Distribution of iduronate 2-sulphate residues in heparan sulphate

Evidence for an ordered polymeric structure

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The structure of human skin fibroblast heparan sulphate has been examined by depolymerization with heparinase, which specifically cleaves highly sulphated disaccharides of structure $\text{GlcNSO}_3 (\pm 6\text{S})-\alpha 1,4\text{IdoA}(2\text{S})$ [*N*-sulphated glucosamine (6-sulphate)- $\alpha 1,4$ -iduronic acid 2-sulphate]. Heparan sulphate contained only a small proportion ($\sim 10\%$) of linkages susceptible to this enzyme. The major products of depolymerization with heparinase were large oligosaccharides with an average molecular mass of 10 kDa ($\text{dp} \sim 40$, where dp is degree of polymerization; for disaccharides, $\text{dp} = 2$ etc.) as assessed by gel filtration on Sepharose CL-6B, compared with a molecular mass of 45 kDa ($\text{dp} \sim 200$) for the intact chains. The large heparinase-resistant oligosaccharides were highly susceptible to depolymerization with the enzyme heparitinase, which cleaves heparan sulphate in areas of low sulphation, where *N*-acetylated disaccharides [$\text{GlcNAc}-\alpha 1,4\text{GlcA}$ (*N*-acetylglucosaminyl- $\alpha 1,4$ -glucuronic acid)] are the predominant structural unit. Further analysis of the location of the heparinase cleavage sites indicated that they were predominantly found in a central position in $\text{GlcNSO}_3-\alpha 1,4\text{IdoA}$ repeat sequences of average length four to seven disaccharides ($\text{dp} 8-14$). These results indicate that heparinase cleaves heparan sulphate in approximately four or five *N*-sulphated domains, each domain containing a cluster of two or three susceptible disaccharides; the domains are separated by long *N*-acetyl-rich sequences that are markedly deficient in sulphate groups. On the basis of these findings a model is proposed which depicts heparan sulphate as an ordered polymeric structure composed of an alternate arrangement of sulphate-rich and sulphate-poor regions. The sulphate-rich regions are likely to be flexible areas of the chain because of their high content of the conformationally versatile IdoA and IdoA(2S) residues. The model has important implications for the biosynthesis and functions of heparan sulphate.

INTRODUCTION

The heparan sulphates are a family of complex linear polysaccharides present on cell surfaces and in the extracellular matrix of most mammalian cells. They are composed of a repeating sequence of disaccharide units consisting of $\alpha\beta$ -linked glucosamine (GlcN) and hexuronate (Hex.A) residues (Lindahl & Höök, 1978; Höök *et al.*, 1984; Gallagher *et al.*, 1986). GlcN is either *N*-acetylated or *N*-sulphated, and, although these substituents are present in approximately equal quantities in the heparan sulphates (Gallagher & Walker, 1985), they are commonly segregated in distinct sequences that vary in length from two to nine disaccharide units (Linker & Hovingh, 1968; Cifonelli, 1968; Kraemer, 1971; Sjöberg & Fransson, 1980; Winterbourne & Mora, 1981; Hampson *et al.*, 1983; Turnbull & Gallagher, 1990). In the *N*-acetylated sequences the Hex.A is glucuronate (GlcA), whereas in the *N*-sulphated regions the C-5 epimer iduronate (IdoA) predominates (Höök *et al.*, 1974; Linker & Hovingh, 1974; Fransson *et al.*, 1980; Delaney & Conrad, 1983; Bienkowski & Conrad, 1984; Guo & Conrad, 1989; Turnbull & Gallagher, 1990). Structural complexity is amplified by substitution with variable patterns of ester (*O*-)sulphate groups, which are largely, though not exclusively, confined to the *N*-sulphated domains (Gallagher *et al.*, 1986; Lindahl *et al.*, 1986; Lindahl & Kjellén, 1987; Turnbull & Gallagher, 1988; Gallagher & Lyon, 1989). This clustering of *N*- and *O*-sulphates creates areas of high negative charge, which are likely to par-

ticipate in electrostatic interactions with proteins and cations in the microenvironment of the cell. High-affinity binding between polysaccharides and protein also occurs, dependent upon specific sulphation patterns. The prototypic structure for such interactions is the pentasaccharide binding site for antithrombin III in heparin, which contains a unique 3-*O*-sulphated GlcNSO_3 residue in addition to a number of other essential substituents (Lindahl *et al.*, 1984). It is also likely that the positions of these specific sequences in the polymer will prove to be important for the expression of biological properties.

In the present paper we have directly addressed the question of the arrangement of the sulphated domains in skin fibroblast heparan sulphate. These investigations represent an extension of earlier studies which showed that the enzyme heparinase cleaves only a small proportion ($\sim 10\%$) of the disaccharide units in this heparan sulphate (Turnbull & Gallagher, 1990). This was a significant observation, since heparinase cleaves specifically at highly sulphated disaccharides of structure $\text{GlcNSO}_3 (\pm 6\text{S})-\alpha 1,4\text{IdoA}(2\text{S})$ [*N*-sulphated glucosamine (6-sulphate)- $\alpha 1,4$ -iduronic acid 2-sulphate], the critical requirement being the IdoA(2S) moiety (Rice & Linhardt, 1989; Linhardt *et al.*, 1990). By determining the molecular-size range of the oligosaccharides produced by heparinase scission of heparan sulphate, and identifying the approximate positions of heparinase cleavage within the *N*-sulphated sequences, we have been able to propose a model for the molecular organization of the polymer chain.

Abbreviations used: dp , degree of polymerization (i.e. for a disaccharide, $\text{dp} = 2$ etc.); Hex.A, hexuronic acid; GlcA, glucuronic acid; IdoA, iduronic acid; IdoA(2S), iduronic acid 2-sulphate; GlcN, glucosamine; GlcNAc, *N*-acetylglucosamine; GlcNSO_3 , *N*-sulphated glucosamine; $\text{GlcNSO}_3(6\text{S})$, *N*-sulphated glucosamine 6-sulphate; ΔGlcA and ΔIdoA , unsaturated glucuronic and iduronic acid residues (since these are products of specific polysaccharide lyase cleavage, it is possible to designate the structure of the hexuronate residue as it would have occurred in the intact polymer; PBS, phosphate-buffered saline (20 mM-sodium phosphate/0.15 M-NaCl, pH 7.4).

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EXPERIMENTAL

Materials

D-[1-³H]Glucosamine (sp. radioactivity 27 mCi/mg) and Na₂³⁵SO₄ (sp. radioactivity 25–40 Ci/mg) were obtained from Amersham International. Heparinase was obtained from Sigma or Seikagaku Kogyo Co. Ltd. (Tokyo, Japan), and heparitinase I and chondroitin ABC lyase from Seikagaku Kogyo Co. Cell-culture media were supplied by Gibco, with the exception of donor calf serum, which was obtained from Flow Laboratories. Bio-Gel P6 (200–400 mesh) was from Bio-Rad, and Sepharose CL-6B, Sephadex G-100 and DEAE-Sephacel were from Pharmacia-LKB. An f.p.l.c. system and Mono-Q columns were obtained from Pharmacia-LKB. Tris, heparin (from porcine intestinal mucosa) and heparan sulphate (from bovine kidney) were all supplied by Sigma. All other reagents and chemicals used were of AnalaR or AristaR grade from BDH.

Cell culture, radiolabelling and preparation of intact heparan sulphate chains

Confluent cultures of adult human skin fibroblasts were maintained at 37 °C (CO₂/air, 1:19) in Eagle's minimal essential medium supplemented with 15% (v/v) donor-calf serum, 2 mM-glutamine, 1 mM-sodium pyruvate, non-essential amino acids, penicillin (100 units/ml) and streptomycin (100 µg/ml).

Cultures were incubated for 72 h with Na₂³⁵SO₄ (10 µCi/ml) and [³H]glucosamine (10 µCi/ml). The medium was removed, and the cell layers carefully washed twice with warm (37 °C) phosphate-buffered saline (PBS). The combined medium and rinsings were centrifuged (200 g for 10 min) and the supernatant stored at –20 °C.

The proteoglycans in the medium were subjected to initial purification by ion-exchange chromatography. Samples were applied to a DEAE-Sephacel column (1 cm × 5 cm) and washed with 0.3 M-NaCl in 20 mM-phosphate buffer, pH 6.8, to elute contaminating proteins and hyaluronic acid. Remaining proteoglycans (principally heparan sulphate and chondroitin/dermatan sulphate) were then eluted with a gradient of 0.3–1 M-NaCl in 20 mM-phosphate buffer. Fractions corresponding to heparan sulphate proteoglycan (eluted at approx. 0.53 M) were collected and desalted on a Sephadex G-25 column (2.5 cm × 40 cm) and freeze-dried. Traces of chondroitin sulphate and dermatan sulphate were removed by treatment of the sample (corresponding to ten 175 cm² culture flasks) with 1 unit of chondroitin ABC lyase for 3 h at 37 °C. The proteoglycans were then digested with Pronase (5 mg/ml) for 4 h at 37 °C. Heparan sulphate chains were recovered by step elution with 1 M-NaCl from a 2 ml DEAE-Sephacel column after washing with 0.3 M-NaCl to elute chondroitin sulphate/dermatan sulphate oligosaccharides and peptide fragments. After dialysis against distilled water the heparan sulphate chains were freeze-dried in preparation for further analysis.

Depolymerization of heparan sulphate glycosaminoglycans

Heparitinase (heparitinase I from Seikagaku Kogyo Co.) was used at a concentration of 20–50 munits/ml in 100 mM-sodium acetate, pH 7.0, containing 0.2 mM-calcium acetate and bovine kidney heparan sulphate (1 mg/ml) as carrier. Samples were incubated at 37 °C for 16 h. Heparinase was used at the same concentration and in the same buffer as heparitinase, but heparin (1 mg/ml) was used as carrier. Samples were incubated at 30 °C for 16 h. In both cases the enzyme was inactivated by heating at 100 °C for 2 min, and parallel control digests, containing unlabelled heparan sulphate or heparin only, were carried out to monitor the progress of the reaction. The increase in A₂₃₂ was measured to ensure that the end point of depolymerization was

reached with each batch of digests. HNO₂ deaminative cleavage was carried out using the low-pH method of Shively & Conrad (1976). Samples were dried by centrifugal evaporation, reconstituted in 10 µl of 1 M-HNO₃ solution and incubated for 15 min at 20 °C. The reaction was stopped by addition of 2 µl of 1 M-Na₂CO₃.

Gel chromatography

Gel chromatography of intact chains and glycosaminoglycan oligosaccharide fragments was performed on Sepharose CL-6B, Bio-Gel P6 and Sephadex G-100 columns (120 cm × 1 cm) in 0.5 M-NH₄HCO₃ eluted at a flow rate of 4 ml/h. Fractions of 1 ml were collected in each case and small aliquots taken for liquid-scintillation counting. Estimates of the size of fragments resolved on Sepharose CL-6B and Sephadex G-100 columns were based on the calibrations published by Wasteson (1971) and Laurent *et al.* (1978).

Cleavage with alkaline borohydride

Samples were adjusted to 50 mM-NaOH/1 M-NaBH₄ and incubated at 45 °C for 24 h. The reaction mixture was neutralized with acetic acid, diluted with double-distilled water and protein-core fragments removed by step elution on a small DEAE-Sephacel column, followed by dialysis and freeze-drying.

F.p.l.c. ion-exchange chromatography

Rapid ion-exchange was achieved using an f.p.l.c. system (Pharmacia); the column used was a Mono-Q HR5/5 anion-exchange type (0.5 cm × 5 cm). The buffer used was 10 mM-Tris/HCl, pH 7.5, containing 6 M-urea (deionized with Amberlite MB-3 monobed resin) and 1% Triton X-100. Samples (1–2 ml in elution buffer) were injected on to the column through a variable-volume injection loop and washed through with 5 ml of buffer. Elution was then achieved with a gradient of 0–1.5 M-NaCl (35 ml total volume) at a flow rate of 1 ml/min, and 0.5 ml fractions were collected and sampled for scintillation counting.

Scintillation counting

Samples (maximum volume 0.3 ml) were mixed with 3 ml of Ready-Value scintillant (Beckman RIIC) and radioactivity determined in a Betatrac 6895 counter (Tracor Analytic). The counting efficiency was determined for ³H and ³⁵S using suitable channel-window settings in order to allow dual-label radioactivity (d.p.m.) calculations to be made.

RESULTS AND DISCUSSION

Depolymerization of skin fibroblast heparan sulphate with heparinase

Dual ³H- and ³⁵SO₄-labelled heparan sulphate proteoglycans were extracted from the medium of adult human skin fibroblasts, and heparan sulphate chains were prepared as peptidoglycans (see the Experimental section). They were eluted as a well-defined peak when subjected to gradient ion-exchange chromatography on a Mono-Q column, being eluted at 0.84 M-NaCl (result not shown).

In order to investigate the distribution of IdoA(2S) residues, heparinase was employed [specificity GlcNSO₃(±6S)-α1,4IdoA(2S); see Rice & Linhardt, 1989; Linhardt *et al.*, 1990]. Heparan sulphate chains were depolymerized with heparinase and fractionated by Bio-Gel P6 chromatography. The results are shown in Fig. 1. Only small amounts of di- and tetra-saccharides were produced by heparinase, and larger oligosaccharides containing resistant internal sequences {i.e. ΔIdoA(2S)-[GlcNR-Hex.A]_n-GlcNSO₃ where R = NAc or NSO₃} were predominant. From the depolymerization profile we calculated that approx.

Fig. 1. Bio-Gel P6 gel filtration of oligosaccharides produced by heparinase depolymerization of heparan sulphate

Heparan sulphate from skin fibroblasts prepared as described in the Experimental section was treated with heparinase and fractionated on a Bio-Gel P6 column (1 cm $\times$ 120 cm). The column was eluted with 0.5 M-NH₄HCO₃ at 4 ml/h and 1 ml fractions were collected for scintillation counting (—, ³H radioactivity; ·····, ³⁵S radioactivity). A series of oligosaccharides ranging from disaccharides (Di; dp 2) and tetrasaccharides (Tetra; dp 4) to larger oligosaccharides (dp $\geq$ 6) was observed, depending on the proportion of susceptible linkages and their distribution in the intact molecule. Oligosaccharides (dp 4 and larger) are generated when the susceptible linkages are separated by disaccharide units containing resistant linkages. The inset shows the scission profile with an expanded scale in order to reveal the proportions of low-molecular-mass products. The large heparinase-resistant oligosaccharides (dp $\geq$ 14) were pooled and freeze-dried for further characterization. Abbreviation: Hexa, hexasaccharide(s).

10% of disaccharides in heparan sulphate contain linkages susceptible to heparinase [for details of the calculation, see Turnbull & Gallagher (1990)]. Very few intermediate-sized oligosaccharides (dp 6–12) were observed (see Fig. 1, inset), most being $\geq$ dp 14 in size. The calculations showed that a high proportion (about 60%) of heparinase-cleavable linkages were spaced apart by large resistant sequences in the intact polymer.

Further characterization of the large heparinase-resistant oligosaccharides

The structural characteristics of the large heparinase-resistant oligosaccharides (a pool of all resistant oligosaccharides of size dp $\geq$ 14; Fig. 1) were investigated further. After alkali treatment to remove residual peptide, their size distribution was analysed by gel filtration on Sepharose CL-6B. Peptide-free intact heparan sulphate chains were also chromatographed on the same column and yielded a symmetrical peak (K_{av} 0.34) corresponding to a molecular mass of approx. 45 kDa (Fig. 2a). By contrast, the oligosaccharides (dp $\geq$ 14) released from the chains by prolonged heparinase treatment were considerably smaller in size, being eluted with a narrow and reproducible radioactivity profile (Fig. 2b; K_{av} 0.64). The estimated mean molecular mass of the fragments was 10 kDa (approx. dp 40), with a range of approx. 7–15 kDa (dp 30–66). However, the Sepharose CL-6B column profile indicated that they may be composed of a number of partially resolved major species with approx. sizes of 7, 11 and 15 kDa (K_{av} of 0.7, 0.62 and 0.56 respectively; see Fig. 2b). In addition, these oligosaccharides were analysed by gel filtration on Sephadex G-100; the elution profile showed a broad peak (K_{av} 0.38) corresponding to a mean size of approx. 10–11 kDa and indicated a similar mixture of discrete major species (results not shown).

Fig. 2. Sepharose CL-6B gel filtration of intact chains and large heparinase-resistant oligosaccharides from heparan sulphate

Intact chains (a) and large heparinase-resistant oligosaccharides (b) (prepared as described in the legend to Fig. 1) were treated with alkaline borohydride and resolved on a Sepharose CL-6B column (1 cm $\times$ 120 cm). The column was eluted with 0.5 M-NH₄HCO₃ at 4 ml/h and 1 ml fractions were collected for scintillation counting (only ³H radioactivity is shown). Size estimates were based on the calibration published by Wasteson (1971).

Exhaustive re-treatment of the large heparinase-resistant oligosaccharides with heparinase did not result in any shift in their Sepharose CL-6B column elution profile. In addition, using 4 M-guanidinium chloride (in 20 mM-phosphate buffer, pH 7) as the eluting buffer resulted in the same elution profile, precluding the possibility that self-associative effects were resulting in an anomalously high molecular-size estimation.

Thus heparinase degrades the 45 kDa heparan sulphate chains to produce predominantly large fragments with a limited molecular-mass range which are one-third to one-sixth the size of the original molecule. These results indicate that a high proportion of the heparinase-sensitive disaccharide units were separated by very large resistant sequences in the intact polymer. In a typical polysaccharide chain the heparinase-sensitive linkages will thus be clustered at four or five sites, the preferred spacing of the sites being approx. 15, 25 and 33 disaccharide units (estimated average disaccharide size $\sim$ 450 Da), corresponding to the approximate sizes (7, 11 and 15 kDa) of the main enzyme-resistant oligosaccharides (Fig. 2b). About 100 disaccharides will be present in the native 45 kDa polymer chain. Since about 10% of these disaccharides were substrates for heparinase, the four or five enzyme-sensitive sites in the chain will each contain an average of two or three susceptible disaccharide units.

Disaccharide sequences adjacent to heparinase-sensitive sites

In order to gain more information on the sequences flanking the heparinase-sensitive sites, the location of GlcNSO₃-IdoA(2S) residues in heparinase-resistant oligosaccharide sequences was analysed. The IdoA(2S) moieties, the critical component of the heparinase substrate, are likely to be located in contiguous N-sulphated sequences because of the close proximity of N- and O-sulphate groups (see the Introduction). One means of preparing

Fig. 3. Separation of heparitinase-resistant oligosaccharides on Bio-Gel P6

Heparan sulphate was treated with heparitinase and fractionated on a Bio-Gel P6 column (1 cm $\times$ 120 cm) as described in the legend to Fig. 1 (only ^3H radioactivity is shown). A series of oligosaccharides (dp numbers given against peaks) ranging from disaccharides (dp 2) and tetrasaccharides (dp 4) to larger resistant sequences (dp 6 to dp ≥ 16) was observed. The 'off-scale' disaccharide peak (2) contained 52% of the total radioactivity. Heparitinase specifically cleaves linkages between hexosamine and GlcA, irrespective of the glucosamine moiety (Linhardt *et al.*, 1990). Oligosaccharides (dp ≥ 4) generated by heparitinase scission contain resistant internal linkages [i.e. $\text{GlcNSO}_3(\pm 6\text{S})-\alpha 1,4\text{IdoA}(\pm 2\text{S})$] and thus represent contiguous sequences of IdoA-containing disaccharides. Fractions corresponding to oligosaccharides dp 4 to dp ≥ 16 were pooled as indicated by the bars, freeze-dried and analysed further by low-pH HNO_2 and heparitinase scission (see Table 1, and Fig. 4).

N-sulphate-rich sequences from heparan sulphate is by treatment with the enzyme heparitinase, which cleaves disaccharides that contain GlcA, but does not cleave *N*-sulphated disaccharides that contain IdoA ($\pm 2\text{S}$) (Linhardt *et al.*, 1990).

Heparitinase extensively degraded the heparan sulphate preparations used in the present study, as indicated previously (Turnbull & Gallagher, 1990); 64% of linkages were cleaved by the enzyme, but a series of relatively small resistant fragments containing from two to eight disaccharide units (dp 4–dp 16) were identifiable in the digestion products after separation on Bio-Gel P-6 (Fig. 3). These oligosaccharides have the general formula:

where R = NAc or NSO_3 .

They thus represent contiguous sequences of IdoA-containing disaccharides. They were highly sensitive to hydrolysis by HNO_2 , the products (assessed by Bio-Gel P6 chromatography) being mainly disaccharides and a small amount of tetrasaccharides (results not shown). The percentage of linkages cleaved was calculated from the profiles for each of the oligosaccharides (dp 4–dp ≥ 16) and varied from 89 to 99%. This confirmed that the internal amino sugars are largely *N*-sulphated, and is consistent with oligosaccharides composed of GlcNSO_3 -IdoA repeat sequences of the type shown above.

In order to assess further the distribution of IdoA(2S) residues, the heparitinase-resistant oligosaccharide preparations (dp 4–dp 14) were also treated with heparitinase and re-chromatographed on Bio-Gel P-6. The results showed a clear correlation between fragment size and the extent of depolymerization. Almost 80% of the dp 14 fragments contained susceptible linkages, whereas only 25% of the dp 8 fragments were degraded (Fig. 4; Table 1). Therefore the content of IdoA(2S) residues increases with increasing number of GlcNSO_3 -IdoA repeats. Heparitinase-

Fig. 4. Heparitinase-susceptibility of IdoA-repeat sequences: Bio-Gel P6 profiles of heparitinase-resistant oligosaccharides depolymerized with heparitinase

Contiguous sequences of IdoA-containing disaccharides (oligosaccharides dp 8–dp 14) were prepared from heparan sulphate as described in Fig. 3. After treatment with heparitinase they were fractionated on a Bio-Gel P6 column (1 cm $\times$ 120 cm) as described in the legend to Fig. 1 (only ^3H radioactivity is shown). In each case the products consisted of a series of fragments ranging from disaccharides up to the size of the intact oligosaccharides. The numbers indicate the dp size of the fragments. The original oligosaccharides were: (a) dp 14; (b) dp 12; (c) dp 10; and (d) dp 8.

resistant oligosaccharides of sizes dp 4 and dp 6 exhibited a very low content of heparitinase-susceptible linkages (Table 1), consistent with their low level of sulphation (an average of 0.9 and 1.3 sulphate groups per disaccharide estimated from $^{35}\text{SO}_4/^{3}\text{H}$ ratios). In addition, heparitinase cleavage of oligosaccharides dp 10–dp 14 generates a relatively low proportion of both disaccharides and oligosaccharides of size dp $n-2$, fragments of intermediate size being the major breakdown products (Fig. 4). This indicates that few are cleaved near their terminal linkages and that IdoA(S) residues are positioned towards the centre of the GlcNSO_3 -IdoA domains. The presence of IdoA(2S) residues in sequences of this type was indicated in previous studies (Cifonelli & King, 1977; Sjöberg & Fransson, 1980). The present results confirm these findings and extend them by demonstrating

Table 1. Heparinase-susceptibility of heparitinase-resistant oligosaccharides

Heparitinase-resistant oligosaccharides were prepared by treatment of intact heparan sulphate with heparitinase followed by Bio-Gel P6 chromatography (see Fig. 3). Resolved oligosaccharides (dp 4–dp 14) were then dialysed (using low-molecular-mass-cut-off dialysis tubing: Spectrapor 6-1000), freeze-dried, cleaved with heparinase and resolved on Bio-Gel P6; the calculations shown are based on the resulting profiles (for dp 8–dp 14, see Fig 4; dp 4 and dp 6 profiles not shown in Fig. 4). The proportion of susceptible linkages was calculated using the formula described previously (Turnbull & Gallagher, 1990).

dp	Disaccharides with susceptible linkages (% of total)	Oligosaccharides remaining intact (%)
4	1.7	98.3
6	2.7	97.0
8	13.2	74.5
10	26.0	45.2
12	29.6	28.9
14	31.6	23.1

Fig. 5. Bio-Gel P6 profile of large heparinase-resistant oligosaccharides depolymerized with heparitinase

Large heparinase-resistant oligosaccharides (a pool of all heparinase-resistant oligosaccharides of size dp ≥ 14 ; Fig. 1) were prepared as described in the legend to Fig. 1. After treatment with heparitinase they were fractionated on a Bio-Gel P6 column (1 cm $\times$ 120 cm) as described in the legend to Fig. 1 (—, ^3H radioactivity; ·····, ^{35}S radioactivity).

Fig. 6. Hypothetical model of the structure of heparan sulphate from human skin fibroblasts

The model proposes that heparan sulphate consists of an alternate arrangement of sulphate-rich and sulphate-poor domains. Heparinase cleavage sites [GlcNSO₃($\pm$ 6S)- α 1,4IdoA(2S)] are found in the sulphated regions within sequences of GlcNSO₃-IdoA repeats. The spacing between these regions would generate the large fragments observed after heparinase scission (Figs. 1 and 2). An extended *N*-acetylated sequence is located adjacent to the protein core in the model, as described previously for this heparan sulphate (Lyon *et al.*, 1987). The regions with a low sulphate content are enriched in *N*-acetylated disaccharides and are sensitive to the enzyme heparitinase (specificity GlcNR- α 1,4GlcA, where R = NAc or NSO₃). The IdoA residues will confer conformational versatility on the sulphated regions, and this property, together with the arrangement of the sulphate groups, will be very important for the functions of heparan sulphate. The spacing of the sulphated regions by the intervening, conformationally restricted *N*-acetyl-rich sequences may also be an important determinant of biological activity, especially if heparan sulphate acts as a template for the biogenesis of the extracellular matrix (see the General discussion section). The model represents a simplified picture of heparan sulphate. There may be variations in the positions and spacing of the sulphated domains and there will be *N*-sulphate groups sparsely distributed in the *N*-acetylated domains. The precise sulphation patterns in the polymer remain to be established. Key to parts of structure: —, *N*-acetylated domains; $\wedge$, *N*-sulphated domains; $\circ$, IdoA repeat sequences; $\uparrow$, 2-*O*-sulphated IdoA moiety; $\downarrow$, heparinase cleavage site.

the preferential location of IdoA(2S) centrally within the GlcNSO₃-IdoA repeats and in longer sequences of these disaccharide units.

Depolymerization of large heparinase-resistant oligosaccharides with heparitinase

The extended disaccharide sequences located between the four or five sites of heparinase cleavage should be enriched in contiguous *N*-acetylated disaccharide sequences. This was confirmed by treatment of the large heparinase-resistant oligosaccharides with heparitinase; they were extensively depolymerized by this enzyme, with disaccharides being the major breakdown product (Fig. 5). The extended GlcNSO₃-IdoA repeat sequences (dp 8–dp 16) that were present in heparitinase digests of intact heparan sulphate (Fig. 3) were markedly depleted (45% of their level in intact chains). In sharp contrast there was a relative increase in the proportion of fragments dp 4 and dp 6 (3.9-fold and 1.3-fold respectively of their levels in the intact chains). These results are consistent with the central cleavage of the GlcNSO₃-IdoA repeat sequences by heparinase during preparation of the large heparinase-resistant oligosaccharides (Fig. 1) and confirm that the IdoA(2S) residues conferring heparinase-sensitivity are preferentially located in a central position within these sequences.

GENERAL DISCUSSION

The principal variables in the molecular fine structure of heparan sulphate are the composition of the hexuronate residues and the content and arrangement of the *O*-sulphate groups (see the Introduction). In the present study we have used heparinase as a specific-cleavage reagent to assess the distribution of GlcNSO₃-IdoA(2S) residues in heparan sulphate from human skin fibroblasts. The results indicate that they are distributed in an ordered manner and provide new insights into the structural order present in heparan sulphate chains. They also raise important questions in relation to polymer biosynthesis and function.

Model for the structure of heparan sulphate

On the basis of the foregoing data we have proposed a structural model in which heparan sulphate is depicted as an ordered polymeric structure composed of an alternate arrangement of highly sulphated and poorly sulphated regions (Fig. 6).

The heparinase-sensitive disaccharides [GlcNSO₃(±6S)-α1,4-IdoA(2S)] are located in relatively short domains consisting of GlcNSO₃-IdoA repeats. These sulphated domains are separated by extended disaccharide sequences which are enriched in GlcNAc-α1,4GlcA units and have a low sulphate content. The existence of an *N*-acetylated sequence contiguous with the protein-linkage region of heparan sulphate was established in a previous study (Lyon *et al.*, 1987), and this finding has been incorporated in the model. The model structure explains why cleavage by heparinase of the polymeric substrate generates a high proportion of large oligosaccharides (Figs. 1 and 2), whereas treatment with heparitinase, which acts mainly on the GlcNAc-α1,4GlcA linkages, produces a high yield of disaccharides and a series of highly sulphated resistant fragments containing the heparinase-cleavage sites (Fig. 3).

The model has some resemblance to an earlier proposal of Cifonelli (1968), who suggested that the presence of *N*-acetylated sequences between *N*-sulphated domains could explain the breakdown pattern of heparan sulphate elicited by hydrolysis with HNO₂. However, we believe that the present study provides the first direct evidence that the sulphated domains are indeed separated by long *N*-acetyl-rich oligosaccharides (Figs. 2b and 5). The data also enable estimates to be made of the molecular-size range of the *N*-sulphated domains (Fig. 3) and of the oligosaccharide spacer sequences positioned between them (Fig. 2b).

The model represents a simplified picture of the molecular structure of heparan sulphate. It is not clear whether the heparinase cleavage sites occur in approximately the same positions in all the polysaccharide chains. The sites are widely and variably spaced, and there could be differences in their actual locations along the polymer chains. Variations in both the number and sizes of the heparinase-resistant domains present in individual chains could result in the evident polydispersity of heparan sulphate. We also have no direct information on the positions of 'mixed' tetrasaccharide sequences composed of alternating *N*-acetylated and *N*-sulphated disaccharides. Such sequences are common in heparan sulphate (see Gallagher & Walker, 1985), and they could be preferentially located at the interfaces of the highly sulphated and poorly sulphated domains. It also remains to be established that the model is applicable to other types of heparan sulphate, although we have preliminary data indicating that heparinase-sensitive disaccharides are restricted to a few widely spaced sites in heparan sulphates from liver tissue and from cultured endothelial cells (M. Lyon, D. Hiscock, J. E. Turnbull & J. T. Gallagher, unpublished work).

The occurrence of sulphated and non-sulphated disaccharides in about equal concentrations in heparan sulphate (Turnbull & Gallagher, 1990) affords greater potential for variability of sulphate concentration along the polymer chain than in any other mammalian glycosaminoglycan. Indeed the alternate arrangement of sulphated and non-sulphated domains appears to be a unique structural characteristic. Corneal keratan sulphate is the only other glycosaminoglycan for which a detailed model structure has been proposed (Oeben *et al.*, 1987). In this model the polysaccharides are composed of a relatively constant domain of monosulphated disaccharide units proximal to the core protein and a variable domain of disulphated units positioned at the non-reducing ends of the chains; the one or two non-sulphated disaccharides are contiguous with the protein-linkage sequence.

Implications of the model for heparan sulphate biosynthesis and function

(a) Biosynthesis. Lindahl and colleagues (Lindahl *et al.*, 1986; Lindahl & Kjellén, 1987) have proposed a model for the synthesis of heparin in the Golgi complex whereby an unsulphated heparan

precursor, pre-assembled on the core protein, traverses extended arrays of distinct membrane-bound modification enzymes. These bring about a sequential and stepwise series of sulphation and epimerization reactions to form the mature heparin. Heparan sulphate appears to be synthesized by a similar mechanism (Levy *et al.*, 1981; Riesenfeld *et al.*, 1982; Silbert & Baldwin, 1984), but the degree of modification is significantly less than in heparin. Our data indicate that the major modification reactions will be confined to specific regions of the chain. This could occur if the individual polymer-modifying enzymes were arranged in the Golgi membranes in a sequential series of discrete clusters rather than in continuous arrays. Structural order may thus be generated in heparan sulphate by a complex and tightly regulated biosynthetic mechanism.

(b) Functions. The proposed model predicts an ordered spacing of the sulphated domains along the polymer chain, and this type of structural order may be critical in conferring specific functional properties. Heparan sulphate binds to many proteins and displays self-affinity interactions [for reviews, see Gallagher *et al.* (1986), Fransson (1989), Gallagher & Lyon (1989) and Ruoslahti (1989)]. The presence of IdoA and IdoA(2S) will confer considerable conformational versatility on the sulphated domains (Sanderson *et al.*, 1987; Casu *et al.*, 1988), whereas the more conformationally restricted GlcNAc-GlcA sequences will tend to maintain an extended overall conformation of the polysaccharide chain. The flexibility of the sulphated regions may facilitate interactions by enabling the molecule to 'mould' to the shape of structural proteins of the extracellular matrix (Casu *et al.*, 1988; Gallagher *et al.*, 1990), perhaps maximizing electrostatic forces that could maintain a stable association. Heparan sulphate on cell surfaces could promote cell adhesion to fibronectin and collagenous substrates by this type of interactive mechanism [for reviews, see Culp *et al.* (1986), Ruoslahti (1988) and Gallagher & Lyon (1989)]. It is even conceivable that the ordered polymeric structure could serve as a template for the biogenesis of extracellular matrices and basement membranes. Heparan sulphate also binds soluble proteins, acting as a catalyst for the antithrombin III-thrombin interaction [reviewed by Marcum & Rosenberg (1989)], and potentiating the activities of lipoprotein lipase, growth factors and perhaps also superoxide dismutase (see Gallagher *et al.*, 1986; Gallagher, 1989). These molecules probably bind to the sulphated regions of heparan sulphate. If specific sequences are required for these interactions (as demonstrated for antithrombin III), then binding will be restricted to specialized areas of the chain. These areas may be suitably disposed on the cell surface to facilitate further interactions of the bound proteins with their substrates or receptors on responding cells. It seems probable that future studies will reveal that the apparently unique molecular design of the polymorphic family of heparan sulphates is adapted to play fundamental roles in controlling the growth, metabolism and functions of mammalian cells.

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