A Novel Sulfated Structure in the Carbohydrate-Protein Linkage Region Isolated from Porcine Intestinal Heparin*

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A preparation of porcine stage 14 intestinal heparin, which contains Ser as a predominant amino acid, was used for isolation of the carbohydrate-protein linkage region of heparin. Two glycoserines were isolated in a molar ratio of 96:4 after an exhaustive digestion with a mixture of bacterial heparinase and heparitinases. Their structures were determined by composition analysis, heparitinase digestion, co-chromatography with an authentic glycoserine on high performance liquid chromatography, and by 500-MHz one- and two-dimensional 1H NMR spectroscopy. The structure of the major one is AglcAβ1-3Galβ1-3Galβ1-4Xylβ1-O-Ser and that of the minor is ΔGlcAβ1-4GlcNAc(6-O-sulfate)α1-4GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-O-Ser. The novel 6-O-sulfated GlcNAc residue was demonstrated to occur in the vicinity of the carbohydrate-protein linkage region. The Gal residues were nonsulfated, in contrast to the sulfated Gal structures recently discovered in the carbohydrate-protein linkage region of chondroitin sulfate proteoglycans. The structural features are discussed in relation to biosynthetic mechanisms of the heparin glycosaminoglycans.

Heparin exerts a variety of biological and biochemical activities such as inhibition of blood coagulation (1), modulation of cellular proliferation (2, 3), potentiation of angiogenesis (4), and interactions with acidic and basic fibroblast growth factors (5–7). It has been established that the anticoagulant activity depends on specific binding of heparin to antithrombin and that the antithrombin-binding site on heparin is a pentasaccharide with specific modifications by sulfation (8).

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However, for other activities the relation with the fine structure of the glycosaminoglycan remains to be established.

The order of the modification reactions to form the antithrombin-binding site is well understood (9). There are two populations of heparin chains, namely one which carries and the other which does not carry the antithrombin-binding site. The biosynthetic selection mechanisms for these chains are unknown. Heparin is synthesized as a proteoglycan to which a number of heparin glycosaminoglycan chains are attached. Interestingly, heparin glycosaminoglycans share the common carbohydrate-protein linkage region GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-O-Ser with other sulfated glycosaminoglycans: heparan sulfate, chondroitin sulfate, and dermatan sulfate (10–12). The question arises how different glycosaminoglycans can be synthesized on the common structure, especially since the chain elongation proceeds stepwise and is governed largely by the substrate specificity of the glycosyltransferases involved.

Recently, we discovered the novel structures GlcAβ1-3Gal(4-O-sulfate)β1-3Galβ1-4Xyl (13, 14) and GlcAβ1-3Gal(6-O-sulfate)β1-3Galβ1-4Xyl (15) in the linkage region of chondroitin 4- and 6-sulfate, respectively. This prompted us to reinvestigate the linkage region of heparin. In the present study we isolated a hitherto unreported sulfated glycoserine from the linkage region using purified heparinase and heparitinases. Part of this work has been presented (16).

EXPERIMENTAL PROCEDURES AND RESULTS7

Isolation of the Linkage Glycoserines—Purified stage 14 heparin from porcine intestine contains only Ser as the predominant amino acid (10). This preparation was exhaustively digested with a mixture of heparinase, heparitinases I and II, and fractionated into fractions I-IV by gel filtration (Fig. 1). The recoveries of Ser in these fractions were 5.9, 9.8, 6.6, and 77.7%, respectively. Fraction I contained dermatan sulfate.

1 The abbreviations used are: Xyl, xylose; UA, uronic acid; ΔUA, 4,5-ununsaturated uronic acid; GalN, galactosamine; GalNAc, 2-deoxy-2-N-acetylamino-D-galactose; ΔDiHS-6S, Δ4,5GlcAβ1-4GlcNAc(6-O-sulfate); ΔDiHS-NS, Δ4,5GlcAβ1-4GlcNAc(6-O-sulfate); ΔDiHS-NS, Δ4,5GlcAβ1-4GlcNβ2-6S-O-disulfate; ΔDiHS-NS, Δ4,5GlcAβ1-4GlcNβ2-6S-O-disulfate; ΔDiHS-tris, Δ4,5GlcAβ1-4GlcNβ2-6S-O-disulfate; HPLC, high performance liquid chromatography. 2 Portions of this paper (including "Experimental Procedures," part of "Results," Figs. 1 and 2, and Tables I–III) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
whereas fraction II contained heparin fragments and dermatan sulfate as judged by cellulose acetate membrane electrophoresis (data not shown). Dermatan sulfate accounts for GaIN in the heparin preparation and represents approximately 5% (w/w) of the total polysaccharides. It likely accounts for most of Ser in fractions I and II, which were not analyzed further. Linkage regions were isolated from fractions III and IV by HPLC.

Glycoserine I—Subfractionation of fraction IV by HPLC yielded glycoserine I with the following conventional structure. The isolation procedure and the structural analysis are described in the "Miniprint."

\[
\Delta \alpha \text{GlcA} \beta 1-3 \text{Gal} \beta 1-3 \text{Gal} \beta 1-4 \text{Xyl} \beta 1-\text{O-Ser}
\]

Glycoserine II—Subfractionation of fraction III by HPLC yielded a number of UV-absorbing peaks (Fig. 3). Approximately 60% of the applied Ser was recovered in the fastest eluting compound, designated glycoserine II, which represent 2.0% of the applied DU. No appreciable amounts of Ser were recovered in the other peaks, most of which are unsaturated tetrasaccharides as judged from the ratio of uronic acid or GlcN to DU. In addition, fraction III was radiolabeled with [\(^3\)H]acetic anhydride and chromatographed by HPLC under the same conditions. It gave one appreciable radiolabeled peak that eluted 2 min later than the original glycoserine II (data not shown), being consistent with acetylation of its Ser residue. These results suggest the presence of only one major glycoserine in fraction III. Preparative HPLC yielded 125 nmol (as Ser) of glycoserine II/100 mg of purified heparin.

Glycoserine II contains 1 mol each of DU, UA, GlcN, and sulfate (Table II). The susceptibility of the isolated glycoserine II to heparinase and various heparitinases was tested in order to dissect it into the subcomponents. The enzymes used have different substrate specificities (18): heparinase produces DDiHS-diS and DDiHS-triS from heparin; heparitinases I and V have similar specificities producing DDiHS-0S, DDiHS-6S, and DDiHS-NS from bovine kidney heparan sulfate; heparitinase II has a broad specificity giving rise to every unsaturated disaccharide listed in Table I from heparin. The results showed that glycoserine II was resistant to heparinase, heparitinases I and II, but susceptible to heparitinase V as judged by the 2-fold increase in UV absorption at 232 nm. Upon HPLC the digest gave rise to an equimolar amount of two components, one was eluting at the position of DDiHS-6S and the other at that of glycoserine I (Fig. 4). This indicates that glycoserine II is composed of DGlca\beta 1-4DGlca\beta 1-6-0-sulfate and GlcA\beta 1-3Gal\beta 1-3Gal\beta 1-4Xyl\beta 1-\text{O-Ser}.

The \(^1\)H NMR spectrum of glycoserine II is depicted in Fig. 5, and the NMR data are given in Table III. The occurrence of an \(\alpha\)GlcNAc residue in the heparin derived linkage glycopeptide instead of \(\beta\)GlcNAc in the reference compound derived from chondroitin sulfate (fraction D-3 in Ref. 13) is reflected by the presence of an \(\alpha\)-anomeric signal at \(\delta \) 5.406. Compared with the spectrum of the reference compound, signals are observed at \(\delta \) 4.446 and \(\delta \) 4.170 which have shifted out of the bulk region under influence of a sulfate group (26).

In the two-dimensional homonuclear Hartmann-Hahn (21) spectrum (Fig. 6) the indicated assignment pathway, starting at the \(\alpha\)GlcNAc H-1, leads to the H-2, H-3, H-4, and H-5 signals of \(\alpha\)GlcNAc. The cross-peaks between the H-5 and the H-6 and H-6' atoms demonstrates that the signals shifted under influence of sulfate can be assigned to the H-6 and H-6' of \(\alpha\)GlcNAc, bearing an O-sulfate at C-6. Full assignment of Xyl and \(\Delta\)GlcA and a partial assignment of the Ser, Gal-2, and Gal-3 residues is possible in the two-dimensional HOHAHA spectrum (Fig. 5, Table III). The structure of glycoserine II is as follows.

\[
\Delta \alpha \text{GlcA} \beta 1-4 \text{GlcN} \alpha 1- \text{4GlcA} \beta 1-3 \text{Gal} \beta 1-3 \text{Gal} \beta 1-4 \text{Xyl} \beta 1- \text{O-Ser}
\]

The differences in the chemical shifts of the Ser and the Xyl H-1 and H-2 protons are probably due to pH variations. Similar differences are found in work on synthetic linkages (compare Refs. 27 and 28).

**DISCUSSION**

In the present study the isolation and characterization of two glycoserines (I and II) from the linkage region of porcine intestinal heparin is described. Glycoserines I and II are derived from two populations that differ in structure in the vicinity of the linkage region. Interestingly, two types of heparin chains occur, namely those which carry and those
FIG. 5. Structural reporter group regions of the resolution-enhanced 500 MHz $^1$H NMR spectra of glycoserine II recorded in $^2$H$_2$O at 15 °C. The numbers and letters in the spectra refer to the corresponding residues in the structures.

which do not carry the antithrombin-binding region. This gives rise to the challenging question whether 6-O-sulfation of GlcNAc is a marker which corresponds to the presence or absence of the antithrombin high affinity heparin. The proportion of the antithrombin high affinity heparin in the stage 14 preparations is approximately 48% as shown by affinity chromatography.3 Thus there appears to be no direct correlation between 6-O-sulfation of the first GlcNAc residue and segregation of the low and high affinity binding sequences. It remains to be investigated whether the lack of phosphate is an authentic feature or an artifact resulting from the isolation procedure.

One of the structural features unique to the linkage region of heparin and heparan sulfate is that the glucosamine units adjacent to the linkage region are regularly N-acetylated (33). It has been suggested that glucosamine units may become 6-O-sulfated only when they are located next to an N-sulfated disaccharide unit (9). If so, the second glucosamine unit which had been bound to glycoserine II would have been N-sulfated. Based on the susceptibility to nitrous acid treatment, the amino group of the second glucosamine unit which had been bound to glycoserine II would have been N-sulfated. In contrast, the second glucosamine unit which had been bound to glycoserine I would not have been N-sulfated (32). Alternatively, a specific GlcNAc 6-O-sulfotransferase may exist which catalyzes 6-O-sulfation of only the innermost GlcNAc next to the linkage region.

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Linkage Region of Porcine Intestinal Heparin

Supplemental Materials:
A Novel Sulfated Structure in the Carbohydrate-Protein Linkage Region Isolated from Porcine Intestinal Heparin

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EXPERIMENTAL PROCEDURES

Materials - Materials were obtained from the following sources: Stage 14 heparin (Hs salt) from American Diagnosica Inc., New York; heparinase I (EC 4.2.2.7) and heparinase II (EC 4.2.2.8) from Nipponkan Co., Tokyo; an standard unsubstituted Gal-3 H-l, and both L8 H-l and H-4 of AGlcA were purchased as previously reported (17). Heparinase II was purified from Flavobacterium sp. (18).

HPLC - Chromatography yielded unsaturated disaccharides which were chromatographed on a column (3 0 145 cm) of Carbohydrate G-200, 9 ml, and eluted at 0.5 ml/min with a mixture of 0.1 M sodium acetate buffer (pH 7.0) and acetate, pH 7.0, containing 2 mM Ca(OAc)2 at 37 °C. Absorbs were measured at 10-30 min intervals for measurement of UV-absorption at 232 nm.

RESULTS

Purified stage 14 heparin was exhaustively digested by a mixture of heparinase I, heparinase I I, and 11 and subjected to gel filtration on a Carbohydrate G-200-m column (3 0 145 cm) of Carbohydrate G-200, 9 ml, and eluted at 0.5 ml/min with a mixture of 0.1 M sodium acetate buffer (pH 7.0) and acetate, pH 7.0, containing 2 mM Ca(OAc)2 at 37 °C. Absorbs were measured at 10-30 min intervals for measurement of UV-absorption at 232 nm.

OTHER ANALYTICAL METHODS

Uronic acid was determined by the carbazole method (22). Enzymic and non-enzymic carboxylic compounds produced by intestinal microflora were spectrophotometrically quantified based on an average molar extinction coefficient of 0.5 at 232 nm (39) caused by the A,6 sites of the uronic acid at the nonreducing end. Analysis of amino sugars and amino acids was carried out on an amino acid analyzer (24). Analysis of sulfate and phosphate was performed after pyridyls by ion chromatography (25).

RESULTS

Purified stage 14 heparin from porcine intestine was exhaustively digested with a mixture of heparinase I, heparinase II, and heparinase III, and fractionated into Fr. I - IV by gel filtration (Fig. 1). Subfractionation of Fr. IV yielded in addition to the peaks which corresponded to authentic unsubstituted disaccharides an extra UV-absorbing peak marked by an arrow (Fig. 2, Table I). The double peaks observed for 3,6DHS GS and 3,6DHS G4 are due to 0, and 0.5 anomers. The compound in the extra peak contained approximately 1 mol of Ser per mol of AGlcA, and it is referred to as Glysaccharide I, representing 50% and 3% of the applied Ser and Gal, respectively. Preparative chromatography yielded 3.8 g of purified 3,6DHS G4 as a single compound. The compound was subjected to 1H-NMR spectroscopy and the obtained data are summarized in Table III. The structure of the representative Glysaccharide II is shown in Fig. 3. The structure of the representative Glysaccharide III is shown in Fig. 4.
**Table I.** Molar ratios of the glycosamines, unsaturated disaccharides and unsaturated trisaccharides produced by heparinase/heparanase digestion. The results obtained from Figs. 2 and 3 are summarized. The calculation is based on AUA at the nonreducing ends.

| Components         | Table I | Glycosamine I | Glycosamine II |
|--------------------|---------|---------------|----------------|
| AUA                |         | 1.00          | 1.00           |
| ADH-OS             | 0.84    | 0.89          |
| ADH-OS             | 1.30    | 0.69          |
| ADH-NS             | 0.79    | 1.87          |
| ADH-NS             | 3.40    | 0.83          |
| ADH-NS             | 2.97    | 2.07          |
| ADH-NS             | 20.44   |               |

**Table II.** Chemical composition of the isolated linkage glycosamines.

| Components | Glycosamine I | Glycosamine II |
|------------|---------------|----------------|
| Ser        | 1.00          | 1.00           |
| AUA        | 0.84          | 0.89           |
| UA*        | 0.69          | 1.87           |
| GlcNAc    | 0.05          | 0.83           |
| Sulfate    | 0.06          | 2.07           |
| Phosphates | >0.01         | nd**           |

*including AUA
**not detected

**Table III.** 1H chemical shifts of structural-reporter group of the constituent linkage disaccharides of Table I. Values of H-4 of 4-keto-3-deoxy-2-sulfamido-2-deoxy-L-galactose-1-sulfate but were actually measured indirectly to sucrose in D$_2$O at 23OC.

| Residue | Reporter groups | Chemical shift |
|---------|-----------------|----------------|
| Ser     | H-6             | 3.90           |
| H-8     | nd              | 3.90           |
| H-9     | nd              | 3.94           |
| H-12    | 4.04            | 4.14           |
| Xyl-1   | H-1             | 4.03           |
| H-2     | 3.96            | 3.99           |
| H-3     | 3.94            | 3.96           |
| H-4     | 3.96            | 4.06           |
| H-5     | 3.96            | 4.06           |
| H-5 eq  | 3.96            | 4.06           |
| Gal-2   | H-1             | 4.20           |
| H-2     | 4.21            | 4.18           |
| H-3     | 4.20            | 4.18           |
| H-4     | 4.21            | 4.18           |
| H-5     | 4.20            | 4.18           |
| Gal-3   | H-1             | 4.20           |
| H-2     | 4.20            | 4.18           |
| H-3     | 4.20            | 4.18           |
| H-4     | 4.20            | 4.18           |
| H-5     | 4.20            | 4.18           |
| Glc-4   | H-1             | 4.20           |
| H-2     | 4.20            | 4.18           |
| H-3     | 4.20            | 4.18           |
| H-4     | 4.20            | 4.18           |
| H-5     | 4.20            | 4.18           |
| GlcNAc-5| H-1             | 4.20           |
| H-2     | 4.20            | 4.18           |
| H-3     | 4.20            | 4.18           |
| H-4     | 4.20            | 4.18           |
| H-5     | 4.20            | 4.18           |
| GlcA-5  | H-1             | 4.20           |
| H-2     | 4.20            | 4.18           |
| H-3     | 4.20            | 4.18           |
| H-4     | 4.20            | 4.18           |
| H-5     | 4.20            | 4.18           |
| n.d.    | not determined  |

n.d.: not determined

* not occurring

**not Glycosamine I values of GlcA-4 are presented**