Structural Studies of Octasaccharides Derived from the Low-Sulfated Repeating Disaccharide Region and Octasaccharide Serines Derived from the Protein Linkage Region of Porcine Intestinal Heparin†

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ABSTRACT: Four octasaccharide serines and three octasaccharides were isolated after heparinase treatment of porcine intestinal heparin. Their structures were characterized by enzymatic digestion in conjunction with HPLC and 500 MHz ^{1}H NMR spectroscopy. Three of the four octasaccharide serines were structurally identical with those isolated previously, whereas one has the unreported structure ΔHexA(2-sulfate)α1−4GlcN(*N*-sulfate)α1−4GlcAβ1−4GlcNAcα1−4GlcAβ1−3Galβ1−3Galβ1−4Xylβ1-*O*-Ser (ΔHexA, GlcN, IdceA, and GlcA represent 4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid, D-glucosamine, L-iduronic acid, and D-glucuronic acid, respectively). The other three octasaccharides were isolated for the first time as discrete structures and shared the common core hexasulfated sequence ΔHexA(2-sulfate)α1−4GlcN(*N*-sulfate)α1−4IdceAα1−4GlcNAcα1−4GlcAβ1−4GlcN(*N*-sulfate)α1−4IdceA(2-sulfate)α1−4GlcN(*N*,6-disulfate) with one or two additional sulfate groups. The octasaccharides which were derived from the low-sulfated repeating disaccharide region of heparin contained the common trisaccharide sequence −4IdceAα1−4GlcNAcα1−4GlcAβ1− [Yamada, S., Yamane, Y., Tsuda, H., Yoshida, K., and Sugahara, K. (1998) *J. Biol. Chem. 273,* 1863−1871], suggesting the programmed biosynthesis of heparin. These octasaccharides are the largest oligosaccharides isolated so far from the low-sulfated irregular region of heparin. Since oligosaccharides larger than a pentasaccharide appear to potentially exhibit binding activities toward growth factors or other functional proteins, they will be useful for investigating the structural requirement for molecular interactions between heparin and/or heparan sulfate and biologically active proteins. During the course of the present structural studies, we evaluated the NMR data accumulated thus far on heparin oligosaccharides and found several interesting rules on chemical shifts of proton signals affected by the neighboring sugar residues and their sulfation, which will be in turn useful for determining structures of unknown heparin and/or heparan sulfate oligosaccharides based on the proton resonances.

Heparin is a glycosaminoglycan composed of 1−4 linked glucosamine and uronic acid residues which are variably sulfated (for reviews, see refs *1*−*3*). The biological roles of heparin are highly diversified, and most of them depend on interactions between heparin and various protein ligands such as growth factors, extracellular matrix proteins, and serine protease inhibitors (for a review, see ref *4*). Binding of proteins to heparin is usually electrostatic in nature, but other types of interactions (lock-and-key binding) may occur as in the cases of antithrombin III (*5*) and basic fibroblast growth factor (*6*), both of which bind with high affinity and specificity to particular pentasaccharide sequences in heparin. Several research groups have attempted to define the specific structural sequences of heparin that provide binding to various heparin-binding ligands. Although minimal heparin sequences required for binding to some proteins have been defined, additional requirements seem to be needed for expressing their biological activities. In fact, some reports suggested that the heparin sequence required to elicit biological actions of antithrombin III (*7*) or basic fibroblast growth factor (*8*) was comprised of ∼18 or ∼12 monosaccharide units, respectively, and thus is more extended than the minimal sequence required for binding.

We have been investigating the basic primary structure of heparin to clarify the structural basis of its various biological activities. Previously, we characterized a number of tetrasaccharides (*9*−*12*) and hexasaccharides (*13*, *14*) from the repeating disaccharide region of heparin and heparan sulfate after extensive digestion with bacterial heparin lyases. To extend the structural studies, we isolated and characterized oligosaccharides larger than the above hexasaccharides. We report herein the isolation and characterization of heparin octasaccharides derived from the protein linkage region and the repeating disaccharide region. Notably, the octasaccharides from the latter region contained hitherto unreported structures. Although several oligosaccharides larger than

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hexasaccharide derived from the repeating disaccharide region of heparin have been isolated and characterized (for a review, see ref *15*), their structural variations are limited and divided into only two groups, one containing the oligosaccharides isolated from the highly sulfated regular region composed of the continuing trisulfated disaccharide unit, −4IdceA(2S)α1−4GlcN(NS,6S)α1−[1] (*16-19*), and the other containing the oligosaccharides from the antithrombin III-binding region carrying the unique GlcN 3-*O*-sulfate (*20*−*23*). In this study, we isolated hitherto unreported octasaccharides from the low-sulfated irregular region of heparin and successfully determined their structures.

During this work, we employed ^{1}H NMR spectroscopy to define the structure of heparin octasaccharides. NMR spectroscopy is a powerful method for characterizing carbohydrate structures (for a review, see ref *24*). Structural parameters, including sugar content, ring conformation, glycosidic linkage, and sulfation pattern, can be determined from analysis of chemical shifts and coupling constants. We have so far made ^{1}H NMR assignments for a large number of heparin-derived oligosaccharides, including tri-, tetra-, penta-, hexa-, and octasaccharides. On the basis of these data, we defined several interesting rules on chemical shifts of proton signals affected by the neighboring sugar residues and their sulfation.

EXPERIMENTAL PROCEDURES

Enzymes and Oligosaccharides. Stage 14 heparin was purchased from American Diagnostica and purified by DEAE-cellulose chromatography as reported previously (*25*). Heparinase (EC 4.2.2.7) and purified heparitinases I (EC 4.2.2.8) and II (no EC number) were obtained from Seikagaku Corp. (Tokyo, Japan). $\Delta^{4,5}$-Glycuronate-2-sulfatase (EC 3.1.6), abbreviated as 2-sulfatase, was purified from *Flavobacterium heparinum* (*26*). The octasaccharide fraction was prepared from stage 14 heparin after heparinase digestion as reported previously (*12*).

Digestion of the Isolated Oligosaccharides with Heparinase, Heparitinases, or $\Delta^{4,5}$-Glycuronate-2-sulfatase. Each isolated oligosaccharide (0.5−1.0 nmol) was digested using 1−5 mIU of heparinase, heparitinase I and/or II, or 2-sulfatase as described previously (*10*, *13*, *25*). Successive enzymatic digestion of a given oligosaccharide with 2-sulfatase and then heparitinase I or II was also carried out as reported previously (*12*). Successive enzymatic digestion of the isolated octasaccharides with heparitinase I and then 2-sulfatase was carried out as follows; each octasaccharide (2.0 nmol) was first incubated with 2 mIU of heparitinase I for 3 h at 37 °C in a total volume of 50 μL of 20 mM acetate/NaOH buffer (pH 7.0) containing 2 mM $Ca(OAc)_2$. One-half (25 μL) of the reaction mixture was analyzed by HPLC on an amine-bound silica column. The other half was mixed with 155 μL of water and 20 μL of 20 mM acetate/NaOH buffer (pH 6.5) containing 0.15% bovine serum albumin and 1 mIU of 2-sulfatase, and then incubated at 37 °C for 2 h. Reactions were terminated by boiling for 1 min, and the reaction mixture was analyzed by HPLC as reported previously (*25*).

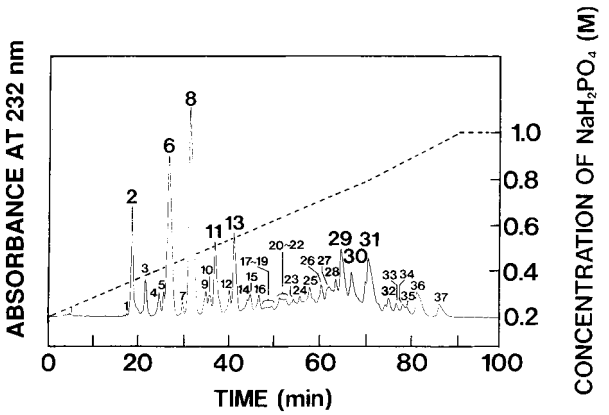

FIGURE 1: HPLC fractionation of the octasaccharide fraction. The octasaccharide fraction obtained from gel filtration on Bio-Gel P-10 was separated into subfractions 5-1−5-37 on an amine-bound silica column using an NaH_2PO_4 gradient (indicated by the dashed line). For experimental details, see Experimental Procedures.

^{1}H NMR Spectroscopy (500 MHz). Oligosaccharides for NMR analysis were fully sodiated using a Dowex 50-X8 (Na^+ form) column (7 mm × 18 mm) and then repeatedly exchanged in ^2H_2O with intermediate lyophilization. ^{1}H NMR spectra (500 MHz) of hexasaccharides were measured on a Varian VXR-500 at a probe temperature of 26 or 60 °C as reported previously (*9*, *27*). Two-dimensional (2D) nuclear Overhauser enhancement spectroscopy (NOESY) experiments were performed with a mixing time of 150 ms (*27*). Chemical shifts are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured indirectly relative to acetone (δ 2.225) in ^2H_2O (*24*).

Other Analytical Methods. Uronic acid was assessed with the carbazole method (*28*). Unsaturated uronic acid was spectrophotometrically quantified on the basis of an average millimolar absorption coefficient of 5.5 at 232 nm (*29*). Amino acids and amino sugars were quantified after acid hydrolysis in 6 M HCl at 110 °C for 20 h or 3 M HCl at 100 °C for 16 h, respectively, using a Beckman 6300E amino acid analyzer (*30*). Capillary electrophoresis was carried out to examine the purity of each isolated fraction in a Waters capillary ion analyzer as reported previously (*11*).

RESULTS

Isolation of the Oligosaccharides. Purified stage 14 heparin from porcine intestine was exhaustively digested with heparinase and fractionated into fractions 1−8 by gel filtration on a column of Bio-Gel P-10 (*12*). Fractions 6−8, which contained hexa-, tetra-, and disaccharides, respectively, were characterized previously (*12*, *14*). In this study, the octasaccharide fraction, fraction 5, was characterized. It was subfractionated by HPLC on an amine-bound silica column into fractions 5-1 and 5-37, as indicated in Figure 1. Eight major fractions, 5-2, -6, -8, -11, -13, -29, -30, and -31, were further purified by rechromatography. They altogether accounted for 60 mol % (as quantified on the basis of ΔHexA) of the oligosaccharides obtained from fraction 5. Fractions 5-6 and -11 turned out to contain dermatan sulfate-derived oligosaccharides (*31*). In this article, analyses of the other fractions are described. Although these individual fractions gave a single peak on HPLC, fractions 5-2, -13, -29, -30,

[1] Abbreviations: COSY, correlation spectroscopy; HOHAHA, homonuclear Hartmann−Hahn; NOESY, nuclear Overhauser enhancement spectroscopy; 2D, two-dimensional; GlcA, D-glucuronic acid; IdceA, L-iduronic acid; HexA, hexuronic acid; ΔHexA, 4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid; NS, 2-*N*-sulfate; 2S, 2-*O*-sulfate; 3S, 3-*O*-sulfate; 6S, 6-*O*-sulfate.

Table 1: Disaccharide Composition Analysis of the Isolated Oligosaccharides[a]

fraction	yield[b]	digestion products
5-2	202	ΔHexA-GlcNAc (104%), ΔHexA(2S)-GlcN(NS) (125%), and ΔHexA-Gal-Gal-Xyl-Ser (110%)
5-8	687	ΔHexA-GlcNAc (96%), ΔHexA(2S)-GlcN(NS,6S) (104%), and ΔHexA-Gal-Gal-Xyl-Ser (101%)
5-13	208	ΔHexA-GlcNAc(6S) (88%), ΔHexA(2S)-GlcN(NS,6S) (94%), and ΔHexA-Gal-Gal-Xyl-Ser (92%)
5-29	262	ΔHexA(2S)-GlcNAc (126%), ΔHexA-GlcN(NS) (126%), ΔHexA(2S)-GlcN(NS) (122%), and ΔHexA(2S)-GlcN(NS,6S) (105%)
5-30	232	ΔHexA-GlcNAc(6S) (101%), ΔHexA-GlcN(NS) (129%), and ΔHexA(2S)-GlcN(NS,6S) (224%)
5-31	353	ΔHexA(2S)-GlcNAc (105%), ΔHexA-GlcN(NS) (105%), and ΔHexA(2S)-GlcN(NS,6S) (256%)

[a] Each oligosaccharide fraction was incubated with a mxture of heparitinases I and II, and the reaction products were characterized by HPLC. Recoveries of the disaccharides were calculated on the basis of the absorption at 232 nm, taking the absorbance of the parent oligosaccharide(s) in each fraction to be 100%, and are shown in parentheses. [b] Nanomoles per 100 mg of heparin.

and -31 were 94, 94, 76, 85, and 73% pure, respectively, when examined by capillary electrophoresis (data not shown). Fraction 5-8 was resolved into two fractions, 5-8-I and -II, by capillary electrophoresis as described below.

Enzymatic Characterization of the Isolated Oligosaccharides. The disaccharide composition of the isolated oligosaccharides was determined by digestion with heparitinase I and/or II, followed by HPLC analysis on an amine-bound silica column. Fractions 5-29, -30, and -31 were degraded into disaccharides by the enzymes, whereas fractions 5-2, -8, and -13 were degraded into approximately 2 mol of disaccharide units and 1 mol of a tetrasaccharide serine derived from the glycosaminoglycan−protein linkage region. Those obtained with fractions 5-8 and -31 are shown as representative chromatograms in Figure 2, and the results are summarized in Table 1. The sequential arrangement of the resultant disaccharide units in each parent oligosaccharide was determined on the basis of the results of enzymatic digestions using heparitinases I and II and $\Delta^{4,5}$-glycuronate-2-sulfatase as well as using 500 MHz ^{1}H NMR analysis.

^{1}H NMR Analysis (500 MHz). All the individual oligosaccharides were analyzed by 500 MHz ^{1}H NMR to confirm the structures proposed by enzymatic analysis. Chemical shifts were assigned by 2D homonuclear Hartmann−Hahn (HOHAHA) and correlation spectroscopy (COSY) analyses as reported for the sulfated oligosaccharides isolated previously from heparin (*9*) and heparan sulfate (*11*). The internal uronic acid residues of each isolated oligosaccharide were unambiguously identified on the basis of the chemical shifts of the anomeric proton signals and the coupling constants $J_{1,2}$. Anomeric proton signals of an αIdceA and a βGlcA residue in heparin and/or heparan sulfate oligosaccharides are observed around δ 5.2−5.0 and 4.7−4.5, respectively (*12*, *32*). The coupling constants $J_{1,2}$ of αIdceA and βGlcA in heparin and/or heparan sulfate oligosaccharides are approximately 3.0 and 8.0 Hz, respectively (*12*, *17*). The saccharide sequences were corroborated by NOESY. The NMR data obtained in this study for the oligosaccharides are summarized in Tables 2 and 3.

Fraction 5-2. As shown in Table 4, amino acid analysis showed that fraction 5-2 contained approximately 1 mol of Ser per mole of ΔHexA. The disaccharide analysis of fraction 5-2 was performed by digestion with heparitinases I and II followed by HPLC. The exhaustive digestion with heparitinases I and II gave rise to equimolar amounts of three unsaturated components, ΔHexA-GlcNAc, ΔHexA(2S)-GlcN(NS), and a nonsulfated glycoserine, ΔHexA-Gal-Gal-Xyl-Ser (glycoserine I in ref *25*) (Figure 2A). These results indicate that fraction 5-2 contains a disulfated octasaccharide serine. Digestion of this fraction with heparitinase II alone

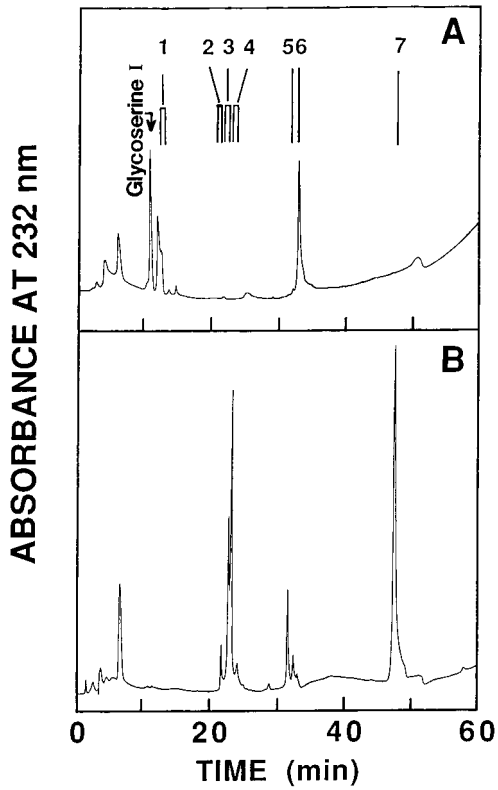

FIGURE 2: HPLC analysis of the heparitinase I and/or II digests of isolated oligosaccharide fractions 5-2 and 5-30. Isolated oligosaccharide fractions 5-2 and 5-30 were digested with heparitinase I and/or II and fractionated by HPLC on an amine-bound silica column: (A) the digest of fraction 5-2 (0.3 nmol) with a mixture of heparitinases I and II and (B) the heparitinase II digest of fraction 5-30 (0.5 nmol). Elution positions of the standard disaccharides isolated from heparin and/or heparan sulfate are indicated at the top of panel A: 1, ΔHexAα1−4GlcNAc; 2, ΔHexAα1−4GlcNAc-(6S); 3, ΔHexA(2S)α1−4GlcNAc; 4, ΔHexAα1−4GlcN(NS); 5, ΔHexAα1−4GlcN(NS,6S); 6, ΔHexA(2S)α1−4GlcN(NS); and 7, ΔHexA(2S)α1−4GlcN(NS,6S). Glycoserine I is the tetrasaccharide serine ΔHexAα1−3Galβ1−3Galβ1−4Xylβ1-*O*-Ser reported previously (*25*). The peaks observed around 5 min were derived from the incubation buffer. For the details of the HPLC conditions, see Experimental Procedures.

produced equimolar amounts of two components, ΔHexA-(2S)-GlcN(NS) and one that eluted near the elution position of the nonsulfated hexasaccharide, ΔHexA-GlcNAc-GlcA-Gal-Gal-Xyl, derived from the glycosaminoglycan−protein linkage region of bovine kidney heparan sulfate (*11*). Hence, the saccharide sequence of the compound in fraction 5-2 is deduced to be ΔHexA(2S)-GlcN(NS)-HexA-GlcNAc-HexA-Gal-Gal-Xyl-Ser. The sequence was confirmed, and the types of internal uronic acid residues were identified by 500 MHz ^{1}H NMR analysis. In the spectrum of fraction 5-2 (results

Table 2: ^{1}H-Chemical Shifts of Structural-reporter-groups of the Monosaccharide Constituents of the Linkage Glycoserines[a]

Residue	Reporter Group	fraction 5-2	fraction 5-8-I	fraction 5-8-II	fraction 5-13
		▲◑□○▭[b]	▲●◇○▭	▲●□○▭	▲●◇◑▭
Ser	Hα	4.220	4.239	4.239	4.178
	Hβ	3.920	3.976	3.976	3.958
	Hβ'	4.000	4.027	4.027	3.958
Xyl-**1**	H1	4.459 (8.0)	4.464 (7.5)	4.464 (7.5)	4.450 (8.0)
	H2	3.352	3.364	3.364	3.362
	H3	3.605	3.605	3.605	3.611
	H4	3.870	3.862	3.862	3.862
	H5ax	3.407	3.400	3.400	3.406
	H5eq	4.119	4.112	4.112	4.114
Gal-**2**	H1	4.531 (8.0)	4.531 (8.0)	4.531 (8.0)	4.532 (8.0)
	H2	3.678	3.681	3.681	3.673
	H3	3.83	3.820	3.820	3.820
	H4	4.189	4.180	4.180	4.188
Gal-**3**	H1	4.658 (8.0)	4.663 (8.0)	4.663 (8.0)	4.662 (7.5)
	H2	3.74	3.746	3.746	3.735
	H3	3.78	3.782	3.782	3.786
	H4	4.155	4.145	4.145	4.160
GlcA-**4**	H1	4.643 (8.0)	4.644 (8.0)	4.644 (8.0)	4.642 (8.0)
	H2	3.404	3.400	3.400	3.416
	H3	3.665	3.678	3.678	3.665
	H4	3.719	3.75	3.75	3.751
GlcNAc-**5**	H1	5.367 (3.5)	5.336 (4.0)	5.359 (3.0)	5.353 (4.0)
	H2	3.862	3.882	3.88	3.913
	H3	3.80	3.782	ND	3.757
	H4	ND[c]	ND	ND	3.70
	H5	ND	ND	ND	3.995
	H6	ND	ND	ND	ND
	H6'	ND	ND	ND	ND
	NAc	2.034	2.033	2.033	2.034
HexA-**6**	H1	4.515 (8.0)	4.929 (2.5)	4.515 (8.0)	4.989 (2.0)
	H2	3.384	3.702	3.391	3.752
	H3	ND	4.088	ND	4.118
	H4	ND	4.059	ND	4.055
	H5	ND	ND	ND	ND
GlcN-**7**	H1	5.570 (4.0)	5.384 (4.0)	5.566 (3.5)	5.345 (4.0)
	H2	3.275	3.274	3.296	3.262
	H3	ND	3.602	3.617	3.617
	H4	ND	3.812	3.81	3.815
	H5	ND	3.977	3.967	3.969
	H6	ND	4.34	ND	ND
	H6'	ND	4.20	ND	ND
	NAc	—[d]	—	—	—
ΔHexA-**8**	H1	5.516 (1.0)	5.485 (2.0)	5.485 (2.0)	5.488 (2.5)
	H2	4.589	4.610	4.610	4.612
	H3	4.233	4.309	4.309	4.307
	H4	5.991	5.971	5.971	5.971

[a] ^{1}H-Chemical shifts of structural-reporter-groups of the monosaccharide constituents of the four linkage oligosaccharides attached to serine are shown. Chemical shifts are given in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured indirectly relative to acetone (δ 2.225 ppm) in ^2H_2O at 26°C (*24*). The estimated error for the values to two decimal places was only ± 0.01 ppm because of partial overlap of signals. That for the values to three decimal places was ± 0.002 ppm. Coupling constants $J_{1,2}$ (in Hz) are given in parentheses.

[b] Symbols represent the following: closed triangle, ΔHexA(2S); open square, GlcA; open diamond, IdceA; open circle, GlcNAc; closed circle, GlcN(NS,6S); circle half-closed on the left side, GlcNAc(6S); circle half-closed on the right side, GlcN(NS); open rectangle, GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-*O*-Ser.

[c] ND, not determined.

[d] —, not occurring.

not shown), two internal uronic acid residues, HexA-**4** and HexA-**6**, were identified as GlcA on the basis of the chemical shift of the anomeric proton signals (δ 4.643 and 4.515, respectively) and the coupling constants $J_{1,2}$ (8.0 and 8.0 Hz, respectively) (Table 2). The structure of the major compound in fraction 5-2 was consistent with the following disulfated octasaccharide serine: fraction 5-2, ΔHexA(2S)α1−4GlcN(NS)α1−4GlcAβ1−4GlcNAcα1−4GlcAβ1−3Galβ1−3Galβ1−4Xylβ1-*O*-Ser.

Fraction 5-8. Fraction 5-8 was resolved into two fractions, 5−8-II and -I, in a molar ratio of 1.0:3.3 when it was examined by capillary electrophoresis (results not shown), indicating that it contains two components with similar structures. However, it could not be separated into each component when chromatographed in a preparative scale and was therefore analyzed without further purification. As shown in Table 4, amino acid analysis showed that fraction 5-8 contained approximately 1 mol of Ser per mole of ΔHexA. The disaccharide analysis of this fraction, carried out by the exhaustive digestion with heparitinases I and II, gave rise to equimolar amounts of ΔHexA-GlcNAc, ΔHexA(2S)-GlcN(NS,6S), and glycoserine I. These results indicate that fraction 5-8 most likely contained two isomeric octasaccharide serines. Heparitinase II digestion of this fraction yielded equimolar amounts of two components, i.e., ΔHexA(2S)-GlcN(NS,6S) and a component eluted at the position of the nonsulfated hexasaccharide serine, ΔHexA-GlcNAc-GlcA-Gal-Gal-Xyl-Ser. Hence, the two compounds in fraction 5-8 share the common saccharide sequence ΔHexA(2S)-GlcN(NS,6S)-HexA-GlcNAc-HexA-Gal-Gal-Xyl-Ser with only a subtle structural difference. The 500 MHz ^{1}H NMR spectrum of fraction 5-8 shows two sets of signals differing in intensity, reflecting the presence of two subcomponents, fractions 5-8-I and -II, in an approximately 3:1 ratio (data not shown). The spectral data of fractions 5-8-I and -II (Table 2) were compared with those of fractions **b**-5-I and -II obtained previously from porcine intestinal heparin (*27*). No significant differences in the spectra were observed between the two sets of subcomponents, indicating that the structures of the two major compounds in fraction 5-8 are as follows with different isomeric uronic acid residues, i.e., GlcA or IdceA as the sixth saccharide residue from the reducing ends: fraction 5-8-I, ΔHexA(2S)α1−4GlcN(NS,6S)α1−4IdceAα1−4GlcNAcα1−4GlcAβ1−3Galβ1−3Galβ1−4Xylβ1-*O*-Ser; and fraction 5-8-II, ΔHexA(2S)α1−4GlcN(NS,6S)α1−4GlcAβ1−4GlcNAcα1−4GlcAβ1−3Galβ1−3Galβ1−4Xylβ1-*O*-Ser.

Fraction 5-13. Amino acid analysis showed that fraction 5-13 contained approximately 1 mol of Ser per mole of ΔHexA (Table 4). Upon HPLC analysis of the double enzyme digestion of this fraction with heparitinases I and II, equimolar amounts of three UV-absorbing peaks of glycoserine I, ΔHexA-GlcNAc(6S), and ΔHexA(2S)-GlcN(NS,6S) were observed, indicating that the major compound in fraction 5-13 was a tetrasulfated octasaccharide serine. When digested with heparitinase II, this compound yielded equimolar amounts of ΔHexA(2S)-GlcN(NS,6S) and a component eluted at the position of the monosulfated glycoserine, ΔHexA-GlcNAc(6S)-HexA-Gal-Gal-Xyl-Ser (glycoserine II previously reported in ref *25*), indicating that the trisulfated disaccharide unit, ΔHexA(2S)-GlcN(NS,6S), is located at the nonreducing terminus. Hence, the saccharide sequence of the compound in fraction 5-13 is ΔHexA(2S)-GlcN(NS,6S)-HexA-GlcNAc(6S)-HexA-Gal-Gal-Xyl-Ser. The sequence was confirmed and the type of the internal uronic acid residues was identified by 500 MHz ^{1}H NMR analysis.

The spectral data for fraction 5-13 (Table 2) were compared with those of fraction **b**-10S-II, which was obtained previously from porcine intestinal heparin after sequential digestion with heparinase and then 2-sulfatase (*27*). No significant differences were observed in the spectra except for the downfield shifts of the signals of especially H-2 belonging to the nonreducing terminal ΔHexA residue. This indicates that the major compound in fraction 5-13 has an additional sulfate group on the C-2 hydroxyl group of the nonreducing ΔHexA residue in the structure for the compound in fractions **b**-10S-II. Therefore, the structure of the compound in fraction 5-13 is deduced to be the following: fraction 5-13, ΔHexA(2S)α1−4GlcN(NS,6S)α1−4IdceAα1−4GlcNAc(6S)α1−4GlcAβ1−3Galβ1−3Galβ1−4Xylβ1-*O*-Ser.

Table 3: ^{1}H-Chemical Shifts of the Constituent Monosaccharides of the Isolated Oligosaccharides Derived from Heparin[a]

Residue	Reporter group	fraction 5-29 ▲◑◆○□◑◆●[b] 8 7 6 5 4 3 2 1	fraction 5-30 ▲●◇◐□◑◆● 8 7 6 5 4 3 2 1	fraction 5-31 ▲●◆○□◑◆● 8 7 6 5 4 3 2 1
GlcN-**1**	H1	5.445 (4.0)	5.445 (3.5)	5.445 (4.0)
	H2	3.257	3.256	3.257
	H3	3.62	3.69	3.688
	H4	3.74	3.74	3.74
	H5	4.131	4.12	4.12
	H6	4.31 - 4.35	4.30 - 4.34	4.30 - 4.34
	H6'	4.31 - 4.35	4.30 - 4.34	4.30 - 4.34
	NAc	—[c]	—	—
HexA-**2**	H1	5.208 (2.0)	5.209 (3.0)	5.210 (3.5)
	H2	4.30	4.305	4.303
	H3	4.184	4.20	4.192
	H4	4.092	4.091	4.092
	H5	ND[d]	ND	ND
GlcN-**3**	H1	5.429 (3.5)	5.428 (3.5)	5.427 (3.5)
	H2	3.257	3.256	3.257
	H3	3.67	3.628	3.65
	H4	3.73	3.73	3.73
	H5	ND	3.96	ND
	H6	3.78 - 3.86	3.78 - 3.86	3.79 - 3.88
	H6'	3.78 - 3.86	3.78 - 3.86	3.79 - 3.88
	NAc	—	—	—
HexA-**4**	H1	4.485 (7.5)	4.487 (7.5)	4.487 (7.5)
	H2	3.374	3.378	3.374
	H3	3.696	3.68	3.696
	H4	3.73	3.75	3.73
	H5	ND	ND	ND
GlcN-**5**	H1	5.333 (3.5)	5.358 (3.5)	5.335 (3.0)
	H2	3.880	3.907	3.897
	H3	ND	ND	ND
	H4	3.86	3.73	3.85
	H5	ND	ND	ND
	H6	ND	4.31	ND
	H6'	ND	ND	ND
	NAc	2.034	2.036	2.034
HexA-**6**	H1	5.197 (3.5)	4.994 (2.0)	5.210 (3.5)
	H2	4.30	3.763	4.303
	H3	4.220	4.124	4.230
	H4	4.037	4.055	4.048
	H5	ND	ND	ND
GlcN-**7**	H1	5.333 (3.5)	5.342 (4.0)	5.329 (3.5)
	H2	3.257	3.262	3.284
	H3	3.67	3.66	3.647
	H4	3.78	3.82	3.82
	H5	ND	4.02	4.011
	H6	3.80 - 3.88	4.30	4.32
	H6'	3.80 - 3.88	4.23	4.23
	NAc	—	—	—
ΔHexA-**8**	H1	5.520 (2.0)	5.492 (2.5)	5.499 (3.0)
	H2	4.601	4.614	4.618
	H3	4.201	4.313	4.297
	H4	5.993	5.973	5.976

[a]Chemical shifts are given in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured indirectly relative to acetone (δ 2.225 ppm) in ^2H_2O at 26°C (*24*). The estimated error for the values to two decimal places was only ± 0.01 ppm because of partial overlap of signals. That for the values to three decimal places was ± 0.002 ppm. Coupling constants $J_{1,2}$ (in Hz) are given in parentheses.

[b]Symbols represent the following: closed triangle, ΔHexA(2S); open square, GlcA; open diamond, IdceA; closed diamond, IdceA(2S); open circle, GlcNAc; closed circle, GlcN(NS,6S); circle half-closed on the left side, GlcNAc(6S); circle half-closed on the right side, GlcN(NS).

[c]—, not occurring.

[d]ND, not determined.

Table 4: Amino Acid Analysis of the Fractions Isolated from the Linkage Region

components	molar ratio fraction 5-2	fraction 5-8	fraction 5-13
ΔHexA	1.00	1.00	1.00
Ser[a]	0.80	0.80	1.09
other amino acids	<0.1	<0.1	<0.1

[a] Values have been corrected for the degradation (5.3%) during acid hydrolysis, which was determined using authentic glycoserine I, and are expressed relative to that for ΔHexA determined on the basis of the absorbance at 232 nm.

Fraction 5-29. Upon HPLC analysis of the heparitinase II digest of this fraction, four major UV-absorbing peaks of ΔHexA-GlcN(NS), ΔHexA(2S)-GlcNAc, ΔHexA(2S)-GlcN-(NS), and ΔHexA(2S)-GlcN(NS,6S) were observed with recoveries of 126, 126, 122, and 105%, respectively, taking the UV absorbance of the parent oligosaccharide in fraction 5-29 to be 100%. These results indicate that the major compound in fraction 5-29 is a heptasulfated octasaccharide composed of equimolar amounts of the four disaccharide constituents corresponding to the above unsaturated units. When digested successively with 2-sulfatase and then heparitinase II, it yielded ΔHexA-GlcN(NS), ΔHexA(2S)-GlcNAc, and ΔHexA(2S)-GlcN(NS,6S) in a molar ratio of 2:1:1 (data not shown), indicating that ΔHexA(2S)-GlcN-(NS) was located at the nonreducing terminus. Heparitinase I digestion of the parent fraction resulted in equimolar

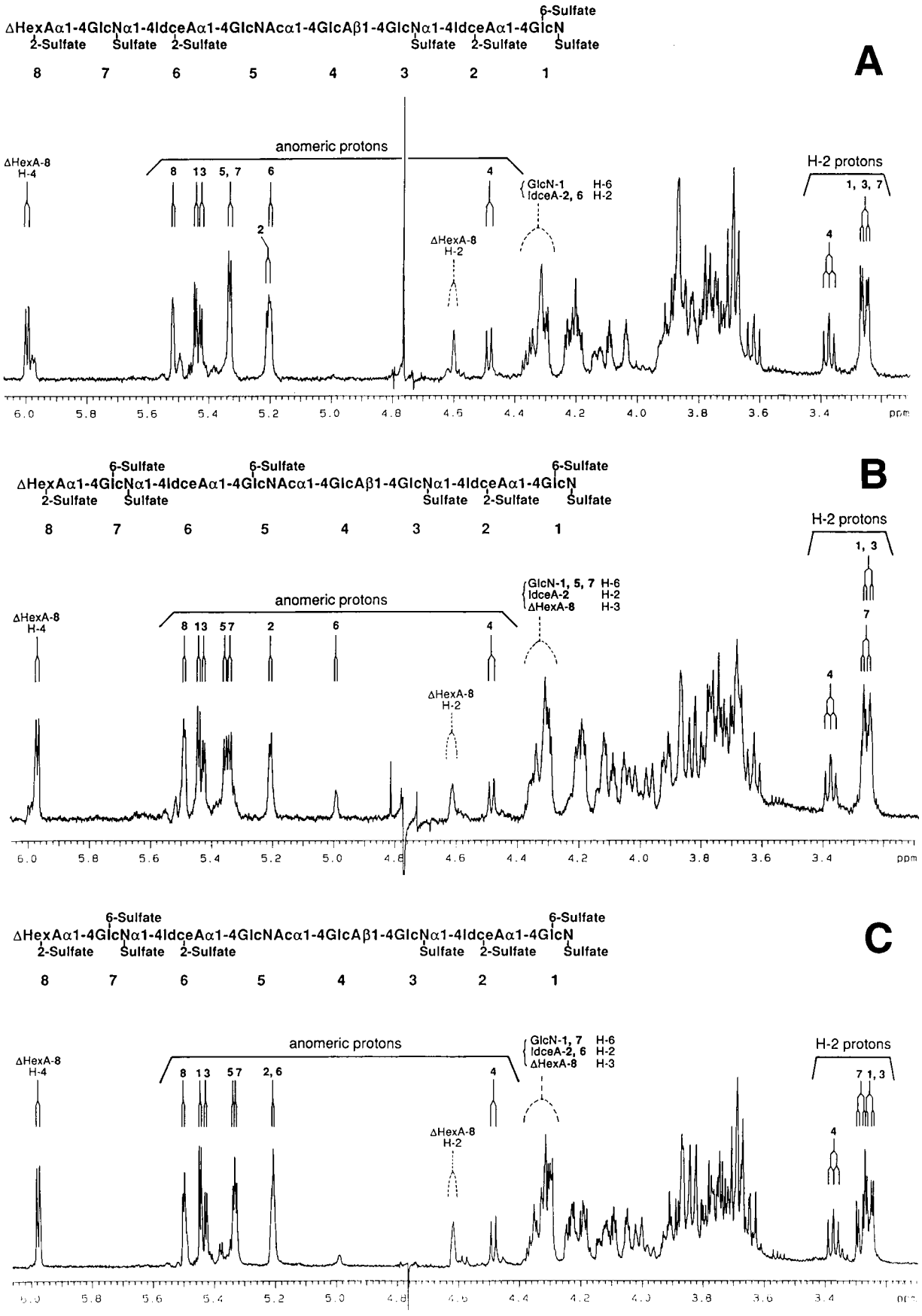

FIGURE 3: One-dimensional 500 MHz ^1H NMR spectra of fractions 5-29, -30, and -31 recorded in ^2H_2O at 26 °C: (A) fraction 5-29, (B) fraction 5-30, and (C) fraction 5-31. The numbers and letters in the spectra refer to the corresponding residues in the structures.

amounts of two unsaturated components that eluted near the elution positions of the tri- and tetrasulfated tetrasaccharides, respectively. Upon successive digestion with heparitinase I and then 2-sulfatase, the presumable trisulfated tetrasaccha-

ride peak was shifted to a position corresponding to the loss of one sulfate group on HPLC, but the presumable tetrasulfated tetrasaccharide was resistant to the action of 2-sulfatase, indicating that the former was derived from the nonreducing side of the parent octasaccharide and that the disaccharide unit located on the nonreducing side of the latter tetrasaccharide was ΔHexA-GlcN(NS). Hence, the disaccharide unit derived from the reducing terminus of the parent octasaccharide was ΔHexA(2S)-GlcN(NS,6S). The above results together indicate that the saccharide sequence of the compound in fraction 5-29 is ΔHexA(2S)-GlcN(NS)-HexA(2S)-GlcNAc-HexA-GlcN(NS)-HexA(2S)-GlcN(NS,6S). The sequence was confirmed and the type of the internal uronic acid residues identified by 500 MHz ^{1}H NMR analysis.

The one-dimensional spectrum and the COSY spectrum of fraction 5-29 are shown in Figure 3A and 4A, respectively. Anomeric proton signals characteristic of 2-sulfated αIdceA and nonsulfated βGlcA were observed around δ 5.2 and 4.5, respectively, whereas no H-1 signals assigned to nonsulfated αIdceA or 2-sulfated βGlcA were observed. Their chemical shifts are known to be approximately 5.0 and 4.6−4.7, respectively (*11*, *12*). The NMR data summarized in Table 3 and the sequential arrangement of the disaccharide units determined by enzymatic analysis indicate that the two 2-sulfated IdceA residues are located at the second and sixth saccharide positions, and that the nonsulfated GlcA residue is located at the fourth saccharide position. Hence, the structure of the major compound in fraction 5-29 is as follows: fraction 5-29, ΔHexA(2S)α1−4GlcN(NS)α1−4IdceA(2S)α1−4GlcNAcα1−4GlcAβ1−4GlcN(NS)α1−4IdceA(2S)α1−4GlcN(NS,6S).

Fraction 5-30. Upon exhaustive heparitinase II digestion, fraction 5-30 yielded ΔHexA-GlcNAc(6S), ΔHexA-GlcN-(NS), and ΔHexA(2S)-GlcN(NS,6S) with recoveries of 101, 129, and 224%, respectively, taking the UV absorbance of the parent oligosaccharide in fraction 5-30 to be 100% (Table 1). This indicates that the major compound in fraction 5-30 is an octasulfated octasaccharide composed of 2 mol of trisulfated disaccharide units corresponding to ΔHexA(2S)-GlcN(NS,6S) and 1 mol each of two monosulfated disaccharide units corresponding to ΔHexA-GlcNAc(6S) and ΔHexA-GlcN(NS). When digested successively with 2-sulfatase and then heparitinase II, it yielded equimolar amounts of ΔHexA-GlcN(NS), ΔHexA-GlcNAc(6S), ΔHexA-GlcN-(NS,6S), and ΔHexA(2S)-GlcN(NS,6S), indicating that 1 mol of ΔHexA(2S)-GlcN(NS,6S) was located at the nonreducing terminus of the parent octasaccharide. Heparitinase I digestion of this fraction resulted in equimolar amounts of ΔHexA-GlcNAc(6S), ΔHexA(2S)-GlcN(NS,6S), and a presumable tetrasulfated tetrasaccharide fragment that was resistant to 2-sulfatase, indicating that the disaccharide unit at the nonreducing terminus of the tetrasaccharide did not contain 2-sulfated ΔHexA, and therefore was not ΔHexA(2S)-GlcN-(NS,6S) but was ΔHexA-GlcN(NS). Hence, the structure of the produced tetrasaccharide is ΔHexA-GlcN(NS)-HexA-(2S)-GlcN(NS,6S). The above results altogether indicate that the major compound in fraction 5-30 was an octasulfated octasaccharide with a saccharide sequence of either ΔHexA-(2S)-GlcN(NS,6S)-HexA-GlcNAc(6S)-HexA-GlcN(NS)-HexA(2S)-GlcN(NS,6S) or ΔHexA(2S)-GlcN(NS,6S)-HexA-GlcN(NS)-HexA(2S)-GlcN(NS,6S)-HexA-GlcNAc(6S). The

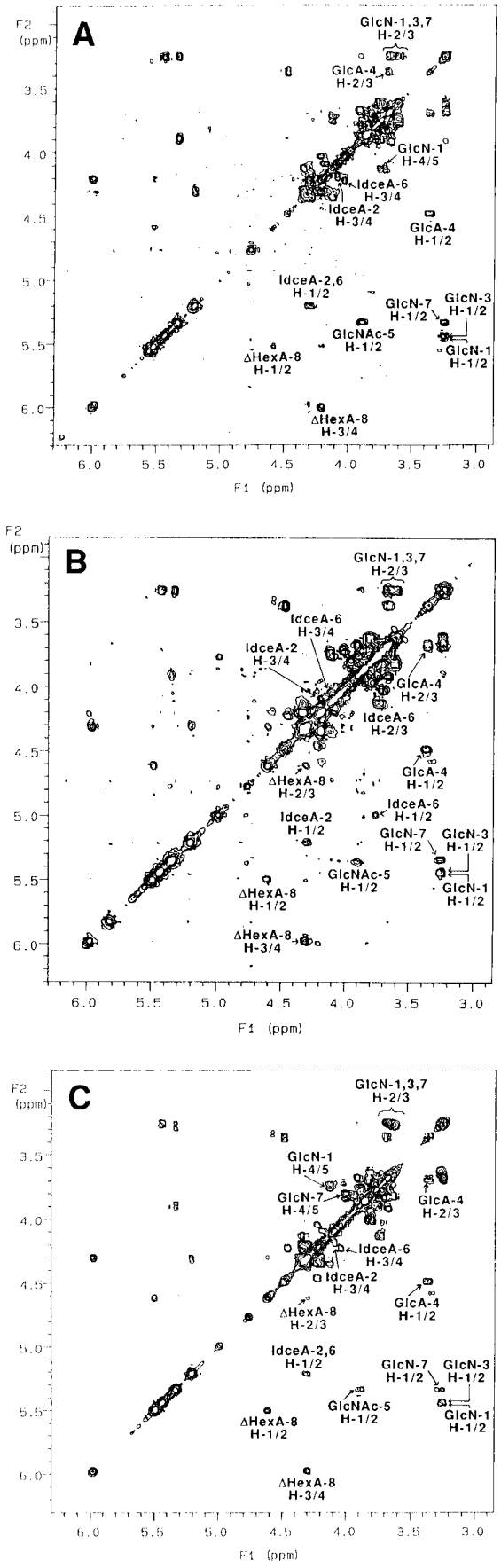

FIGURE 4: COSY spectra of fractions 5-29, -30, and -31 recorded in ^2H_2O at 26 °C: (A) fraction 5-29, (B) fraction 5-30, and (C) fraction 5-31.

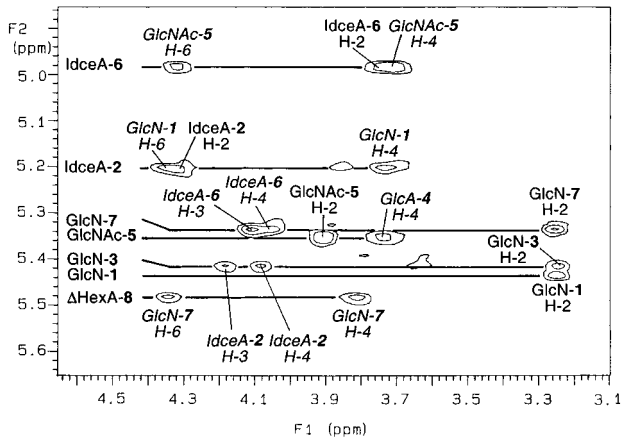

FIGURE 5: 2D NOESY spectrum of fraction 5-30 recorded in ^2H_2O at 26 °C. Anomeric protons are denoted with horizontal lines. Transglycosidic NOEs on the H-1 tracks are italic.

sequence and the type of the internal uronic acid residues were identified by 500 MHz ^{1}H NMR analysis.

The three internal uronic acid residues of the octasaccharide in fraction 5-30 were identified as GlcA, nonsulfated IdceA, and 2-sulfated IdceA, respectively, on the basis of the corresponding chemical shifts (δ 4.487, 4.994, and 5.209) of the anomeric proton signals and the coupling constants $J_{1,2}$ (7.5, 2.0, and 3.0 Hz) (Figures 3B and 4B and Table 3). The saccharide sequence of the major compound in this fraction was unequivocally established on the basis of the NOEs between ΔHexA-**8** H-1 and GlcN-**7** H-4/H-6, between GlcN-**7** H-1 and nonsulfated IdceA H-3/H-4, between nonsulfated IdceA H-1 and GlcNAc-**5** H-4/H-6, between GlcNAc-**5** H-1 and GlcA H-4, between GlcA H-1 and GlcN-**3** H-4/H-6, between GlcN-**3** H-1 and 2-sulfated IdceA H-3/H-4, and between 2-sulfated IdceA H-1 and GlcN-**1** H-4/H-6 (Figure 5). These NMR data and the data from the enzymatic analysis showed that the 2-sulfated IdceA, nonsulfated GlcA, and nonsulfated IdceA residues are located at the second, fourth, and sixth saccharide positions from the reducing terminus, respectively. Hence, the structure of the major compound in fraction 5-30 is as follows: fraction 5-30, ΔHexA(2S)α1−4GlcN(NS,6S)α1−4IdceAα1−4GlcNAc(6S)α1−4GlcAβ1−4GlcN(NS)α1−4IdceA(2S)α1−4GlcN(NS,6S).

Fraction 5-31. Upon exhaustive heparitinase II digestion, fraction 5-31 yielded ΔHexA(2S)-GlcNAc, ΔHexA-GlcN(NS), and ΔHexA(2S)-GlcN(NS,6S) with recoveries of 105, 105, and 256%, respectively, taking the UV absorbance of the parent oligosaccharide in fraction 5-31 to be 100% (Figure 2B). The results indicate that the major compound in fraction 5-31 is an octasulfated octasaccharide composed of 2 mol of trisulfated disaccharide units corresponding to ΔHexA(2S)-GlcN(NS,6S) and 1 mol each of two monosulfated disaccharide units corresponding to ΔHexA(2S)-GlcNAc and ΔHexA-GlcN(NS). When digested successively with 2-sulfatase and then heparitinase II, it yielded equimolar amounts of ΔHexA-GlcN(NS), ΔHexA(2S)-GlcNAc, ΔHexA-GlcN(NS,6S), and ΔHexA(2S)-GlcN(NS,6S), indicating that 1 mol of ΔHexA(2S)-GlcN(NS,6S) was located at the nonreducing terminus of the parent octasaccharides. Heparitinase I digestion of this fraction resulted in equimolar amounts of two unsaturated components, both of which were assumed to be tetrasulfated tetrasaccharides on the basis of their elution positions. Upon successive digestion with heparitinase I and then 2-sulfatase, one of the presumable tetrasulfated tetrasaccharide peaks was shifted to a position corresponding to the loss of one sulfate group on HPLC but the other tetrasulfated tetrasaccharide peak was resistant to the 2-sulfatase action, indicating that the former was derived from the nonreducing side of the parent octasaccharide and that the disaccharide unit located on the nonreducing side of the latter tetrasaccharide was ΔHexA-GlcN(NS). Hence, the disaccharide unit at the reducing terminus of the parent octasaccharide is ΔHexA(2S)-GlcN(NS,6S). The above results altogether indicate that the saccharide sequence of the compound in fraction 5-31 is ΔHexA(2S)-GlcN(NS,6S)-HexA-(2S)-GlcNAc-HexA-GlcN(NS)-HexA(2S)-GlcN(NS,6S). The saccharide sequence was confirmed and the type of the internal uronic acid residues was identified by 500 MHz ^{1}H NMR analysis as described below.

In the spectrum of fraction 5-31 (Figures 3C and 4C and Table 3), anomeric proton signals of 2-sulfated αIdceA and nonsulfated βGlcA were observed around δ 5.2 and 4.5, respectively, but those of nonsulfated αIdceA and 2-sulfated βGlcA were not. These NMR data and the sequential arrangement of the disaccharide units determined by enzymatic analysis indicated that 2-sulfated IdceA residues were located at the second and sixth saccharide positions, and a nonsulfated GlcA residue was at the fourth saccharide position. Hence, the structure of the major compound in fraction 5-31 is as follows: fraction 5-31, ΔHexA(2S)α1−4GlcN(NS,6S)α1−4IdceA(2S)α1−4GlcNAcα1−4GlcAβ1−4GlcN(NS)α1−4IdceA(2S)α1−4GlcN(NS,6S).

Relationship between ^{1}H Resonances and Saccharide Structural Features. 2D HOHAHA and COSY are useful methods for summarizing some rules about the relationship between ^{1}H resonances and saccharide structural features. In general, H-1, -2, and -3 signals of each constituent monosaccharide residue of oligosaccharides are easily identified in these spectra since they are separated from the bulk region which contains H-4, -5, and -6 signals. We compiled the ^{1}H NMR data using the cross-peaks in 2D HOHAHA and COSY spectra and extracted some rules by systematic comparison of the data. When the cross-peaks between the H-1 and H-3 signals of ΔHexA in the 2D HOHAHA spectra of about 40 oligosaccharides larger than tetrasaccharide were plotted (Figure 6A), we found that the ^{1}H resonances were divided into four groups on the basis of differences in the modification of the ΔHexA and the GlcN next to the ΔHexA. The anomeric proton signals of ΔHexA residues in the ΔHexA-GlcN, ΔHexA-GlcN(6S), or ΔHexA(2S)-GlcN-(±6S) sequence were observed between δ 5.06 and 5.08, δ 5.14 and 5.16, or δ 5.49 and 5.53 ppm, respectively. The H-3 signals of ΔHexA residues in the ΔHexA(2S)-GlcN or ΔHexA(2S)-GlcN(6S) sequence were observed between δ 4.20 and 4.24 or δ 4.29 and 4.33 ppm, respectively. These results indicated that the H-1 resonances of ΔHexA are influenced by *O*-sulfate substitutions at C-2 of the ΔHexA and at C-6 of the GlcN on the reducing side, but not influenced differentially by the N-acetylation or N-sulfation of the amino group of the adjacent GlcN residues. Therefore, the presence of a 6-*O*-sulfate group on the GlcN residue adjacent to ΔHexA can be deduced on the basis of the H-1 and H-3 chemical shifts of ΔHexA. When plots similar to those shown in Figure 6 were also constructed for GlcA or

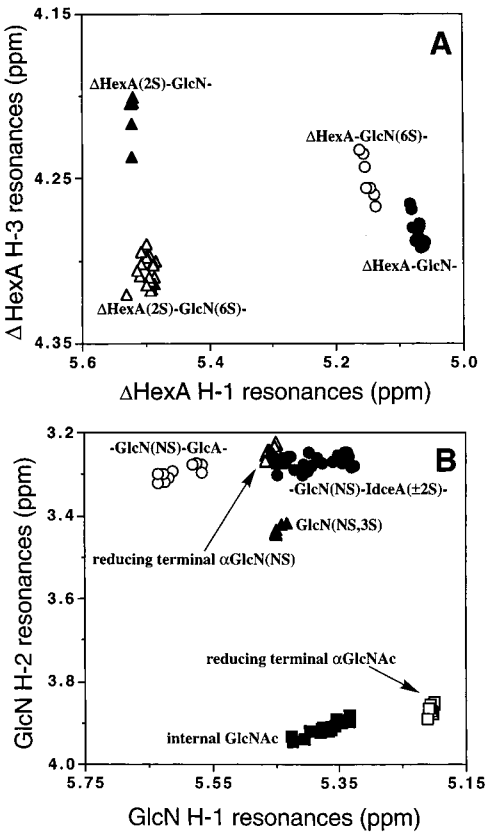

FIGURE 6: Relationship between saccharide sequences and the ^{1}H resonances for ΔHexA or GlcN residues. (A) The cross-peaks between H-1 and H-3 signals of the ΔHexA residues in 2D HOHAHA spectra of oligosaccharides. Various symbols represent the ^{1}H signals of ΔHexA residues in the following sequences: (●) ΔHexA-GlcN, (○) ΔHexA-GlcN(6S), (▲) ΔHexA(2S)-GlcN, and (△) ΔHexA(2S)-GlcN(6S). (B) The cross-peaks between H-1 and H-2 signals of the GlcN residues in 2D HOHAHA or COSY spectra of oligosaccharides. Various symbols represent the ^{1}H signals of the GlcN residues in the following sequences: (■) the internal GlcNAc, (□) the α-anomer of the reducing terminal GlcNAc, (●) GlcN(NS)-IdceA(±2S), (○) GlcN(NS)-GlcA, (△) the α-anomer of the reducing terminal GlcN(NS), and (▲) GlcN(NS,3S).

IdceA (results not shown), one useful effect was noticed; the chemical shift of the H-3 of the GlcA residue in a GlcN(NS)-GlcA sequence was consistently shifted 0.1−0.2 ppm downfield as compared to the H-3 resonances in a GlcNAc-GlcA sequence. Hence, the modification of N-sulfation or N-acetylation of the amino group of the GlcN residue on the nonreducing side of the GlcA can be identified on the basis of the chemical shifts of the GlcA H-3.

The relationship between ^{1}H resonances of GlcN and modifications in the vicinity of the GlcN residue was also analyzed using the cross-peaks in 2D HOHAHA and COSY spectra obtained from about 40 oligosaccharides larger than a trisaccharide. As a representative, the cross-peaks between α-anomeric proton and H-2 signals of GlcN residues of oligosaccharides were plotted in Figure 6B. Although the reducing terminal sugar residue of an oligosaccharide in solution is generally in equilibrium between α- and β-anomers, the heparan sulfate and/or heparin oligosaccharides containing N-sulfated glucosamine at the reducing terminus exist predominantly (approximately 90%) as an α-anomer (*33*−*35*). Therefore, the proton signals for the β-anomer were not analyzed in this study. The cross-peaks shown in Figure 6B were clustered into several groups depending on the differences in the modifications of the vicinity of the GlcN residue. The α-anomeric proton signals for the reducing terminal GlcNAc residues were observed between δ 5.20 and 5.21 ppm and those for the internal GlcNAc residues between δ 5.33 and 5.43 ppm. The anomeric proton signals of GlcN(NS) residues in the GlcN(NS)-GlcA sequence were observed between δ 5.57 and 5.64 ppm, and those in the GlcN(NS)-IdceA(±2S) sequences or those of the reducing terminal GlcN(NS) residues were observed between δ 5.33 and 5.47 ppm. These results indicate that the chemical shifts of the GlcN(NS) anomeric proton differ by 0.1−0.3 ppm depending upon whether GlcN(NS) is linked to GlcA. The α-anomeric proton resonances of GlcN(NS) residues in GlcN(NS)-IdceA(±2S) sequences occurred in the upfield relative to those of reducing terminal GlcN(NS) residues (Figure 6B, closed circle and open triangle), although they partially overlapped. Neither 6-O-sulfation of a GlcN residue nor the type of adjacent uronic acid on the nonreducing side of a GlcN residue influenced the H-1 and H-2 signals of the GlcN(NS) residue. However, 3-O-sulfation of the GlcN(NS) residue resulted in a downfield shift of its H-2 and H-3 signals of 0.10−0.23 and 0.67−0.96 ppm, respectively, as reported previously (*9*). Thus, on the basis of the anomeric proton and H-2 chemical shifts of the GlcN residue, it can be identified whether the GlcNAc residue is located at the reducing end, whether the GlcN(NS) residue is 3-O-sulfated, and whether a GlcA residue is present on the reducing side of the GlcN(NS) residue.

DISCUSSION

Among the structures isolated in this study, the octasaccharide serine found in fraction 5-2 and the three octasaccharides found in fractions 5-29, -30, and -31 were isolated for the first time as discrete structures. The structure of the octasaccharide serine in fraction 5-2 has hitherto been unreported probably due to its smaller proportion in the starting heparin as compared with the octasaccharide serines isolated previously. Since the protein linkage region is first constructed in biosynthesis of heparin glycosaminoglycan, possible differences in the structure of the linkage region may influence that of the repeating disaccharide region to be synthesized thereafter. It remains to be determined, however, whether biologically active domain structures such as the binding domain for antithrombin III are embedded indeed in specific subclass chains which may be classified by different linkage structures as previously discussed in detail (*25*, *27*).

Since the isolated octasaccharides were resistant to heparinase which cleaves most glucosaminidic linkages in the highly sulfated region (*12*, *36*, *37*), they must be derived from the low-sulfated irregular blocks of the repeating disaccharide region of heparin. The octasaccharides shared the common hexasulfated octasaccharide core sequence ΔHexA(2S)-GlcN(NS)-IdceA-GlcNAc-GlcA-GlcN(NS)-IdceA(2S)-GlcN(NS,6S) (*14*) with additional sulfate groups. The common sequence shows that the central low-sulfated tetrasaccharide is sandwiched by highly sulfated disaccharides, HexA(2S)-GlcN(NS), on the nonreducing and reducing sides, which should reflect the unrevealed control mechanism of heparin biosynthesis as discussed previously (*14*). So far, only a limited number of octasaccharides have been isolated from heparin, two being derived from the highly sulfated

regular region and six from the antithrombin III-binding region. Since oligosaccharides larger than a pentasaccharide appear to potentially exhibit binding activities toward growth factors or other functional proteins (*5*, *6*, *22*, *38*, *39*), the octasaccharides isolated in this study are large enough to exhibit binding activities toward functional proteins. Although none of the isolated oligosaccharides contain the known functional domains for binding to antithrombin III (*5*, *22*) or basic fibroblast growth factor (*6*), it remains to be determined whether binding domains for certain growth factors or biologically active proteins are embedded in the isolated octasaccharides. The isolated octasaccharides will be useful for investigating the structural requirement for molecular interactions between heparin and/or heparan sulfate and biologically active proteins.

^{1}H NMR spectroscopy is a powerful structural methodology which can reveal various structural features of sugar chains (*24*), including isomer types of sugar residues, saccharide sequences, and sulfation patterns, and has been applied to the analysis of glycosaminoglycans as well. Horne and Gettins (*17*) discussed the effects of sulfation on the ^{1}H resonances using the NMR data obtained from heparin-derived oligosaccharides in detail. However, the number of oligosaccharides used was only six, and all were derived from the highly sulfated regular region of heparin. In this study, the relationship between ^{1}H resonances and saccharide sequences was systematically analyzed using approximately 40 oligosaccharides isolated so far, which range in size from tri- to octasaccharides derived from the repeating disaccharide region (for a review, see ref *15*), after digestion with bacterial eliminases, and those NMR data allowed us to evaluate the effects of saccharide sequences on the chemical shifts and to develop some interesting rules which should be useful for assigning protons of larger sulfated oligosaccharides.

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