Structures of Five Sulfated Hexasaccharides Prepared from Porcine Intestinal Heparin Using Bacterial Heparinase

STRUCTURAL VARIANTS WITH APPARENT BIOSYNTHETIC PRECURSOR-PRODUCT RELATIONSHIPS FOR THE ANTITHROMBIN III-BINDING SITE*

Hiromi Tsuđa, Shuhei Yamada, Yukari Yamanę, Keiichi Yoshida, John J. Hopwood, and Kazuyuki Sugahara

From the Department of Biochemistry, Kobe Pharmaceutical University, Higashinada-kō, Kobe 658, the Tokyo Research Institute of Seikagaku Corp., Higashi-yamato-shi, Tokyo 207, Japan, and the Yysosomal Diseases Research Unit, Department of Chemical Pathology, Adelaide Children's Hospital, 72 King William Road, North Adelaide, South Australia 5006, Australia

Porcine intestinal heparin was extensively digested with Flavobacterium heparinase and size-fractionated by gel chromatography. Subfractionation of the hexasaccharide fraction by anion exchange high pressure liquid chromatography yielded 10 fractions. Six contained oligosaccharides derived from the repeating di-saccharide region, whereas four contained glycosaminoglycan-protein linkage region. The latter structures were reported recently (Sugahara, K., Tsuda, H., Yoshida, K., Yamada, S., de Beer, T., and Vliegenthart, J. F. G. (1995) J. Biol. Chem. 270, 22914–22923). In this study, the structures of one tetra- and five hexasaccharides from the repeat region were determined by chemical and enzymatic analyses as well as 500-MHz 2H NMR spectroscopy. The tetrasaccharide has the hexasulfated structure typical of heparin. The five hexa- heptasulfated hexasaccharides share the common core pentasulfated structure \( \text{HexA}(2S)\alpha_1-4\text{GlcN}(\text{NS,6S})\alpha_1-4\text{IdoA}\alpha_1-4\text{GlcA}\beta_1-4\text{GlcN}(\text{NS}) \) with one or two additional sulfate groups \( \Delta\text{HexA}, \text{GlcN}, \text{IdoA}, \text{GlcA} \), and \( \text{GlcG} \) represent 4-deoxy-\( \alpha \)-L-threo-hex-4-enepyranosyluronic acid, \( \alpha \)-L-glucosamine, \( \alpha \)-L-iduronic acid, and \( \alpha \)-L-glucuronic acid, whereas \( 2S, 6S \), and NS stand for 2-O-, 6-O-, and 2-N-sulfate, respectively. Three components have the following hitherto unreported structures: \( \Delta\text{HexA}(2S)\alpha_1-4\text{GlcN}(\text{NS,6S})\alpha_1-4\text{GlcA}\alpha_1-4\text{GlcN}(\text{NS,6S}) \), \( \Delta\text{HexA}(2S)\alpha_1-4\text{GlcN}(\text{NS,6S})\alpha_1-4\text{IdoA}\alpha_1-4\text{GlcA}\alpha_1-4\text{GlcN}(\text{NS,6S}) \), and \( \Delta\text{HexA}(2S)\alpha_1-4\text{GlcN}(\text{NS,6S})\alpha_1-4\text{IdoA}(2S)\alpha_1-4\text{GlcN}(\text{NS,6S}) \). Two of the five hexasaccharides are structural variants derived from the antithrombin III-binding sites containing 3-O-sulfated GlcN at the reducing termini with or without a 6-O-sulfate group on the reducing N-3-disulfated GlcN residue. Another contains the structure identical to that of the above heptasulfated antithrombin III-binding site fragment but lacks the 3-O-sulfate group and therefore is a pro-form for the binding site. Another has an extra sulfation group on the internal IdoA residue of this pro-form and therefore can be considered to have diverged from the binding site in the biosynthetic pathway. Thus, the isolated hexasaccharides in this study include the three overlapping pairs of structural variants with an apparent biosynthetic precursor-product relationship, which may reflect biosynthetic regulatory mechanisms of the binding site.

Heparin is a highly sulfated, linear polysaccharide that has various biological activities such as inhibition of blood coagulation (Marcum and Rosenberg, 1989), modulation of cellular proliferation (Clowes and Karnovsky, 1977; Thornton et al., 1983), potentiation of angiogenesis (Folkman and Ingber, 1989), and interactions with various growth factors (Maciag et al., 1984; Shing et al., 1984; Klagsbrun and Shing, 1985; Nakamura et al., 1986). The basic polymeric structure of heparin is an alternating repeat sequence of the disaccharide units \( \rightarrow 4\text{IdoA}\alpha_1-4\text{GlcA}\beta_1-4\text{GlcN}(\text{NS}) \), which can be variably sulfated (for reviews see Rodén (1980), Gallagher and Lyon (1989), and Lindahl (1989)). It is synthesized on a serine residue of a protein core named “serglycin” through a specific structure, the so-called carbohydrate-protein linkage region: \( \text{GlcA}\beta_1-3\text{Gal}\beta_1-3\text{Gal}\beta_1-4\text{Xyl}\beta_1-1\text{O-Ser} \) (Lindahl and Rodén, 1965). Although the principal structure of the repeating disaccharide region, known as the regular region (Casu, 1985), is composed of the major trisulfated disaccharide unit, \( \rightarrow 4\text{IdoA}(2\text{-sulfate})\alpha_1\rightarrow 4\text{GlcN}(\text{N,6-disulfate})\alpha_1\rightarrow \), undersulfation and substantial structural variability are observed in the irregular region, which is distributed along the chain flanked by the regular region and accounts for approximately one-quarter of the heparin polysaccharide chain. The structural variability is often the basis of a wide variety of domain structures with a number of biological activities ascribed to heparin.

Recent structural studies of the binding domains to ATIII (For review see Lindahl (1989)) and basic fibroblast growth factor (Maccarana et al., 1993) are the best known examples showing the relationships between the complicated fine structures and biological functions. The ATIII-binding site requires a minimal pentasaccharide sequence uniquely 3-O-sulfated on the central GlcN residue. This specific pentasaccharide has been shown to be primarily responsible for the anticoagulant activity of heparin (Lindahl et al., 1983). It has also been demonstrated that the binding domain to basic fibroblast growth factor requires a 2-O-sulfated IdoA residue and N-sulfated GlcN residue(s) for its specific interaction with the...
growth factor. Some structural variability has been observed within both binding sequences (Lindahl et al., 1984; Yamada et al., 1993; Maccarana et al., 1993).

We have been studying the basic primary structure of heparin to clarify the structural basis of its various biological activities. Previously, we demonstrated its structural variability by isolating six glycosamines from the carbohydrate-protein linkage region (Sugahara et al., 1992, 1995) and a number of tetrasaccharides from the repeating disaccharide region of porcine intestinal heparin after extensive digestion with bacterial heparin lyases (Yamada et al., 1993, 1994, 1995). In this study, we isolated and characterized five heparan sulfate structures from the repeating disaccharide region of the same heparin preparation after extensive enzymatic digestion to investigate the structure beyond the above tetrasaccharide sequences. These included three hitherto unreported heparan sulfate structures and structural variants with an apparent biosynthetic precursor-product relationship for the ATIII-binding site.

**EXPERIMENTAL PROCEDURES**

Materials—Stage 14 heparin was purchased from American Diagnostica (New York, NY) and purified by DEAE-cellulose chromatography as reported previously (Sugahara et al., 1992). Heparan sulfate (EC 2.2.2.7) and purified heparitinases I (EC 4.2.2.8) and II (no EC number) were obtained from Sekagaku Corp. (Tokyo, Japan). 4,5-Glucuronate-2-sulfate (EC 3.1.6.), abbreviated as 2-sulfate, and heparitinase V (no EC number) were purified from Flavobacterium heparinum (McLean et al., 1984) and Flavobacterium sp. H202 (Yoshida et al., 1989), respectively. Sephadex gels were from Pharmacia Biotech Inc., and Bio-Gel resins were from Bio-Rad. NaNH₂ (15 Ci/mmol) was supplied by American Radiolabeled Chemicals, Inc. (St. Louis, MO). 4-Methylumbelliferyl-α-D-L-iduronide was from Sigma, and p-nitrophenyl-β-D-glucuronide was from Nakalai Tesque (Kyoto, Japan). Seven standard unsaturated disaccharides were prepared from heparin as reported previously (Yamada et al., 1992). Standard heparin disaccharides were prepared by deaminative deavage (Shively and Conrad, 1976a, 1976b) were gifts from Dr. H. E. Conrad, University of Illinois. β-Glucuronidase and 4,5-HexA (EC 3.2.1.31) purified to homogeneity from Ampullaria (freshwater apple shell) hepatopancreas (Tsukada and Yoshino, 1987) was obtained from Tokyo Zouki Chemical Co., Tokyo. Human liver α-D-iduronidase (EC 3.2.1.76) was purified as reported previously (Freaman and Hopwood, 1992).

Preparation and Purification of Hexasaccharides—Stage 14 heparin was purified by anion exchange chromatography and digested with heparitinase, and the digest was fractionated into fractions a–d by gel filtration as described previously (Sugahara et al., 1995). Fractions c and d contained tetra- and disaccharides, respectively, which were derived from the repeating disaccharide region as characterized by HPLC (data not shown). Fraction a contained larger oligosaccharides and glycosamines/glycopeptides that were derived from the glycosaminoglycan-protein linkage region. Fraction b containing mainly heparan sulfate was subfractionated by HPLC on an anion-bound silica column at a flow rate of 1 ml/min using a stepwise gradient of NaH₂PO₄ solution at pH 7.4. Samples were collected at 10-min intervals for radioactivity measurement in an Aloka LSC-700 liquid scintillation counter. Individual disaccharide peaks were identified by comparison with authentic heparan disaccharides as reported previously (Bienkowski and Conrad, 1985).

500-MHz 1H NMR Spectroscopy—Hexasaccharides for NMR analysis were fully sodiated using a Dowex 50-X8 (Na⁺ form) column (7 × 18 mm) and then repeatedly exchanged in H₂O with intermediate lyophilization. 500-MHz 1H NMR spectra of hexasaccharides were measured on a Varian VXR-500 at a temperature of 26 °C as reported previously (Yamada et al., 1993). 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 H₂O (Vliegenthart et al., 1983).

**RESULTS**

Isolation of the Oligosaccharides—Purified stage 14 heparin from porcine intestine was exhaustively digested with heparinase and fractionated into fractions a–d by gel filtration on Cellulofine GCL-90 mm (Sugahara et al., 1995). Amino sugar and uronic acid analyses showed that fraction b contained approximately 3 mol each of GlcN and HexA/mmol of βHexA (data not shown), i.e., heparan sulfate. Fraction b was subfractionated by HPLC on an anion-bound silica column into Fr. b-1 to b-26

---

1 The abbreviations used are: HPLC, high pressure liquid chromatography; IDoA, l-iduronic acid; HEXa, hexuronic acid; ΔHexA or H3HexA, 4-deoxy-4-epi-l-hexuronic acid; anMAG, 2-anhydro-3-O-methylglucosamine; ΔDIHS-0.5, ΔHexA(4,1)-GlCNAC; ΔDIHS-6S, (4→8)HexA(4,1)-GlCNAC(6-sulfate); ΔDIHS-NS, ΔHexA(1,4)-GlCNAC(N-sulfate); ΔDIHS-dis, ΔHexA(1,4)-GlCNAC(N6-disulfate); ΔDIHS-6S, ΔHexA(2,1)-GlCNAC(N-sulfate); ΔDIHS-tris, ΔHexA(2,1)-GlCNAC(N6-disulfate); N5, 2-N-sulfate; N2, O-sulfate; N3, 3-O-sulfate; N5, 6-O-sulfate; ATIII, antithrombin III, Fr., fraction(s).
(Sugahara et al., 1995). Nine major fractions, Fr. b-5, -6, -10, -15, -17, -19, -20, -22, and -24, were further purified by rechromatography. They altogether accounted for 77 mol % (as DiHexA) of the oligosaccharides obtained from fraction b. Fr. b-5, -6, and -10 contained glycoserines derived from the glycosaminoglycan-protein linkage region as reported previously (Sugahara et al., 1995). The other fractions, Fr. b-15, -17, -19, -20, -22, and -24, were subjected to structural analysis below in this study. These individual fractions gave a single peak on HPLC but were approximately 95, 99, 54, 66, 96, and 75% pure, respectively, when examined by capillary electrophoresis (Fig. 1). Fr. b-17 contained the previously reported hexasulfated tetrasaccharide DiHexA(2S)GlcNAc(6S)-IdoA(2S)-1-4GlcN(NS,65)α1-4GlcN(NS,65) (Linker and Hovingh, 1984) as confirmed by 1H NMR spectroscopy, where 25, 65, and NS represent 2-O-, 6-O-, and 2-N-sulfation, respectively (data not shown). Apparently, this tetrasaccharide was partitioned into the heparin fraction due to its heavily sulfated structure. The amounts of the analyzed fractions isolated from 100 mg of the starting purified heparin are summarized in Table I.

Enzymatic Analysis—The disaccharide compositions of the isolated oligosaccharide fractions were determined by digestion with heparitinase(s) and/or heparinase, followed by HPLC on an amine-bound silica column. All the above oligosaccharide fractions except for Fr. b-17 were enzymatically degraded into approximately 3 mol of disaccharide units or 1 mol each of a tetrasaccharide unit. As representative chromatograms, those obtained with Fr. b-15 and b-24 are shown in Figs. 2 and 3, respectively. Fr. b-15 yielded DiHS-6S, DiHS-diS2, and DiHS-triS upon heparitinase I digestion as shown in Fig. 2A; their recoveries were 113, 105, and 124%, respectively, when taking the UV absorbance of the parent oligosaccharide(s) in Fr. b-15 as 100% (Table I). Thus, the major component in this fraction was a hexasulfated hexaamidase composed of a monosulfated, a disulfated, and a trisulfated disaccharide unit. Its sensitivity to 2-sulfatase was examined to localize the DiHexA(2S)-containing disaccharide unit DiHS-triS in the hexasaccharide sequence. The enzyme acts only on the DiHexA(2S) structure at the nonreducing end (McLean et al., 1984). When Fr. b-15 was successively digested with 2-sulfatase and then heparitinase I, it yielded DiHS-6S and DiHS-diS2 with recoveries of 92 and 204%, respectively, indicating that DiHS-triS was located at the nonreducing end and converted to DiHS-diS2 by successive digestion (Fig. 2B). When digested with heparitinase V (Yoshida, et al., 1989), Fr. b-15 gave rise to equimolar amounts of DiHS-diS2 and a presumable tetrasulfated tetrasaccharide (Fig. 2C), indicating that DiHS-diS2 was derived from the other terminus, i.e. the reducing end. Therefore, the structure of the compound in Fr. b-15 is DiHexA(2S)-GlcNAc(6S)-HexA-GlcNAc(6S)-HexA-GlcN-(NS,65).

The major component in Fr. b-24 accounted for 75% of the UV-absorbing materials in this fraction as judged by capillary electrophoresis (Fig. 1E). Exhaustive digestion of this fraction with a mixture of heparinase and heparitinase I yielded DiHS-triS, DiHS-diS2, and DiHS-diS1 with recoveries of 158, 96, and 125%, respectively (Fig. 3A). Upon incubation with heparinase only, Fr. b-24 was degraded into two unsaturated components, DiHS-triS and a component that eluted near the

**TABLE I**

| Fr. | Yield | Oligosaccharides formed |
|-----|-------|-------------------------|
| b-15 | 625 | ΔDiHS-6S (113%), ΔDiHS-diS2 (105%), ΔDiHS-diS3 (24%) and ΔDiHS-triS (124%) |
| b-17 | 851 | ΔDiHS-triS (213%) |
| b-19S | 437 | ΔDiHS-diS1 (282%) |
| b-20 | 369 | ΔDiHS-diS1 (33%), ΔDiHS-diS2 (21%), ΔDiHS-triS (109%) and HexA-GlcNAc(6S)-GlcA-GlcN(NS,3,5) (62%) |
| b-22 | 1458 | ΔDiHS-triS (122%) and HexA-GlcNAc(6S)-GlcA-GlcN(NS,3,5) (106%) |
| b-24 | 494 | ΔDiHS-diS1 (125%), ΔDiHS-diS2 (30%), ΔDiHS-diS3 (96%) and ΔDiHS-triS (158%) |

*a* nmol/100 mg heparin.

*b* The amount of the parent fraction b-19 was 310 nmol/100 mg heparin.

*c* Identified based upon the co-chromatographic behavior with the previously isolated tri- or tetrasulfated tetrasaccharide structure, respectively, derived from the ATIII-binding site of heparin (Yamada et al., 1993).
corresponding to $\Delta$DiHS-diS$_1$, i.e. $\Delta$HexA-GlcN(NS,6S)-HexA-GlcN(NS,6S)-HexA-GlcN(NS,6S)-HexA-GlcN(NS,6S). Consequently, the structure of the major component in the parent fraction b-19 was $\Delta$HexA-(2S)-GlcN(NS,6S)-HexA-GlcN(NS,6S)-HexA-GlcN(NS,6S). Heparitinase I digestion of both Fr. b-20 and -22 resulted in two unsaturated components, the trisulfated disaccharide $\Delta$DiHS-triS and a component that eluted near the elution position of the tri- or tetrasulfated tetrasaccharide (data not shown). Recoveries of the di- and tetrasaccharide components from Fr. b-20 or -22 were 109 and 62% or 122 and 106%, respectively (Table I). The lower recoveries of the presumable tetrasaccharide components of these fractions compared with those of their counterpart disaccharides suggested that the excess disaccharides were derived from minor components in these fractions consistent with the results of capillary electrophoresis. The presumable tetrasaccharide components from both Fr. b-20 and -22 were resistant to heparinase and heparitinases I and II (data not shown), probably due to the 3-O-sulfation of the reducing GlcN as reported previously for the ATIII-binding site-derived tetrasaccharides (Yamada et al., 1993). The presumable tetrasaccharides were co-chromatographed on HPLC with the authentic tetrasaccharides containing 3-O-sulfated GlcN residue (Yamada et al., 1993), demonstrating that the tri- and tetrasulfated tetrasaccharides derived from Fr. b-20 and -22 were identical to $\Delta$HexA-GlcNAc(6S)-GlcA-GlcN(NS,3S) and $\Delta$HexA-GlcNAc(6S)-GlcA-GlcN(NS,3S), respectively.

Sensitivities of the compounds in Fr. b-20 and -22 to 2-sulfatase were examined to characterize the sequential arrangement of the constituent di- and tetrasaccharide units. After 2-sulfatase digestion, Fr. b-20 and -22 gave a single peak on HPLC, which eluted approximately 10 min earlier than the corresponding parent compound, indicating that the major compound in each fraction had a sulfate group on the C-2 position of the $\Delta$HexA residue at the nonreducing terminus. Therefore, the structures of the major compounds in Fr. b-20 and -22 were $\Delta$HexA(2S)-GlcN(NS,6S)-HexA-GlcNAc(6S)-HexA-GlcNAc(6S)-GlcA-GlcN(NS,3S) and $\Delta$HexA(2S)-GlcN(NS,6S)-HexA-GlcNAc(6S)-GlcA-GlcN(NS,3S), respectively.

HPLC Analysis of the Di- and Tetrasaccharides Formed by HNO$_2$/NaB$_3$H$_4$ Treatment—To identify the internal uronic acid residues in the hexasaccharides of the isolated fractions, nitrous acid degradation products of each fraction were analyzed by HPLC. Bacterial lyase treatment converts the original structures of internal uronic acid, GlcA and IdoA in the oligosaccharides into the common 4,5-unsaturated, 4-deoxy-threo-4-enepyranosyluronic acid. In contrast, nitrous acid treatment preserves the original uronic acid structures despite loss of an N-sulfate group and production of an artificial structure, anhydromannitol, at the reducing end of the resultant oligosaccharides (Shively and Conrad, 1976a, 1976b).

The resultant di- and/or tetrasaccharide(s) from each hexasaccharide fraction were isolated by gel filtration on Bio-Gel P-2 and were analyzed by HPLC. These fractions obtained from Fr. b-15, -20, or -22 were confirmed to contain a disaccharide and a tetrasaccharide component as judged from their elution positions on HPLC (data not shown) (Bienkowski and Conrad, 1985). The tetrasaccharide component presumably derived from the reducing side of each of these original hexasaccharides was subjected to digestion with human liver $\alpha$-iduronidase (Freeman and Hopwood, 1992) and Ampullaria $\beta$-glucuronidase (Tsukada and Yoshino, 1987) to identify the uronic acid residues at the nonreducing termini. After $\alpha$-iduronidase digestion, a part (40, 57, or 60%) of the tetrasaccharide peak derived from Fr. b-15, -20, or -22 eluted 7–14 min earlier than the corresponding parent compound on HPLC. As representative

Fig. 3. HPLC analysis of the enzyme digests of Fr. b-24. Fr. b-24 (0.25 nmol) was digested with a mixture of heparitinase I and heparinase (A), heparinase (B), heparitinase I (C), or 2-sulfatase and then heparitinase I successively (D) as described under “Experimental Procedures.” For the HPLC conditions, see the legend to Fig. 2.
The five sulfated hexasaccharide structures isolated in this study share the pentasulfated hexasaccharide backbone \( \Delta \text{HexA}(25)\text{-anM}_{65} \) with one or two additional sulfate groups on GlcN-1, GlcN-3, or IdoA-4. Two of these, Fr. b-15 and -22, have been isolated previously (Linhardt et al., 1992), whereas the other three (Fr. b-19, -20, and -24) were isolated recently for the first time as discrete structures in this study. All the hexasaccharides contain the common trisulfated disaccharide unit on their nonreducing sides and the GlcN(5S) residue at their reducing termini, reflecting the substrate specificity of heparinase used for digestion of the starting heparin. These structural features are in good agreement with the established
TABLE II

| Residue | Reporter group | Fr. b-15 | Fr. b-195 | Fr. b-20 | Fr. b-22 | Fr. b-24 |
|---------|----------------|----------|----------|----------|----------|----------|
| GlcN-1  | H1 5.465 (3.5) | 5.464 (3.5) | 5.45 (ND) | 5.449 (3.5) | 5.464 (3.5) |
|         | H2 3.253        | 3.270     | 3.43     | 3.435     | 3.262     |
|         | H3 3.716        | 3.718     | ND       | 4.488     | 3.725     |
|         | H4 ND           | 3.70      | ND       | 4.015     | 3.682     |
|         | H5 4.052        | 4.15      | ND       | 4.227     | 4.144     |
|         | H6 4.34         | 4.340     | ND       | 4.42      | 4.33      |
|         | H6' 4.34        | 4.340     | ND       | 4.42      | 4.32      |
|         | NAc —           | —         | —        | —         | —         |
| GlcA-2  | H1 4.566 (8.0) | 4.603 (8.0) | 4.61 (ND) | 4.524 (8.0) | 4.576 (7.5) |
|         | H2 3.255        | 3.38      | 3.26     | 3.359     | 3.337     |
|         | H3 3.717        | 3.86      | 3.64     | 3.664     | 3.716     |
|         | H4 3.803        | 3.80      | 3.78     | 3.773     | 3.808     |
|         | H5 3.920        | ND        | ND       | 3.92      | ND        |
| GlcN-3  | H1 5.378 (3.5) | 5.634 (4.0) | 5.363 (4.0) | 5.375 (3.5) | 5.380 (4.0) |
|         | H2 3.912        | 3.298     | 3.90     | 3.910     | 3.924     |
|         | H3 3.773        | 3.696     | 3.78     | 3.759     | 3.76      |
|         | H4 ND           | 3.73      | ND       | ND        | 3.77      |
|         | H5 ND           | 4.00      | ND       | ND        | 3.971     |
|         | H6 ND           | 4.46      | ND       | ND        | ND        |
|         | H6' ND          | 4.16      | ND       | ND        | ND        |
|         | NAc 2.038       | —         | 2.035    | 2.038     | 2.035     |
| HexA-4  | H1 5.024 (ND) | 4.607 (2.0) | 4.989 (2.0) | 5.011 (2.5) | 5.184 (3.0) |
|         | H2 3.779        | 3.38      | 3.758    | 3.769     | 4.035     |
|         | H3 4.130        | 3.86      | 4.115    | 4.115     | 4.190     |
|         | H4 4.049        | 3.80      | 4.050    | 4.048     | 4.103     |
|         | H5 ND           | ND        | ND       | ND        | ND        |
| GlcN-5  | H1 5.339 (4.0) | 5.624 (3.5) | 5.345 (4.0) | 5.341 (4.0) | 5.435 (4.0) |
|         | H2 3.253        | 3.319     | 3.25     | 3.247     | 3.272     |
|         | H3 3.646        | 3.674     | 3.69     | 3.624     | 3.625     |
|         | H4 3.820        | 3.86      | 3.82     | 3.814     | 3.81      |
|         | H5 3.958        | 4.02      | 3.98     | 3.972     | 4.038     |
|         | H6 4.34         | 4.46      | 4.32     | 4.34      | 4.33      |
|         | H6' 4.15        | 4.16      | 4.20     | 4.18      | 4.24      |
|         | NAc —           | —         | —        | —         | —         |
| ∆HexA-6 | H1 5.494 (2.0) | 5.141 (6.5) | 5.490 (2.5) | 5.492 (2.0) | 5.500 (2.0) |
|         | H2 4.598        | 4.260     | 4.613    | 4.608     | 4.619     |
|         | H3 4.312        | 3.772     | 4.302    | 4.396     | 4.290     |
|         | H4 5.968        | 5.786     | 5.972    | 5.971     | 5.979     |

a Symbols represent the following: open triangle, ΔHexA; closed triangle, ΔHexA(2S); open square, GlcA; open diamond, IdoA; closed diamond, IdoA(2S); closed circle, GlcN(NS,6S); circle half-closed on the left side, GlcNAc(6S); circle half-closed on the right side, GlcN(3S); double underline, GlcN(3S).

b ND, not determined.

c —, not occurring.
Specificity of heparinase that cleaves the glucosaminidic linkage in the GlcN(1\textasciitilde4)IdoA(2S) sequence in a polymer (Linker and Hovingh, 1984; Merchant et al., 1985) and that in the GlcN(1\textasciitilde4)IdoA(2S) sequence in small oligosaccharides (Yamada et al., 1994, 1995).

Heparin and heparan sulfate have been demonstrated to exhibit various biological activities (Kjelle\textcommasec{}n and Lindahl, 1991). Especially, their specific interactions with various growth factors have recently attracted much attention. However, the functional domain structures elucidated to date are limited to only a few examples, including the minimum pentasaccharide sequences for ATIII binding and basic fibroblast growth factor. Although the hexasaccharide in Fr. b-20 contains an IdoA(2S) residue in its sequence, it lacks the disaccharide extension GlcA\textbeta1\texttextasciitilde4GlcNS(NS,5,6S) of the basic fibroblast growth factor binding sequence on the nonreducing side. It remains to be determined whether the binding domains to the other growth factors or biologically active proteins are embedded in the isolated hexasaccharides.

The most interesting structural feature of the isolated hexasaccharides is that they include three overlapping pairs of structural variants with an apparent biosynthetic precursor-product relationship for the ATIII-binding site. Structurally, the hexasaccharide in Fr. b-15 is a pro-form of that in Fr. b-22. The former lacks the 3-sulfate group on GlcN of the latter. Likewise, the hexasaccharide in Fr. b-20 is a pro-form of that in Fr. b-22, the former lacking the 6-sulfate of GlcN of the latter. The hexasaccharide in Fr. b-24 contains an IdoA(2S) residue in its sequence, which lacks the disaccharide extension GlcA\textbeta1\texttextasciitilde4GlcNS(NS,5,6S) of the basic fibroblast growth factor binding sequence on the nonreducing side.

The hexasaccharides isolated in this study appear to be large enough to potentially exhibit binding activities toward growth factors or other functional proteins. None of them, however, showed ATIII-mediated inhibition of factor Xa as examined according to Morita et al. (1977). Although the hexasaccharides in Fr. b-20 and -22 contain a 3-sulfated GlcN residue at their reducing termini, they lack a part of the minimum pentasaccharide sequence on their reducing sides, i.e. IdoA(2S)\textbeta1\texttextasciitilde4GlcNS(NS,6S). The isolated hexasaccharides are not expected to contain the functional domains for binding to basic fibroblast growth factor. The hexasaccharides, except for that in Fr. b-24, do not have an IdoA(2S) residue essential for binding to basic fibroblast growth factor. Although the hexasaccharide in Fr. b-24 contains an IdoA(2S) residue in its sequence, it lacks the disaccharide extension GlcA\textbeta1\texttextasciitilde4GlcNS(NS,5,6S) of the basic fibroblast growth factor binding sequence on the nonreducing side. It remains to be determined whether the binding domains to the other growth factors or biologically active proteins are embedded in the isolated hexasaccharides.

Only about one-third of chains of commercial porcine intestinal heparin contain an ATIII-binding site and have a high affinity for ATIII. The critical 3-O-sulfation of GlcN required for the ATIII-high affinity concludes the biosynthesis of the ATIII-binding site (Kusche et al., 1988). Based upon the isolation of a precursor tetrasaccharide sequence, which lacked the 3-O-sulfate group from heparin chains with ATIII-low affinity, Kusche et al. (1990) proposed that essentially each low affinity chain would contain a potential but not utilized 3-O-sulfation site. Linhardt et al. (1992) challenged this hypothesis, proposing that the existence of a low affinity heparin may not simply be the result of the incomplete action of 3-O-sulfotransferase in
Sulfated Hexasaccharides from Heparin

the final step, but rather some earlier step involved in the formation of the precursor sites may be primarily responsible for high and low ATIII affinity heparins. Recently, Razi and Lindahl (1995) proposed an intriguing hypothesis that the 3-O-sulfotransferase may be inhibited by a sulfated saccharide sequence outside the 3-O-sulfate acceptor region based upon the observation that an octasaccharide fraction isolated from ATIII-low affinity heparin, unlike low affinity heparin polysaccharide (Kusche et al., 1990), yielded high affinity components following incubation with a GlcN 3-O-sulfotransferase preparation.

The multiple pro-form structures demonstrated in this study probably do not represent precursors and products but rather reflect structural variants that have diverged during the modification reactions in the as yet unresolved but precisely programmed biosynthetic scheme. Practically, they will be valuable acceptor substrates for sulfotransferases to investigate such biosynthetic mechanisms by which the production of specific functional carbohydrate sequences is regulated. For example, it would be of interest to examine whether the hexasaccharide structures in Fr. b-15 and -20 serve as acceptor substrates for IdoA 2-O-sulfotransferase and GlcN 6-O-sulfotransferase (Habuchi et al., 1995) to produce the hexasaccharide structures found in Fr. b-24 and -22, respectively. It will be intriguing to find out whether the hexasaccharide structures in Fr. b-24 and -22 serve as acceptor substrates for sulfotransferase as to investigate such biosynthetic pathways.

They also will be useful for characterizing heparin/heparan sulfate-degrading enzymes including both the bacterial heparinase/heparitinases and mammalian heparanases. The former enzymes are essential tools for structural studies (Yoshida et al., 1989), whereas the latter have been implicated in tumor metastasis (for review see Nakajima et al., 1995), T cell adhesion (Gilat et al., 1995), and chemotactic functions (Hoogewerf et al., 1995).

Acknowledgments—We thank Dr. Makiko Sugiuira (Kobe Pharmaceutical University) for recording the NMR spectra and Nariko Fujikawa for excellent technical assistance.

REFERENCES

Bárcu, T., Loréau, J.-C., Petitou, M., Michelson, S., and Choay, J. (1989) J. Cell. Physiol. 140, 538–548
Bienkowski, M. J., and Conrad, H. E. (1985) J. Biol. Chem. 260, 356–365
Bitter, M., and Muir, H. (1962) Anal. Biochem. 4, 330–334
Casu, B. (1985) Adv. Carbohydr. Chem. Biochem. 43, 51–134
Clowes, A., and Karnovsky, M. (1977) Nature 265, 625–626
Folkman, J., and Ingber, D. E. (1989) in Heparin (Lane, D. A., and Lindahl, U., eds) pp. 317–333, Edward Arnold, London
Freeman, C., and Hopwood, J. J. (1992) Biochem. J. 282, 899–908
Gallagher, J. T., and Lyon, M. (1989) in Glycoproteins and Glycolipids (Sharon, N., Lis, H., Duskin, D., and Kahane, I., eds) pp. 330–331
Habuchi, H., Habuchi, O., and Kimata, K. (1995) J. Biol. Chem. 270, 4172–4179
Hoogewerf, A. J., Leone, J. W., Reardon, I. M., Howe, W. J., Asa, D., Heinrikson, R. L., and Ledbetter, S. R. (1995) J. Biol. Chem. 270, 3268–3277
Horne, A., and Gettins, P. (1992) Carbohydr. Res. 225, 43–57
Ishihara, M. (1994) Glycobiology 4, 817–824
Kjellén, L., and Lindahl, U. (1993) Annu. Rev. Biochem. 60, 443–475
Klagburg, M., and Shing, Y. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 805–809
Kusche, M., Bäckström, G., and Lindahl, U. (1988) J. Biol. Chem. 263, 15474–15484
Kushe, M., Torri, G., Casu, B., and Lindahl, U. (1990) J. Biol. Chem. 265, 17670–17676
Lindahl, U. (1989) in Heparin (Lane, D. A., and Lindahl, U., eds) pp. 159–189, Edward Arnold, London
Lindahl, U., and Rödén, L. (1965) J. Biol. Chem. 240, 2821–2826
Lindahl, U., Bäckström, G., and Thunberg, L. (1983) J. Biol. Chem. 258, 9826–9830
Lindahl, U., Thunberg, L., Bäckström, G., Riesenfeld, J., Nordling, K., and Björk, J. (1986) J. Biol. Chem. 261, 12368–12374
Linkhardt, R. J., Wang, H., Loganathan, D., and Bae, J. (1992) J. Biol. Chem. 267, 2380–2387
Linker, A., and Hovingh, P. (1984) Carbohydr. Res. 127, 75–94
Lyon, M., Deakin, J. A., Mizuno, K., Nakamura, T., and Gallagher, J. T. (1994) J. Biol. Chem. 269, 11216–11223
Maccarana, M., Casu, B., and Lindahl, U. (1993) J. Biol. Chem. 268, 2398–23905
 Merchant, Z. M., Kim, Y. S., Rice, K. G., and Linhardt, R. J. (1985) Biochem. J. 229, 369–377
Morita, T., Kato, H., Iwana, S., Takada, K., Kimura, T., and Sakakibara, S. (1977) J. Biol. Chem. 252, 1495–1498
Nakamura, T., Teramoto, H., and Ichihara, A. (1986) J. Biol. Chem. 261, 1495–1498
Shively, J. E., and Conrad, H. E. (1976a) Proc. Natl. Acad. Sci. U. S. A. 73, 6489–6493
Shively, J. E., and Conrad, H. E. (1978) J. Biol. Chem. 253, 11267–11275
Rodén, L. (1980) The Biochemistry of Glycoproteins and Proteoglycans (Lennarz, W. J., ed) pp. 267–371, Plenum Publishing Corp., New York
Shively, J. E., and Conrad, H. E. (1979a) Science 206, 565–569
Shively, J. E., and Conrad, H. E. (1979b) Science 206, 565–569
Sugahara, K., Okamoto, H., Nakamura, M., Shiibahara, S., and Yamashina, I. (1987) Arch. Biochem. Biophys. 258, 391–403
Sugahara, K., Yamada, S., Yoshida, K., de Ward, P., and Vliegenthart, J. F. G. (1984) J. Biol. Chem. 261, 1528–1533
Sugahara, K., Toho-oaka, R., Yamada, S., Kho, K.-H., Morris, H. R., and Del, A. (1994) Glycobiology 4, 535–544
Sugahara, K., Tsuda, H., Yoshida, K., Yamada, S., de Beer, T., and Vliegenthart, J. F. G. (1995) J. Biol. Chem. 270, 22914–22923
Thornton, S. C., Mueller, S. N., and Levine, E. M. (1983) Science 222, 623–625
Tsukada, T., and Yashiro, M. (1987) Comp. Biochem. Physiol. 86B, 565–569
Vliegenthart, J. F. G., Dorland, L., and Van Halbeek, H. (1983) Adv. Carbohydr. Chem. Biochem. 41, 209–274
Yamada, S., Yoshida, K., Sugiuira, M., and Sugahara, K. (1992) J. Biol. Chem. 267, 440–447
Yamada, S., Yoshida, K., Sugiuira, M., Sugahara, K., Kho, K.-H., Morris, H. R., and Del, A. (1994) Glycobiology 4, 535–544
Yamada, S., Tsuda, H., Yoshida, K., Yamada, S., de Beer, T., and Vliegenthart, J. F. G. (1995) J. Biol. Chem. 270, 22914–22923
Yamada, S., Murakami, T., Tsuda, H., Yoshida, K., and Sugahara, K. (1995) J. Biol. Chem. 270, 8656–8705
Yamada, T., Saita, H., Habuchi, O., and Suzuki, S. (1968) J. Biol. Chem. 243, 1523–1535
Yoshida, K., Mihazono, H., Tawada, A., Kikuchi, H., and Morikawa, K. (1989) Proc. Xth Int. Symp. Glycoconjugates, Jerusalem, Israel (Sharon, N., Lis, H., Duskin, D., and Kahane, I., eds) pp. 330–331

Downloaded from http://www.jbc.org/ by guest on July 23, 2018
