Determining Heparan Sulfate Structure in the Vicinity of Specific Sulfotransferase Recognition Sites by Mass Spectrometry*

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Sulfated motifs on heparan sulfate (HS) are involved in various extracellular processes from cell signaling to enzymatic regulation, but the structures of these motifs are obscure. We have developed a strategy to determine the structure of sulfotransferase recognition sites which constitute these motifs. Stable isotope is first introduced into specific sites on HS with HS sulfotransferases and the modified HS is then digested into oligosaccharides of differing sizes. The overlapping oligosaccharides containing the introduced stable isotope are identified by changes in the m/z profiles by mass spectrometry, and their relationships are elucidated. In this way, the HS structures in the vicinity of the sulfotransferase recognition site are quickly determined and groups on precursor structures of HS that direct the action of HS sulfotransferases are pinpointed.

Heparan sulfate (HS)† is one major polysaccharide found on proteoglycans. HS chains are first synthesized in the Golgi apparatus as repeated units of the disaccharide of a glucuronic acid and an N-acetylated glucosamine (GlcA-GlcNAc). The glucuronic acid can be epimerized to iduronic acid (IdoA). Incomplete sulfation at the 2-O positions of the uronic acids, and the 3-O, 6-O, and N positions of the glucosamine by various sulfotransferases results in structural diversity within HS (1, 2). The sulfate groups usually cluster in small regions and form sulfated motifs.

The sulfated motifs can bind to growth factors, cytokines, and morphogens as well as proteases in the extracellular matrix and regulate their activities (3); thus HS plays critical roles in organ development (4–6), morphogenesis (7, 8), angiogenesis (2), blood coagulation (2), inflammation (1), wound healing, and cancer progression (9). These sulfated motifs also play roles in pathological processes, such as functioning as receptors for viral entry (10–13).

Although HS plays important roles in various biological processes, the structures of these biological motifs are obscure, due to the difficulties involved in obtaining homogeneous components and determining their structures. Cloning, expressing, and sequencing biopolymers have tremendously advanced our understanding of DNA and protein, but no similar methods are available for studying HS.

In this report, we describe a novel strategy that permits us to quickly determine specific HS structures in the vicinity of the sulfotransferase recognition site for 3-OST-4. HS 3-OSTs are rare modification enzymes that help to generate binding sites for proteins such as antithrombin III (2) and herpes simplex virus glycoprotein D (11). 3-OST-4 is specifically expressed in brain tissues and may play a role in neuronal development (14). The delineation of sulfated motifs on the HS chain of proteoglycans will shed light on how cells interact with extracellular proteins, respond to extracellular signals, and initiate signaling cascade beginning on the cell membrane (15). This strategy relies on introducing a stable isotope of sulfate into specific sites on the HS chain by 3-OST-4. The mass-labeled HS is then digested into oligosaccharides and the overlapping oligosaccharides containing the stable isotope are identified by changes in their masses.

MATERIALS AND METHODS

Bovine kidney heparan sulfate, heparinase, and heparitinase I and II were obtained from Seikagaku America (Falmouth, MA). These enzymes were reconstituted at 0.3 milliunits/μl according to the manufacturer’s directions. Iduronate-2-sulfatase was from ProZyme (San Leandro, CA). Heparan sulfate sulfotransferases 3-OST-4 and 6-OST-1 were cloned, expressed, and purified using a Baculovirus expression system as described previously (14, 16). 35S and 33S stable isotopes were from Isotecs Corp. (Columbia, MD). PAPS was prepared as described previously (16).

Sample Preparation—Bovine kidney heparan sulfates were labeled with sulfotransferases in the presence of 35S, 33S, or [35S]PAPS. The labeling reaction was carried out in a 20-μl reaction with 10 μg of HS, 2 μl of 2× buffer (50 mM MES (pH 7.0), 1% (w/v) Triton X-100, 5 mM MgCl2, 5 mM MnCl2, 2.5 mM CaCl2, 0.075 mg/ml protease chloride, 1.5 mg/ml bovine serum albumin), 1 μl of 2 mM [35S]PAPS, or 2 μl of [35S]PAPS (about 1.0 × 107 cpm) and 20 ng of expressed pure sulfotransferase. The reaction was incubated at 37 °C for 2 h, and the modified HS was purified by a DEAE column. The HS sample was loaded onto 0.2 ml of DEAE affinity matrix in a small purification column, washed with 2 ml of 0.25 M NaCl, 20 mM NaAc (pH 6.0), and eluted with 0.4 ml of 1× NaCl, 20 mM NaAc (pH 6.0). The HS was then precipitated with 1 ml of ethanol and centrifuged at 14,000 × g for 30 min in a 4 °C room. Because the incorporation of [35S]PAPS into HS can be easily monitored, labeling with [35S]PAPS was performed in a parallel fashion. The labeled HS samples were then subjected to heparan sulfate lyase digestion at 37 °C for 2 h to completion according to Seikagaku’s protocols. The digestion buffer contained 40 mM ammonium acetate (pH 7.0), 1 mM CaCl2, and 1 milliunit of enzyme. The digestion of the 35S-labeled HS was analyzed by polyacrylamide gel electrophoresis and the digestion of the 33S- or 35S-labeled HS was analyzed by liquid chromatography-coupled mass spectrometry (LC/MS).

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‡The abbreviations used are: HS, heparan sulfate; 3-OSTs, 3-O-sulfotransferases; GlcA, glucuronic acid; IdoA, iduronic acid; AUA, Δ14-5-uronic acid; GlcNAc, N-acetylg glucosamine; GlcNS, N-sulfated glucosamine; 2S, 2-O-sulfate; 6S, 6-O-sulfate; 3S, 3-O-sulfate; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; dp, degree of polymerization, or number of saccharide units, e.g. dp2 = disaccharide; MES, 4-morpholineethanesulfonic acid; LC/MS, liquid chromatography-coupled mass spectrometry.

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Determined Heparan Sulfate Structure

\[ \text{Formula, } (C_{12}H_{19}O_{10}N_{1})_p(SO_3)_q(COCH_3), \]

Fig. 1. Oligosaccharide structures and its m/z profiles. A, an oligosaccharide structure generated by heparan sulfate lyases. The oligosaccharide has a formula of \((C_{12}H_{19}O_{10}N_{1})(SO_3)_q(COCH_3)_p\). B, the number of disaccharides; \(q\), number of the sulfate groups; \(t\), number of the acetyl groups. The \textit{wavy lines} indicate the uncertainty of the epimerization. B, \textit{in vitro} stable isotope incorporation changes the m/z profile of an oligosaccharide. The natural existence of multiple stable isotopes of carbon, hydrogen, oxygen, and sulfur cause the multiple peaks in an m/z profile. The distance between the neighboring peaks in a profile equal to \(1/w\). The \textit{in vitro} incorporation of one \(^{33}\text{S}\) or one \(^{34}\text{S}\) increases the second or the third peak respectively. \(w\), molecular mass; \(z\), the number of negative charges.

RESULTS

Calculation of the Molecular Weights and m/z Ratios of HS Oligosaccharides—The molecular weight of an HS oligosaccharide is the total weight of its functional groups and backbone structure (Fig. 1A). Based on major isotopes of \(^{12}\text{C}\), \(^{1}\text{H}\), \(^{16}\text{O}\), \(^{14}\text{N}\), and \(^{33}\text{S}\), the basic disaccharide GlcA-GlcNH\(_2\) (C\(_{12}\)H\(_{19}\)O\(_{10}\)N\(_1\)) has a molecular mass of 337.09; one sulfation increases the mass by 79.96 (-SO\(_3\)-); one acetylation (-COCH\(_3\)-) increases the mass by 42.01. Therefore, the molecular mass \((w)\) of an oligosaccharide with a formula of \((C_{12}H_{19}O_{10}N_{1})_p(SO_3)_q(COCH_3)_t\) should be as follows,

\[
W = 337.09p + 79.96q + 42.01t \quad \text{(Eq. 1)}
\]

where \(p\), number of disaccharide units; \(q\), number of sulfates; \(t\), number of acetyl groups.

For an oligosaccharide with \(z\) negative charges due to loss of protons, the mass/charge ratio \((m/z)\) will be as follows,

\[
m/z = (w - z)/z \quad \text{(Eq. 2)}
\]

The m/z values of the observed oligosaccharides in our study were calculated with Equations 1 and 2 (Table I).

Because of the existence of minor natural stable isotopes, such as \(^{13}\text{C}\), \(^{18}\text{O}\), \(^{15}\text{N}\), \(^{33}\text{S}\), and \(^{34}\text{S}\), the actual molecular mass of an oligosaccharide is a composite of \(w\), \(w + 1\), \(w + 2\), \(w + 3\), etc. Subsequently, the m/z value will also be a cluster of \((w - z)/z\), \((w + 1)/z\), \((w + 2 - z)/z\), \((w + 3 - z)/z\), etc., with neighboring values 1/z unit apart (Fig. 1B). Therefore, the distance between the neighboring peaks in an m/z profile reveals the number of the negative charge \(z\) on the molecule. Because the intensity of
the peaks in a m/z profile is dependent on the natural abundance of each isotope, the ratio among the individual peaks is also a constant. In vitro incorporation of 33S or 34S will enhance the second or third peak respectively due to 1 or 2 mass units increase over the major isotope 35S (Fig. 1B).

**LC/MS Study of 3-OST-4-modified Heparan Sulfate—HS** samples were first labeled with stable isotope 34S or radioisotope 35S by 3-OST-4 (16) and then digested with various heparan sulfate lyases. The digested of the 35S-labeled HS were analyzed by PAGE (Fig. 2). It was found that heparitinase II alone generated 35S-labeled hexasaccharide (dp6) and tetrasaccharide (dp4); heparitinase was able to convert the dp6 to dp4; and heparanase was able to convert the dp4 to disaccharide (dp2). The sizes of the labeled oligosaccharides were determined with defined oligosaccharides (18).

In a parallel fashion, the digests of the stable isotope 34S-labeled HS were analyzed by LC/MS and all the 34S-labeled oligosaccharides were located on the HPLC chromatograms by examining the m/z profiles (Fig. 3). It was noted that actually two heaxasaccharides (peaks a and b) and two tetrasaccharides (peaks c and d) were labeled with 34S in the heparitinase II digestion (Fig. 3A, upper panel). Mass profiles showed two heaxasaccharides with regular m/z of 685.62 and 725.62 and the two tetrasaccharides of 496.08 and 536.06, respectively (Fig. 3B). These results identified the two heaxasaccharides as dp5–4S1Ac (four sulfates and one acetyl group) and dp6–5S1Ac (five sulfates and one acetyl group) and the two tetrasaccharides as dp4–4S and dp4–5S (four or five sulfates, respectively) (Table I). Addition of heparitinase to the digestion converted the two heaxasaccharides to the two tetrasaccharides (Fig. 3A, middle panel). The further addition of heparinase converted the two tetrasaccharides to two disaccharides (peaks e and f) (Fig. 3A, lower panel). The disaccharide in peak e exhibited a regular m/z of 576.04 and z of 1 and was then identified as dp2–3S (three sulfates). The disaccharide in peak f exhibited a regular m/z of 785.19 and z of 1 and was then identified as a quasi-complex between dp2–4S (four sulfates) and dibutylamine, an ion-pairing reagent with the molecular mass of 129.15 (785.19 − 129.15 = 656.04) (Table I). Due to its high charge density, the dp2–4S has a strong tendency to form complexes with dibutylamine.

When the above experiments were repeated with 33S labeling, the oligosaccharides identified above showed 33S incorporation (Fig. 3C).

**Determining the Variable Sulfation on the Oligosaccharides—** Two disaccharides with three or four sulfates, two tetrasaccharides with four or five sulfates and two hexasaccharides with four or five sulfates were observed to contain 3-OST-4 introduced 33S. It was obvious that one particular sulfation was variable. To identify the site of this sulfation, HS samples were first modified by 3-OST-4, and then modified by other sulfotransferases (16), both in the presence of [35S]PAPS. The doubly modified HS samples were digested with heparan sulfate lyases and analyzed by LC/MS. It was found that 6-OST-1 was able to convert the dp2–3S to the dp2–4S and the dp4–3S to the dp4–4S (Fig. 4). The enhancement of the peaks at 789.17 and 538.05 in the m/z profiles of the dp2–4S and dp4–5S indicated the incorporation of the second 34S into the dp2–3S and dp4–4S, respectively. This experiment showed that a 6-O-sulfate on the oligosaccharides was variable for the sulfotransferase activity of 3-OST-4.

**Determining the Structure of the 33S-Labeled Oligosaccharides—** Two disaccharides, dp2–3S and dp2–4S, contained the 3-OST-4-incorporated 33S. Because only four positions on an HS disaccharide (2-O, 3-O, 6-O, and the N) can be sulfated by sulfotransferases, the dp2–4S must be fully sulfated. The fact that 6-OST-1 was capable of adding one sulfate group onto the dp2–3S indicates that the 6-O position in the disaccharide was not sulfated. Therefore, the two disaccharides have the following structure.

\[ \Delta UA2S-GlcNS3S \leq 6S \]

**Structure 1**

Two tetrasaccharides dp4–4S and dp4–5S contained in vitro incorporated 34S. Because these two tetrasaccharides could be further converted into the two previously identified dp2–3S and dp2–4S by heparinase digestion, the only explanation for this phenomenon is that the dp4–4S and dp4–5S each lost a monosulfated disaccharide during enzymatic digestion. This monosulfated disaccharide is likely to be \[ \Delta UA-GlcNS \], because this is a common disaccharide found in enzyme cleaved HS (1)

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**Table I**

| The calculated m/z values of some common HS oligosaccharides |

| \( p \) | \( q \) | \( t \) | \( z \) |
|---|---|---|---|
| 1 | 0 | 378.10 |
| 1 | 1 | 416.05 |
| 2 | 0 | 458.06 |
| 3 | 0 | 755.96 |
| 4 | 0 | 655.92 |
| 5 | 0 | 1072.97 |

The calculations are based on the major stable isotopes of 12C, 1H, 16O, 14N, and 32S with the equations:

\[ m = (w - x)z + (w - y)z + 79.98 + 42.01t \]

where \( p \), number of disaccharides; \( q \), number of sulfates; \( t \), number of acetyl groups; \( z \), number of negative charges. Binding of dibutylamine molecule (molecular weight, 129.15) to an oligosaccharide will increase m/z by 129.15/z. The m/z values of the oligosaccharides that could contain 3-OST-4-incorporated sulfate are in bold face.

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**Fig. 2.** Digestions of the 33S incorporated HS by different heparan sulfate lyases. The digestions were separated on a 12% PAGE gel. The sizes of the 33S-labeled oligosaccharides were determined by comparing with oligosaccharides of defined sizes.
and no monosulfated free amine containing disaccharide has been reported so far. Since heparinase cuts the GlcNS-H11006 6S-IdoA2S linkage (19), the tetrasaccharides should have the following structure.

\[
\text{STRUCTURE 2}
\]

The order of the disaccharides in the tetrasaccharides was also confirmed by treatment with the exo-enzyme iduronate-2-sulfatase, which removed one sulfate only from the dp2–3S or dp2–4S (Fig. 5) but not from the tetrasaccharides, indicating that the dp2–3S and dp2–4S should locate at the reducing sides of the tetrasaccharides. The oligosaccharides in peaks g and h were identified as dp2–2S and a dp2–3S, as they had regular \( m/z \) of 496.04 and 576.02, respectively (Table I). Because these two disaccharides were derived from the \( \Delta \text{UA2S-GlcN3S6S} \) by taking off the 2-O-sulfate, they should have the structures of \( \Delta \text{UA-GlcN3S6S} \) and \( \Delta \text{UA2S-GlcN3S6S} \), respectively, and were eluted at slightly different time points.

Two hexasaccharides dp6–4S1Ac and dp6–5S1Ac contained in vitro incorporated \( ^{34}\text{S} \) and they could be converted by heparitinase to the previously identified dp4–4S and dp4–5S. It is apparent that each hexasaccharide lost one acetylated disaccharide \( \Delta \text{UA-GlcN3Ac} \) (dp2–1Ac) during the enzymatic digestion. Because heparitinase cuts the GlcN3Ac-H11006 6S-GlcA linkage (20), the two hexasaccharides should have the following structure.

\[
\text{STRUCTURE 3}
\]

**DISCUSSION**

We have developed a strategy to rapidly obtain the structure of HS in the vicinity of a specific sulfotransferase recognition site. This site is labeled with a stable sulfur isotope by the sulfotransferase. The modified HS is then digested with enzymes in a controlled manner to oligosaccharides of different sizes. The overlapping oligosaccharides containing the incorporated isotope are identified by mass spectrometry. The precise mass measurement also establishes the sizes of the oligosaccharides and the number of sulfate and acetyl groups on each oligosaccharide. The disaccharide arrangement in the oligosaccharides can be distinguished by substrate specificities of the heparan sulfate lyases and with the help of exoglycosidases and sulfatases. In this manner, the structure in the vicinity of the introduced isotope can be deduced. The size of the sequence obtained is dependent on the conditions and methods used for HS cleavage. More than one stable isotope and enzyme can be employed to explore the structural information on HS; for example, \( ^{33}\text{S} \) can be incorporated by one sulfotransferase and \( ^{34}\text{S} \)
by another. In this way, the distance between the two recognition sites, and thus the relationship between the two sulfotransferases, can be established.

Since most, if not all, heparan sulfate sulfotransferases have been cloned recently and all these enzymes can be assumed to possess different substrate specificities, it should be possible to obtain the structures in the vicinity of the recognition sites of all the sulfotransferases. The results will reveal the substrate specificities of those enzymes as well as how different critical groups direct the action of the subsequent sulfotransferases and eventually how the biological relevant motif structures on heparan sulfate are generated. Recently, we have also developed methods to in vitro synthesize functional HS structures with the cloned enzymes (16, 21, 22). With these technology advances, it is possible for us to define the biological functions of HS motifs by synthesizing the defined HS structures.

It is interesting to note that the upstream half of the hexasaccharide structures ΔUA-GlcNAc-GlcA-GlcNS-IdoA2S-GlcNS3S:6S is non-sulfated and the downstream half is sulfated. At the disaccharide level, 3-OST-4 recognizes heavily sulfated disaccharides and generates tri- and tetra-sulfated disaccharides. Tetra-sulfated disaccharide is the most heavily sulfated disaccharide in HS and is also found in 3-OST-5 generated products (23). The biological significance of this disaccharide is under further investigation. The 6-O-sulfate that resides in the same glucosamine residue where 3-O-sulfation occurred was found to be variable, indicating that this particular sulfate is not required for 3-OST-4 recognition. The fact that this 6-O-sulfate was also found variable in 3-OST-1 and 3-OST-3 recognition (10) suggests that 3-O-sulfotransferases do not make contacts with this 6-O-sulfate during substrate recognition.

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We have also investigated the heparan sulfate structure at the vicinity of 3-OST-1 recognition site. Our current data (data not shown) confirmed the previously reported tetrasaccharide structure HUA-GlcNac6S-GlcA-GlcNS3S (24). Comparing with the tetrasaccharide structure at 3-OST-4 recognition site (HUA-GlcNS-IdoA2S-GlcNS3S6S), we found that the two internal sugar residues in the tetrasaccharides are different. We surmise that an N-acetylated glucosamine and a following GlcA are involved in directing the action of 3-OST-1, whereas an N-sulfated glucosamine and a following 2-O-sulfated IdoA are involved in the action of 3-OST-4. The differences on the HS structures recognized by the two sulfotransferases indicate that they have different biological functions.

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