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Substrate specificities of mouse heparan sulphate glucosaminyl 6-O-sulphotransferases

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Glycosaminoglycan heparan sulphate interacts with a variety of proteins, such as growth factors, cytokines, enzymes and inhibitors and, thus, influences cellular functions, including adhesion, motility, differentiation and morphogenesis. The interactions generally involve saccharide domains in heparan sulphate chains, with precisely located O-sulphate groups. The 6-O-sulphate groups on glucosamine units, supposed to be involved in various interactions of functional importance, occur in different structural contexts. Three isoforms of the glucosaminyl 6-O-sulphotransferase (6-OST) have been cloned and characterized [H. Habuchi, M. Tanaka, O. Habuchi, K. Yoshida, H. Suzuki, K. Ban and K. Kimata (2000) J. Biol. Chem. **275**, 2859–2868]. We have studied the substrate specificities of the recombinant enzymes using various O-desulphated poly- and oligosaccharides as substrates, and using adenosine 3′-phosphate 5′-phospho[^{35}S]sulphate as sulphate donor. All three enzymes catalyse 6-O-sulphation of both -GlcA-GlcNS- and -IdoA-GlcNS- (where GlcA represents D-glucuronic acid, NS the N-sulphate group and IdoA the L-iduronic acid) sequences, with preference for IdoA-containing targets, with or without 2-O-sulphate substituents. 6-OST1 showed relatively higher activity towards target sequences lacking 2-O-sulphate, e.g. the -GlcA-GlcNS- disaccharide unit. Sulphation of such non-O-sulphated acceptor sequences was generally favoured at low acceptor polysaccharide concentrations. Experiments using partially O-desulphated antithrombin-binding oligosaccharide as the acceptor revealed 6-O-sulphation of N-acetylated as well as 3-O-sulphated glucosamine residues with each of the three 6-OSTs. We conclude that the three 6-OSTs have qualitatively similar substrate specificities, with minor differences in target preference.

Key words: antithrombin, glycosaminoglycan, glucosaminyl 6-O-sulphotransferase, heparan sulphate, heparin, O-sulphation.

INTRODUCTION

Heparan sulphate (HS) proteoglycans are sulphated polysaccharides covalently attached to defined core proteins. HSPGs are ubiquitously expressed in most animal tissues. They are found at the cell surface and also in the extracellular matrix. Biosynthesis of heparan sulphate (HS) is a multi-step process involving a large number of enzymes (reviewed in [1–4]). Nascent HS chains are covalently attached to serine residues in the core protein. The HS–protein linkage region consists of the tetrasaccharide sequence glucuronic acid–galactose–galactose–xylose, which is formed by the action of four specific enzymes [4,5]. A single *N*-acetyl-D-glucosamine (GlcNAc) residue is then added to the linkage region followed by the alternating additions of D-glucuronic acid (GlcA) and GlcNAc residues to the non-reducing end of the growing polymer. Concomitant with elongation, the chains are modified by N-deacetylation and N-sulphation of GlcNAc, epimerization of GlcA at C-5 to L-iduronic acid (IdoA) units and finally introduction of O-sulphate groups at C-2 of uronic acid units and at C-6 and C-3 of D-glucosamine (GlcN) residues. The following enzymes are involved in the modification: four glucosaminyl N-deacetylase/N-sulphotransferases [6–9], three glucosaminyl 6-O-sulphotransferases (6-OSTs) [10] and five glucosaminyl 3-OSTs [11,12] but only one GlcA C-5 epimerase [13–15] and one hexuronic acid 2-OST [16,17]. The modification reactions are generally incomplete, such that the resultant polysaccharides vary in structure in a tissue-specific manner. It is believed that the fine structure of HS is determined through a regulated expression of the modifying enzymes. Heparin, a highly sulphated variant of HS, is exclusively synthesized by connective-tissue-type mast cells, where it is stored in cytoplasmic granules [18]. An extensive modification of heparin results in the formation of extended regions of -IdoA2S-GlcNS6S- (where 2S represents 2-O-sulphate group and NS the N-sulphate group) repeating disaccharide units. HS is generally less modified than heparin and has a more diverse structure, containing essentially three types of domain structures [19–21]. These are unmodified non-sulphated N-acetylated regions with GlcA residues, contiguous N-sulphated regions that are also 2-O- and 6-O-sulphated and contain both GlcA and IdoA units, and ‘mixed sequences’ of alternating N-sulphated and N-acetylated disaccharide units. The mixed sequences, similar to the N-sulphated domains, contain GlcA, IdoA and 6-O-sulphated residues, but appear to lack 2-O-sulphate groups [19]. Although the main features of HS biosynthesis have been outlined (see [4]), several aspects, including the subcellular organization of the biosynthetic enzymes, the mechanism of regulation of HS fine structure and the properties of the individual enzymes involved, remain unclear.

6-O-sulphate groups occur on glucosamine residues mainly in -GlcA/IdoA-GlcNS6S-, -GlcA-GlcNS3S6S-, -GlcA/IdoA-GlcNAc6S- and -GlcA2S/IdoA2S-GlcNS6S- disaccharide units.

Abbreviations used: $aMan_R$, 2,5-anhydro-D-mannitol (formed by reduction of terminal 2,5-anhydromannose residues using $NaBH_4$); AT, antithrombin; HS, heparan sulphate; GlcA, D-glucuronic acid; GlcN, D-glucosamine; GlcNAc, *N*-acetyl-D-glucosamine; IdoA, L-iduronic acid; NS, N-sulphate group; 6-OST, 6-O-sulphotransferase; 2S, 2-O-sulphate group; PAPS, adenosine 3′-phosphate 5′-phosphosulphate; downstream and upstream refer to constituents linked to the reducing and non-reducing ends respectively of a reference monosaccharide residue.

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Table 1 Saccharides used as [^{35}S]sulphate acceptors

Information regarding composition is mainly based on the analysis of disaccharides generated by treatment with HNO_2 (pH 1.5)/$NaBH_4$ [19] and in some cases by NMR spectroscopy. Abbreviations: DMSO, treatment with DMSO/methanol for the indicated time periods (see the Experimental section); cNS, chemical N-sulphation; MTSTFA, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide; hydrazinolysis, treatment with hydrazine/hydrazine sulphate; ND, not determined.

	Modification	IdoA-GlcNS*/IdoA ± 2S-GlcNS (%)	GlcA/total HexA (%)	Most abundant disaccharide unit in contiguous N-sulphated regions
Heparin 2,6-ODS	DMSO 8 h, 93 °C; re-cNS	ND	9	-IdoA-GlcNS-
Heparin 6-ODS-A	DMSO 2 h, 93 °C; re-cNS	52	9	-IdoA-GlcNS-
Heparin 6-ODS-B	MTSTFA	13	9	-IdoA2S-GlcNS-
HS native	None	ND	15	-IdoA2S-GlcNS-
HS 6-ODS	DMSO 2 h, 93 °C; re-cNS	17	15	-IdoA2S-GlcNS-
K5 50 % NS	Hydrazinolysis; cNS	–	100	-GlcA-GlcNS/Ac-*
K5 100 % NS	Hydrazinolysis; cNS	–	100	-GlcA-GlcNS-
K5 100 % NS 50 % epi	Hydrazinolysis; cNS; C-5 epimerization	–	∼ 50	-GlcA/IdoA-GlcNS-
AT10/12 ODS	DMSO 2 h, 93 °C; re-cNS	ND	ND	See Figure 4

* Corresponds to the N-acetylated as well as N-sulphated regions.

In a previous study, the acceptor requirements of the mice forms of 6-OSTs were investigated by determining the amounts of [^{35}S]sulphate incorporated into various polysaccharide substrates [10]. The 6-O-sulphate acceptor properties of these substrates differed with the three enzymes in such a manner as to suggest qualitative differences in target preference. To explore this possibility, we have determined the structures of the actual sulphate acceptor sequences, for each enzyme, in their reactions with a variety of substrate saccharides. Products were analysed with regard to the C-5 configuration of hexuronic acid units adjacent to 6-O-sulphation targets and also with regard to potential effects of pre-existing 2-O-sulphate groups. Moreover, an oligosaccharide derived from the antithrombin (AT)-binding region of heparin was used to examine 6-O-sulphation of N-acetylated and 3-O-sulphated GlcN residues.

We conclude that the recombinant mouse 6-OST1, 6-OST2 and 6-OST3 can all (a) catalyse 6-O-sulphation of both N-sulphated and N-acetylated GlcN units; (b) sulphate GlcNS residues downstream (the terms 'upstream' and 'downstream' refer to constituents linked to the non-reducing and reducing ends respectively of a reference monosaccharide residue [22]) of GlcA as well as IdoA in mixed -GlcNS-GlcA-GlcNS- and -GlcNS-IdoA-GlcNS- target sequences, although with strong preference for the latter acceptor; and (c) catalyse 6-O-sulphation of GlcNS3S residues.

MATERIALS AND METHODS

Materials

Bovine lung heparin (Upjohn) and pig intestinal HS (a gift from G. van Dedem, Diosynth, The Netherlands) were desulphated by treatment with DMSO/methanol (9:1, v/v) for 2 h (6-O-desulphation) or 8 h (2-O- and 6-O-desulphation) at 93 °C [23,24]. A deca-dodecasaccharide with high affinity for AT was prepared from pig intestinal mucosa heparin by a procedure involving partial deaminative cleavage, reduction with $NaBH_4$ and affinity chromatography of the products on AT–Sepharose. Oligosaccharides with high affinity for AT were size-fractionated by gel filtration on Sephadex G-50 (superfine grade; Amersham Biosciences) and eluted with 1 M NaCl. The recovered deca-dodecasaccharide fraction was desalted by gel chromatography [Sephadex G-15 in 10 % (v/v) EtOH] and then desulphated for 2 h as described above. These desulphated products were all re-N-sulphated by treatment with trimethylamine–sulphur trioxide [25], yielding O-desulphated, re-N-sulphated saccharides. Selectively 6-O-desulphated bovine lung heparin, retaining all N- and 2-O-sulphate groups, prepared by treatment with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide [26], was supplied by Camilla Westling (University of Uppsala, Uppsala, Sweden). Capsular polysaccharide from *Escherichia coli* K5, with the structure [-(GlcA β1,4-GlcNAc α1,4)-]$_n$ and derivatives in which approx. 50 % or approx. 100 % of the N-acetyl groups had been chemically replaced by N-sulphate groups, was supplied by Benito Casu (Ronzoni Institute, Milan, Italy). N-sulphated K5 polysaccharide, in which approx. 50 % of the GlcA residues had been converted into IdoA by enzymic epimerization at C-5 of GlcA, was provided by Pasqua Oreste (Ricerche Sperimentali Montale, Montale, Italy). An overview of saccharide substrates is provided in Table 1. Adenosine 3′-phosphate 5′-phospho[^{35}S]sulphate ([^{35}S]PAPS) was prepared as described in [27] or by incubating ATP sulphurylase (Sigma), adenosine 5′-phosphosulphate kinase (a gift from Irwin H. Segel, University of California, Davis, CA, U.S.A.), ATP (Boehringer Mannheim, Mannheim, Germany) and inorganic pyrophosphatase (Sigma) with carrier-free [^{35}S]sulphate (NEN, Boston, MA, U.S.A.) [28]. The resultant [^{35}S]PAPS was purified by anion-exchange chromatography on DEAE-Sephacel (Amersham Biosciences, Uppsala, Sweden). Unlabelled PAPS was purchased from Sigma. HexA-[^{3}H]aMan$_R$ disaccharides (where HexA represents unspecified hexuronic acid and aMan$_R$ the 2,5-anhydro-D-mannitol formed by the reduction of terminal 2,5-anhydromannose residues using $NaBH_4$), with and without O-sulphate groups in different positions, were used as reference compounds [29].

Isolation of recombinant 6-OSTs

The 6-OST1, 6-OST2 and 6-OST3 enzymes were expressed in COS-7 cells as FLAG fusion proteins and isolated on an anti-FLAG affinity column, essentially as described in [30]. COS-7 cells (1.5×10^6), precultured for 24 h in 100 mm culture dishes, were transfected with 13 μg of pFLAG-CMV2-m6-OST1, -OST2 and -OST3 respectively, using TransFact (Promega, Mannheim, Germany) according to the manufacturer's instructions. After incubation in Dulbecco's modified Eagle's medium containing 10 % (v/v) foetal calf serum and antibiotics for 72 h, the cell layers were washed with Dulbecco's modified Eagle's medium

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alone, scraped and homogenized in 2 ml of 10 mM Tris/HCl (pH 7.2), 0.5 % (w/v) Triton X-100, 0.15 M NaCl, 20 % (v/v) glycerol, 10 mM $MgCl_2$ and 2 mM $CaCl_2$. The homogenates were subjected to stirring for 1 h and then centrifuged at 10000 ***g*** for 30 min. FLAG fusion proteins in the supernatant (cell extract) were isolated by anti-FLAG M2 (Sigma) affinity chromatography according to the method described by the manufacturer. The amounts of purified enzyme proteins were estimated by immunoblotting. Purified enzyme preparations were separated by SDS/PAGE (10 % gel), transferred on to PVDF membranes (Immunovilon; Millipore, Eschborn, Germany) and probed with an anti-FLAG peptide antibody BIOM2 (Sigma). Known amounts of the FLAG-tagged proteins were used as standards to estimate the relative amount of purified enzymes.

Enzymic ^{35}S-sulphation of polysaccharides with purified recombinant 6-OSTs

Sulphation of chemically modified oligo- and polysaccharides was achieved by incubating 500 nmol/ml (as disaccharide units; approx. 250 μg) of polysaccharide acceptors (unless otherwise indicated) with 200 ng/ml purified recombinant 6-OSTs in 0.05 M imidazole/HCl (pH 6.8), containing 75 μg/ml protamine chloride and 1 μM [^{35}S]PAPS (1 nmol/ml) at 37 °C for 20 min [30]. The incubation volumes varied between 50 and 200 μl in separate experiments. After incubation, the reaction was stopped by heating at 100 °C for 1 min. Chondroitin sulphate was added as carrier (0.1 μmol of GlcA) and the resultant ^{35}S-labelled polysaccharides were separated from unchanged [^{35}S]PAPS or free [^{35}S]sulphate by precipitation with ethanol containing 1.3 % (w/v) potassium acetate and 0.5 mM EDTA, followed by gel chromatography on a Fast desalting column (Amersham Biosciences).

Analysis of ^{35}S-labelled saccharides

Deaminative cleavage with nitrous acid was performed at pH 1.5 (cleavage of glucosaminidic linkages at N-sulphated GlcN units), followed by reduction with $NaBH_4$, and the deamination products were fractionated by gel chromatography on Sephadex G-15 (1 cm × 180 cm) equilibrated with 0.2 M NH_4HCO_3. Fractions corresponding to di- and tetrasaccharides were collected, repeatedly freeze-dried, and the disaccharides were further separated by anion-exchange HPLC (see below). Tetrasaccharides containing a GlcNAc residue were N-deacetylated by hydrazinolysis (treatment with hydrazine/hydrazine sulphate) in 250 μl of hydrazine hydrate (30 % water; Fluka, Buchs, Switzerland) containing 1 % (w/v) hydrazine sulphate at 95 °C for 5 h [31]. Excess hydrazine was evaporated and further co-evaporated twice with 0.5 ml of water. The N-deacetylated product was re-isolated by gel chromatography on a column of Sephadex G-15 in 0.2 M NH_4HCO_3, freeze-dried and cleaved with nitrous acid at pH 3.9 (cleavage of N-unsubstituted glucosamine residues), followed by reduction with $NaBH_4$. The ^{35}S-labelled disaccharides formed were analysed by anion-exchange HPLC using a Whatman Partisil-10 SAX column, eluted with KH_2PO_4 as described in the legend to Figure 1.

All substrates and also the fully N-sulphated species generated some labelled material that was eluted as tetrasaccharides. This phenomenon is due to 'anomalous ring contraction', i.e. loss of the N-sulphate group and contraction of the pyranose ring without cleavage of the glycosaminyl bond during the deamination step [31,32]. 'Ring contraction products' generally represented 5–15 % of the total radioactivity. Before further analysis of tetrasaccharides, any component generated by 'anomalous ring contraction' was cleaved by mild acid hydrolysis (25 mM H_2SO_4, 80 °C for 30 min).

For enzymic sequence analysis, ^{35}S-labelled di- and tetrasaccharides were first separated into mono-O- and di-O-sulphated species by high-voltage paper electrophoresis on Whatman no. 3MM paper in 1.6 M formic acid (pH 1.7, 40 V/cm). After drying, guide strips were cut into 1 cm segments that were analysed for radioactivity by liquid-scintillation counting. The tetra- and disaccharides were recovered by elution from the paper with water. Purified di- or tetrasaccharides were incubated with iduronate 2-sulphatase, α-L-iduronidase and/or glucosaminyl 6-sulphatase (Oxford GlycoSciences, Abingdon, Oxon, U.K.) in a total volume of 25 μl of 50 mM sodium acetate (pH 5.0). The samples were incubated for 16 h according to the manufacturer's instructions.

RESULTS

Previous experiments [10] showed that the three different recombinant mice 6-OSTs catalyse the transfer of labelled sulphate from [^{35}S]PAPS into a variety of acceptor polysaccharides. The present study was undertaken to define more precisely the substrate requirements of the different enzymes, by analysing the ^{35}S-labelled structures resulting from incubations with the substrates given in Table 1. These saccharides cover a wide range of structures with various N-substituents (N-acetyl versus N-sulphate), hexuronic acid compositions and position of O-sulphate groups. Under standard incubation conditions (see the Experimental section) ^{35}S incorporated into the various polysaccharide acceptors ranged between 3.7×10^4 and 38×10^4 c.p.m./0.1 μmol of acceptor disaccharide units (results not shown for distinct enzyme–substrate combinations).

6-O-sulphation of polysaccharide substrates

The 6-O-^{35}S-labelled polysaccharides were characterized by deaminative cleavage (pH 1.5), followed by reduction of the products with $NaBH_4$, and recovery of the resultant labelled oligosaccharides by gel chromatography. Fully N-sulphated saccharide regions yield disaccharides, whereas N-acetylated GlcN units resist deamination and therefore give rise to tetrasaccharides or larger fragments [33]. Disaccharides typically accounted for 75–95 % of the ^{35}S-labelled products, the remainder appearing in the elution position of tetrasaccharides (results not shown). Higher yields of tetrasaccharides were obtained in incubations with HS substrate or AT-binding oligosaccharide. Tetrasaccharides derived from the latter substrate were subjected to structural analysis, with the aim of assessing 6-O-sulphation of GlcNAc units (see below).

Analysis of ^{35}S-labelled disaccharides revealed that all three 6-OST isoforms preferentially recognize -GlcNS-IdoA ± 2S-**GlcNS**- target structures (target GlcN unit in boldface) rather than the GlcA-containing isomeric sequence. Thus > 90 % of the 6-O-[^{35}S]sulphate incorporated into O-desulphated heparin and HS consistently appeared adjacent to an upstream IdoA unit (Figure 1, Table 2), although distinct GlcA-aMan$_R$6^{35}S peaks were observed. Indeed, using partially or fully N-sulphated K5 polysaccharide (composed of -GlcA-GlcNR- repeating disaccharide units) as acceptor yielded, for each 6-OST, GlcA-aMan$_R$6^{35}S as the only deamination product generated in significant amounts. Since O-desulphated heparin contains more IdoA than GlcA, whereas GlcA is the sole hexuronic acid component in the K5 polysaccharide, these results are inconclusive regarding substrate

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Table 2 Disaccharides generated by deaminative cleavage of O-^{35}S-sulphated polysaccharides

Polysaccharide substrates were incubated with [^{35}S]PAPS and the indicated 6-OSTs. The resultant ^{35}S-labelled samples were degraded to disaccharides (deamination at pH 1.5 followed by reduction of products with $NaBH_4$), which were analysed by anion-exchange HPLC (see the Experimental section). The values for heparin 6-ODS-A and HS 6-ODS are means ± mean deviation for 2–4 independent incubations. Values in parentheses refer to incubations with lysates of HEK-293 cells overexpressing 6-OST3 (for details regarding transfections, see [17,39]). ND, not determined.

	Deamination products (% of total 6-O-[^{35}S]disaccharides)								
	GlcA-aMan$_R$6^{35}S			IdoA-aMan$_R$6^{35}S			IdoA2S-aMan$_R$6^{35}S		
Polysaccharide substrate	6-OST1	6-OST2	6-OST3	6-OST1	6-OST2	6-OST3	6-OST1	6-OST2	6-OST3
Heparin 2,6-ODS	4	1	< 1	94	95	95	2	4	4
Heparin 6-ODS-A	3 ± 2	3 ± 2	2 ± 1 (3)	86 ± 3	83 ± 4	82 ± 2 (60)	11 ± 2	14 ± 6	16 ± 3 (37)
Heparin 6-ODS-B	–	–	– (2)	–	–	– (28)	–	–	– (70)
HS 6-ODS	7.5 ± 0.5	3	1 ± 0.5 (2)	23 ± 5	11	17 ± 2 (26)	69 ± 5	86	82 ± 2 (72)
HS native	< 5	ND	< 1	< 5	ND	< 1	94	ND	99
K5 50 % NS	100	100	100	–	–	–	–	–	–
K5 100 % NS	100	100	100	–	–	–	–	–	–
K5 100 % NS, 50 % epi	15	7	5	85	93	95	–	–	–

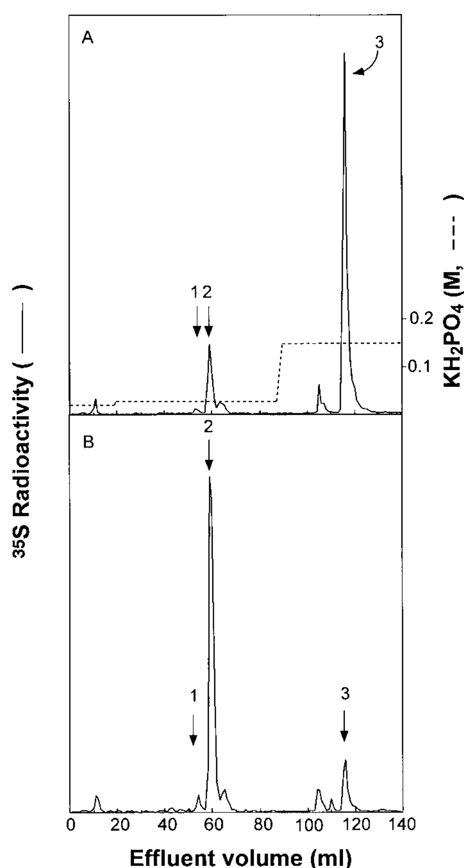

Figure 1 Anion-exchange HPLC of ^{35}S-labelled disaccharides obtained after deaminative cleavage of labelled polysaccharides

Chemically O-desulphated polysaccharides, HS 6-ODS (**A**) and heparin 6-ODS-A (**B**), were incubated with [^{35}S]PAPS and recombinant 6-OST3 (see the Experimental section). Labelled polysaccharide was isolated and degraded by HNO_2 (pH 1.5) and the resultant disaccharides were reduced and recovered by gel filtration. Samples of isolated disaccharides were analysed on a Partisil-10 SAX column eluted at the rate of 1 ml/min with KH_2PO_4 solutions of stepwise increasing concentration (as indicated by the broken line in **A**). Mono-O-sulphated disaccharides were eluted with 0.026 M and di-O-sulphated disaccharides with 0.15 M KH_2PO_4. The elution positions of standard disaccharides are indicated by arrows: 1, GlcA-aMan$_R$6S; 2, IdoA-aMan$_R$6S; 3, IdoA2S-aMan$_R$6S.

preferences for the 6-OSTs. We therefore decided to test a substrate based on N-sulphated K5 polysaccharide, in which approximately half of the GlcA units had been converted into IdoA by incubation of GlcA with C-5 epimerase. The predominant labelled disaccharide was IdoA-aMan$_R$6^{35}S, although GlcA-aMan$_R$6^{35}S was invariably present. Notably, the polysaccharides incubated with 6-OST1 yielded consistently higher proportions of GlcA-aMan$_R$6^{35}S when compared with the corresponding polysaccharides incubated with 6-OST2 or 6-OST3 (Table 2).

We further considered the effect of IdoA 2-O-sulphation on adjacent GlcNS 6-O-sulphation. A completely 2,6-O-desulphated, re-N-sulphated heparin, obtained through a process involving exhaustive treatment with methanolic DMSO (heparin 2,6-ODS in Table 1), yielded almost exclusively IdoA-aMan$_R$6^{35}S on enzymic 6-O-^{35}S-sulphation and very little IdoA2S-aMan$_R$6^{35}S, as expected (Table 2). A preparation (heparin 6-ODS-A) subjected to similar desulphation but for a shorter time period (Table 1) likewise produced primarily the mono-6-O-^{35}S-sulphated disaccharide, although the proportion of IdoA2S-aMan$_R$6^{35}S was higher than the extensively O-desulphated heparin. The low level of disulphated disaccharide was initially puzzling, since the O-desulphation procedure used to generate heparin 6-ODS-A had been aimed at preferential removal of 6-O-sulphate groups, while retaining most of the IdoA 2-O-sulphate groups. On the other hand, HS 6-ODS that had been subjected to the same O-desulphation procedures yielded more IdoA2S-aMan$_R$6^{35}S than IdoA-aMan$_R$6^{35}S (Table 2). The identity of IdoA2S-aMan$_R$6^{35}S was ascertained by digestion with iduronate 2-sulphatase (see the Experimental section), which resulted in quantitative conversion of the di-O-sulphated disaccharide into IdoA-aMan$_R$6^{35}S (Figure 2). These results were largely similar for all three 6-OST isoforms. Compositional analysis of O-desulphated acceptor polysaccharides provided a clue to the discrepant yields of IdoA2S-aMan$_R$6^{35}S disaccharides. Most of the IdoA units in heparin 6-ODS-A were found to be non-sulphated, whereas approx. 80 % of the IdoA residues in the N-sulphated domains of HS 6-ODS were 2-O-sulphated (Table 1). Apparently, 6-O-desulphation of heparin had been accompanied by appreciable 2-O-desulphation, whereas the HS (for unknown reason) had undergone more selective 6-O-desulphation. The different ratios of IdoA-aMan$_R$6^{35}S/IdoA2S-aMan$_R$6^{35}S observed after enzymic 6-O-sulphation thus largely reflect the disaccharide composition of the respective acceptor sequences.

To corroborate these findings, 6-O-desulphation of heparin was accomplished by a different method [treatment with

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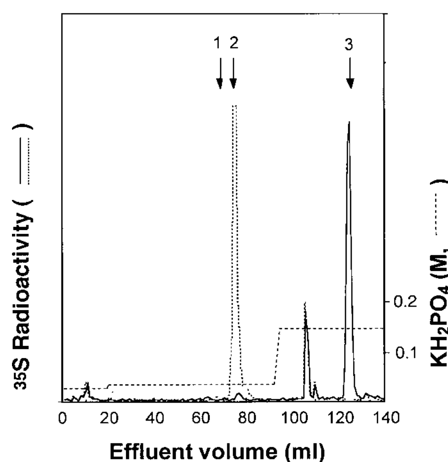

Figure 2 Anion-exchange HPLC of ^{35}S-labelled di-O-sulphated disaccharides before and after digestion with iduronate 2-sulphatase

[^{35}S]Disaccharides isolated after the incubation of 6-OST1 with HS 6-ODS and [^{35}S]PAPS were separated into mono-O- and di-O-sulphated species by high-voltage electrophoresis (see the Experimental section). Purified di-O-sulphated disaccharide was analysed by anion-exchange HPLC before and after iduronate 2-sulphatase digestion. The broken line represents digested disaccharide and the solid line represents the undigested sample. The reference compounds indicated by arrows 1–3 are the same as described in the legend to Figure 1.

N-methyl-*N*-(trimethylsilyl)trifluoroacetamide], which does not affect 2-O-sulphate (nor N-sulphate) groups [26]. Compositional analysis of the product (heparin 6-ODS-B; Table 1) indicated that IdoA units remained largely 2-O-sulphated. Cell lysates of 6-OST3-overexpressing 293 cells were used as enzyme source in parallel incubations of the novel heparin 6-ODS-B and the acceptors, heparin 6-ODS-A and HS 6-ODS, analysed previously (Table 2). In contrast with heparin 6-ODS-A, the more selectively 6-O-desulphated heparin 6-ODS-B yielded more IdoA2S-aMan$_R$6^{35}S than IdoA-aMan$_R$6^{35}S, similar to the HS 6-ODS. Taken together, the data in Tables 1 and 2 show that the proportions of the two 6-O-^{35}S-sulphated disaccharides essentially reflect the degree of 2-O-sulphation of the corresponding acceptor polysaccharides. All three 6-OST isoforms thus readily sulphate GlcNS residues immediately downstream of 2-O-sulphated as well as non-sulphated IdoA units.

Results shown in Table 2 were obtained using 500 μM (as disaccharide units) polysaccharide acceptors, essentially at saturating conditions (cf. Figure 7 in [10]). To elucidate any dependence of substrate specificity on substrate concentrations, HS 6-ODS was selected for experiments with different amounts of acceptor (100–500 μM disaccharide units). Incorporation of ^{35}S increased with increasing concentrations of HS 6-ODS when incubated with 6-OST1 and 6-OST3. By contrast, 6-OST2 afforded higher incorporation at 250 μM when compared with 500 μM acceptor concentration (results not shown). Similar aberrant behaviour was seen previously, using completely O-desulphated heparin as acceptor [10]. With all three 6-OST isoforms, lowering the acceptor concentration resulted in a shift from 2-O-sulphated -IdoA2S-GlcNS- towards non-O-sulphated -IdoA-GlcNS- or -GlcA-GlcNS- target disaccharide units (Figure 3). Nevertheless, 6-OST1 remained clearly distinguished from the other two at all acceptor concentrations tested, by its relative preference for non-O-sulphated target structures. Thus the relatively more abundant formation of GlcA-aMan$_R$6^{35}S and the less pronounced formation of IdoA-aMan$_R$6^{35}S in samples incubated with 6-OST1 as compared with samples incubated with 6-OST2 and 6-OST3 was maintained at all acceptor concentrations tested.

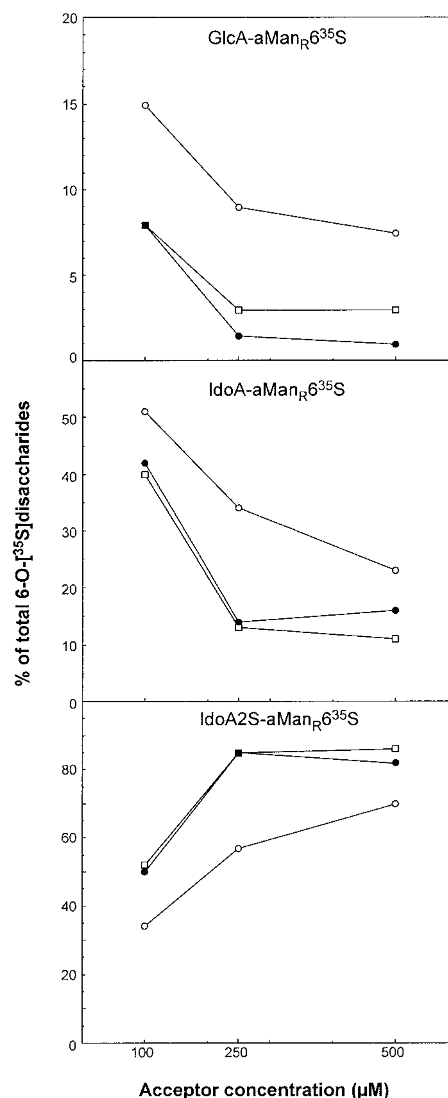

Figure 3 Effect of acceptor polysaccharide concentration on target selection by 6-OST isoenzymes

Recombinant 6-OST1 (○), 6-OST2 (□) and 6-OST3 (●) were incubated with HS 6-ODS at the concentrations indicated (refer to disaccharide units) and [^{35}S]PAPS. Labelled polysaccharide was isolated and degraded with HNO_2 (pH 1.5) and the resultant disaccharides were reduced, recovered by gel chromatography and analysed by anion-exchange HPLC as described in the legend to Figure 1. The proportions of each indicated disaccharide species are expressed in relation to total labelled disaccharides.

6-O-sulphation of oligosaccharide derived from the AT-binding region

It is not known whether the three mouse 6-OSTs are capable of 6-O-sulphating GlcNAc residues or a GlcNS residue that carries a 3-O-sulphate group. The partially O-desulphated sequence shown in Figure 4(A) represents a hypothetical 6-O-sulphate acceptor that may be explored to address these aspects of 6-OST substrate specificity. The AT-binding pentasaccharide sequence in heparin and HS corresponds to units 1–5 in Figure 4(A), although with largely 6-O-sulphated GlcN residues. If unit 3 is 6-O-^{35}S-sulphated, deaminative cleavage at GlcNS residues would yield two types of fragments, depending on whether the GlcN unit 1 is N-acetylated (R=-$COCH_3$) or N-sulphated (R=-SO_3^-). If unit 1 is N-sulphated, the ^{35}S-labelled unit 3 will be recovered in a

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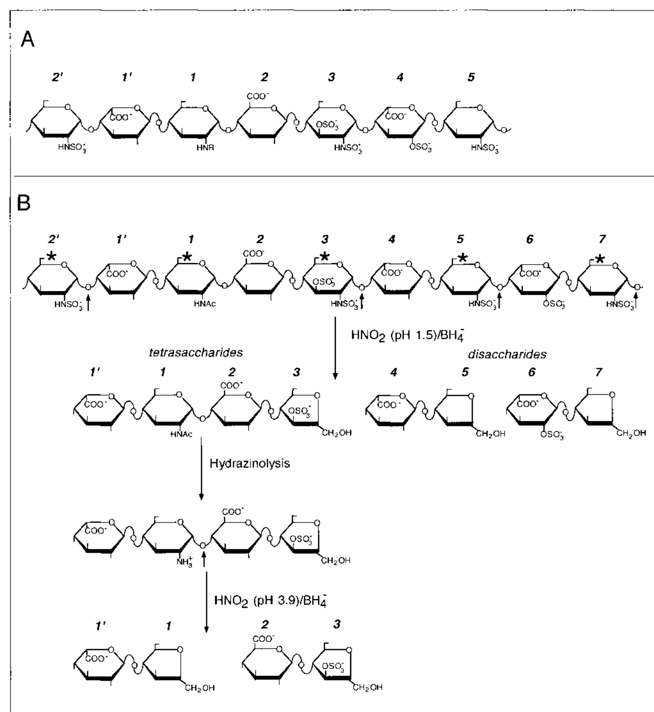

Figure 4 Structure of partially O-desulphated AT-binding heparin oligosaccharide

(**A**) A partially O-desulphated heparin structure encompassing the AT-binding pentasaccharide sequence (units 1–5) and an adjacent upstream disaccharide residue (units 2′–1′). The O-desulphation procedure was aimed at removing most of the 6-O-sulphate groups, while retaining most of the 2-O- and 3-O-sulphate groups. R=-$COCH_3$ or -SO_3^-. (**B**) A scheme of the chemical cleavage steps used in the structural analysis of the enzymically ^{35}S-labelled partially O-desulphated AT-binding heparin oligosaccharide. The potential 6-O-sulphate acceptor sites are indicated by asterisks.

disaccharide. On the other hand, if unit 1 is N-acetylated then unit 3 will form part of a tetrasaccharide encompassing units 1′–3.

A partially O-desulphated AT-binding 10/12-mer containing an appreciable proportion (approx. 45 %) of the initial 3-O-sulphate groups was used as a substrate for recombinant 6-OSTs. After enzymic 6-O-^{35}S-sulphation of the partially O-desulphated AT-binding substrate, labelled oligosaccharide was recovered and deaminated at pH 1.5 (see Figure 4B), and labelled di- and tetrasaccharides were separated by gel filtration (results not shown). Similar to the labelled disaccharides obtained using partially O-desulphated heparin and HS as substrates, the disaccharide fractions derived from the AT-binding region contained major components of IdoA-aMan$_R$6^{35}S and IdoA2S-aMan$_R$6^{35}S (Figure 5). However, contrary to the products of polysaccharide 6-O-^{35}S-sulphation, the disaccharides from the AT-binding region also contained substantial proportions of the labelled 3,6-di-O-sulphated disaccharide, GlcA-aMan$_R$ 3S6^{35}S. The identity of this component was established by digestion with β-glucuronidase, which eliminated the disaccharide and yielded a new labelled component at the elution position of aMan$_R$3S6S (Figure 5). Notably, although the (-GlcNS)-GlcA-GlcNS3S- disaccharide unit clearly could serve as substrate for all three 6-OSTs, the yield of labelled 3,6-di-O-sulphated product was highest with 6-OST1 (Table 3).

The tetrasaccharide fractions were treated by mild acid hydrolysis to eliminate any products of ‘anomalous ring contraction’ (see the Experimental section). The final tetrasaccharide fractions (13–20 % of the ^{35}S incorporated by the different 6-OSTs)

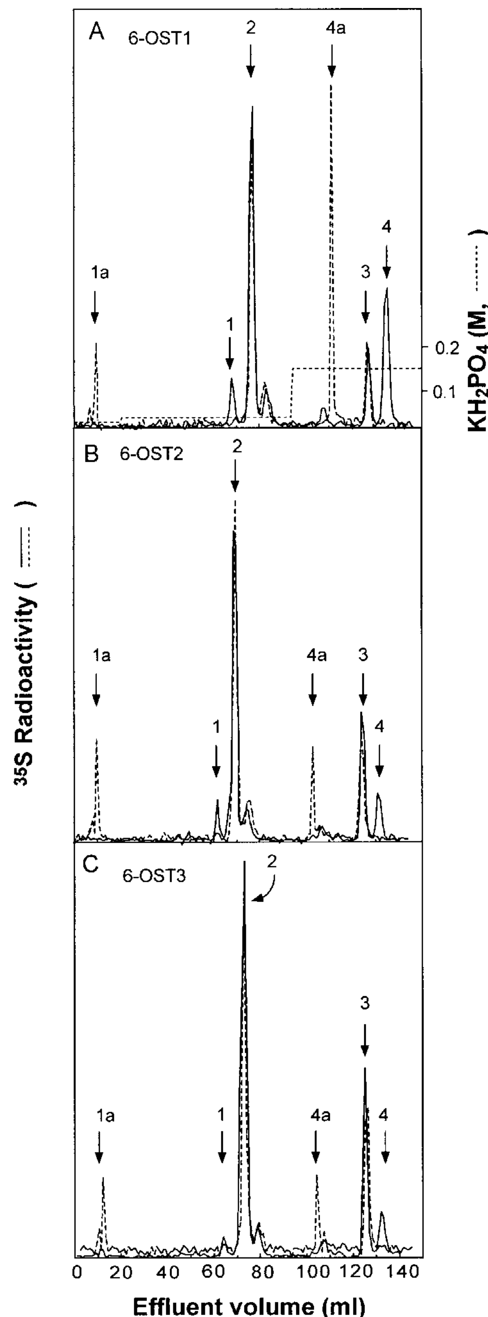

Figure 5 Anion-exchange HPLC of ^{35}S-labelled disaccharides derived from the AT-binding region of heparin

Heparin deca-dodecasaccharide with high affinity for AT was partially O-desulphated and incubated with [^{35}S]PAPS and recombinant 6-OST enzymes (see the Experimental section). Labelled oligosaccharide was isolated and degraded by HNO_2 (pH 1.5) and the resultant disaccharides were reduced, recovered by gel chromatography and analysed on a Partisil-10 SAX HPLC column as described in the legend to Figure 1. Elution patterns indicated by a dotted line refer to samples that had been digested with β-glucuronidase before HPLC analysis. The elution positions of standard disaccharides are indicated by arrows: 1, GlcA-aMan$_R$6S; 1a, aMan$_R$3S- or 6S; 2, IdoA-aMan$_R$6S; 3, IdoA2S-aMan$_R$6S; 4. GlcA-aMan$_R$3S6S; 4a, aMan$_R$3S6S.

were cleaved into disaccharides by N-deacetylation, followed by deamination at pH 3.9 (Figure 4B). Anion-exchange HPLC showed prominent peaks of GlcA-aMan$_R$3S6^{35}S (Figure 6), demonstrating that also the 3-O-sulphated disaccharide unit downstream of an N-acetylated GlcN residue, thus in a -GlcNAc-GlcA-GlcNS3S- sequence, provides an acceptor site for all three

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Table 3 Products formed on deaminative cleavage of O-^{35}S-sulphated oligosaccharides derived from the antithrombin-binding region

^{35}S-Labelled oligosaccharide was deaminated at pH 1.5, followed by reduction of products with $NaBH_4$. The resultant disaccharides (Di) were analysed by anion-exchange HPLC (see the Experimental section). The values are means ± mean deviation for 2–4 independent incubations. The labelled tetrasaccharide generated by deamination at pH 1.5 was N-deacetylated and degraded to disaccharides (Tetra → Di) by deamination at pH 3.9. The products were reduced and analysed by HPLC. For additional information, see the text. n.d., none detected.

	Deamination products (%)							
	GlcA-aMan$_R$6^{35}S		IdoA-aMan$_R$6^{35}S		IdoA2S-aMan$_R$6^{35}S		GlcA-aMan$_R$3S6^{35}S	
Isoenzyme	Di	Tetra → Di	Di	Tetra → Di	Di	Tetra → Di	Di	Tetra → Di
6-OST1	7.5 ± 0.5	38	51.5 ± 0.5	15	12 ± 0.1	n.d.	29 ± 1	47
6-OST2	5.5 ± 0.5	32	66 ± 1	55	18 ± 2	n.d.	10.5 ± 0.5	13
6-OST3	3.0 ± 0.3	38	62 ± 1	40	26 ± 1	n.d.	9.0 ± 0.1	22

6-OSTs. Again, 6-O-[^{35}S]sulphation of the already 3-O-sulphated GlcNS unit, as related to that of non-O-sulphated residues, was most pronounced for 6-OST1 (Table 3).

In addition to GlcA-aMan$_R$3S6^{35}S, cleavage of the tetrasaccharide fractions consistently yielded both GlcA-aMan$_R$6^{35}S and IdoA-aMan$_R$6^{35}S disaccharides (Figure 6, Table 3). The formation of the latter component is of particular significance, since it provides proof for 6-O-^{35}S-sulphation of the N-acetylated GlcN unit 1 (see Figure 4). A GlcA residue immediately downstream of an N-acetylated GlcN unit is not a substrate for the GlcA C-5 epimerase and, thus, will invariably retain D-gluco configuration [34]. Therefore the IdoA-aMan$_R$6^{35}S disaccharide must have been derived from saccharide units 1′–1 in the intact 10/12-mer (Figure 4). Notably, IdoA units may be formed by inversion of C-5 configuration during hydrazinolysis, but only along with cleavage of the adjacent glucosaminidic bond [31]. Since the N-deacetylated tetrasaccharides were re-isolated after hydrazinolysis, such interconversion of configuration could not be applied to the GlcA unit 2.

To corroborate further GlcNAc 6-O-sulphation, labelled tetrasaccharide (generated by incubation with 6-OST2) was subjected to enzymic sequence analysis. Before enzyme digestion, the ^{35}S-labelled tetrasaccharide was fractionated by high-voltage paper electrophoresis, and a largely di-O-sulphated fraction was recovered (results not shown). This step was required to separate enzymically 6-O-^{35}S-sulphated products from the bulk of unmodified substrate, which was found to inhibit exoenzyme digestion (results not shown). Anion-exchange HPLC (MonoQ column) of the purified tetrasaccharide revealed a major peak of labelled di-O-sulphated species, along with a smaller amount of tri-O-sulphated tetrasaccharide (Figure 7A). Digestion with α-iduronidase produced a small shift in elution position (caused by removal of unit 1′ from tetrasaccharide 1′–3; see Figure 4) (results not shown). However, subsequent digestion with 6-*O*-sulphatase resulted in a major release of label from the oligosaccharide fractions and concomitant formation of inorganic [^{35}S]sulphate (Figure 7B). This 6-O-[^{35}S]sulphate group could only have been released from GlcNAc unit 1.

DISCUSSION

Numerous studies point to important roles for 6-O-sulphate groups in regulating biological activities of HS. Thus not only the overall degree of 6-O-sulphation but also the location of 6-O-sulphate groups has been implicated in the control of events as diverse as growth factor action and protease inhibition [21,33]. The 6-O-sulphate groups in HS occur in different structural contexts. Approximately half the total 6-O-sulphate in HSs substitutes contiguous N-sulphated domains, often in juxtaposition to 2-O-sulphated hexuronic acid units. The remaining portion is found in domains of alternating N-acetylated and N-sulphated disaccharide units, which largely lack 2-O-sulphate groups [19]. Both N-acetylated and N-sulphated GlcN residues can be 6-O-sulphated; moreover, 6-O-sulphation is variably combined with 3-O-sulphation of the same GlcNS unit. It has been tempting to assume that 6-O-sulphation of such widely divergent target sequences would be catalysed by different 6-OST isoforms, and the present study was undertaken to address this possibility.

The three 6-OST isoforms were previously found to differ markedly in their preference for HS-related polysaccharide acceptors of different compositions [10]. These characteristics were expressed through the amounts of ^{35}S incorporated, but were tacitly assumed to reflect distinct preferences in acceptor structures. To resolve the matter at the disaccharide acceptor level, we identified target structures for the three enzymes, using a variety of poly- and oligosaccharide substrates. Remarkably, no qualitative differences were found; similar target sites were utilized by all three enzymes. Thus the 6-OSTs were all capable of 6-O-sulphating N-sulphated as well as N-acetylated GlcN residues. All were capable of 6-O-sulphating GlcNS immediately downstream of GlcA units, although IdoA-containing disaccharide units, whenever present, were the preferred targets. Moreover, all 6-OSTs utilized GlcNS acceptors adjacent (downstream) to 2-O-sulphated as well as non-sulphated IdoA residues (Tables 2 and 3). These results were obtained at acceptor concentrations well above the K_m values as reported in [10]. At lower acceptor concentrations, using the HS 6-ODS substrate, all three 6-OSTs appeared to attack increasingly non-O-sulphated target sequences as well (Figure 3). The stronger preference for 2-O-sulphated acceptor disaccharides at substrate saturation probably reflects a higher affinity of 2-O-sulphated substrates for 6-OSTs, as demonstrated recently using a panel of oligosaccharide substrates (P. Jemth, E. Smeds, A.-T. Do, H. Habuchi, K. Kimata, U. Lindahl and M. Kusche-Gullberg, unpublished work). Nevertheless, 6-OST1 differed from 6-OST2 and 6-OST3 by its relative preference at all acceptor concentrations tested for disaccharide units lacking 2-O-sulphate groups, in particular -GlcA-GlcNS- (Figure 3).

The functional role of GlcN 6-O-sulphation has been studied in particular detail in relation to the AT-binding region in heparin and HS. Two O-sulphate groups, at C-6 of unit 1 and at C-3 of unit 3, are essential for high-affinity binding of the saccharide to AT (Figure 4). Previous experiments with O-desulphated oligosaccharide acceptor showed that AT-binding ability was

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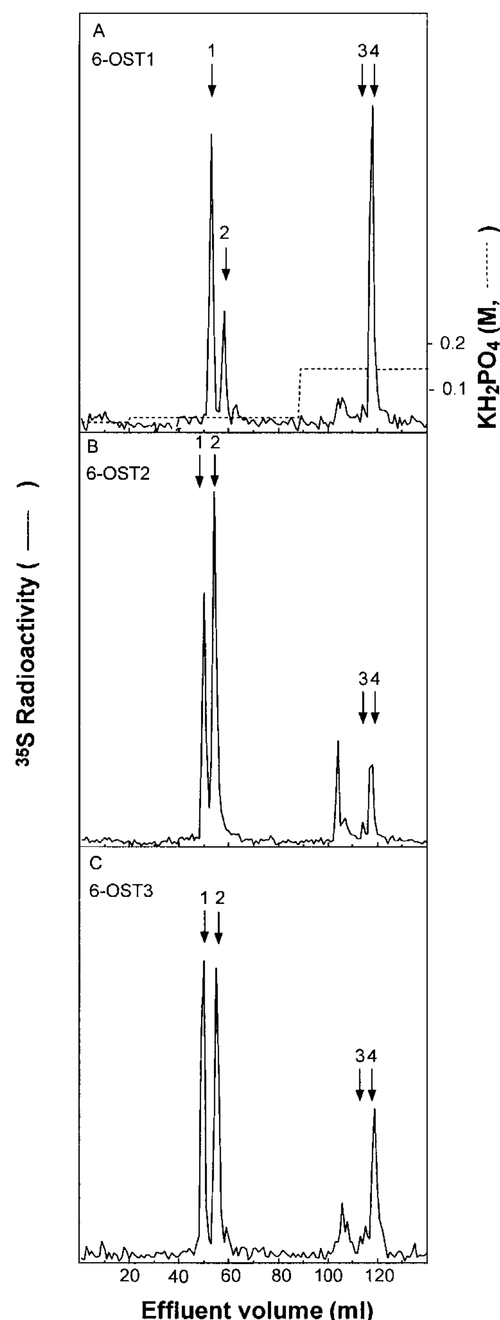

Figure 6 Cleavage of ^{35}S-labelled tetrasaccharides derived from the AT-binding region

A deca-dodecasaccharide with high affinity for AT was partially O-desulphated and incubated with [^{35}S]PAPS and recombinant 6-OST enzymes (see the Experimental section). Labelled oligosaccharide was isolated and degraded by HNO_2 (pH 1.5), and the ^{35}S-labelled tetrasaccharides obtained were N-deacetylated by hydrazinolysis and then cleaved to disaccharides by deamination at pH 3.9 (see the Experimental section). Products were analysed on a Partisil-10 SAX column as described in the legend to Figure 1. The reference compounds indicated by arrows 1–4 are the same as described in the legend to Figure 5.

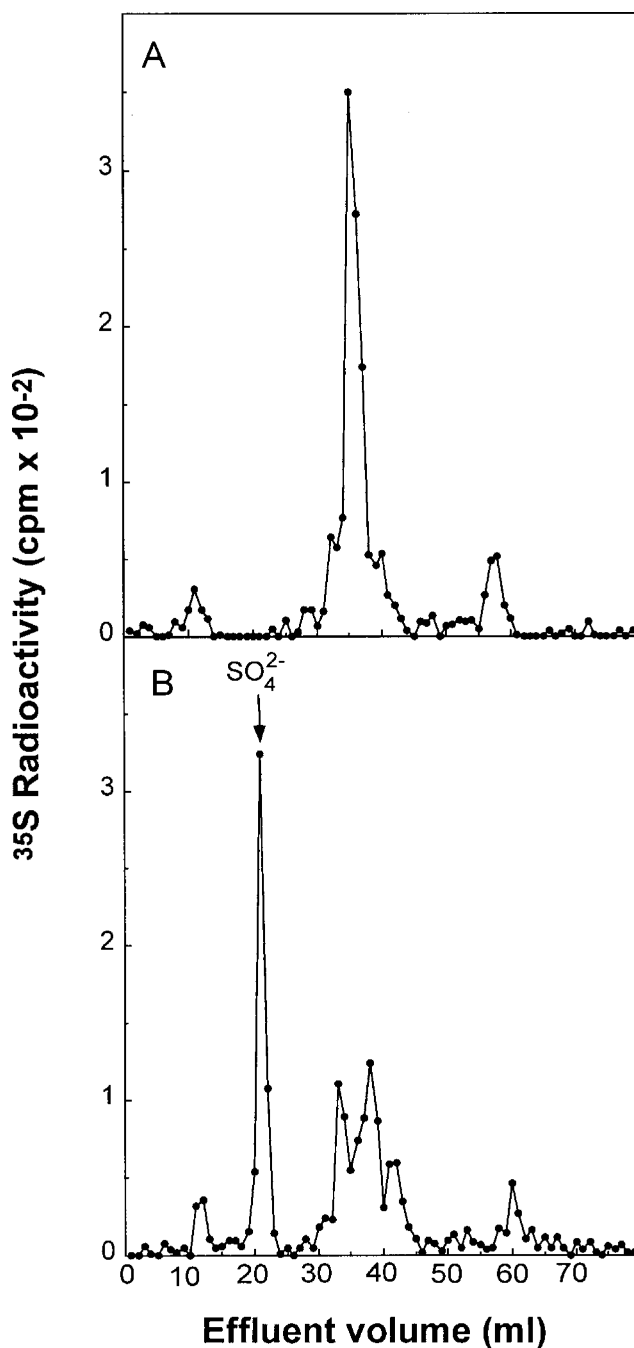

Figure 7 Enzymic sequence analysis of ^{35}S-labelled tetrasaccharide

A largely di-O-sulphated tetrasaccharide, 6-O-^{35}S-sulphated by incubation with 6-OST2, was analysed on a MonoQ HPLC column (**A**) before and (**B**) after digestion with α-iduronidase followed by digestion with 6-*O*-sulphatase. The minor peak at 57 ml in (**A**) corresponds to tri-O-sulphated tetrasaccharide. The column was eluted with a linear gradient (100 ml) extending from 0 to 1 M NaCl, pH 3.0 (pH adjusted with HCl). Effluent fractions of 1 ml were analysed for radioactivity. SO_4^{2-}, the elution position of standard inorganic sulphate.

acquired on enzymic O-sulphation at the two critical sites [35,36]. Recent studies using cell mutants and transfected recombinant O-sulphotransferases [37] or synthetic oligosaccharide acceptors [38] indicate that these sites can be independently sulphated. 6-O-sulphation of the 3-O-sulphated unit 3 is optional and is of little importance to AT binding. Zhang et al. [37] showed that N-acetylated unit 1 (Figure 4) may be 6-O-sulphated by 6-OST1. Our present results demonstrate that 6-OST2 and 6-OST3, in addition to 6-OST1, can all 6-O-sulphate GlcNAc unit 1 as well as the N- and 3-O-sulphated unit 3 (Figures 5 and 6, Table 3). In fact, 6-OST2 and 6-OST3 show a relative preference for the N-acetylated target compared with 6-OST1. The low activity of

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6-OSTs towards GlcNAc residues reported previously [10] may be due to the use of acceptors that lack structural features required for efficient substrate recognition.

Functional significance of the 6-OST isoforms remains unclear. Aspects for consideration include differential expression of the various enzymes. In the adult mouse, 6-OST1 is thus preferentially expressed in liver, and 6-OST2 in brain and spleen, whereas 6-OST3 shows diffuse expression in all the tissues examined [10]. Variable expression of the 6-OSTs may serve to generate HS species with different degrees of 6-O-sulphation in different organs [39]. It is possible that the small differences in substrate specificity observed *in vitro* may play an important role *in vivo*. On a more speculative note, the various 6-OST species may selectively associate with other HS biosynthetic enzymes, or with non-enzyme auxiliary proteins, in such a manner as to generate the different spectra of 6-O-sulphation observed in HS from diverse tissues and cells. Further work is needed to understand the functional significance of the intriguing difference in acceptor preference displayed by the 6-OSTs in incubations with polysaccharide acceptors of different structures [10]. Finally, it is important to compare the various 6-OSTs in reactions with more elaborately designed oligosaccharide substrates. The isoforms may conceivably differ in their preference for acceptor sequences with different dispositions of preformed 2-O-sulphate or even 6-O-sulphate groups, in ways that may significantly influence the fine structure of the final product. So far, no targeted mutations in any of the 6-OSTs have been reported. 6-OST-deficient mice will probably shed more light on the roles of the respective isoforms in generating HSs with different structures, thereby influencing HS interactions with various proteins.

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