digestion (Fig. 4, lane b) indicated that the periodate-oxidation products corresponding to the first three repeating disaccharide positions (see also Fig. 3) were relatively scarce, suggesting that these repeats generally contain GlcA. (This result does not exclude the presence of IdoA- SO_4 in these positions but, as shown below, that is highly unlikely.)

Furthermore, brief oxidation of end-labelled dermatan sul-

phate resulted in the formation of a component that migrated to the same position as 3-(4-hydroxyphenyl)propionylated and radiolabelled Ser (Fig. 4, lane a). This component was obtained from both phosphatase-treated and untreated dermatan sulphate and it was the major remaining fragment after 6 h of oxidation (results not shown). Apparently, Xyl residues in pig skin dermatan sulphate are non-phosphorylated. There were minor components in positions near that of Gal-Gal-Xyl-Ser(-BHR), suggesting that the preceding GlcA residue was oxidized to some extent.

Comparison between partial degradations with chondroitin lyases and oxidation-elimination

Dermatan sulphate preparation DS-36, which contains approx. seven or eight GlcA residues per chain (M_n 14 500; GlcA/IdoA ratio 1:3; Fransson *et al.*, 1979) was obtained after papain proteolysis, 3-(4-hydroxyphenyl)propionylated, radiolabelled and examined after various partial degradations. The results are summarized and presented as densitometric scans in Fig. 5. Partial digestions with chondroitin ABC lyase afforded the patterns shown in Figs. 5(a) and 5(b). Fragments ranging in size from the linkage region (with one repeat) to at least 20 repeats appeared with approximately equal intensity. In contrast, the patterns obtained after partial degradation with chondroitin AC-I lyase at GlcA (Fig. 5c) and after a 1 h oxidation cleavage of IdoA (Fig. 5d) suggested that GlcA was more prevalent in positions 1–3 and 5–10 from the linkage region than elsewhere. It should be noted that periodate oxidation also resulted in cleavage of Xyl and that both IdoA-SO₄ and GlcA remained intact. However, the results indicate that non-sulphated IdoA was more common towards the non-reducing end.

Partial degradations with testicular hyaluronidase

Testicular hyaluronidase preferentially cleaves clustered GlcA-GalNAc repeats in dermatan sulphate, which makes it more selective than chondroitin AC-I lyase (Fransson, 1985). Dermatan sulphate preparation DS-36 (obtained after papain proteolysis) was 3-(4-hydroxyphenyl)propionylated, radiolabelled and degraded partially by testicular hyaluronidase followed by electrophoresis (Fig. 6). Two preparations were used, one of which had been treated with alkaline phosphatase before derivative formation. As shown in Fig. 6, untreated dermatan sulphate (Fig. 6a) and alkaline-phosphatase-treated dermatan sulphate (Fig. 6b) afforded very similar patterns. These fragments should have the general structure

where n corresponds to the number of disaccharide repeats (see also Fig. 3). The shortest fragment migrated like the standard GlcA-GalNAc(-SO₄)-GlcA-Gal-Gal-Xyl-Ser (LR in Fig. 6), and components ranging from this position ($n = 1$) up to approx. $n = 20$ could be identified. In addition, there was a band in the decasaccharide position that did not correspond to the regular pattern. This could be an undersulphated version of a fragment with $n = 2$. Furthermore, the intensity of the bands varied, particularly after short incubation times, suggesting that clustered GlcA-GalNAc repeats were distributed periodically in a non-random fashion. The densitometric scan of the track obtained after a 2 min incubation (Fig. 6c) indicated again that GlcA was prevalent in positions 1–3, 7–12 and possibly also between 20 and 30 from the linkage region.

As testicular hyaluronidase has transglycosylative activity, fragments could theoretically be extended by a few repeats, which would give erroneous results. To prevent such extensions, non-reducing terminal GlcA was removed by carrying out the digestions with testicular hyaluronidase in the presence of a large

Fig. 7. Densitometric scans of electrophoretograms of end-labelled dermatan sulphate partially degraded with testicular hyaluronidase in the absence (a and c) or in the presence (b and d) of an excess of β -glucuronidase

Dermatan sulphate preparation DS-36 was 3-(4-hydroxyphenyl)-propionylated and radiolabelled as described in the Experimental section. Samples were partially degraded with testicular hyaluronidase as described in the legend to Fig. 6 in the absence or in the presence of 5000 units of β -glucuronidase. After electrophoresis and autoradiography the tracks were scanned by videodensitometry. The tracks are (a and b) digestions for 1 h or (c and d) digestions for 4 h. LR indicates the position of linkage-region fragment GlcA-GalNAc(-SO₄)-GlcA-Gal-Gal-Xyl-Ser (Fransson, 1968).

excess of β -glucuronidase. The results showed (Fig. 7) that the banding patterns were essentially unaffected by the presence of β -glucuronidase (cf. Figs. 7a and 7c with Figs. 7b and 7d) except that the fragments that terminate with GalNAc (Figs. 7b and 7d) migrated slightly faster than the regular ones (Figs. 7a and 7c).

General discussion

Here we present a procedure for the sequence analysis of dermatan sulphate. This glycosaminoglycan is generally present in small proteoglycans (PG-S1 or biglycan and PG-S2 or decorin) in only one or two positions (see Heinegård & Sommarin, 1987; Ward *et al.*, 1987; Ruoslahti, 1988; Choi *et al.*, 1989; Melching & Roughley, 1989). Dermatan sulphate is linked via Ser in sequences such as Glu-Ala-Ser-Gly-Ile-Gly, and after exhaustive proteolysis preparations containing mainly the residual Ser residue can easily be obtained. The amino group of the Ser residue was used for 3-(4-hydroxyphenyl)propionylation with the Bolton-Hunter reagent. We have attempted to maintain a high degree of derivative formation but generally kept the extent of radioiodination to approx. 10^6 c.p.m./ μ g to minimize undesirable side effects. If only small amounts of dermatan sulphate are

available the specific radioactivity can probably be increased further. The procedure described in the present study should be useful for analysing the glycosaminoglycan chains of other proteoglycans provided that they are relatively sparsely substituted and that acidic amino acids as well as *N*- or *O*-linked oligosaccharides near the linkage Ser residue can be removed.

Extensive degradation of reducing end-labelled pig skin dermatan sulphate with chondroitin ABC lyase produced a linkage-region fragment of the proposed structure $\Delta\text{HexA-GalNAc}(\text{-SO}_4)\text{-GlcA-Gal-Gal-Xyl-Ser}(\text{-BHR})$. This fragment was eroded stepwise, first by chondroitin AC lyase (either type I or II) to $\Delta\text{HexA-Gal-Gal-Xyl-Ser}(\text{-BHR})$, then by HgCl_2 to $\text{Gal-Gal-Xyl-Ser}(\text{-BHR})$, and finally to $\text{Xyl-Ser}(\text{-BHR})$ by β -galactosidase. As most of the Gal could be removed by this enzyme, there are probably very few Gal-SO_4 residues (Sugahara *et al.*, 1988). The Xyl residues in the linkage region of pig skin dermatan sulphate are largely unsubstituted, because they were extensively cleaved by periodate oxidation even before treatment with alkaline phosphatase. It is possible that only newly synthesized dermatan sulphates carry phosphorylated Xyl residues (Glössl *et al.*, 1986).

The pig skin dermatan sulphate used in this study comprises chains with various GlcA contents, probably ranging from one to eight residues per chain (Fransson *et al.*, 1979). The present results confirm that the position next to Gal-Gal-Xyl-Ser is always occupied by GlcA (Fransson, 1968). The next three disaccharide repeats also seem to be GlcA-rich, whereas positions 4, 5 and possibly 6 often have IdoA. In the next four or five repeats GlcA is again more common. Clustered GlcA-GalNAc repeats seem to peak at positions 1–3, 8–9 and again around 25. Although the dermatan sulphates analysed here were probably derived from more than one type of proteoglycan secreted from different types of cells, the results indicate that the GlcA-GalNAc repeats have a periodic and non-random distribution. The pattern may be more distinct when the analysis is applied to glycosaminoglycan chains from a specific site in a particular proteoglycan made by one cell type at a defined stage of maturation. By determining the GlcA content in reducing end-labelled fragments of different sizes produced by chemical depolymerization of pig skin dermatan sulphate Uchiyama & Nagasawa (1987) arrived at similar conclusions to those described here. It should be pointed out, however, that there must be chains that contain IdoA in nearly all of the available positions. Moreover, we have not determined the location of IdoA-SO_4 .

Coupling of the reducing end of heparin (Radoff & Danishefsky, 1984; Rosenfeld & Danishefsky, 1988) or the peptide portion of heparan sulphate proteoglycans (Lyon *et al.*, 1987) to other reagents (^{125}I iodotyramine) or to gel matrices have been used to study the structural features near the linkage region to protein. In heparan sulphate this segment of the chain contains an extended *N*-acetylated sequence (Lyon *et al.*, 1987). In heparin, the first antithrombin-binding sequence is 20 disaccharide units away from the carbohydrate-protein linkage (Rosenfeld & Danishefsky, 1988). Other studies indicate that the building blocks in heparin are generally non-random (Linhardt *et al.*, 1989). Especially, repeats that have not been fully processed during biosynthesis occur in an ordered fashion. Moreover,

regions of heparin that are processed in a special manner, e.g. the antithrombin-binding sequence, are markedly non-random when the intact heparin chains of a heparin proteoglycan are examined (Jacobsson *et al.*, 1986). However, heparin fragments generated by endoglucuronidase action do not seem to have an ordered distribution of the antithrombin-binding sites (Linhardt *et al.*, 1989; Oscarsson *et al.*, 1989).

This work was supported by grants from the Swedish Medical Research Council, National Board for Technical Development, Cancer Trust Foundation, The J. A. Persson Foundation, Borgvall Foundation, Kock Foundation, Österlund Foundation, Gustaf V:s 80-Års Fond and the Medical Faculty, University of Lund. We are grateful to Professor D. Heinegård, Dr. A. Malmström, Dr. I. Carlstedt, Dr. L. Cöster, Dr. A. Lindblom and Dr. A. Schmidtchen for valuable support, advice and discussions and to Birgitta Jönsson for artwork.

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