Very-high-field n.m.r. studies of bovine lung heparan sulphate oligosaccharides produced by nitrous acid deaminative cleavage

¹³C-n.m.r. study of methylene resonances: degree and positions of C-6 sulphation

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(Received 8 November 1982/Accepted 10 February 1983)

Oligosaccharides with the general structure UA-(GlcNAc-GlcUA)_n-aManOH ($m = 1-5$) (where UA represents uronic acid, GlcNAc *N*-acetylglucosamine, GlcUA glucuronic acid and aManOH anhydromannitol) were prepared from low-sulphated heparan sulphates of bovine lung origin by nitrous acid deaminative cleavage followed by reduction. Analysis of the methylene signals in the 100 MHz ¹³C-n.m.r. spectrum of the tetrasaccharide ($m = 1$) shows that, whereas the extent of C-6 *O*-sulphation in the GlcNAc is approx. 65%, in the aManOH [formerly a GlcNSO₃ (*N*-sulphoglucosamine) residue in the parent heparan sulphate] it is only approx. 10%. In the higher oligosaccharides ($m = 2-5$) the gross extent of C-6 *O*-sulphation of GlcNAc residues falls systematically with increasing oligosaccharide size, whereas that in the aManOH residues remains below 10%. There is also evidence that the C-6 *O*-sulphation of the GlcNAc residues is confined to the GlcNAc residue adjacent to the non-reducing terminal uronic acid residue. It is therefore tentatively proposed that the GlcNAc in the sequence -GlcNSO₃-UA-GlcNAc- might be a favoured substrate for the 6-*O*-sulphotransferase. It is concluded that in the low-sulphated heparan sulphates GlcNSO₃ residues that do not occur in (GlcNSO₃-UA)_n blocks tend to have a significantly smaller extent of C-6 *O*-sulphation than do GlcNAc residues that occur in -GlcNSO₃-UA-GlcNAc-GlcUA-GlcNSO₃-sequences.

The heparan sulphates are glycosaminoglycans that are structurally related to the blood anti-coagulant heparin, and they are found in at least two proteoglycan forms. Heparan sulphates probably occur on the surfaces of all mammalian cells (Kraemer, 1971) as small proteoglycans with hydrophobic protein cores (see, e.g., Oldberg *et al.*, 1979), but distinct large proteoglycans of molecular weight 1×10^6 – 2×10^6 have been found in bovine lung gas-exchange tissue and pleura by Radhakrishnamurthy *et al.* (1980).

Heparan sulphate chains have been shown to self-associate (Fransson *et al.*, 1980a), and this property may contribute to specific cell–cell contact phenomena. Furthermore, self-association is dependent

on the presence of particular saccharide sequences (Fransson *et al.*, 1980b; Fransson, 1981), and heparan sulphates from the surfaces of virus-transformed cells may show diminished charge density and no ability to self-associate (Fransson *et al.*, 1981).

Comparatively little is known about the biosynthesis of heparan sulphates, but it is believed to resemble that of heparin (Rodén, 1980), which has been extensively researched (Lindahl & Höök, 1978; Lindahl *et al.*, 1981). In heparin biosynthesis, the initial polymerization product ('heparan') is [(1→4)-β-D-glucuronyl-(1→4)-α-D-N-acetylglucosaminyl]_n, which is then subjected to *N*-deacetylation and subsequent *N*-sulphation. Epimerization of glucuronate to iduronate may only occur in residues that are adjacent to and on the reducing side of an *N*-sulphoglucosamine residue. Sulphates are incorporated at the 2-position of iduronate and then the 6-position of glucosamine residues (Jacobsson & Lindahl, 1980). In heparan sulphate biosynthesis it must be assumed that a smaller extent of *N*-deacetylation takes place and consequently the

Abbreviations used: HS-*n*, heparan sulphate oligosaccharides containing *n* residues; UA, uronic acid; IdUA, α-L-iduronic acid; GlcUA, β-D-glucuronic acid; GlcNAc, 2-acetamido-2-deoxy-α-D-glucose (*N*-acetylglucosamine); GlcNSO₃, 2-sulphamido-2-deoxy-α-D-glucose (*N*-sulphoglucosamine); aManOH, 2,5-anhydro-D-mannitol.

extent of further modifications is limited in comparison with that in heparin.

There have been several n.m.r. studies of heparan sulphates (Dietrich *et al.*, 1974; Ayotte *et al.*, 1980; Huckerby & Nieduszynski, 1982), and Scott & Heatley (1982) have proposed a hydrogen-bonding scheme for heparan sulphate chains in solution.

In the present study the extents and residue positions of 6-sulphation are examined in oligosaccharides produced by nitrous acid deaminative cleavage of heparan sulphate chains from bovine lungs.

Experimental

Materials

Heparin and heparin by-products from bovine lung were supplied by Dr. W. E. Lewis (Glaxo Operations, Runcorn, Cheshire, U.K.). Sephadex G-25 (superfine grade) was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden, Bio-Gel P-10 (200–400 mesh) was from Bio-Rad Laboratories, Richmond, CA, U.S.A., and Amberlite mixed-monobed resin MB-3 (analytical grade) was from BDH Chemicals, Poole, Dorset, U.K. KB^3H_4 was purchased from Amersham International, Amersham, Bucks., U.K.

Preparative methods

Heparin by-products were fractionated by the method of Rodén *et al.* (1972). The crude material (40 g) was dissolved in 5% calcium acetate/0.5 M-acetic acid (2 litres) and fractionated with ethanol, the glycans precipitated at between 18% and 36% (v/v) ethanol being recovered. The heparan sulphates were precipitated as cetylpyridinium complexes, which were then redissolved in 2 M-NaCl. On lowering of the salt concentration the material precipitated at between 0.3 M- and 1.0 M-NaCl was recovered by ethanol precipitation, and was subsequently used as the heparan sulphate parent material for nitrous acid digestion.

Degradative methods

Complete deaminative cleavage of GlcNSO₃ glycosidic linkages in the heparan sulphate preparation was achieved by the following method. Heparan sulphate (5 g) was dissolved in water (50 ml), and additions of 1 M-citric acid (200 ml) and 2 M-NaNO₂ (25 ml) were made. After being stirred, the mixture was left for 1 h at room temperature. Excess nitrous acid was removed by careful addition of ammonium sulphamate (0.14 g/ml; 50 ml). Terminal anhydromannose residues produced by the reaction were reduced overnight under alkaline conditions with KB^3H_4 , followed by a further overnight treatment with NaBH_4 .

Heparan sulphate oligosaccharides were

recovered from the resulting mixture by addition of ethanol (4 vol.). This produced a lower, uronic acid-containing, layer, which was desalted on a Sephadex G-25 column (size, 133 cm × 3.0 cm; eluent, distilled water; flow rate, 38 ml/h).

Fractionation of oligosaccharides

The desalted oligosaccharide mixture was fractionated by gel filtration on a column of Bio-Gel P-10 (200–400 mesh) of size 151 cm × 2.6 cm and operated at a constant hydrostatic head (275 cm H₂O). The eluent was 0.2 M-NH₄HCO₃ and 7.2 ml fractions were collected. The effluent was analysed for uronic acid (Brown, 1946) and for ³H label by liquid-scintillation counting. Fractions were pooled in accordance with the following K_{av} values for each oligosaccharide size: HS-4 (0.72–0.81); HS-6 (0.60–0.67); HS-8 (0.50–0.55); HS-10 (0.43–0.45); HS-12 (0.37–0.39). Bovine serum albumin and Cl[−] were used as V_0 (void volume) and V_t (total volume) markers respectively. The proportions of individual oligosaccharides as determined by uronic acid content were as follows: HS-4 (24%), HS-6 (13%), HS-8 (8%), HS-10 (6%), HS-12 (5%), HS > 12 (15%); 29% of the total uronic acid in the preparation was removed along with the salts as material of approximately disaccharide size. Oligosaccharides were converted into the sodium form by passage through a 10 ml column of Amberlite MB-3 at 7°C followed by titration to pH 6–7 with NaOH.

¹³C-n.m.r. spectroscopy

Preliminary ¹³C-n.m.r. spectra were obtained with a JEOL FX-100 spectrometer equipped with a 10 mm variable-temperature probe operating at 25.05 MHz.

High-field ¹³C-n.m.r. spectra were determined on a Bruker WH 400 instrument with 5 mm and 10 mm variable-temperature probes operating at 100.6 MHz.

Oligosaccharide samples (30–150 mg) were buffered to pH 7 with phosphate and dissolved in ²H₂O (0.5 ml for 5 mm probe; 1.1 and 1.5 ml for the 25 MHz and 100 MHz 10 mm probes respectively). Spectra were recorded at 60°C, with 3-trimethylsilyl[²H₄]propionic acid sodium salt as internal reference (Huckerby, 1983).

Results

The heparan sulphate oligosaccharides prepared by nitrous acid deaminative cleavage have the general structure UA-(GlcNAc-GlcUA)_m-aManOH (as shown in Scheme 1) where $m = 1-5$ (tetra- to dodeca-saccharide, fractions HS-4 to HS-12). As no resonances ($\delta \sim 60.7$ p.p.m.) corresponding to C-2 in N-sulphoglucosamine residues are observed in any

of the oligosaccharide spectra, it may be concluded that the deaminative cleavage is complete. It is assumed from biosynthetic considerations that residue 1 (Scheme 1) may be either iduronate or glucuronate, as it was previously linked at C-4 to an *N*-sulphoglucosamine residue, and similarly that all other uronic acids must be glucuronate. The main features of the ^{13}C -n.m.r. spectrum for fraction HS-4 are shown in Fig. 1. It is clear that the tetrasaccharide fraction contains a mixture of variously substituted species, and because of this complexity a full analysis is not attempted here. The present study is concerned with an examination of the methylene resonances, which lie between δ 62 and

71 p.p.m. Expansions of this region are shown for fractions HS-4 to HS-12 in Fig. 2, and spectral parameters are given in Table 1.

In a heparan sulphate disaccharide fraction (spectrum not shown) two resonances are observed at $\delta \sim 64$ p.p.m. (CH_2OH) and a further band at $\delta \sim 70.8$ p.p.m. ($\text{CH}_2\text{OSO}_3^-$), which must all arise from aManOH residues. On this basis, methylene signals from the other oligosaccharides may be classified as originating from either terminal aManOH or from GlcNAc residues. For all of the oligosaccharides an aManOH residue gives rise to two major methylene carbon resonances at $\delta \sim 64.05$ p.p.m. of almost equal relative intensity,

Fig. 1. 100 MHz ^{13}C -n.m.r. spectrum for heparan sulphate tetrasaccharide fraction

The left-hand portion shows anomeric resonances (δ 98–106 p.p.m.) and the right-hand section depicts the main ring resonances (δ 62–90 p.p.m.). Carbonyl and methyl resonances are omitted.

Scheme 1. Representation of nitrous acid cleavage of heparan sulphate and structure of a typical tetrasaccharide sequence

$\text{R} = \text{CO}_2^-$ and $\text{R}' = \text{H}$ for GlcUA; $\text{R} = \text{H}$ and $\text{R}' = \text{CO}_2^-$ for IdUA; $\text{X} = \text{H}$ or SO_3^- .

Table 1. ^{13}C -n.m.r. spectral parameters of heparan sulphate oligosaccharides

Oligosaccharide fraction	δ (p.p.m.)				% of C-6 O-sulphation of GlcNAc*
	aManOH		GlcNAc		
	C-1-OH C-6-OH	C-6-OSO ₃ ⁻	C-6-OH	C-6-OSO ₃ ⁻	
HS-4	{64.06 64.26}	70.46	{62.77 62.94}	{68.79 69.03}	65
HS-6	{64.09 64.28}	70.37	{62.78 + shoulder}	{68.79 69.04}	34
HS-8	{63.98 64.18}	70.40	{62.64 62.82}	{68.70 68.95}	21
HS-10	{64.05 64.25}	70.45	{62.71 62.90}	{68.75 69.00}	13
HS-12	{64.11 64.31}	~70.5	{62.78 62.95}	{68.78 69.06}	9

* Percentage of 6-O-sulphation calculated from peak areas for the resonances at $\delta \sim 62.8$ p.p.m. and ~ 68.9 p.p.m.

together with a small resonance at $\delta \sim 70.4$ p.p.m. (see Table 1). The latter resonance may be assigned to C-6 derived from a 6-O-sulphated *N*-sulphoglucosamine residue in the parent material. These assignments concur with the findings obtained by Kosakai & Yosizawa (1981) from oligosaccharides of a similar nature derived from whale heparin. The oligosaccharides ($m \geq 1$) all show extra methylene resonances, found at $\delta \sim 62.8$ p.p.m. and ~ 68.9 p.p.m. that may be assigned to the methylene groups in C-6-unsulphated and C-6-O-sulphated GlcNAc residues respectively.

It is important to determine whether the experimental conditions employed in nitrous acid cleavage may also hydrolyse 6-sulphate residues, because this would invalidate n.m.r. quantification of these extents of sulphation. In a parallel investigation of bovine lung heparin oligosaccharides produced by a similar nitrous acid cleavage, it was shown that the aManOH residue in the disaccharide fraction is almost completely 6-sulphated, as indicated by the unit resonances both for sulphated C-6 and unsulphated C-1 at δ 70.85 and 63.95 p.p.m. respectively (see Fig. 3).

It may be assumed that the relative intensities for sulphated and non-sulphated methylene resonances within a given residue provide a direct measure for the extent of sulphation. Throughout the heparan sulphate oligosaccharide series (HS-4 to HS-12) the extent of 6-sulphation on the aManOH residue is about 10% or less. In contrast, in the tetrasaccharide the extent of 6-sulphation on the GlcNAc residue is about 65%. It may be noted that in the tetrasaccharide (see Scheme 1) the extent of 6-sulphation is considerably higher in the parent heparan sulphate at residue 2 (GlcNAc) relative to

that at residue 4 (GlcNSO₃). Furthermore, the resonance at $\delta \sim 68.9$ p.p.m. (from sulphated C-6 in residue 2) has a bimodal character with components of relative intensity about 3:2. In the tetrasaccharide, the overall extent of 6-sulphation on the aManOH residue is only about 10%. Thus 90% of the aManOH residues are unsulphated, and this proportion of ester sulphation cannot account for the bimodality seen. However, residue 1 may be either glucuronate or iduronate, and it is reasonable to assume that the intensity distribution (at residue 2) represents the degree of epimerization at residue 1 for those structures in which residue 2 is 6-sulphated.

Discussion

Inspection of C-6-OSO₃ resonances in GlcNAc at $\delta \sim 68.9$ p.p.m. throughout the heparan sulphate oligosaccharide series (see Fig. 2 and Table 1) shows a consistently bimodal structure. Thus two, and only two, chemical environments are being sensed by this resonance. In principle, as the oligosaccharide series is ascended the 6-sulphate groups could be randomly distributed among GlcNAc residues. If this were the case the resultant methylene resonances would no longer be expected to show just two signals. It should be noted that the relative proportions of the two components at $\delta \sim 68.9$ p.p.m. remain approximately constant throughout the oligosaccharide series. This could tentatively be explained if it were assumed that these proportions reflected a constant iduronate/glucuronate ratio at residue 1. This explanation would only be viable if the resonance under consideration originated entirely from residue 2. The

Fig. 2. 100 MHz ^{13}C -n.m.r. spectra detailing methylene resonances for the heparan sulphate oligosaccharides. The resonances between δ 62 and 71 p.p.m. are shown for the following oligosaccharide fractions: (a) HS-4; (b) HS-6; (c) HS-8; (d) HS-10; (e) HS-12.

hypothesis that the total 6-sulphation on GlcNAc is confined to residue 2 is supported by the data for total percentage extents of 6-sulphation on GlcNAc. If it is assumed that all of this sulphate is located at 65% occupancy on residue 2 (as is the case for fraction HS-4) and no sulphate is located elsewhere, then the total calculated extents of 6-sulphation for the higher oligosaccharides are 32%, 22%, 16% and 13%, in very good agreement with the observed values (see Table 1).

It is clear that, in the tetrasaccharide, the extent of 6-sulphation is higher in the GlcNAc (residue 2) than in the aManOH (residue 4), which in the parent polymer corresponded to a GlcNSO₃. If the hypo-

thesis discussed above is correct, then the extents of 6-sulphation at residue 2 always exceed those at residue n (where n is the number of residues in the oligosaccharide HS-4 to HS-12). Could it be that the 6-*O*-sulphotransferase for heparan sulphates particularly favours the GlcNAc in -GlcNSO₃-UA-GlcNAc-sequences?

It is noteworthy that the sequence IdUA-GlcNAc(6OSO₃)-GlcUA is known to be part of the antithrombin-binding site in heparin (Lindahl *et al.*, 1981), and sequences such as GlcNSO₃-IdUA-GlcNAc-GlcUA-GlcNSO₃-IdUA are believed to comprise contact zones for chain-chain associations in heparan sulphates (Fransson, 1981).

Fig. 3. 100 MHz ^{13}C -n.m.r. spectrum for the disulphated disaccharide nitrous acid cleavage product from bovine lung heparin

Resonances between δ 60 and 110 p.p.m. are shown. The resonances at δ 63.95 and 70.85 p.p.m. are assigned to the unsulphated C-1 and sulphated C-6 methylene carbon atoms of aManOH respectively.

Conclusion

In the low-sulphated heparan sulphates from bovine lung the GlcNSO₃ residues that do not occur in (GlcNSO₃-UA)_n blocks tend to have a small extent (approx. 10%) of C-6 O-sulphation, but GlcNAc residues that occur in -GlcNSO₃-UA-

GlcNAc-GlcUA-GlcNSO₃-sequences on average have a greater extent (approx. 65%) of C-6 O-sulphation.

We thank the Science and Engineering Research Council and Glaxo Operations Ltd. for a C.A.S.E. studentship (to P. N. S.). Support from the Science and Engineering Research Council for use of their 400 MHz n.m.r. facility is gratefully acknowledged. We are indebted to Dr. B. E. Mann and Dr. C. Spencer for assistance and valuable discussions.

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