Structure of a Dermatan Sulfate Hexasaccharide That Binds to Heparin Cofactor II with High Affinity*

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Dermatan sulfate increases the rate of inhibition of thrombin by heparin cofactor II (HCII) ~1000-fold by providing a catalytic template to which both the inhibitor and the protease bind. Dermatan sulfate is a linear polymer of D-glucuronic acid (GlcA) or L-iduronic acid (IdoA) alternating with N-acetyl-D-galactosamine (GalNAc) residues. Heterogeneity in dermatan sulfate results from varying degrees of O-sulfation and from the presence of the two types of uronic acid residues. To characterize the HCII-binding site in dermatan sulfate, we isolated the smallest fragment of dermatan sulfate that bound to HCII with high affinity. Dermatan sulfate was partially N-deacetylated by hydrazinolysis, cleaved with nitrous acid at pH 4, and reduced with [3H]NaBH4. The resulting fragments, containing an even number of monosaccharide units with the reducing terminal GalNAc converted to [3H]2,5-anhydro-d-talitol (ATalH), were size-fractionated and then chromatographed on an HCII-Sepharose column. The smallest HCII-binding fragments were hexasaccharides, of which ~6% bound. Based on ion-exchange chromatography, the bound material appeared to comprise a heterogeneous mixture of molecules possessing four, five, or six sulfate groups per hexasaccharide. Subsequently, hexasaccharides with the highest affinity for HCII were isolated by overloading the HCII-Sepharose column. The high-affinity hexasaccharides were fractionated by strong anion-exchange chromatography, and one major peak representing ~2% of the starting hexasaccharide was isolated. The high-affinity hexasaccharide was cleaved to disaccharides that were analyzed by anion-exchange chromatography, paper electrophoresis, and paper chromatography. A single disulfated disaccharide, IdoA(2-SO4)GalNAc(4-SO4) was observed, indicating that the hexasaccharide has the following structure: IdoA(2-SO4)GalNAc(4-SO4) → IdoA(2-SO4)GalNAc(4-SO4) → IdoA(2-SO4)GalNAc(4-SO4) → ATalH(4-SO4). Since IdoA(2-SO4)GalNAc(4-SO4) comprises only ~5% of the disaccharides present in intact dermatan sulfate, clustering of these disaccharides must occur during biosynthesis to form the high-affinity binding site for HCII.

Glycosaminoglycans present on the surface of cells or in the extracellular matrix interact with heparin cofactor II (HCII) and antithrombin III (ATIII) to inhibit proteolytic enzymes involved in blood coagulation (1, 2). HCII and ATIII are homologous members of the “serpin” family of serine protease inhibitors that circulate in plasma at micromolar concentrations. The serpins act as suicide substrates to inhibit their target proteases. For example, thrombin attacks the Leu444Ser445 peptide bond near the C-terminal end of HCII (3), and a stable 1:1 complex is formed in which the protease is inactive. Dermatan sulfate and heparin increase the rate of formation of the thrombin-HCII complex ~1000-fold (4). The stimulatory effect appears to involve the formation of a ternary complex in which both the inhibitor and the protease bind via ionic interactions to a single glycosaminoglycan chain (5). Thrombin is the only protease known to be inhibited rapidly by HCII in the presence of glycosaminoglycans (6). In contrast, ATIII inhibits all of the proteases of the intrinsic coagulation pathway (particularly factors IXa, Xa, and thrombin) (7). Inhibition of these proteases by ATIII is stimulated by heparin but not by dermatan sulfate (4).

Dermatan sulfate proteoglycans synthesized by fibroblasts activate HCII and may regulate thrombin activity in the local environment of these cells (8). Synthesis of dermatan sulfate occurs by addition of alternating D-glucuronic acid (GlcA) and N-acetyl-D-galactosamine (GalNAc) residues to a core protein structure (9). Many of the GlcA residues become epimerized at C-5 to yield L-iduronic acid (IdoA). Subsequently, O-sulfation may occur at the C-4 or C-6 positions of GalNAc or at the C-2 position of IdoA. Because the epimerization and sulfation reactions are incomplete, the structure of dermatan sulfate is heterogeneous. Griffith and Marbet (10) reported that a subpopulation of dermatan sulfate molecules bound when applied to a column of immobilized HCII. The bound molecules were eluted with 0.25–0.50 M NaCl and were 4.0–5.5 times more active than the unfractionated material in stimulating thrombin inhibition by HCII. Subsequently, we prepared fragments of dermatan sulfate by partial chemical depolymerization and found that the smallest fragments that stimulated thrombin inhibition by IICII were dodecasaccharides (11). When applied to a column of immobilized HCII, 27% of the dodecasaccharides bound. The specificity of the bound dodecasaccharides was 4.3 times higher than the unfractionated material, whereas the unbound dodecasaccharides were essentially inactive. Thus, current evidence suggests that binding of dermatan sulfate to HCII is

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1 The abbreviations used are: HCII, heparin cofactor II; ATIII, antithrombin III; serpin, serine protease inhibitor; GlcA, D-glucuronic acid; GalNAc, N-acetyl-D-galactosamine; IdoA, L-iduronic acid; GalN, D-galactosamine; ATalH, 2,5-anhydro-D-talitol; Chromozym TH, tosyl-Gly-Pro-Arg-p-nitroanilide; HPLC, high-performance liquid chromatography.
Dermatan sulfate was partially N-deacetylated with hydrazine/hydrazine sulfate and then reacted with nitrous acid, yielding ATal in place of each N-deacetylated residue. ATal, the hydrazone of iduronic acid, R-\text{NHNH}_2, is shown. IdoA, the hydrazide of iduronic acid, contains a positive reaction in the carbazole assay resulting in a sharp peak at elution position 0.

required for inhibition of thrombin and that dermatan sulfate molecules vary in their ability to bind to HCII. In the present study, we have determined the structure in porcine skin dermatan sulfate to which HCII binds with highest affinity.

EXPERIMENTAL PROCEDURES

RESULTS

Depolymerization of Dermatan Sulfate—Oligosaccharides of various chain lengths were prepared by the hydrazinolysis/deamination reaction sequence outlined in Fig. 1 (15, 16). Dermatan sulfate was partially N-deacetylated with hydrazine/hydrazine sulfate and then reacted with nitrous acid, which quantitatively cleaves the glycosidic linkages following each N-deacetylated residue, yielding ATal in place of each GalN residue, and to convert the hydrazides back to the original uronic acids (15). The ATal residues were then reduced with [\text{H}]NaBH₄ to label each fragment at its reducing terminus. The most common repeating disaccharide unit in porcine skin dermatan sulfate, IdoA-GalNAc(4-SO₄), is shown. IdoA₆, the hydrazide of iduronic acid, contains an even number of monosaccharide units with an intact uronic acid at the nonreducing terminus and 2,5-anhydro-p-talose (ATal) at the reducing terminus in place of the N-galactosamine residue. The oligosaccharide mixture was then reduced with NaBH₄ to convert the ATal to 2,5-anhydro-d-talitol (ATal₆). In most preparations, [\text{H}]NaBH₄ was used to uniformly label the reducing termini of the oligosaccharides. The advantages of this method of depolymerization compared with enzymatic or other chemical methods are that cleavage is random (16), epimerization of the uronic acid residues does not occur (15), and the O-sulfate substituents are not affected (20).

The oligosaccharides were fractionated on a P-10 gel filtration column, and the concentration of dermatan sulfate in each fraction was determined by the carbazole assay for uronic acid (Fig. 2, open circles). The elution positions are plotted in relation to blue dextran and [\text{C}]glucose, which were included in each sample to insure proper alignment of the peaks from different runs. The numbers above each peak in the figure indicate the chain lengths of the oligosaccharides based on the elution position of [\text{H}]disaccharides produced by complete depolymerization of dermatan sulfate (Fig. 2, closed circles). The small [\text{H}]-labeled peak that eluted after the major disaccharide peak in this sample contained no uronic acid, and paper chromatography showed that it consisted mostly of ATal₃(4-SO₄) (data not shown).

Binding of Oligosaccharides to HCII-Sepharose—[\text{H}]Labeled dermatan sulfate oligosaccharides were prepared as outlined in Fig. 1 and chromatographed on the P-10 column. The peaks of radioactivity corresponded exactly to the peaks detected by the carbazole assay (data not shown). The center fractions of each [\text{H}]oligosaccharide peak were pooled and deacetylated in preparation for HCII binding assays. A sample (0.2 nmol) of each size-fractionated [\text{H}]oligosaccharide was applied to an HCII-Sepharose column in buffer containing 0.05 M NaCl and eluted with a linear salt gradient. The bound oligosaccharides eluted between 0.1 and 0.6 M NaCl. The percentage of each sample bound to the HCII column is shown in Table I. The smallest fragments that bound were hexasaccharides. As the chain length increased from 6 to 16 monosaccharide units, the percentage bound increased from 6 to 34%, whereas 82% of intact dermatan sulfate bound to the

FIG. 1. Partial deaminative cleavage of dermatan sulfate. Dermatan sulfate was treated with anhydrous hydrazine containing 10 mg/ml hydrazine sulfate for 1 h at 100 °C, resulting in partial N-deacetylation of GalNAc residues and conversion of some of the uronic acid residues to hydrazides (15). The molecules were then treated with nitrous acid at pH 4 to cleave to glycosidic linkages following each N-deacetylated residue, yielding ATal in place of each GalN residue, and to convert the hydrazides back to the original uronic acids (15). The ATal residues were then reduced with [\text{H}]NaBH₄ to label each fragment at its reducing terminus. The most common repeating disaccharide unit in porcine skin dermatan sulfate, IdoA-GalNAc(4-SO₄), is shown. IdoA₆, the hydrazide of iduronic acid, contains an even number of monosaccharide units with an intact uronic acid at the nonreducing terminus and 2,5-anhydro-p-talose (ATal) at the reducing terminus in place of the N-galactosamine residue.

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FIG. 2. Size fractionation of dermatan sulfate oligosaccharides. Dermatan sulfate was treated with hydrazine/hydrazine sulfate for 1 h, cleaved with nitrous acid, and reduced with NaBH₄. The resulting oligosaccharides were mixed with blue dextran and [\text{C}]glucose standards and fractionated on a 1.5 × 198-cm P-10 column in aqueous 10% ethanol containing 1 M NaCl. The dermatan sulfate concentration of each fraction was determined by the carbazole assay (C). Alternatively, dermatan sulfate was treated with hydrazine/hydrazine sulfate for 10 h, cleaved with nitrous acid, and reduced with [\text{H}]NaBH₄. The resulting [\text{H}]disaccharides were fractionated on the P-10 column as above and an aliquot of each fraction was counted for [\text{H}] (○). The data are plotted as elution volumes relative to the two internal standards. The number of monosaccharide units per oligosaccharide is indicated above each peak. Blue dextran produces a positive reaction in the carbazole assay resulting in a sharp peak at elution position 0.
column. Thus, most of the intact dermatan sulfate chains contained at least one HCII-binding site. When the unbound hexasaccharides and octasaccharides were reapplied to the column in 0.05 M NaCl, >90% of each fraction flowed through (data not shown), indicating that the column had quantitatively absorbed all of the molecules capable of binding to HCII.

Isolation of the High-affinity Hexasaccharide—Analysis of the bound hexasaccharides on a Partisil 10 SAX column showed a heterogeneous mixture of molecules that appeared to contain four, five, or six sulfate groups per hexasaccharide (data not shown), whereas the unbound molecules appeared to contain only three sulfate groups per hexasaccharide (see below). To isolate the hexasaccharides with the highest affinity for HCII, we deliberately overloaded the HCII-Sepharose column with [3H]hexasaccharides. To prepare the starting material for this experiment, dermatan sulfate was partially depolymerized by the hydrazinolysis/deamination procedure, and the oligosaccharides were size-fractionated on the P-10 column prior to reduction. Depolymerization of 48 mg of dermatan sulfate resulted in 4–6 mg of oligosaccharides in each of the major peaks. The center fractions of the hexasaccharide peak were pooled (~5 mg), reduced with [3H]NaBH₄, and re-chromatographed on the same column to remove 3H-labeled contaminants introduced during the reduction as well as any tetrasaccharides or octasaccharides that may have been pooled along with the major hexasaccharide peak. The center fractions of the re-chromatographed hexasaccharide peak were pooled, desalted, and concentrated. A portion of the 3H-labeled hexasaccharides (1.7 mg = ~1.3 μmol) was applied to the HCII-Sepharose column, which contained ~0.5 μmol of HCII, and eluted with a NaCl gradient as shown in Fig. 3 (panel A). About 4% of the [3H]hexasaccharides bound to the column and eluted as a single broad peak centered at 0.25 M NaCl (Bound-1). When Bound-1 was reapplied to the column, 95% of the material bound (data not shown); the material that did not bind the second time was presumed to represent the tail of Flow Through-1 that had not reached baseline before the start of the gradient. About 2% of Flow Through-1 bound when reapplied to the column (Fig. 3, panel B), confirming that the column had been overloaded initially.

Anion-exchange Chromatography of Hexasaccharides—The Flow Through-1, Bound-1, and Bound-2 [3H]hexasaccharides pooled from the HCII-Sepharose column, as well as unfractonated [3H]hexasaccharides, were chromatographed on a Partisil 10 SAX ion-exchange column as shown in Fig. 4. Most of the unfractonated [3H]hexasaccharides (panel A) eluted as a group of peaks at 0.40 M KH₂PO₄, whereas the remainder eluted in three distinct groups at 0.50, 0.65, and 0.75 M KH₂PO₄. Because ~90% of the disaccharides in porcine skin dermatan sulfate are monosulfated (15, 16), the major group of [3H]hexasaccharides in the unfractonated material (group 3 in panel A) should contain three sulfate groups per hexasaccharide. Each group of peaks eluting at a higher KH₂PO₄ concentration was assumed to contain one more sulfate per hexasaccharide than the preceding group and has been labeled accordingly in Fig. 4. As described below, we have confirmed that the major peak in the group 6 position contains three disulfated disaccharides.

Flow Through-1 (Fig. 4, panel B) had an elution pattern very similar to the unfractonated sample except for a reduction in the group 6 peak. In agreement with this finding, Bound-1 (panel C) eluted as one major peak at the group 6 position (containing 78% of the radioactivity) and several smaller peaks at the group 5 position. Thus, the HCII-Sepharose column preferentially bound one major [3H]hexasaccharide. Bound-2 (panel D) eluted as several large peaks with 64% of the [3H]hexasaccharides in group 5 and 36% in group 6. When the group 5 and 6 peaks isolated by Partisil 10 SAX chromatography were applied separately to the HCII-Sepharose column, the group 5 [3H]hexasaccharides eluted between 0.21 and 0.27 M NaCl (data not shown), whereas the group 6 [3H]hexasaccharides eluted as a narrow peak centered at 0.30 M NaCl (see Fig. 10). These results suggest that the group 6 [3H]hexasaccharide binds to HCII with a higher affinity than any of the other hexasaccharides. Removal of most of the high-affinity (group 6) [3H]hexasaccharide from Flow Through-1 allowed [3H]hexasaccharides with lower affinity to bind to the HCII-Sepharose column (panel D). Further experiments indicated that essentially all of the [3H]hexasaccharides in groups 4, 5, and 6 bound to the HCII-Sepharose column in 0.05 M NaCl, whereas none of the group 3 [3H]hexasaccharides bound (data not shown).

To confirm the chain length of the high-affinity [3H]hexasaccharide, the group 6 peak was isolated by Partisil 10 SAX chromatography of the Bound-1 pool as shown in Fig. 4 (panel

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**Table I**

Binding of dermatan sulfate [3H]oligosaccharides to HCII Sepharose

Dermatan sulfate [3H]oligosaccharides (100,000 cpm) 2-16 monosaccharide units in length or 0.5 mg of intact dermatan sulfate were applied to a 1 X 5.4-cm column of HCII-Sepharose equilibrated in 50 mM NaCl, 50 mM Tris-HCl, pH 7.4. The column was washed in the same buffer, and the bound material was eluted with a linear NaCl gradient. A 500-μl aliquot of each fraction was counted for 3H or subjected to the carbazole assay (in the case of intact dermatan sulfate) to determine the percentage bound.

<table>
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<tr>
<th>Monosaccharide units</th>
<th>% bound</th>
</tr>
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<tbody>
<tr>
<td>2</td>
<td>≤0.03</td>
</tr>
<tr>
<td>4</td>
<td>≤0.03</td>
</tr>
<tr>
<td>6</td>
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<tr>
<td>8</td>
<td>8.4</td>
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<td>10</td>
<td>10.9</td>
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<tr>
<td>12</td>
<td>16.1</td>
</tr>
<tr>
<td>14</td>
<td>25.4</td>
</tr>
<tr>
<td>16</td>
<td>33.8</td>
</tr>
<tr>
<td>Intact</td>
<td>81.7</td>
</tr>
</tbody>
</table>

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**Fig. 3.** HCII affinity chromatography of [3H]hexasaccharides. In panel A [3H]hexasaccharides (1.7 mg) were applied to a 1 x 5.4-cm column of HCII-Sepharose equilibrated in 50 mM NaCl, 50 mM Tris-HCl, pH 7.4. The column was washed in the same buffer, and the bound hexasaccharides (Bound-1) were eluted with a linear NaCl gradient as shown (-- -- --). A 2-μl sample of each fraction (2 ml) was counted for 3H (○). The flow-through material (Flow Through-1) was pooled, desalted, concentrated, and reapplied to the same column (panel B). The bound hexasaccharides (Bound-2) were eluted as in panel A. The percentage of radioactivity found in each peak is indicated in the figure.
Dermatan Sulfate Hexasaccharide

FIG. 4. Ion-exchange chromatography of [3H]hexasaccharides. Unfractionated hexasaccharides (panel A) and hexasaccharides prepared by HiII-Sepharose chromatography as in Fig. 3 (Flow Through-1, panel B; Bound-1, panel C; and Bound-2, panel D) were chromatographed on a Partisil 10 SAX HPLC column as described under “Experimental Procedures.” The hexasaccharides were eluted with a linear KH2PO4 gradient as shown (---). 3H radioactivity (-) was measured with a flow detector. The peaks eluted in four groups as indicated at the top of the figure. The number above each group of peaks represents the apparent degree of sulfation of the hexasaccharides contained in those peaks.

Structure of the High-affinity Hexasaccharide—To determine the composition of the high-affinity [3H]hexasaccharide, the molecule was cleaved to disaccharides by exhaustive hydrazinolysis/deamination as described under “Experimental Procedures.” The products were divided into two equal portions for reduction. One sample was reduced with unlabeled NaBH4 so that only the disaccharide present at the reducing terminus of the original [3H]hexasaccharide would be labeled. The other sample was reduced with [3H]NaBH4 so that the internal disaccharides would be labeled in addition to the reducing terminal disaccharide. Following reduction, the samples were desalted, concentrated, and chromatographed on a Micropak AX-5 ion-exchange column eluted by isocratic steps of KH2PO4. Fig. 6 (panel A) shows the elution positions of a mixture of [3H]disaccharide standards derived from porcine skin dermatan sulfate. The NaBH4-reduced (panel B) and [3H]NaBH4-reduced (panel C) disaccharide samples derived from the high-affinity [3H]hexasaccharide each eluted as a single peak in the third isocratic step (120 mM), suggesting that the high-affinity [3H]hexasaccharide is composed of three identical disaccharide units. Although the [3H]disaccharides eluted in the same isocratic step as the standard IdoA(2-SO3)2ATala(4-SO3), the peaks tended to elute earlier from the Micropak AX-5 column as the column aged. When the [3H]disaccharides derived from the high-affinity [3H]hexasaccharide were mixed with an equal amount of 3H-labeled IdoA(2-SO3)2ATala(4-SO3) and chromatographed on the
Micropak AX-5 column, a single narrow peak of radioactivity was detected, indicating that the repeating disaccharide co-eluted with IdoA(2-SO₄)→ATala(4-SO₄) (data not shown).

The following experiments confirmed that the [³H]disaccharide derived from the high-affinity [³H]hexasaccharide has the structure IdoA(2-SO₄)→ATala(4-SO₄).

1) To determine the degree of sulfation, samples of the NaBH₄-reduced and [³H]NaBH₄-reduced disaccharides were analyzed by paper electrophoresis in comparison with [³H] disaccharide standards containing zero, one, or two sulfate groups (Fig. 7). Both of the labeled samples derived from the high-affinity hexasaccharide migrated to the same position as the disulfated standard, indicating that the disaccharide units of the hexasaccharide each contain two sulfate groups.

2) Conrad (21) previously reported conditions under which acid hydrolysis of the GlcA β1→3 linkage occurs much more slowly than hydrolysis of IdoA α1→3. We determined the time course of acid hydrolysis of the [³H]NaBH₄-reduced disaccharide under similar conditions (see "Experimental Procedures"). At each time point, the [³H]-labeled products were analyzed by paper chromatography in two solvent systems and quantified. The products were identified by comparison with [³H]-labeled standards that were chromatographed simultaneously. The percentage of the total radioactivity found in each of the [³H]-labeled products is plotted versus the migration distance toward the anode.

In summary, depolymerization of the high-affinity [³H] hexasaccharide yields a single type of disaccharide, IdoA(2-SO₄)→ATala(4-SO₄), which is derived from IdoA(2-SO₄)→GalNAc(4-SO₄). Therefore, the high-affinity [³H]hexasaccharide has the structure shown in Fig. 9A. As indicated in Table II, the repeating disaccharide unit of the high-affinity hexa-

**Fig. 7.** Paper electrophoresis of [³H]disaccharides derived from the high-affinity [³H]hexasaccharide. In panel A, disaccharide standards containing zero (IdoA→ATA)₄, one (IdoA→ATA)₄(4-SO₄) or two (IdoA(2-SO₄)→ATala(4-SO₄)) sulfate groups were electrophoresed on Whatman 3MM paper in a 3 x 5 cm lane as described under "Experimental Procedures." The number of sulfate groups in each of the standards is indicated at the top of the panel. NaBH₄-reduced (panel B) and [³H]NaBH₄-reduced (panel C) disaccharides derived from the high-affinity [³H]hexasaccharide were electrophoresed on the same paper. Each lane was cut into 1 cm segments that were counted for [³H]. The radioactivity in each segment is plotted versus the migration distance toward the anode.

**Fig. 8.** Acid hydrolysis time course of the [³H]disaccharide derived from the high-affinity [³H]hexasaccharide. The [³H]NaBH₄-reduced disaccharides derived from the high-affinity [³H] hexasaccharide were dissolved in 0.5 M H₂SO₄, covered with mineral oil, and heated at 100 °C. At the times shown, samples were removed, neutralized, and analyzed by paper chromatography in solvent systems 1 and 2 as described under "Experimental Procedures." The percentages of the total radioactivity in each of the hydrolysis products were determined at each time point. The products identified were: the starting disaccharide IdoA(2-SO₄)→ATala(4-SO₄) (O), IdoA→ATala (C), ATala(4-SO₄) (E), and ATala (A).
FIG. 9. Structure of the high-affinity hexasaccharide. A, the deduced structure of the high-affinity hexasaccharide is shown. The reducing terminal ATala(4-SO₄) is a product of the deaminative cleavage reaction and corresponds to GalNAc(4-SO₄) in the intact polysaccharide. B, comparison of oligosaccharide structures resulting from deaminative cleavage and Smith degradation (see text for discussion). UA, uronic acid; C₄, 4-carbon remnant of uronic acid.

TABLE II

<table>
<thead>
<tr>
<th>Disaccharide composition of porcine skin dermatan sulfate</th>
<th>This study</th>
<th>Shaklee and Conrad (Ref. 15)</th>
<th>Hopwood and Muller (Ref. 16)</th>
</tr>
</thead>
<tbody>
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<td>Unsulfated</td>
<td>0.9</td>
<td>0</td>
<td>1.8</td>
</tr>
<tr>
<td>GlcA → ATala(4-SO₄)</td>
<td>1.8</td>
<td>2</td>
<td>1.0</td>
</tr>
<tr>
<td>GlcA → ATala(6-SO₄)</td>
<td>16.9</td>
<td>17</td>
<td>11.2</td>
</tr>
<tr>
<td>IdoA → ATala(4-SO₄)</td>
<td>75.4</td>
<td>72</td>
<td>75.0</td>
</tr>
<tr>
<td>IdoA(2-SO₄) → ATala(4-SO₄)</td>
<td>5.0</td>
<td>9</td>
<td>4.5</td>
</tr>
<tr>
<td>Total</td>
<td>100.0</td>
<td>100</td>
<td>93.5</td>
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DISCUSSION

The present experiments were designed to identify the binding site (or sites) for HCII in porcine skin dermatan sulfate. Our approach involved deaminative cleavage of partially N-deacetylated dermatan sulfate to yield oligosaccharides of various chain lengths, followed by affinity chromatography of the oligosaccharides on HCII-Sepharose. The data support the following conclusions. 1) The smallest oligosaccharides that bind tightly to HCII in the presence of 0.05 M NaCl are hexasaccharides. 2) Hexasaccharides that contain three sulfate groups per molecule (~94% of the total hexasaccharides) do not bind to HCII. 3) In contrast, hexasaccharides that contain four, five, or six sulfate groups per molecule (~6% of the total hexasaccharides) bind to HCII with various affinities. 4) The hexasaccharide having the highest affinity for HCII is composed of three repeating IdoA(2-SO₄)GalNAc(4-SO₄) disaccharides, with the reducing terminal GalNAc(4-SO₄) converted to ATala(4-SO₄) by the cleavage procedure. 5) IdoA(2-SO₄)GalNAc(4-SO₄) disaccharides are not distributed randomly in the dermatan sulfate polymer, but are clustered during biosynthesis to form the HCII-binding site. 6) The high-affinity hexasaccharide is at least 20 times less active than intact dermatan sulfate in a thrombin inhibition assay with HCII, implying that longer chains are required for efficient stimulation of the HCII-thrombin reaction.

Previously, we prepared oligosaccharides from dermatan sulfate by Smith degradation (periodate oxidation followed by mild acid hydrolysis) and found that the smallest oligosaccharides that bound to HCII were octasaccharides rather than...
hexasaccharides (11). In the Smith degradation procedure, cleavage occurs at unsulfated IdoA residues, resulting in oligosaccharides that contain an intact GalNAc residue at the non-reducing terminus and a 4-carbon remnant of IdoA at the reducing terminus. As indicated in Fig. 9B, hexasaccharides produced by Smith degradation resemble hydrazinolysis/deamination hexasaccharides except for the presence of only two intact uronic acid residues. Since Smith degradation hexasaccharides do not bind to HCII, the presence of 3 uronic acid residues may be required for binding to HCII.

Little information was available previously concerning the structural requirements for binding of dermatan sulfate to HCII. Scully et al. (22) reported that dermatan sulfate and chondroitin sulfate preparations with various disaccharide compositions had different thrombin inhibitory activities with HCII. They showed that porcine skin dermatan sulfate composed mainly of IdoA+GalNAc(4-SO4) had less activity than hagfish dermatan sulfate enriched in IdoA(2-SO4, or 3-SO4)→GalNAc(4,6-diSO4). In contrast, porcine skin dermatan sulfate was more active than chondroitin sulfate H, which contains primarily IdoA→GalNAc(4-SO4). They concluded that 2-O-sulfation of IdoA increases activity with HCII while 6-O-sulfation of the GalNAc(4-SO4) residues mediate the relatively nonspecific binding of heparin and other polyanions (36-38). The presence of IdoA(2-SO4) residues in the high-affinity hexasaccharide distinguishes this molecule from the common tri-sulfated hexasaccharide IdoA→GalNAc(4-SO4)→IdoA→GalNAc(4-SO4)→IdoA→ATah(4-SO4) that does not bind to HCII (Fig. 4, panel B). Further experiments are required to determine whether the 2-O-sulfate groups of IdoA form ionic bonds with arginine residues in the dermatan sulfate-binding site of HCII.

REFERENCES

EXPERIMENTAL PROCEDURES

Materials — Dermatan sulfate (chondroitin sulfate B) from porcine skin was purchased from Sigma. The dermatan sulfate (100 mg) in H2O was treated with an equal volume of nitric acid at pH 1.5 (prepared as described by Shively and Conrad (12) for 1 h at room temperature to degrade the sulfuric acid. The material was neutralized with 1 M Na2CO3 dissolved in water) and was used for uronic acid determination. The samples were then centrifuged, dialyzed against water, and lyophilized.

Preparation of Dermatan Sulfate Disaccharide and Monosaccharides Standards — Dermatan sulfate disaccharide standards were prepared using methods described by Shively and Conrad (15). Disaccharides produced by exhaustive hydrolysis/elimination of the sulfated disaccharide (described below) were fractionated on a Mono S 3/10 column (Pharmacia) and lyophilized. The disaccharides were isolated and identified by their electrophoretic mobility in polyacrylamide gel electrophoresis and analyzed by their degree of sulfation. The disaccharides were also hydrolyzed with acid for various times, and the products were analyzed by paper chromatography as described below. The free acids of the disaccharides were identified as well as the intermediates produced, and were identified for each disaccharide. When compared to published data, the results of those experiments confirmed the structural assignments for each standard.

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Dermatan Sulfate Hexasaccharide

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Nitrous Acid Depolymerization of the High-affinity Hexasaccharide -- The high-affinity (6-90,000 amu) was dried in a 100 µl Reacti-Vial, and 20 µl of ethylenediamine containing 0.5 mg of hydroxylamine was added. The sample was treated at 90°C for 1 h (partial N-desacetylation) or 10 h (complete N-desacetylation), cooled on ice, dried under a stream of N₂, and lyophilized overnight. The sample was then incubated with 20 µl of nitrous acid at pH 4 (prepared by mixing 2.5 ml of 0.5 M NaOH with 1 ml of 1 M H₃PO₄, 12, 13) for 30 min at room temperature, and cooled on ice. The pH was adjusted to 8.5 with 3-10 µl of 0.2 M Na₂CO₃, pH 10.2. One half of the sample was reduced with 10 µl of 0.5 M NaBH₄ in 0.2 M Na₂CO₃, pH 10.2, for 2 h at 0°C. The other half of the sample was reduced with 10 µl of 1 M NaBH₄ in 0.2 M Na₂CO₃, pH 10.2, for 0.5 h at 0°C followed by 2.5 µl of 0.5 M unlabeled NaBH₄ for an additional 1 h at 0°C. Enzyme NaBH₄ was destroyed in a hood by the addition of 5 µl of 3 M H₂SO₄. The samples were dried under a stream of N₂ and redissolved in 200 µl of H₂O three times, then neutralized with 5-10 µl of 1 M Na₂CO₃ and desalted.

Paper Electrophoresis and Paper Chromatography -- Paper electrophoresis and paper chromatography were run as described by Shalakee and Conrod (13). Paper electrophoresis was performed on Whatman 3MM paper (3 x 57 cm lanes) in borate buffer (pH 8.9) at 2000 volts. Approximately 100 µg of [3H]hexasaccharide was applied per lane. Descending paper chromatography was performed by two methods: system 1, Whatman no. 1 paper (3 x 57 cm lanes) developed in 1-butanol:acetic acid:1 M NH₄OH (3:2:1, by volume); system 2, Whatman no. 1 paper (melamine formamide) developed in 1-butanol:acetic acid:1 M NH₄OH (3:2:1, by volume). About 4000 cpm of acid hydrolysate samples were applied per lane. After development, the rates were cut into 1 cm segments that were counted for radioactivity.

Acid Hydrolysis Time Course -- The isolated hexasaccharide (100,000 amu) was mixed with (14C)isocitrate (17,000 cpm) and dried in a 60 x 50 mm glass tube. The sample was desiccated in 0.5 M H₂SO₄, covered with a layer of mineral oil, and heated in a dry bath at 100°C. Aliquots (5 µl) were removed at various time points, cooled on ice, and neutralized with 7 µl of 1 M Na₂CO₃. A portion of each aliquot was analyzed by paper chromatography in systems 1 and 2. The migration distances of the [3H]hexasaccharide products relative to the (14C)isocitrate internal standard were compared to those of unlabeled standards run simultaneously.

Inhibition of Thrombin by Hepatitis C B11 in the Presence of Dermatan Sulfate Oligosaccharides -- The rate of inhibition of thrombin by HCV in the presence of dermatan sulfate oligosaccharides was determined by mixing 10 µl of unlabeled reduced oligosaccharides (0.3740 µg/ml H₂SO₄, 80 µl of 1 mg/ml polyethylene glycol, 0.15 M NaCl, 0.02 M Tris-HCl, pH 7.5) with 14C-thrombin (3 µl of 400 mU/ml thrombin) (added last) in a disposable polystyrene cuvette. After a 60-s incubation at room temperature, 500 µl of 96 µM Chromozym TR in TSP/PEG was added, and the absorbance at 405 nm was measured continuously for 100 s. The rate of change of absorbance was proportional to the concentration of active thrombin remaining in the incubation. No inhibition occurred in control experiments in which thrombin was pre-incubated with HCV in the absence of dermatan sulfate nor did inhibition occur when thrombin was incubated with dermatan sulfate alone over the range of concentrations tested.
Structure of a dermatan sulfate hexasaccharide that binds to heparin cofactor II with high affinity.
M M Maimone and D M Tollefsen


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