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Chemical modification of glycosaminoglycans. Sulphation of heparan sulphate derivatives obtained by periodate oxidation/borohydride reduction

José Kovensky & Alicia Fernández Cirelli[1]

Departamento de Química Orgánica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellón II, Ciudad Universitaria, 1428, Buenos Aires, Argentina

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Two heparan sulphates, of different *N*- and *O*-sulphate content and iduronic/glucuronic acid ratio (**HS I** and **HS II**), were submitted to partial periodate oxidation, borohydride reduction and subsequent *O*-sulphation. The sulphated derivatives showed increased anticoagulant activities by APTT assay and were significantly degraded by heparinase. © 1997 Published by Elsevier Science Ltd. All rights reserved.

INTRODUCTION

Sulphated glycosaminoglycans (GAG) bind to a variety of plasma proteins such as antithrombin III (AT III), heparin cofactor II (HC II), lipoprotein lipase (LPL) and low-density lipoproteins (LDL). Increasing charge density of the GAG is usually associated with stronger protein binding. However, the binding properties of chondroitin sulphate (CS), dermatan sulphate (DS), heparan sulphate (HS) and heparin to those proteins are not only related to their charge density, but also to the L-iduronic acid (IdoA) content of the GAG chain (Casu, 1991; Lindahl & Pejler, 1987). The unique property of IdoA to exist in more than one equi-energetic conformation would result in more flexible GAG chains in IdoA-rich polysaccharides than those containing the more rigid D-glucuronic acid (GlcA) residues, allowing a better exposition of anionic groups to basic protein sites (Casu *et al.*, 1988).

Glycol splitting, by periodate oxidation of all non-sulphated GlcA and IdoA residues, was reported to increase the LPL-releasing activity of heparin (Casu *et al.*, 1986) and the antithrombotic activity of DS (Mascellani *et al.*, 1989–1990). On the other hand, van Boeckel *et al.* (1988) prepared a pentasaccharide corresponding to the AT III binding site of heparin which retained the anti-factor Xa activity, although it contained an open-chain fragment instead of an uronic acid residue.

Our previous studies, on the chemical modification of GAG, include salicylic acid, carboxymethyl and sulphated derivatives (Kovensky & Fernández Cirelli, 1993, 1994, 1995). We now report the preparation and properties of modified heparan sulphates, obtained by partial periodate oxidation–borohydride reduction and subsequent sulphation. The natural abundance and the wide distribution of HS make this polysaccharide a suitable starting material for the synthesis of modified glycosaminoglycans.

EXPERIMENTAL

Materials

Heparin and heparan sulphate **HS II** were kindly given by Syntex Argentina S.A. Heparan sulphate **HS I** was fractionated from commercial heparin by-products as previously reported (Kovensky *et al.*, 1990). Heparinase was purchased from Sigma Chemical Co. (USA). ^{13}C-NMR of **HS I**: δ 175·7 (CO), 104·4 (C-1 GlcA), 101·7 (C-1 IdoA), 99·9–98·8 (C-1 GlcN), 67·4 (C-6 O-SO_3^-), 62·4 (C-6 OH), 59·0 (C-2 GlcNSO_3^-), 54·6 (C-2 GlcNAc), 23·5 (acetyl). A similar spectrum was obtained for **HS II**, with changes in signal intensities.

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Analytical methods

Sulphate and carboxyl groups were determined by conductimetry (Casu & Gennaro, 1975). Uronic acids estimation by carbazol–borate method, agarose gel electrophoresis in 0·04 M barium acetate, heparinase depolymerization and anticoagulant activities (APTT) were performed as before (Kovensky *et al.*, 1990; Fraidenraich y Waisman & Fernández Cirelli, 1992). ^{13}C-NMR spectra were recorded in a Varian XL-100 spectrometer operating at 25·2 MHz; samples were dissolved in D_2O–H_2O (1:1).

Periodate oxidation/borohydride reduction of HS I

Heparan sulphate **HS I** (400 mg) was dissolved in water (60 ml), and sodium metaperiodate (856 mg) was added. Initial pH of the mixture was 5·0, but no buffer was added. After stirring for 24 h in the dark, ethyleneglycol (1 ml) was added and the solution was stirred for 2 h. The product was recovered (387 mg) after dialysis and evaporation under reduced pressure.

The periodate oxidized product (220·6 mg) was dissolved in water (22 ml) and 441·2 mg of sodium borohydride was slowly added with continuous stirring. After 3 h at room temperature, the pH of the mixture was adjusted to 5·0 with acetic acid, and then to 8·0 with M NaOH. After dialysis and evaporation 220·3 mg of compound **2** was obtained. ^{13}C-NMR of **2**: δ175·6 (**C**O), 104·4 (C-1 GlcA), 101·7 (C-1 IdoA), 99·9–98·4 (C-1 GlcN), 67·4 (C-6 O-SO_3^-), 62·4 (C-6 OH), 61·1 (**C**H_2OH), 58·8 (C-2 GlcNSO_3^-), 54·6 (C-2 GlcNAc), 23·3 (acetyl).

When the same sequence was performed on **HS II**, compound **5** was obtained in a similar yield as compound **2**. The only differences in signal intensity were observed when comparing the ^{13}C-NMR spectra of **2** and **5**, in accordance with the different structural features of the starting materials.

Sulphation of compound 2

Sulphation was performed by the method of Ogamo *et al.* (1989). Compound **2** (95·9 mg) was converted into the tributylammonium salt and dissolved in anhydrous DMF (11 ml). Sulphur trioxide–triethylamine complex (0·5 g, Nair & Bernstein, 1987) was added. After 90 min at room temperature, cold water (12 ml) was added, the pH of the mixture was adjusted to 8·0 and dialysed. Evaporation under reduced pressure yielded 98·6 mg of the sulphated product **3**. ^{13}C-NMR of **3**: δ 175·6 (**C**O), 104·4 (C-1 GlcA), 101·5 (C-1 IdoA), 99·9–98·3 (C-1 GlcN), 67·4 (C-6 O-SO_3^- and **C**H_2O-SO_3^-), 58·8 (C-2 GlcNSO_3^-), 54·5 (C-2 GlcNAc), 23·3 (acetyl).

On sulphation, compound **5** yielded **6** with similar spectroscopic characteristics as those observed for compound **3**.

RESULTS AND DISCUSSION

Since the splitting of all non-sulphated uronic acid residues was reported to have abolished the AT III affinity of heparin (Casu *et al.*, 1986; Choay, 1989), an incomplete periodate oxidation reaction was performed on two heparan sulphates of different *N*- and *O*-sulphate content and iduronic/glucuronic acid ratio (**HS I** and **HS II**, Table 1). In both cases, the oxidized residues accounted for about 20% of total uronic acid units, as estimated by the carbazol–borate method (Table 2). The signal at 61·1 ppm (**C**H_2OH), in the ^{13}C-NMR spectrum of compounds **2** and **5**, accounted for the primary hydroxyl groups arising from the reduction of the aldehyde groups formed by glycol splitting by periodate oxidation.

Our previous studies on sulphation of HS (Kovensky & Fernández Cirelli, 1991) indicated that O-sulphation occurs mainly at primary hydroxyl groups, i.e. O-6 of

Table 1. Comparative data for HS I and HS II

	GlcNSO_3^-/ GlcNAc[a]	GlcN 6OSO_3^-/ 6OH[a]	IdoA/GlcA[b]
HS I	30:70	30:70	30:70
HS II	40:60	60:40	50:50

[a]Estimated from ^{13}C-NMR spectra.
[b]Estimated from the carbazol/orcinol ratio (Fransson *et al.*, 1980).

Table 2. Analytical data, anticoagulant activities and heparinase depolymerization of heparan sulphate derivatives

	SO_3^-(%)[a]	COO^-(%)[a]	SO_3^-/COO^-[a]	COO^-(%)[b]	APTT (U/mg)	A_{230} (48 h)	Heparinase (% degr)[c]
1 HS I	11.1	7.04	0.87	8.45	8.3	0.097	< 5
2 oxi/red *1*	n.d.	n.d.	n.d.	6.73	8.1	0.104	< 5
3 sulphated *2*	24.9	7.20	1.90	n.d.	21.7	2.400	58
4 HS II	16.8	7.44	1.26	7.90	155	0.970	24
5 oxi/red *4*	16.9	7.42	1.25	6.30	152	1.714	42
6 sulphated *5*	24.3	7.20	1.86	n.d.	217	3.178	77
7 heparin	32.8	7.19	2.51	n.d.	167	4.111	100

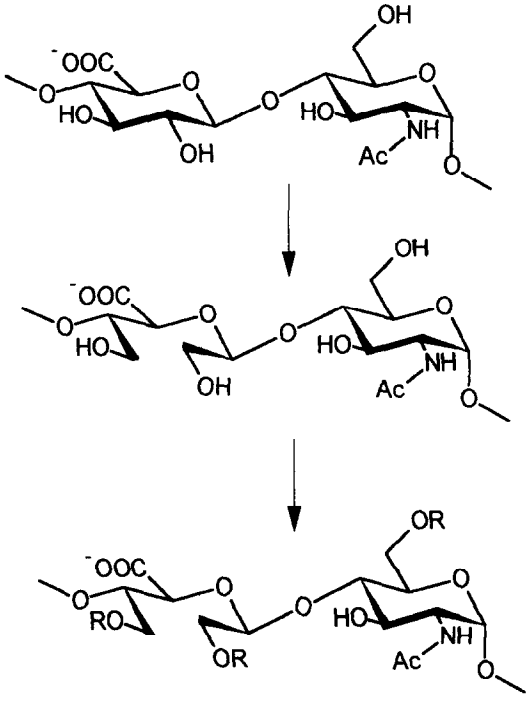
[a]Conductimetric titration.
[b]Carbazole/borate.
[c]100% degradation was arbitrary assigned to heparin.
n.d.: non determined.

glucosamine residues. Similar results were reported for O-desulphated heparins (Ogamo *et al.*, 1989). Therefore, sulphation of **2** and **5** is likely to occur not only at the C-6 of glucosamine residues, but also at the primary centers originated by periodate oxidation–borohydride reduction.

O-Sulphation of tributylammonium salts of **2** and **5** with sulphur trioxide–triethylamine complex in DMF gave **3** and **6**, respectively. Sulphate content was found to be 24·9% for **3**, and 24·3% for **6** (Table 2). After the sulphation step, product **3** was analysed by agarose gel electrophoresis. Compound **3** showed a slightly increased mobility due to its higher anionic charge. Band broadening, which would appear as a consequence of either chain depolymerization or inhomogeneous charge distribution, was not observed.

The sulphated products **3** and **6** were also analyzed by ^{13}C-NMR spectroscopy. A drastic diminution in the intensity of the signals at 61–62 ppm was observed, together with a higher intensity of resonances between 67 and 68 ppm, corresponding to O-sulphation at primary hydroxyl groups (Scheme 1).

On the other hand, chemical modifications were followed by APTT assay in order to visualise the possible effects on the biological activity. Periodate oxidation between C-2 and C-3 of uronic acid residues (mainly GlcA) did not affect the starting anticoagulant potency, measured by the APTT assay, which appeared to be relatively independent on the integrity of all uronic acid units (or at least GlcA units) of the GAG chain. After O-sulphation, about a three-fold increase in the anti-



Scheme 1.

coagulant activity was observed for **3**. The sulphation of compound **5** also produces an important increase from 152 to 217 units/mg (Table 2). Our previous studies on HS indicated that only a moderate enhancement of the anticoagulant potency (1–1·5-fold) can be achieved by direct O-sulphation of HS. Moreover, natural high sulphated heparan sulphates from rat liver tissues exhibit low values in APTT assays (Kovensky *et al.*, 1990). Therefore, the strong increase observed by sulphation of oxi HS could be a consequence of the introduction of sulphate groups in sensitive positions, i.e. at the open-chain residues (O-2 and/or O-3 of original GlcA units) in addition to the enhanced flexibility of the polysaccharide chain that results from the replacement of a rigid GlcA unit by an open-chain fragment in several points of the macromolecule.

Recently (Nader *et al.*, 1989–1990) it was reported that periodate-oxidized heparins were also good substrates for heparinase, an specific enzyme for the major disaccharide in heparin. We assayed the susceptibility of heparin, and starting and modified heparan sulphates to heparinase depolymerization. While **HS I** and **HS II** were not significantly degraded, depolymerization was observed for the oxi/red product of **HS II** (compound **5**). In **HS I** the low values make it difficult to obtain definite conclusions. The depolymerization of compound **5** may be rationalized in terms of a higher flexibility after splitting of one fifth of the initial uronic acid units. On the other hand, the sulphated product **3** was actually depolymerized to the extent of 58% of that obtained for heparin, while for **6** this value was near 80%. These results would suggest that the O-sulphated open-chain residues could adopt a proper conformation to allow substrate–enzyme recognition. Such a three-dimensional arrangement would be a consequence of both O-sulphation and the extra flexibility obtained upon splitting of the C2—C3 bond. This finding becomes more meaningful taking into account that periodate oxidation had only affected a limited proportion of total uronic acid residues of the starting GAG chain.

The above results make this chemical modification useful for modifying the biological activity of heparan sulphates, very abundant in animal tissues, although more specific biological tests are required, moreover since similar results were obtained starting from heparan sulphates of different structural characteristics. From a structural point of view, these modified heparan sulphates may serve as model compounds to study the conformational aspects of GAG–protein interactions.

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