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Further evidence that periodate cleavage of heparin occurs primarily through the antithrombin binding site

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Dedicated to Professor Derek Horton on the occasion of his 70th birthday to celebrate his important contributions to the field of Carbohydrate Chemistry

Abstract

Porcine mucosal heparin was fragmented into low-molecular-weight (LMW) heparin by treatment of periodate-oxidized heparin with sodium hydroxide, followed by reduction with sodium borohydride and acid hydrolysis. Gradient polyacrylamide gel electrophoresis analysis showed a mixture of heparin fragments with an average size of eight disaccharide units. 1D ^{1}H NMR showed two-thirds of the *N*-acetyl groups were lost on periodate cleavage, suggesting cleavage had occurred at the glucopyranosyluronic acid (Glc*p*A) and idopyranosyluronic acid (Ido*p*A) residues located within and adjacent to the antithrombin III (ATIII) binding site. The *N*-acetyl glucopyranose (Glc*p*NAc) residue was lost on workup. The Glc*p*A residue, within the ATIII binding site, is on the non-reducing side of the *N*-sulfo, 3, 6-*O*-sulfo glycopyranosylamine (Glc*p*NS3S6S) residue. Thus, periodate cleaved heparin should be enriched in Glc*p*NS3S6S residues. Two-dimensional correlation spectroscopy (2D COSY) confirmed that LMW heparin prepared through periodate cleavage contained Glc*p*NS3S6S at its non-reducing end. As expected, this LMW heparin also showed reduced ATIII mediated anti-factor IIa and anti-factor Xa activities.

1. Introduction

Heparin is a glycosaminoglycan composed of *O*-sulfo and *N*-acetyl or *N*-sulfo (1→4)-linked glucosamine and uronic acid residues. While heparin exhibits a wide variety of biological activities, its most clinically important activity is as an anticoagulant. Excessive anticoagulation, however, can lead to hemorrhage and occasionally death. A reduction in molecular weight can reduce heparin's anticoagulant activity while maintaining its antithrombotic activity, and provides a rationale for the use of LMW heparins.[1,2] Such LMW heparins also can be used in applications where anticoagulation is undesirable.[3] LMW heparins generally have molecular weights ranging from 2000 to 8000 with an average molecular weight of ~6000.[2] LMW heparins are claimed to have reduced hemorrhagic side effects (a higher safety/efficacy ratio) in addition to more predictable pharmacological action, sustained antithrombotic activity and improved bioavailability.[1,2]

Commercial glycosaminoglycan (GAG) heparin is polydisperse with an average molecular weight of 12 000 and contains <15 wt% chains having MW ≤ 5000.[4] The content in low molecular weight chains in commercial heparin can be enriched by solvent precipitation or by gel-permeation chromatography.[5] However, because of the small percentage of LMW chains present in commercial heparin, the yields are low and the resolution of these fractionation methods is poor. Thus, commercial LMW heparins are prepared by enzymatic or chemical depolymerization of heparin.[6] Hep-

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arin can be depolymerized enzymatically under very mild conditions using polysaccharide lyases. Commercially available heparin lyase I (heparinase) is capable of acting on linkages within heparin in an endolytic fashion through a β-eliminative cleavage mechanism.[7] This enzymatic reaction can be chemically mimicked by esterification of the carboxyl group of the uronic acid residue and treating the resulting heparin ester with base.[8,9] Both of these eliminative depolymerizations are used to prepare commercial LMW heparins.[6]

Oxidative depolymerization of heparin has also been used commercially to prepare LMW heparins. The glucosamine residue in heparin is susceptible to nitrosation forming an unstable *N*-nitroso derivative that on rearrangement can lose nitrogen and form a carbocation at the C-2 position. Nucleophilic attack by the ring oxygen on this center results in ring contraction and cleavage of the adjacent glycosidic linkage.[9–11] Heparin can be oxidatively broken down using hydrogen peroxide, giving rise to hydroxyl radicals that react with sugar residues and degrade them to 1-, 2- and 3-carbon fragments, resulting in LMW heparins.[9,12]

A final oxidative process that relies on Smith degradation,[13] and periodate oxidation, followed by borohydride reduction and mild acid hydrolysis, has been less thoroughly investigated. The unsulfated glucuronic acid and iduronic acid residues in heparin have vicinal diol functionality and thus, are susceptible to periodate oxidation.[9,13] The goal of the current study is to establish the precise location of periodate cleavage within the heparin polymer.

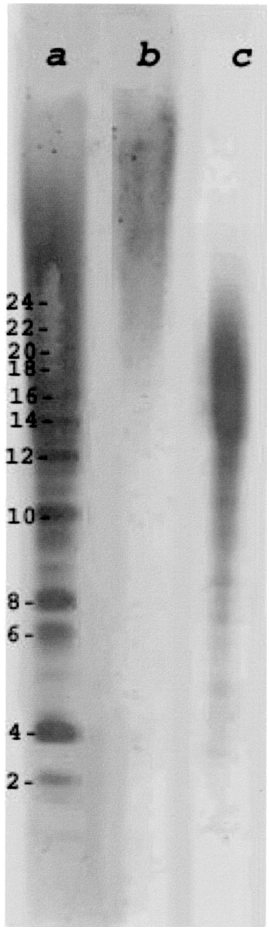

Fig. 1. Gradient polyacrylamide gel electrophoresis analysis of heparin and periodate fragmented heparin. Lane **a** contains a standard mixture of heparin oligosaccharide prepared from bovine lung heparin using heparin lyase. The degree of polymerization of selected bands within this mixture (determined based on standards) are shown to the right of this lane. For conditions used in these analyses, see the Experimental section. Lane **b** corresponds to porcine intestinal heparin starting material. Lane **c** shows the heparin treated with sodium periodate, sodium hydroxide, sodium borohydride and hydrochloric acid.[17]

2. Results and discussion

The structure of the pharmaceutical heparin, sodium salt, starting material was first confirmed by ^{1}H NMR spectroscopy (not shown). Porcine mucosal heparin was fragmented into a low molecular weight heparin by treatment with sodium periodate, sodium hydroxide, sodium borohydride and hydrochloric acid. Periodate oxidatively cleaves the C-2–C-3 bonds of the unsulfated glucuronic and iduronic acid residues, affording a ring-opened residue containing aldehyde groups at C-2 and C-3. This residue is quite labile to base, and thus treatment with sodium hydroxide results in partial fragmentation of the heparin chain. Reduction of terminal residues of the fragments, as well as the residual uncleaved polysaccharide, was then accomplished by treatment with sodium borohydride. Upon reduction, it is quite labile to acid, and thus glycosidic bonds were hydrolyzed with hydrochloric acid. The molecular weight of the LMW heparin was next examined by polyacrylamide gel electrophoresis (PAGE) as shown in Fig. 1. The parent porcine mucosal heparin had an average molecular weight $\sim 12\,000$, while the resulting heparin fragments had an average degree of polymerization (dp) of 16, corresponding to a low molecular weight heparin of average molecular weight ~ 5000. The 1D ^{1}H NMR of this LMW heparin was next determined (Fig. 2). Integration of the *N*-acetyl peak (not shown) with respect to Ido*p*A2S H-1 was 0.73 for the heparin starting material and 0.26 for the heparin product, suggesting that two-thirds of *N*-acetyl groups were lost on periodate cleavage. Porcine intestinal mucosal heparin has chains with high affinity for ATIII that contain an antithrombin III (ATIII) binding site[14] and chains with low ATIII affinity that contain an identical site (ATIII binding site precursor[15]), which lack the critical 3-*O*-sulfo group. Most of the periodate-sensitive residues (Glc*p*A and Ido*p*A) in porcine intestinal mucosal heparin reside in the ATIII binding site

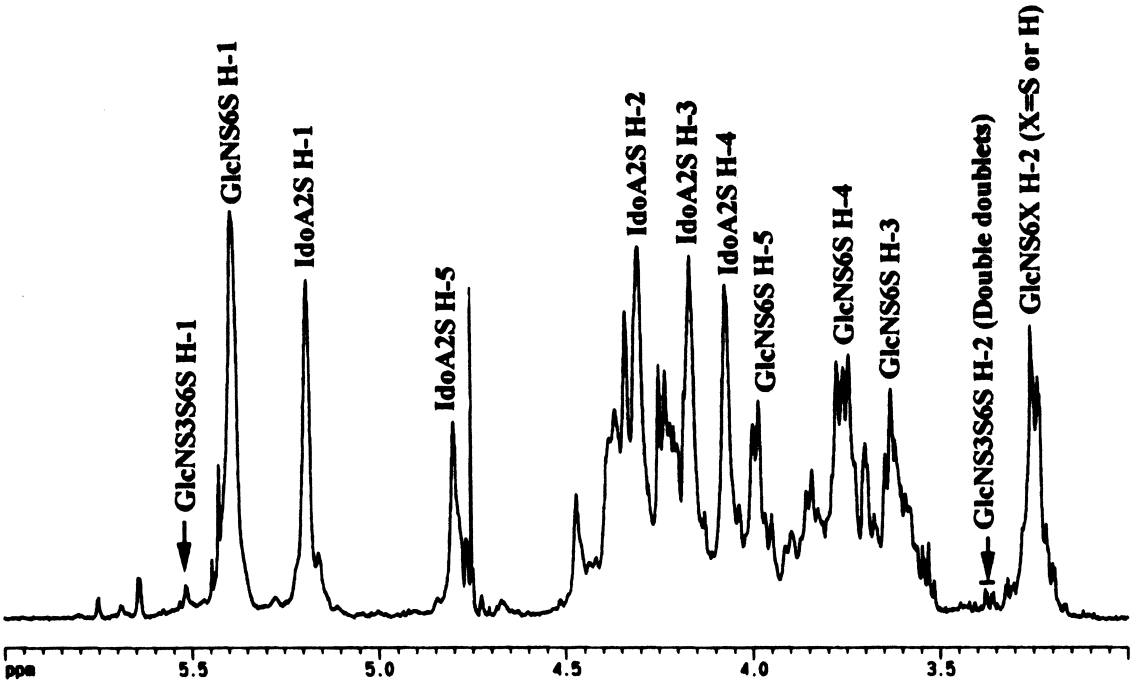

Fig. 2. One-dimensional 600 MHz ^{1}H NMR spectra for the LMW heparin after periodate oxidation.

and the ATIII binding site precursor. Approximately 50% of the *N*-acetyl groups are localized within the ATIII pentasaccharide binding site.[15] Thus, it appeared that periodate cleavage was both selective to these sites and released the *N*-acetyl-containing residues into a fragment that was lost on workup. Based on the known structure of the ATIII binding site, cleavage at a Glc*p*A residue should expose the Glc*p*NS3S6S residue from the ATIII binding site (and Glc*p*NS6S from the ATIII binding site precursor) at the non-reducing end (NRE) of the products. To confirm the positioning of the cleavage site within the ATIII binding site, the 1D ^{1}H NMR spectrum (Fig. 2) of the LMW heparin obtained through periodate cleavage was assigned using 2D COSY (Fig. 3). Assignment of the anomeric proton shows no Glc*p*A or Ido*p*A residues are present by the absence of signals at 3.38 and 3.69 ppm, respectively. These signals are clearly observed in the spectrum of the heparin starting material (not shown). This demonstrates that in addition to a 66% decrease in GlcN*p*Ac, a complete loss of vicinal diol containing Glc*p*A and Ido*p*A residues result from periodate treatment (Fig. 4). Next, we turned our attention to the Glc*p*NS3S6S residue found in the center of the intact heparin ATIII binding site. This residue is difficult to observe in heparin because it occurs only once in every three chains of average molecular weight $\sim$12 000, corresponding to $\sim$1 Glc*p*N residue in 50. In spectrum of the synthetic ATIII-binding pentasaccharide, however, this residue is easily assigned. The synthetic pentasaccharide, containing a single internal Glc*p*NS3S6S residue, showed a shift of 3.96 ppm for its H-4 signal. This compares well with the chemical shift of 4.02 ppm of the same, albeit small peak, observed in the ^{1}H NMR spectrum of heparin (not shown). In contrast, the LMW heparin prepared by periodate oxidation

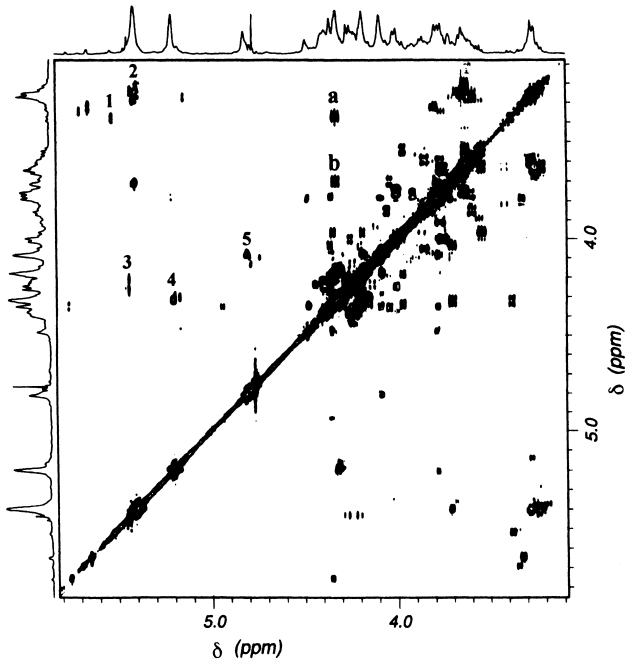

Fig. 3. 2D-relayed COSY spectrum. The major COSY cross-peaks are: a, Glc*p*NS3S6S (H-2/H-3); b, Glc*p*NS3S6S (H-3/H-4); 1, Glc*p*NS3S6S (H-1/H-2); 2, Glc*p*NS6X (H-1/H-2); 3, Glc*p*NAc6X (H-1/H-2); 4, Ido*p*A2S (H-1/H-2); and 5, Ido*p*A2S (H-4/H-5). The numbers are placed on top of the cross-peaks.

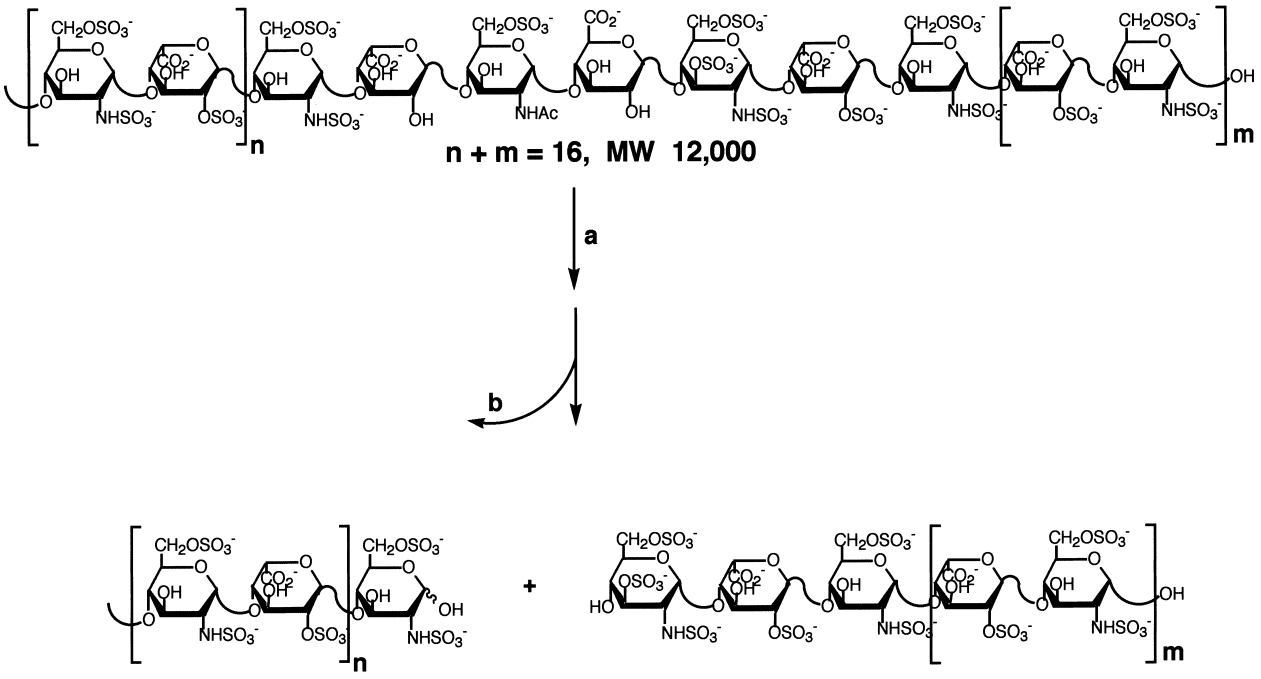

Fig. 4. Preparation of a LMW heparin derivative. a. Periodate oxidation and fragmentation of heparin; b. removal of the Glc*p*NAc-containing dialyzate.

shows a signal corresponding to the Glc*p*NS3S6S H-4 at 3.71 ppm (Figs. 2 and 4). The characteristic shifts for the non-reducing end Glc*p*NS3S6S residue in this LMW heparin are virtually identical to those reported in the literature (Table 1).[16] Typically a proton adjacent to a glycosidic bond is shifted 0.2 to 0.28 ppm downfield, suggesting that the LMW heparin prepared by periodate oxidation contains a Glc*p*NS3S6S residue only at its non-reducing end. No internal Glc*p*NS3S6S is seen in this sample consistent with the absence of an intact ATIII binding site. As expected, and in contrast to heparin, this LMW heparin demonstrated no measurable anticoagulant activity as determined by ATIII-mediated anti-Xa and anti-IIa amidolytic assays[17,18].

In conclusion, the location of Glc*p*A and Ido*p*A residues in the ATIII pentasaccharide binding site can be definitely established by periodate cleavage. Furthermore, this method represents one of the best approaches for preparing LMW heparins having negligible anticoagulant activity for alternate applications, such as the regulation of complement activation, atherogenesis, smooth muscle proliferation, anti-infective and growth factor binding activities.[3,19,20]

3. Experimental

General methods.—Normal human plasma (NHP) was collected from healthy volunteers. Anti-factor Xa activity was determined using a Coatest LMW heparin/heparin kit (Chromogenix, Mölndal, Sweden). ^2H_2O (99.9%), sodium periodate was from Aldrich Chemical Co. (Milwaukee, WI). Spectra/Por dialysis tubing [1,000-3,500 molecular weight cut-off (MWCO)] was from Spectrum (Houston, TX). Fourier-transform ^1H NMR analysis spectra were recorded on Bruker DRX-400 and 600 MHz instruments and Me_4Si or the residual solvent signal was used as internal standards.

Periodate-oxidized heparin fragments.—Heparin was fragmented by periodate oxidation based on a modification of an earlier procedure.[17] Briefly, heparin, sodium salt (20 g, 1.43 mmol) was dissolved in 175 mL of distilled water. The pH was adjusted to 5.0 using 1 N HCl. $NaIO_4$ (15 g, 0.070 mol), dissolved in 500 mL water, was added in a single portion with stirring. The pH was readjusted to 5.0 using 1 N HCl and left for 24 h at 4 °C in the dark. The solution was dialyzed against 4 volumes of water (with one change of water) for 15 h at 4 °C.

To the approximately 1.5 L of solution obtained after dialysis, 32 mL of 10 N NaOH was added. The solution

Table 1
Chemical shifts of a non-reducing terminal Glc*p*NS3S6S residue

Proton	Observed δ (ppm)	Reported δ (ppm)[16]
H-1	5.52	5.53
H-2	3.38	3.41
H-3	4.31	4.34
H-4	3.71	3.72

was stirred at room temperature for 3 h. To prevent the development of colored products, this step was done in the dark.

$NaBH_4$ (1 g, 0.026 mol) was added in one portion, and the approximately 1.5 L of solution was stirred for 4 h. The pH was then adjusted to 4.0 using 37% HCl, and the solution was stirred for an additional 15 min. The solution was neutralized to pH 7.0 using 1 N NaOH and NaCl (32.8 g, 0.56 mol) followed by the addition of 2.54 L ethanol. The solution was left for 3 h without stirring, and the precipitate was recovered by centrifugation ($22\,000 \times g$) for 20 min. The precipitate, recovered by decantation, was suspended in 400 mL absolute ethanol. The solution was filtered using a Buchner funnel, and the recovered solids were left to dry for 5 h under vacuum affording 14.2 g of product.

The product was dissolved in 190 mL of water. NaCl (2.8 g, 0.05 mol) was added, and the pH was adjusted to 3.5 using 1 N HCl. The volume was adjusted to 280 mL using water. Absolute ethanol (240 mL) was added with stirring. The solution was stirred 15 min and then left without stirring for 10 h at room temperature. After decanting, the precipitate was recovered and dissolved in water. The ethanol was removed by rotary evaporation under reduced pressure, and the residue was freeze-dried affording 10 g of LMW heparin.

NMR sample preparation.—For ^1H NMR spectroscopy, approximately 10 mg of each sample was exchanged by lyophilization three times from 0.5-mL portions of 99.9% ^2H_2O before being redissolved in ^2H_2O for NMR analysis. Chemical shifts are reported relative to Me_4Si at 0.00 ppm.

Gradient PAGE analysis.—Gradient polyacrylamide gel electrophoresis (PAGE) was performed on a 32-cm vertical slab gel unit PROTEAN II equipped with Model 1000 power source from Bio-Rad (Richmond, CA). Polyacrylamide linear gradient resolving gels (14×28 cm), 12–22%) total acrylamide) were prepared and run as previously described.[4] Molecular sizes of the oligosaccharide samples were determined by comparing with a banding ladder of heparin oligosaccharide standards prepared from bovine lung heparin.[4] Oligosaccharides were visualized by Alcian blue staining.

Anti-factor Xa and anti-factor IIa activities.—Briefly, LMW heparin and heparin standard were diluted in normal human plasma. Chromogenic Xa substrate S-2732 (Suc-Ile-Glu(γ-Piperidyl)-Gly-Arg-pNA) 2.9 mM in 50 mM Tris, 7.5 μM EDTA, pH 8.4 buffer (200 μL), was added to 25 μL of plasma containing sample and 200 μL of bovine Factor Xa (1.25/mL). After mixing, the reaction was incubated for 8 min at 37 °C, and 200 μL of 20% aqueous acetic acid was added. Residual Factor Xa was then determined by measuring absorbance at 405 nm. Anti-factor IIa activity was determined by incubating 50 mL of LMW heparin in NHP diluted 4-fold with water with 50 mL of human thrombin (12 NIH units/mL) at 37 °C for 30 s. Then, 50 mL (2.5 mmol/mL) of Chromogenic TH (ethylmalonyl-Pro-Arg-*p*-nitroanilide hydrochloride) was added, and the amidolytic thrombin activity was measured at 405 nm. Measurements were performed on an ACL 300 plus from Instrumentation (Lexington, MA) and calculated in comparison with USP Heparin Reference Standard (K-3) supplied by U.S. Pharmacopeial Convention (Rockville, MD).[21]

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