Heparan Sulfate d-Glucosaminyl 3-O-Sulfotransferase-3A Sulfates N-Unsubstituted Glucosamine Residues

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3-O-Sulfation of glucosamine by heparan sulfate d-glucosaminyl 3-O-sulfotransferase (3-OST-1) is the key modification in anticoagulant heparan sulfate synthesis. However, the heparan sulfates modified by 3-OST-2 and 3-OST-3A, isoforms of 3-OST-1, do not have anticoagulant activity, although these isoforms transfer sulfate to the 3-OH position of glucosamine residues. In this study, we characterize the substrate specificity of purified 3-OST-3A and its influence on the sulfate level. The 3-OST-3A enzyme was purified from SF9 cells infected with recombinant baculovirus containing 3-OST-3A cDNA. Two 3-OST-3A-modified tetrasaccharides were purified from the 3-O-[35S]sulfated heparan sulfate that was digested by heparin lyases. These tetrasaccharides were analyzed using nitrous acid and enzymatic degradation combined with matrix-assisted laser desorption/ionization-mass spectrometry. Two novel tetrasaccharides were discovered with proposed structures of ΔUA2S-GlcNS-IdoUA2S-[35S]GlcNH23S6S and ΔUA2S-GlcNS-IdoUA2S-[35S]GlcNH23S6S. The results demonstrate that 3-OST-3A sulfates N-unsubstituted glucosamine residues, and the 3-OST-3A modification sites are probably located in defined oligosaccharide sequences. Our study suggests that oligosaccharides with N-unsubstituted glucosamine are precursors for sulfation by 3-OST-3A. The intriguing linkage between N-unsubstituted glucosamine and the 3-O-sulfation by 3-OST-3A may provide a clue to the potential biological functions of 3-OST-3A-modified heparan sulfate.

Heparan sulfates (HS)† are negatively charged polysaccharides with 1→4-linked sulfated glucosamine and uronic acid repeating disaccharide units. HS are present on the cell surface as well as in the extracellular matrix and bind to proteins involved in anticoagulation, angiogenesis (1), viral infection (2), and monocyte adhesion (3). The biosynthesis of HS includes the formation of a polysaccharide backbone by d-glucuronyl and N-acetyl-d-glucosaminyltransferase (4) followed by serial sulfation and epimerization reactions. The enzymes responsible for the sulfation and epimerization of HS polysaccharide have been cloned, including heparan sulfate N-deacetylase/N-sulfotransferase, heparan sulfate 2-sulfotransferase, heparan sulfate 6-sulfotransferase, heparan sulfate d-glucosaminyl 3-O-sulfotransferase (3-OST-3), and d-glucuronyl C5-epimerase (5–10). Despite the recent success in cloning HS biosynthetic enzymes, the mechanisms for generating HS with defined monosaccharide sequences are still poorly understood. However, several biological functions of HS are believed to be dictated by unique sulfated saccharide sequences, and the HS biosynthetic enzymes regulate the synthesis of these oligosaccharides (11). Indeed, 3-OST-1 is up-regulated at the transcriptional level in F9 cells to control the biosynthesis of anticoagulantly active HS (12). HS modifying HS with 3-O-sulfated anticoagulantly inactive HS, which are thought to affect cell differentiation (11).

HS contains defined antithrombin-binding sites with the structure -GlcNSorAc6S-GlcUA-GlcNS3S-6S-IdoUA2S-GlcNS6S- (12, 13). Within the pentasaccharide, 3-O-sulfation to form GlcNS6S±6S is one of the critical modifications that confers antithrombin binding affinity (14). This critical 3-O-sulfation is performed by 3-OST-1 (EC 2.8.2.25) (10, 15). By using purified 3-OST-1, we found that there are six antithrombin-binding sites in a single HS. HS chain, suggesting that the synthesis of HS is a highly organized process requiring a specific biosynthetic pathway (16).

The different isoforms of 3-O-sulfotransferase sulfate unique disaccharides and generate HS with different biological functions (17). These isoforms have more than 60% homology in the sulfotransferase domain (18). The isoforms are expressed at different levels in various human tissues (18), suggesting their importance in making tissue-specific HS.

Given that 3-O-sulfation of glucosamine made by 3-OST-1 is critical for synthesizing HS and is a rare modification in any given HS (10, 15, 19), we are interested in determining the biological functions of 3-OST-3 and 3-OST-2 modified HS.
this paper, we characterize the substrate specificity of heparan sulfate 3-O-sulfotransferase 3A (3-OST-3A) at the tetrasaccharide level using purified enzyme. 3-OST-3A transfers sulfate to the 3-OH position of N-unsubstituted glucosamine (GlcNH$_2$) as determined by MALDI-MS coupled with nitrous acid and enzy- matic degradations. Our results suggest an intriguing link- age between the GlcNH$_2$ residue and 3-O-sulfation by 3-OST-3A.

**EXPERIMENTAL PROCEDURES**

**Materials**

pCDNA3 plasmid containing 3-OST-3A was prepared as described previously (17). [35S]PAPS was prepared by incubating 0.4 mCi/ml [35S]Na$_2$SO$_4$ (Amersham, Piscataway, NJ) and 18 mCi ATP with 6 mg/mL dialyzed yeast extract (Sigma) as described previously (15). Iduronate 2-sulfat- urylase using the method described in our previous publication (17) with oligonucleotide was synthesized (by GeneLink) with the sequence of expression vector (Life Technologies, Inc.). First, a double-stranded expression vector was constructed by inserting the honeybee melittin (20).

**Preparation of 3-OST-3A Baculovirus Expression Plasmid**

Construction of the Baculovirus Expression Vector—The baculovirus expression vector was constructed by inserting the honeybee melittin signal sequence (S-GAATAG with a pcDNA3 template plasmid containing 3-OST-3A (17). The PCR product was cleaved by EcoRI and cloned into an EcoRI-digested pMel-Bac-C vector (Invitrogen). The resulting vector served as PCR template using the 5’ primer GAATAG with a pcDNA3 template plasmid containing 3-OST-3A (17).

expression vector (Life Technologies, Inc.). First, a double-stranded oligonucleotide was synthesized (by GeneLink) with the sequence of encoding a (His)$_n$ sequence with BamHI and EcoRI co-adhesive ends at the 5’ and 3’ ends, respectively. This oligonucleotide was inserted into BamHI/EcoRI-digested pMEL-Bac-C vector (Invitrogen). The resulting vector served as PCR template using the 5’ primer ATATGCTTATGAGAAGTGCTAATGCGCTTAA-PO$_4^{-}$ and 3’ primer CAACAACGCACAGAATCTAGC. The PCR product, consisting of honeybee melittin signal and the (His)$_n$ sequence, was digested with EcoRI and BamHI and cloned into an RsrII/EcoRI-cleaved pFastBac-HTa vector (Life Technologies, Inc.). The product and the anticipated reading frame were confirmed by sequencing (Nucleic Acid/ Protein Research Core Facility, The Children’s Hospital of Philadelphia).

Construction of 3-OST-3A Baculovirus Expression Plasmid—Se- creted 3-OST-3A was constructed by removing the 43 amino acids corresponding to the signal transmembrane segment and 3-OST-3A was obtained by a PCR reaction using a 5’ primer ATATGCTTATGAGAAGTGCTAATGCGCTTAA-PO$_4^{-}$ and a pcDNA3 reverse 3’ primer GCATTAGTGTTGCTAATGCGCTTAA-PO$_4^{-}$ with an EcoRI site (underlined) and a pcDNA3 forward 5’ primer CAACAACGCACAGAATCTAGC. The PCR product, consisting of honeybee melittin signal and the (His)$_n$ sequence, was digested with EcoRI and BamHI and cloned into an RsrII/EcoRI-cleaved pFastBac-HTa vector (Life Technologies, Inc.). The product and the anticipated reading frame were confirmed by sequencing (Nucleic Acid/ Protein Research Core Facility, The Children’s Hospital of Philadelphia).

**Expression and Purification of 3-OST-3A**

Expression of 3-OST-3A—3-OST-3A recombinant baculovirus was prepared from 3-OST-3A baculovirus expression plasmid using the Bac-to-Bac Baculovirus Expression system (Life Technologies, Inc.) according to the manufacturer’s protocol. Exponentially growing Sf9 cells (3 to 4 x 10$^7$/mL) were infected with 25 µl (2 x 10$^7$ plaque forming units/mL) of recombinant viral stock solution. The cell medium (40 mL/T175 flask) was changed to serum-free medium (SFM-900, Life Technologies, Inc.) 48 h after infection. The medium was harvested every 24 h for 4 days. The harvested medium was centrifuged at 10,000 x g for 15 min, and CHAPS was added to a final concentration of 0.6%. This solution was frozen in liquid nitrogen and stored at -80 °C for subsequent purification.

Measurement of 3-OST-3A Activity—We determined 3-OST-3A activity using the method described in our previous publication (17) with minor modifications. Briefly, a 50-µl reaction contained 200 µg/mL bovine kidney HS (ICN), 1 x 10$^5$ cpm of [35S]PAPS, 75 µg/mL promethine chloride, 400 µg/mL chondroitin sulfate C, 50 mM MES, pH 7.0, 10 mM MnCl$_2$, 5 mM MgCl$_2$, 100 mM NaCl, 120 µg/mL bovine serum albumin, and 1% Triton X-100 (v/v). The reaction mixture was incubated at 37 °C for 1 h. The [35S]HS was isolated by DEAE chromatography.

**Purification of Recombinant 3-OST-3A Enzyme**—The entire purified enzyme was carried out at 4 °C. The harvested medium was mixed with Tris-HCl to a final concentration of 10 mM, adjusted to pH 8 with 1 N NaOH, and centrifuged. The supernatant was mixed with an equal volume of cold 10 mM Tris, pH 8.0, and then loaded on a heparin-agarose column (0.5 x 8 cm, Sigma), which was equilibrated with 200 mM NaCl in TCG buffer using 14,000 MWCO tubing (Spectrum). The dialyzed solution was loaded on a 3’,5’-ADP-agarose column (5 x 8 cm, Sigma), which was equilibrated with 200 mM NaCl in TCG buffer at a flow rate of 0.2 ml/min. The column was washed with 6 ml of TCG buffer containing 200 mM NaCl and eluted with a linear gradient of NaCl from 200 to 1000 mM in 80 ml of TCG buffer. The fractions (66 ml) containing 3-OST-3A activity were pooled and dialyzed against 200 mM NaCl in TCG buffer at a flow rate of 0.2 ml/min. The column was washed with 6 ml of TCG buffer containing 200 mM NaCl and eluted with a linear gradient of NaCl from 200 to 1000 mM in 12 ml of TCG buffer followed by an isocratic elution with 12 ml of 1000 mM NaCl. The fractions (9 ml) containing 3-OST-3A activity were pooled. A portion of 3’,5’-ADP-agarose column purified material (1 ml) was further fractionated by gel permeation chromatography on a GPC-HPLC. The eluted fraction was equilibrated with a buffer containing 25 mM MOPS, 2% glycerol, 0.6% CHAPS, 1000 mM NaCl, pH 7.0, at a flow rate of 0.5 ml/min at room temperature (15). The GPC-HPLC purified enzymes were frozen in liquid nitrogen and stored at -80 °C for analysis by SDS-PAGE.

The GPC-HPLC purified material (24 ng) was [35S]-labeled with Bolton-Hunter reagent (NEZ Life Science Products) as described previously (15), and the [125$I$]-labeled protein (0.1 ng, around 1,000 cpm) was analyzed on a 12% precast SDS-PAGE gel (FMC) along with 14C-labeled IgG anti-chicken IgY (Aves Laboratories). The immunocomplex was detected using ECL chemiluminescence substrate (Amersham Pharma- cia Biotech) and Biomax MS film (Kodak).

**Determination of the Structures Tetra A and Tetra B**

Preparation of 3-OST-3A 35S-Labeled HS—Purified 3-OST-3A (30 ng) was mixed with 1 µg of unlabeled HS isolated from 33-cells and 10 µl [35S]PAPS (5 x 10$^5$ cpm) in the enzyme reaction buffer described above, omitting protamine chloride and chondroitin sulfate. Twenty minutes were pre pared to obtain a sufficient amount of tetrasaccharides for structural analysis. The [35S]HS was degraded with a mixture of heparin lyases, including heparinase I, heparinase II, and heparinase IV as described previously (16). The degraded HS was fractionated on a P-6 column (0.75 x 200 cm) (16). The fractions that eluted in the tetrasaccharide region were pooled and purified on a silica-based polyamine HPLC column (PAM-HPLC).

**Enzymatic and Nitrous Acid Degradation of Tetrasaccharides**—The digestion conditions for 4,5-glycuronate 2-sulfate were previously described (21). The conditions for nitrous acid degradation at both high pH (pH 4.0) and low pH (pH 1.5) and the conditions for digestion with iodoacetate-2-sulfate and -6-iduronidase were also reported in a prior publication (17).

Analysis of Tetrasaccharides and 35S-Disaccharides by Capillary Electrophoresis—The analysis of the Tetra A and Tetra B on capillary
electrophoresis with UV 232-nm detection followed a method previously outlined (25). Briefly, analysis of purified tetrasaccharides A and B was completed on a Hewlett-Packard 3D CE unit by using a uncoated fused silica capillary (inner diameter, 75 μm; L = 80.5 cm). Analyses were measured using an extended path length capillary. The electrolyte was a solution of 10 mM dextran sulfate and 50 mM Tris-phosphate, pH 2.5. Separations were carried out at 30 kV with reverse polarity. A hydrodynamic injection was performed; total injection was calculated to be 58 nl.

**Matrix-assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS)**—The sample preparation of purified oligosaccharides for MALDI-MS followed the procedures reported previously (25). MALDI-MS spectra were acquired in the linear mode by using a PerSeptive Biosystem Voyager Elite reflectron time-of-flight instrument fitted with a 337-nm nitrogen laser. Mass spectra were calibrated by using the signals for protocatechuic (Arg-Gly-Asp)-Arg and its complex with a hexasaccharide standard of the sequence Id0-A2S-GlcNS6S-IdoA2S-GlcNS6S-IdoA2S-AnManNS6S. The Determination of the Amount of N-Unsubstituted Glucosamine in HS—The amount of N-unsubstituted glucosamine was determined by using o-phthalaldehyde as described by Roth (27). To validate this method, we have determined the amount of N-unsubstituted glucosamine in the HS from bovine kidney (ICN). We found that the level of N-unsubstituted glucosamine of the HS from bovine kidney (ICN) is 1.5% (w/w), which is very close to the published value of 1.6% (w/w) by Toida et al. (28) using the same method.

**RESULTS**

**Purification of Recombinant 3-OST-3A**—The secreted form of 3-OST-3A was expressed in Sf9 insect cells as described under “Experimental Procedures.” 3-OST-3A was purified 161-fold with a 16.7% yield from serum-free media using a heparin-Toyopearl Gel 650M column and a 3.5'ADP-agarose column (Table I). The 125I-labeled purified protein migrated as a doublet band at a molecular mass of 39,000 Da on 12% SDS-PAGE (Fig. 1, panel A). The molecular mass of the purified 3-OST-3A is very close to the molecular mass (42,850 Da) calculated from the amino acid sequence (18). We have also carried out Western analysis of the purified 3-OST-3A and obtained a very similar band pattern to the 125I-labeled 3-OST-3A, suggesting that both bands contain the epitope peptide sequence (Fig. 1, panel B). Taken together, our results suggest that the preparation of 3-OST-3A is pure. The doublet band on SDS-PAGE is probably due to differential proteolytic cleavage or different post-translational modifications (29).

The purified protein exhibited heparan sulfate 3-O-sulfotransferase activity and sulfated the same disaccharides as 3-OST-3A expressed in COS-7 cells (data not shown). We have also determined the substrate specificity of the purified 3-OST-3A with regard to various glycosaminoglycans. We have observed that the amount of [35S]sulfate transferred to heparan sulfate is 50–200-fold greater than other glycosaminoglycans, indicating that heparan sulfate is the preferred substrate for 3-OST-3A among glycosaminoglycans (Table II). It is interesting to note that heparin, chemically very similar to HS but with a higher sulfation level and greater content of iduronic acid, is not a substrate for 3-OST-3A.

**Determination of the Structures of Tetra A and Tetra B—3-O-[35S]-Sulfated HS was prepared by incubating the unlabeled HS from 33-cells and purified 3-OST-3A and [35S]PAPS as described under “Experimental Procedures.” We have modified a total of 20 μg of HS by using purified 3-OST-3A enzyme in order to obtain sufficient amount of 3-O-sulfated oligosaccharides for structural analysis. The [35S]HS was exhaustively digested with a mixture of heparin lyases including heparinase, heparitinase I, II, and IV and was fractionated on Bio-Gel P-6 (Fig. 2, panel A). 60–70% of the [35S]counts eluted as tetrasaccharide and 13–31% eluted as hexasaccharide, and no [35S]disaccharide was observed. The tetrasaccharide portion was further purified by PAMN-HPLC to yield three [35S]-labeled components. Two of these were designated as Tetra A and Tetra B (Fig. 2, panel B). Tetra A accounted for 27–35%, and Tetra B accounted for 32–33% of the [35S]counts that were applied to the PAMN-HPLC column. The third [35S] component (about 6% of total loaded [35S] counts on PAMN-HPLC) was [35S]sulfate as judged by analysis on gel permeation chromatography- and RPIP-HPLC. We have obtained 5.3 × 10^5 dpm of Tetra A, which is equivalent to 54 pmol, and 9.4 × 10^5 dpm of Tetra B, which is equivalent to 94 pmol, given that the specific radioactivity of [35S]PAPS is 4.5 Ci/mmol. Such amounts of Tetra A and Tetra B permitted us to analyze these tetrasaccharides by capillary electrophoresis using a UV detector and MALDI-MS.
To confirm the purity, Tetra A and Tetra B were analyzed on capillary electrophoresis using a UV-232 nm on-line detector (25, 30). Each tetrasaccharide showed a single symmetrical peak (Fig. 3). Our data suggest that the obtained Tetra A and Tetra B are pure. We have also collected the UV peak from capillary electrophoresis to determine the 35S radioactivity within the eluent. We have found that the collected UV peak (less than 1 \( \mu l \)) contains 103 and 74\% of injected 35S radioactivity for Tetra A and Tetra B, respectively. Therefore, it suggests that the material with UV 232 nm absorbance indeed represents the radioactively labeled tetrasaccharide. In addition, the intensities of the UV peaks on capillary electrophoresis are consistent with the amount of the analytes estimated by the specific radioactivity of \([35S]\)sulfate. Taken together, our data suggest that both radioactively labeled Tetra A and Tetra B are sufficiently pure for MALDI-MS analysis. Furthermore, the migration times of Tetra A and Tetra B on capillary electrophoresis are consistent with those of a tetrasulfated and a pentasulfated oligosaccharide, respectively (25, 30).

To determine the molecular weight of Tetra A and Tetra B, both compounds were analyzed by MALDI-MS using a synthetic peptide (Arg-Gly)\(_{19}\)-Arg as a complexing agent. MALDI-MS has proved to be able to determine the molecular weight of heparin oligosaccharides within 0.03\% without significant loss of sulfate (25, 31). Internal calibration with the peptide yielded a molecular ion signal of the protonated 1:1 complex at \( m/z \) 5220.2 and 5299.8 for Tetra A and Tetra B, respectively (Fig. 4, panels A and B). After subtracting the contribution of the protonated peptide (\( m/z \) 4225.4 and 4226.1 respectively), the molecular mass of Tetra A was calculated to be 995 Da, very close to the theoretical value (994 Da) for a tetrasulfated tetrasaccharide (\( \Delta UUA\text{UA}(\text{GlcN})_2(\text{SO}_3\text{H})_4, C_{24}H_{38}N_2O_{32}S_4 \)).\(^2\) The molecular mass of Tetra B was calculated to be 1074 Da, identical to the theoretical value for a pentasulfated tetrasaccharide (\( \Delta UUA\text{UA}(\text{GlcN})_2(\text{SO}_3\text{H})_5, C_{24}H_{38}N_2O_{35}S_5 \)).\(^2\)

We found that both Tetra A and Tetra B were susceptible to nitrous acid degradation at pH 4.0. Under these conditions, nitrous acid reacts with the free amino group of glucosamine residues and converts the glucosamine to 2,5-anhydromannose (32). As shown in Fig. 5, the retention times of Tetra A and Tetra B were shifted from 32.5 to 28.5 min (Fig. 5, panels Aa and Ab) and from 42.5 to 36.5 min (Fig. 5, panels Ba and Bb) on PAMN-HPLC, respectively, after high pH (pH 4.0) nitrous acid degradation followed by sodium borohydride reduction. The shifts in retention times of Tetra A and Tetra B after high pH nitrous acid treatment suggest that both tetrasaccharides contain an \( N \)-unsubstituted glucosamine (GlcNH\(_2\)) residue, based upon the nitrous acid cleavage specificity as described previously (32). This conclusion was strengthened with analysis by MALDI-MS as described below.

To prove further that the shift in retention times on PAMN-HPLC is due to deamination of GlcNH\(_2\) residue, we determined the molecular masses of nitrous acid-treated Tetra A and Tetra B after high pH nitrous acid treatment. Their molecular masses were determined to be 980 Da\(^3\) and 1060 Da, respectively, by MALDI-MS (spectra not shown). For the nitrous acid-treated Tetra A, the obtained molecular weight is again very close to the theoretical value (979 Da) for a tetrasulfated tetrasaccharide with an anhy-\(^3\) We calculated the molecular weights of Tetra A and Tetra B based on 35S, because [35S]sulfate represents less than 0.1\% of total sulfate.
dromannitol at the reducing end (\(\text{DUAUAGlcN(AnMan)(SO}_3\text{H)}_4\), C\(\text{24H}_{37}\text{NO}_3\text{S}\)\(_4\)). Similarly, the obtained molecular weight of high pH nitrous acid-treated Tetra B is very close to the theoretical value (1059 Da) for a pentasulfated tetrasaccharide with an anhydromannitil residue at the reducing end (\(\text{DUAUAGlcN(AnMan)(SO}_3\text{H)}_5\), C\(\text{24H}_{38}\text{NO}_3\text{S}\)\(_5\)). A 15- and 14-Da reduction in molecular mass for Tetra A and Tetra B, respectively, demonstrated that a deamination did occur at the reducing end in both tetrasaccharides after nitrous acid treatment at pH 4.0, given that 15 Da is the theoretical reduction resulting from deamination.\(^4\) Therefore, Tetra A and Tetra B each contain a GlcNH\(_2\) residue at the reducing end. It is important to note that the HS from 33-cells contains 4.3% (w/w) N-unsubstituted glucosamine as determined by measuring the amount of free amino group by using o-phthalaldehyde.

We determined the position and number of sulfate groups on each residue within the tetrasaccharide by \(\Delta^{4.5}\)-glucuronate 2-sulfatase and low pH nitrous acid digestion as illustrated in Fig. 6, panel B. We observed that the retention time of Tetra A was shifted from 32 to 23 min on PAMN-HPLC after \(\Delta^{4.5}\)-glucuronate 2-sulfatase digestion (Fig. 6, panels Aa and Ab). This result suggests that a 2-O-sulfate group is on the \(\Delta^{\text{UA}}\) residue at the nonreducing end, based on the substrate specificity of \(\Delta^{4.5}\)-glucuronate 2-sulfatase (21, 33). The sulfatase-treated Tetra A (as illustrated in Fig. 6, panel Ba and panel Bc), a trisulfated tetrasaccharide, was then degraded by nitrous acid at pH 1.5, yielding a \(\text{35S}\) labeled product that migrated as a disaccharide on a Bio-Gel P-2 column (data not shown). The latter result suggests that a sulfate group is at the NH position of the glucosamine residue adjacent to \(\Delta^{\text{UA}}\)2S, because nitrous acid reacts with N-sulfate group of glucosamine residues at pH 1.5 (32). The \(\text{35S}\)-labeled product co-eluted with IdoUA2S-[\(\text{35S}\)]GlcNH\(_3\) standard on RPIP-HPLC as characterized previously (17) (Fig. 6, panel Ac), Taken together, our data suggest that Tetra A has a structure of \(\text{DUA}^{\text{2S}}\)-GlcNS-IdoUA2S-[\(\text{35S}\)]GlcNH\(_3\)3S.

We used a similar strategy to characterize Tetra B (Fig. 7, panel B). One sulfate was found at the 2-O-position of the \(\Delta^{\text{UA}}\) residue, because Tetra B was sensitive to \(\Delta^{4.5}\)-glucuronate 2-sulfatase digestion (Fig. 7, panel Aa and panel Ab). Another sulfate group was found at the NH position of the glucosamine residue adjacent to \(\Delta^{\text{UA}}\)2S, because Tetra B was degraded to \(\text{35S}\)-disaccharide by nitrous acid at pH 1.5 as judged by P-2 gel chromatography (data not shown). However, the resulting \(\text{35S}\)-disaccharide did not co-elute with any known disaccharide standard on RPIP-HPLC (Fig. 7, panel Ac). We treated this disaccharide with iduronate 2-sulfatase and generated a \(\text{35S}\)-labeled monosaccharide (Fig. 7, panel Ae). The resultant \(\text{35S}\)-monosaccharide co-eluted with [\(\text{3H}\)]AnMan3S6S standard (Fig. 6, panel Ae). Therefore, the structure of the

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\(^3\) Another component with a molecular mass of 995 Da was also observed in the MALDI-MS analysis of the high pH nitrous acid-treated Tetra A. This component has the same molecular weight as the intact Tetra A, suggesting that it is probably the by-product of the nitrous acid treatment.

\(^4\) Deamination should result in a loss of molecular mass of 17 Da. The subsequent sodium borohydride reduction increases the molecular mass by 2 Da. Therefore, the net loss of molecular mass after high pH nitrous acid treatment is 15 Da.
35S-disaccharide is IdoUA2S-[3-35S]AnMan3S6S. Taken together, our data demonstrated that Tetra B has a structure of \(\Delta\)UA2S-GlcNS-IdoUA2S-[3-35S]GlcNH23S6S.

**DISCUSSION**

In the present study, we investigated the substrate specificity of 3-OST-3A at the tetrasaccharide level. This enzyme was previously identified as a heparan sulfate 3-O-sulfotransferase, and the substrate specificity was characterized at the disaccharide level (17, 18). In order to lay the foundation for studying the biological functions of 3-OST-3A, it is necessary to define the substrate specificity of 3-OST-3A at the oligosaccharide level. Furthermore, it is important to utilize purified recombinant enzyme in those studies to eliminate the potential confounding effects of other heparan sulfate sulfotransferases. We expressed 3-OST-3A enzyme in Sf9 cells and purified the recombinant enzyme from serum-free medium. We then 35S-sulfated HS by using purified enzyme and [35S]PAPS and digested the product with a mixture of heparin lyases to obtain Tetra A and Tetra B. The structures of Tetra A and Tetra B were determined to be \(\Delta\)UA2S-GlcNS-IdoUA2S-GlcNH23S and \(\Delta\)UA2S-GlcNS-IdoUA2S-GlcNH23S6S, respectively. Neither of these two structures has been reported previously. We found a trisulfated disaccharide with a structure of IdoUA2S-[3-35S]AnMan6S3S in the low pH nitrous acid-degraded 3-OST-3A-modified [35S]HS. This disaccharide was not identified in our previous study (17), because this component was not eluted from a C18-HPLC column with the conditions used. We have now identified this novel disaccharide and found that it was part of the structure of Tetra B.

Our results demonstrated that the [35S]sulfate-labeling sites of Tetra A and Tetra B are at the 3-OH position of the N-unsubstituted glucosamine residue (GlcNH2), proving that 3-OST-3A transfers sulfate to GlcNH2 and GlcNH26S residues. We identified GlcNH2 residues in Tetra A and Tetra B based upon high pH (pH 4.0) nitrous acid degradation followed by the analysis on PAMN-HPLC and MALDI-MS. Our data indicate that deamination of GlcNH23S or GlcNH23S6S residue also occurs at pH 1.5. It is worthwhile to note that N-unsubstituted glucosamine residues react with nitrous acid at pH 4.0, and N-sulfated glucosamine residues react with nitrous acid at pH...
1.5 (32). However, such specificity is not absolute, as nitrous acid reacts with N-unsubstituted glucosamine and N-sulfated glucosamine at pH 3, although at a slower rate (34). Furthermore, the conclusions of the specificity study of nitrous acid degradation are based on model compounds that do not contain 3-O-sulfation. Because that 3-O-sulfate group is located adjacent to the free amino group that reacts with nitrous acid, it is possible that 3-O-sulfate affects the reactivity of the nearby amino group to nitrous acid at low pH. Indeed, we have found that glucosamine 3-O-sulfate monosaccharide is susceptible to nitrous acid treatment at pH 1.5 to yield 2,5-anhydromannitol 3-O-sulfate, although the amount of such product is about 20-fold less than that of the monosaccharide treated by nitrous acid at pH 4.0. Because the appropriate oligosaccharide standards containing the GlcNH23S±6S residue are not available, we cannot completely mimic the low pH nitrous degradation of the GlcNH23S±6S residue within Tetra A and Tetra B and within 3-OST-3A-modified HS polysaccharide. Nevertheless, the susceptibility of glucosamine 3-O-sulfate monosaccharide to low pH nitrous acid degradation suggests that the GlcNH23S±6S residue within the tetrasaccharides and HS polysaccharides is susceptible to low pH nitrous acid treatment. Taken together, our data suggest that the specificity of low pH nitrous degradation toward 3-O-sulfated glucosamine residue is not as stringent as the specificity toward 6-O-sulfated or nonsulfated glucosamine.

The content of GlcNH2 in heparan sulfate is usually very low. Toida et al. (28) have demonstrated that GlcNH2 makes up 1–7% (w/w) of the HS isolated from various bovine and porcine tissues. They have isolated a tetrasaccharide and a hexasaccharide containing GlcNH2 with a structure of GlcUA-GlcNS6S-GlcNH2 and GlcUA-GlcNS6S-GlcUA-GlcNAc, respectively, from heparitinase I-digested porcine intestinal mucosa heparan sulfate (boldface letters are used to emphasize the positions of the GlcNH2 residues within the oligosaccharides). Their data suggest that the GlcNH2 is bracketed by a GlcUA on the reducing and nonreducing end, and the GlcNH2 residue is located in the boundary between low and high sulfated regions (28). However, our data indicated that the GlcNH2 residue sulfated by 3-OST-3A is in a highly sulfated domain containing 1.5 and 2.0 sulfate groups per disaccharide, respectively, which is as much as 2.5- to 3.0-fold of the average sulfation level in the HS from 33-cells (23). Furthermore, the

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5 Glucosamine 3-O-sulfate (100 µg, from Sigma) was incubated with nitrous acid at high (pH 4.0) and low pH (pH 1.5) as described under “Experimental Procedures” followed by sodium [3H]borohydride (2 mCi, 25 mCi/mmol) reduction. The products were desalted on Bio-Gel P-2. We obtained 3.8 × 104 cpm of [3H]-labeled monosaccharide from high pH nitrous acid-treated glucosamine 3-O-sulfate and 3.6 × 104 cpm of [3H]-labeled monosaccharide from low pH nitrous acid-treated glucosamine 3-O-sulfate. The [3H]-labeled monosaccharide (about 9,000 cpm) was analyzed on RPIP-HPLC using an elution condition described in our prior publication (17). We have found that nearly 95% of [3H] counts from high pH nitrous acid-treated glucosamine 3-O-sulfate is [3H]AnMan3S, as judged by co-eluting with the standard on RPIP-HPLC. We have also found that about 5% of [3H] counts from low pH nitrous acid-treated glucosamine 3-O-sulfate is [3H]AnMan3S, and 95% of the [3H] counts was eluted at nonsulfated monosaccharide region. The [3H]-labeled material that was eluted at nonsulfated monosaccharide is probably the ring contraction by-product (34).
GlCNH₂ residue sulfated by 3-OST-3A is linked by Id0UA2S at the nonreducing end. These structural differences around the GlCNH₂ residue are probably due to the following factors: 1) different fine structures are present in the HS from different tissues and species (35); and 2) 3-OST-3A merely sulfates a minor portion of the total GlCNH₂ residues, suggesting that GlCNH₂ is present in multiple complex structural contexts in HS. Indeed, heparin from porcine intestinal mucosa is not a substrate for 3-OST-3A (Table II), despite the fact that heparin contains a small percentage of GlCNH₂ residues (36).

The unique tetrasaccharide structures isolated from 3-OST-3A-modified HS suggest that the enzyme recognizes specific oligosaccharide precursor structures containing GlCNH₂ residues. The mechanism for the biosynthesis of GlCNH₂ has not been established. However, Van den Born et al. (37) have postulated that GlCNH₂ is the by-product of heparan sulfate N-deacetylase/N-sulfotransferase modification. Because the enzyme exhibits both N-deacetylation and N-sulfation activities, it may exert only one activity under certain conditions, such as low concentration of PAPS (38–40). However, it is also possible that a special isoform of heparan sulfate N-deacetylase/N-sulfotransferase might be responsible for synthesizing GlCNH₂ given that several isoforms have been reported (5, 6, 41, 42). Alternatively, a unique endolytic sulfamidase (N-sulfatase) hydrolyzes the sulfate from the GlCN residue in a specific saccharide sequence to form GlCNH₂, as suggested by Van den Born et al. (37). In any of the above cases, our observation indicates that a portion of the GlCNH₂ is probably located in a defined oligosaccharide sequence that serves as a precursor for sulfation by 3-OST-3A. It suggests that the generation of GlCNH₂ residues is a preprogrammed process during the biosynthesis of HS. It is interesting to note that the GlCNH₂6S residue was also found to be a part of a precursor structure of 3-OST-3A. The synthesis of the GlCNH₂6S may also require a special isoform of heparan sulfate 6-O-sulfotransferase.

The tetrasaccharide precursor structures for heparan sulfate 3-OST-1 have been identified to be ΔUA-GlcNAc=6S-ΔUA-GlcNS=6S by Zhang et al. (16). Comparing these tetrasaccharides with the structures sulfated by 3-OST-3A, we have noted that these two isoforms sulfate completely different oligosaccharide structures. However, the amino acid sequence of the sulfotransferase domains of 3-OST-1 and 3-OST-3A is 60% homologous. Therefore, it is obviously important to understand which part of the enzyme molecule dictates the substrate specificities of heparan sulfate 3-O-sulfotransferases.

HS with GlCNH₂ residues has been demonstrated to be involved in L-selectin binding. It has been reported that heparinase-sensitive polysaccharide from calf pulmonary artery endothelium is responsible for binding to L-selectin (43). Subsequently, it has been reported that the level of GlCNH₂ is enriched in L-selectin binding HS (44). An N-unsubstituted glucosamine residue is within the L-selectin-binding site, because high pH (pH 4.0) nitrous acid-treated HS has significantly reduced affinity for L-selectin. It is also very interesting to note that HS with GlCNH₂ residues has been identified in rat kidney, and the GlCNH₂ can be specifically recognized by the monoclonal antibody JM 403 (37). This antibody has been reported to be able to affect the permeability of glomeruli, which might be caused by blocking a specific saccharide sequence within HS (45). Neither the L-selectin nor JM 403 antibody-binding sites has been characterized so far. Because 3-OST-3A sulfates GlCNH₂ residues, it will be extremely interesting to determine whether 3-OST-3A modification of HS leads to the formation of L-selectin-binding sites or affects the permeability of the glomeruli.