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Note

Modifications under basic conditions of the minor sequences of heparin containing 2,3 or 2,3,6 sulfated D-glucosamine residues

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Abstract

The ^{1}H NMR chemical shift assignments are reported for 2,3-di- or 2,3,6-tri-sulfated glucosamine in a sample of high affinity (for antithrombin III) heparin in three distinct environments. Following treatment in strong basic conditions the disappearance of these signals and the appearance of new signals consistent with the formation of *N*-sulfo-2,3-aziridine containing aminosugar residues is demonstrated. © 1997 Elsevier Science Ltd.

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1. Introduction

Heparin is a polysaccharide which has found widespread use as an anticoagulant and antithrombotic agent and consists predominantly of alternating 1,4 linked α-L-iduronic or β-D-glucuronic acid and α-D-glucosamine residues. Most of the iduronic acid residues are sulfated in the 2-position, most of the glucosamine residues carry *N*- and 6-*O*-sulfation while small proportions bear *N*-acetyl groups at C-2. In addition, there are a number of residues containing 3-*O*-sulfated D-glucosamine, most notably 2,3,6-tri-sulfated D-glucosamine in the pentasaccharide sequence (A–G–A*–I–A) which is responsible for binding to antithrombin [1] but which is also dispersed in the polysaccharide. In heparin or heparan sulfate samples, these 2,3,6-tri-sulfated residues have been found between non-sulfated iduronic acid and 2-*O*-sulfated iduronic acid or between glucuronic acid and 2-*O*-sulfated iduronic acid. The 2,3-di-sulfated glucosamine unit has also been reported between two iduronic acid 2-*O*-sulfated residues [2].

Many studies have been undertaken to try to correlate a number of biological activities with the pattern of substitution within heparin employing degradative techniques and/or NMR [2–4]. A number have also concerned the formation of epoxide groups in 2-*O*-sulfated iduronic acid units in heparin or heparan sulfate following treatments in strong basic conditions and their subsequent opening to form either iduronic or galacturonic acid residues [5–7]. In some

Abbreviations: A, I, and G refer to aminosugar, iduronic acid and glucuronic acid, respectively; A-*N* refers to the *N*th hydrogen or carbon atom of the aminosugar residue and I-*N* to the *N*th hydrogen or carbon atom of the iduronic acid residue; More specifically, A*-*N* refers to 2,3 or 2,3,6 sulfated aminosugar residues and A^{+*}-*N* refers to the same residues after aziridine formation has been induced

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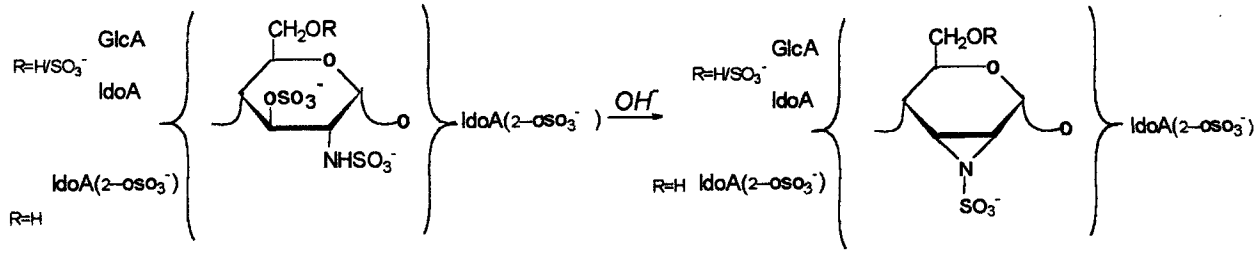

Scheme 1.

of these, attempts were made to correlate various activities with structural changes which, in the absence of detailed analyses of the minor sequences of the polymers, were assumed to consist of modifications to the iduronic acid residues.

Following our recent observations of the formation of *N*-sulfated aziridine groups in a sample of oversulfated heparin which resulted from treatment in strongly basic conditions we investigated the possibility of a similar reaction occurring during the treatment of intact heparin in similar conditions which are often employed as a method of selectively de-sulfating I-2 [5–7]. The formation of aziridine groups proceeds via internal nucleophilic attack of the sulfamino nitrogen at C-2 of the 2,3,6-tri-sulfated glucosamine units upon C-3 resulting in the loss of the sulfate group at C-3 and the inversion of stereochemistry at C-3 [8]. We chose for these experiments a sample of high affinity heparin (for antithrombin III) which had previously been purified by affinity chromatography and in which there was evidence by ^{1}H NMR of relatively high levels of 2,3-di- or 2,3,6-tri-

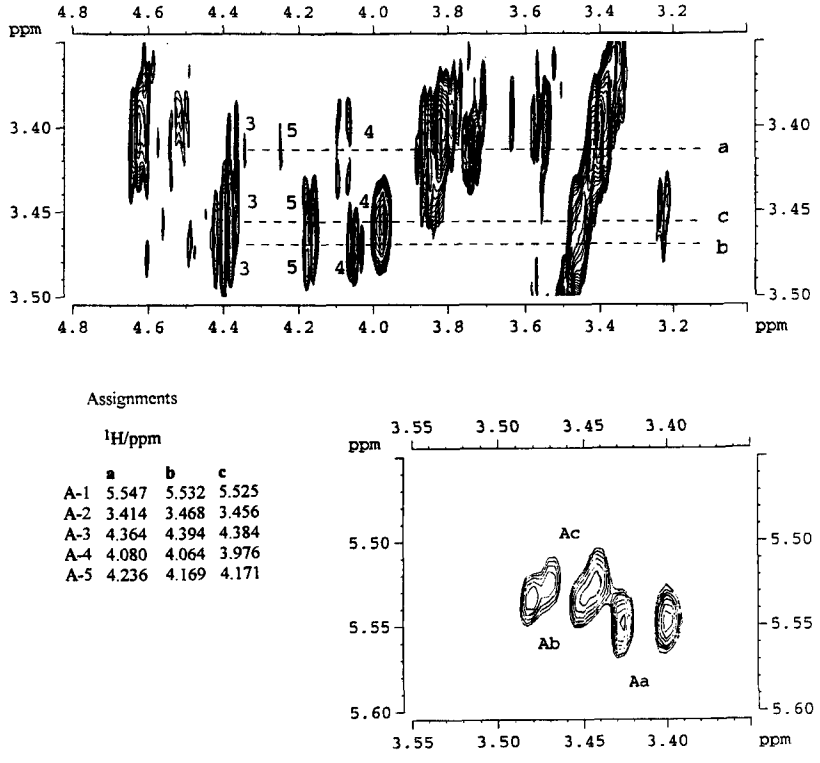

Fig. 1. Detail of the 500-MHz ^{1}H NMR spectra of high affinity heparin showing (above) correlations in the TOCSY spectrum for A*-3 to A*-5 and (below) the COSY spectrum showing correlations between A*-1 and A*-2 for 2,3-di- or 2,3,6-tri-sulfated glucosamine residues in three different environments. Assignments for A*-1 to A*-5 are given in the table (inset).

sulfated glucosamine residues. Here, we present evidence for the conversion of glucosamine 2,3-di- or 2,3,6-tri-sulfate residues in this sample into the *N*-sulfo-2,3-aziridine derivative by a treatment in basic conditions [8] (see Scheme 1).

2. Results and discussion

Evidence for the formation of the *N*-sulfo-aziridine group is based on a detailed ^{1}H and ^{13}C NMR analysis of the intact high affinity heparin both before and after de-sulfation at I-2 and also of an unfractionated heparin derivative which was subjected to the same reaction conditions [5].

The signals arising from tri-sulfated glucosamine residues in three distinct magnetic environments in a sample of high affinity heparin have been assigned by ^{1}H COSY and TOCSY spectra and are shown in Fig. 1. At least one of these, labelled Ac in Fig. 1 presumably arises from the A–G–A*–I–A sequence and is not observed in a comparable spectrum of low affinity heparin (results not shown). Other possible sequences include I_{2OH}–A*–I_{2S} or G–A*–I_{2S} outside the ATIII binding pentasaccharide sequence or in the case of 2,3-di-sulfated glucosamine, I_{2OH}–A*–I_{2S}, I_{2S}–A*–I_{2S}, or G–A*–I_{2S} [2].

Details of the ^{1}H COSY and HMQC spectra of a sample of high affinity heparin which has been subjected to I-2 de-*O*-sulfation under strong basic conditions, together with the ^{1}H and ^{13}C assignments for the aminosugar signals arising from aziridine formation are shown in Fig. 2. The typical ^{13}C NMR spectrum of a sample of I-2 de-*O*-sulfated heparin (from unfractionated starting heparin) showing the signals arising from the aziridine containing aminosugar residue is shown in Fig. 3.

A sample of over-sulfated heparin in which aziridine formation has been induced [8] was subjected to deaminative degradation in nitrous acid but no degradation of the aziridine function was apparent while the small quantities of intact glucosamine present in the material prior to treatment were no longer detectable by ^{1}H or ^{13}C NMR (results not shown).

Perlin et al. [9] reported attempts to study the effects of basic treatment on a model monosaccharide, methyl 2-deoxy-2-sulfamino-3-*O*-sulfo-α-D-glucopyranoside, having considered the possibility of the modification of tri-sulfated glucosamine residues in heparin. They concluded that no change was detectable in the monosaccharide but did not study the polymer in detail and reported only major sequence assignments. The reason for this failure to form the aziridine group remains unclear, but may be explained by the slightly weaker conditions used by Perlin (pH 11.8 and 12.5) or a degree of conformational constraint may be necessary for the reaction to proceed which is present in the case of the polymer but not in the case of the monosaccharide. Linhardt et al. [4] reported the results of an enzymatic degradation of intact heparin with heparin lyase I (E.C. 4.2.2.7.) and the subsequent characterisation of the oligosaccharide fragments so obtained. In this case, they found evidence of 2,3-di- or 2,3,6-tri-sulfated

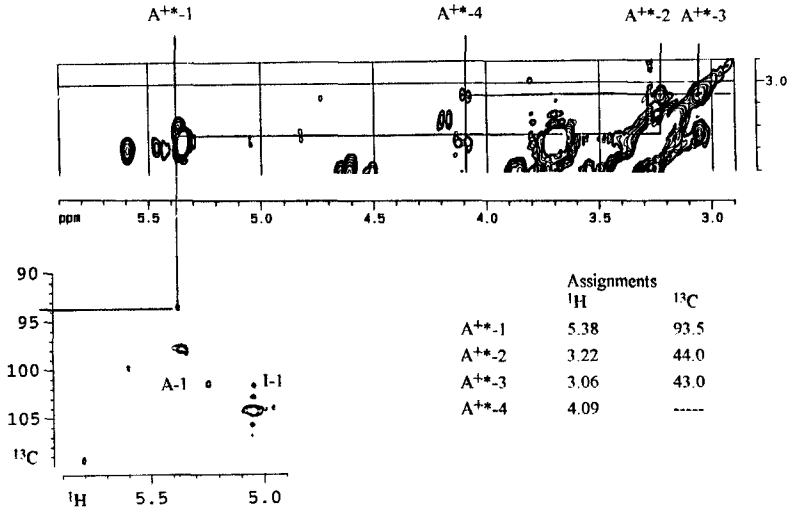

Fig. 2. (Upper) Detail of the 500-MHz ^{1}H COSY NMR spectrum of high affinity heparin in which aziridine formation and I-2 de-*O*-sulfation have been induced by treatment with base. (Lower) Detail of the 500-MHz HMQC spectrum showing ^{1}H–^{13}C correlation for A^{+*}-1 to A^{+*}-4. Assignments are given in the table.

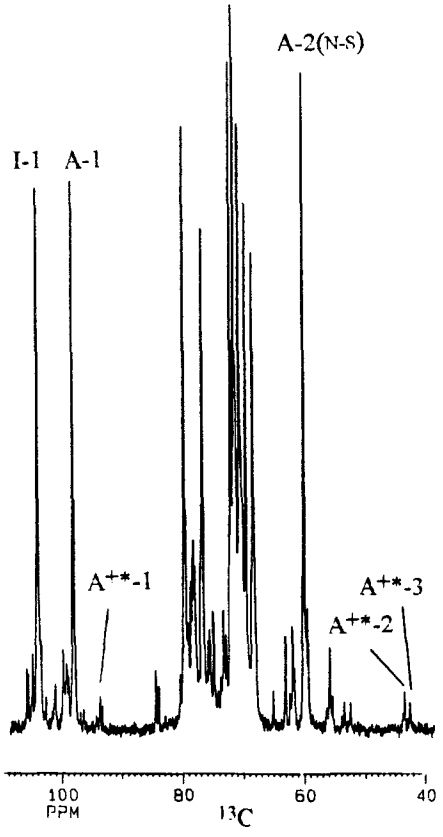

Fig. 3. ^{13}C NMR spectrum at 100 MHz of a sample of heparin in which aziridine formation and I-2 de-*O*-sulfation has been induced showing A^{+*}-1 to A^{+*}-3 signals.

glucosamine in the products. In a later work [7], the same group reported the results of a comparable study on heparin after it had been subjected to alkaline treatment to form a number of modified uronic acid residues. In this case, the degradation (employing the same enzyme) proceeded to about 21% completion but no evidence was found for the presence of 2,3,6-tri-sulfated glucosamine residues in any of the product fractions studied.

In a more recent study, Holme et al. [10] reported attempts to systematically study these reactions in basic conditions and their effects on the APTT, anti-Xa and anti-IIa activities of heparin. Their investigation also included analyses of the disaccharide products formed by deaminative cleavage. The polymers which were subjected to strong basic conditions showed a lower quantity of the degradation product arising from glucuronic acid linked to glucosamine 2,3,6-tri-sulfate.

The observations of Holme et al. [10] and Linhardt et al. [7] may be explained by the formation of an aziridine containing aminosugar residue under basic conditions. The aziridine group has proved to be resistant to deaminative degradation in nitrous acid conditions and would not therefore be expected to appear in the disaccharide degradation products obtained by Holme et al. but to remain in the oligosaccharide fractions in a similar way to sequences containing *N*-acetylated glucosamine residues. In the work of Linhardt et al., the fact that no 2,3,6-tri-sulfated glucosamine residues were found in the enzymatic degradation products may be explained by the formation of aziridine in these aminosugar residues which are then resistant to digestion by heparin lyase I. It has not proved possible to test this hypothesis because we have found that in samples of heparin treated with base, the formation of epoxide accompanies that of aziridine groups. A direct comparison between the susceptibility to enzymatic degradation of intact heparin and heparin which includes sequences of aziridine groups and unmodified iduronic acid 2-*O*-sulfate could not therefore be made.

Samples of heparin in which I-2 de-*O*-sulfation and aziridine formation had been induced were practically devoid of activity (20 U/mg compared with 200 U/mg for intact heparin) in an anti-factor Xa assay. This loss of activity should not however be solely attributed to the removal of sulfate groups in iduronic acid but may also be related to modifications in the 2,3,6-tri-sulfated glucosamine residues.

The formation of the aziridine group should be considered in any analyses of the structure–activity relationships of heparin samples which have been treated in basic conditions, in particular, those involving de *O*-sulfation reactions employing lyophilisation or heating in sodium hydroxide or other strong bases.

3. Experimental

Formation of I-2 de-O-sulfated and N-sulfated aziridine containing residues.—Typically, a sample of heparin (200 mg) was dissolved in 0.1 M NaOH (20 mL), frozen and lyophilised essentially as described [5,8]. After lyophilisation was complete, the sample was dissolved in a small vol of distilled water and neutralised (dil HCl), purified and prepared for NMR analysis as described below.

Deaminative degradation of aziridine containing over-sulfated heparin with nitrous acid.—Deamination was carried out by a slight modification of the published method [11]. A sample of aziridine containing over-sulfated heparin (300 mg) [8] was treated with a soln of nitrous acid (0.43 M, 10 mL) maintaining pH2 (by addition of 1.0 M HCl) for 90 mins at 20

°C. After neutralisation (dil NaOH), the products were purified and analysed by NMR as described below.

Purification and preparation of compounds for NMR experiments.—After the above reactions had been completed, the polysaccharide components were precipitated with a large vol of EtOH satd with NaOAc and left to stand overnight at 4 °C. The precipitates were retrieved by filtration, washed with cold EtOH, the filtrate dissolved in a small vol of distilled water and dialysed (6–8 kDa cut-off) against distilled water. After dialysis, the soln was concd to a small vol by rotary evaporation and subjected to cation-exchange on a column of Dowex 50 W (H^+ form) followed by careful neutralisation (dil NaOH) and then purified by gel chromatography on a Sephadex G-25 column (125 × 1.25 cm) eluting with an EtOH–degassed distilled water mixture (1:10 v/v). Fractions were collected monitoring for the presence of polysaccharide material or salts by their absorbance at 215 nm. The samples containing polysaccharide materials were then again subjected to cation-exchange on a column of Dowex 50W resin (H^+ form), carefully neutralised (dil NaOH) and dried by rotary evaporation, exchanged three times with D_2O and finally dissolved in 0.5 mL of D_2O in a 5-mm tube (or in 2.0 mL in a 10-mm tube for the monodimensional ^{13}C spectra) and used for NMR analyses. In all cases, the pD of the solns were carefully adjusted to 8.0 (dil NaOD). Typically, 15–20 mg of purified polysaccharide was employed in the COSY, TOCSY, and HMQC experiments and 100–150 mg was used for the mono-dimensional ^{13}C spectra. In all NMR experiments, high quality D_2O containing low paramagnetic impurities was employed.

Anti-factor Xa assay.—Tests for anti-factor Xa activity were performed using a chromogenic end-point assay according to the method described [12] employing an accucolour heparin kit (Sigma).

NMR experimental procedure.—The ^1H (COSY, TOCSY, and HMQC) spectra were obtained at 500 MHz with a Bruker AMX 500 spectrometer equipped with a 5-mm $^1H/X$ inverse probe and the ^{13}C spectra were obtained at 100 MHz using a Bruker AMX 400 instrument equipped with a 10-mm broad-band probe. The chemical shift values were recorded downfield from external trimethylsilyl propionate (TSP) as standard at 40 °C. Mono-dimensional ^{13}C spectra were recorded using the standard Bruker sequence with powergated proton decoupling utilising signal enhancement by the nuclear Overhauser effect. Proton spectra were assigned with the use of double quantum filtered COSY spectra with gradient enhancement [13] and were recorded employing a Bruker BGU (unit-z) with a maximum strength of 50 $G \cdot cm^{-1}$. These gradient strengths were determined empirically to be the maximum required to completely eliminate the HDO signal in the sample. Data were acquired using 8 scans per series in 1K × 512W data points with zero filling in F1 and a sine squared function was applied before Fourier transformation.

TOCSY spectra.—^1H TOCSY spectra were obtained using the gradient-enhanced version of HOHAHA [14] employing MLEV-17 as the spin-lock pulse. Data were collected as 1K × 512 matrices with 16 scans per evolution time (t_1) increment. Spectral width was 2000 Hz in both $\omega 1$ and $\omega 2$ and 60 mS of spin-lock was applied. A 1 mS sine-bell gradient pair with a maximum amplitude of 5 and -5 $G \cdot cm^{-1}$ were used.

HMQC spectra.—^1H–^{13}C chemical shift correlations were obtained with ^1H detection via gradient enhanced heteronuclear multiple-quantum coherence spectra [15] which employed a square wave with a maximum amplitude of 20, 5, -20, and 5 $G \cdot cm^{-1}$. The first value was optimised to obtain a good presaturation of the HDO signal in the sample. 16 Scans were collected for each series in 1K × 256W data points before processing and were zero filled to 2K × 512W by application of a shifted sine squared function prior to Fourier transformation.

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