Domain Structure of Heparan Sulfates from Bovine Organs

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Marco Maccarana, Yoshiyuki Sakura, Akira Tawada, Keiichi Yoshida, and Ulf Lindahl

From the Department of Medical and Physiological Chemistry, Uppsala University, The Biomedical Center, S-751 23 Uppsala, Sweden, and the Tokyo Research Institute of Säkagaku Corporation, Hisagiyamato-shi, Tokyo 207, Japan

Samples of heparan sulfate, isolated from bovine aorta, lung, intestine, and kidney, were degraded by digestion with a mixture of heparitinases or by treatment with nitrous acid, with or without previous N-deacetylation. Analysis of the resulting oligosaccharides showed that the various heparan sulfate samples contained residues with NaBH₄; GlcA, D-glucuronic acid; IdoA, L-iduronic acid; HexA, unspecified hexuronic acid; GlcNAc, 2-deoxy-2-acetamido-D-glucose; HexNAc, 2-acetamido-2-deoxy-D-glucose; HPLC, high performance liquid chromatography; -SO₃, sulfate group; -OSO₃, ester sulfate group.

The sulfated glycosaminoglycans generally occur in the tissues as proteoglycans, in which the polysaccharide chains are covalently bound to a core protein (reviewed in Ref. 1). Heparan sulfate (HS) proteoglycans are abundant on cell surfaces and in the extracellular matrices of most tissues and have been attributed a multitude of biological activities. These effects are mostly believed to reflect binding of HS chains to specific proteins (2–4). While the physiological or pathophysiological significance of such interactions is subject to increasing attention, their structural basis is generally unclear. Thus, with the exception of the antithrombin-binding pentasaccharide sequence in heparin/HS (reviewed in Ref. 5) and more recently described, still incompletely defined, binding regions for some growth factors (6–10), little is known regarding the saccharide sequences involved in protein binding. In fact, since most HS-binding proteins bind to heparin, i.e., the most densely sulfate-substituted member of the glycosaminoglycan family, the interactions are often tacitly assumed to be nonspecific in character. On the other hand, indirect observations imply an appreciable degree of specificity in interactions between HS and various proteins such as cytokines (11) or selectins (12), and it has been proposed that specific protein-binding regions that occur in “hidden” form in heparin are selectively expressed by different HSs (3, 4, 8).

The sequence characteristics of HSs produced by different cells, or occurring in different tissues, are poorly defined. No methodology is yet available for routine sequence analysis of HS chains. Furthermore, the problem is complicated by the inherent heterogeneity of even highly purified HS preparations. The biosynthetic reactions required to generate different HS sequences have been defined and include the formation of a GlcAβ1,4-GlcNAcα1,4-n polysaccharide, which is subsequently modified through O-deacytation/N-sulfation of GlcNAc units, C-5 epimerization of GlcA to IdoA units, and O-sulfation at different positions. The O-sulfate substitution may occur at C-2 of IdoA and some GlcA units, and at C-6 (to some extent C-3) of GlcN units (3). Due to the sequential order of these reactions, and the substrate specificities of the enzymes involved, the initial distribution of N-sulfate groups will heavily influence the location of IdoA units and O-sulfate groups as well. N-Sulfate and residual N-acetyl groups tend to form block structures (13, 14). Generally, the degree of N-sulfation correlates with the IdoA and O-sulfate contents of a heparin/HS type polysaccharide (15). However, few attempts have been made to assess, in a more comprehensive fashion, the overall domain structure of HS, with regard to N-substituents as well as the products of “downstream” polymer-modification reactions. The present study was initiated to obtain such information, by structural analysis of HSs from different bovine organs.

EXPERIMENTAL PROCEDURES

Materials—Preparations of HS were obtained in principle as described (16). Briefly, the various samples were isolated from bovine aorta, lung, intestine, and kidney by a procedure involving protease digestion of boiled and minced tissue, alkali treatment, removal of nucleic acids by low pH precipitation and nuclease digestion, ion-exchange chromatography, and removal of galactosaminoglycans by digestion with chondroitinase ABC. The ion-exchange chromatography used to recover HSs from aorta, lung, and intestine was done using a column of Dowex 1-X2, that was first washed to eliminate material dissociated from the resin in 0.75 M NaCl and then eluted with 1.0 M (aorta) or 1.25 M (lung, intestine) NaCl. The kidney HS was separated into two fractions, preparation A that was eluted between 0.7 M and 1.1 M NaCl, and preparation B that was eluted between 1.1 M and 1.25 M NaCl. The M₉, ranges for the various preparations were estimated to ~12–44 × 10⁶ (aorta), ~9–30 × 10⁶ (lung), ~10–30 × 10⁶ (intestine), ~9–21 × 10⁶ (kidney A) and ~10–32 × 10⁶ (kidney B) by gel permeation HPLC using chondroitin sulfates as standards (data not shown).
Hydrazine hydrate (36% water) and hydrazine sulfate were purchased from Fluka and Merck, respectively. NaB\textsubscript{3}H\textsubscript{4} (16 Ci/ml/mmol) was obtained from Amersham Corp. Bio-Gel P-10, superfine grade, was obtained from Bio-Rad.

Methods—Compositional analysis of HS was performed using Flavobacterium heparinum HS lyases. About 100 µg of HS was digested with a mixture of heparinases I and II (EC number: EC 4.2.2.7 and EC 4.2.2.8; 20 milliunits each; Sekagaku) in 40 µl of 20 mM acetic buffer, pH 7.0, 2 mM calcium acetate, at 37°C for 2 h. The reaction was terminated by boiling for 30 s. The yield of resultant unsaturated disaccharides was estimated by gel permeation HPLC using connected columns of TSK-gel 4000, 3000, 2500 PWXL, equipped with a refractive index monitor. The disaccharides were separated further on a CarboPac PA-1 HPLC column ( Dionex), eluted with a LiCl gradient as described in the legend to Fig. 2, and monitored by absorbance at 230 nm, essentially as described (17).

Deaminative cleavage of HS with nitrous acid was done according to two alternative procedures. In one approach, 0.5 mg of polysaccharide was treated with 500 µl of HNO\textsubscript{2} reagent at pH 1.5, followed by reduction of the products with 0.5 mCi of NaB\textsubscript{3}H\textsubscript{4}, essentially as described (18). Under these conditions, N-sulfated GlcN units are selectively attacked, with cleavage of the corresponding glucosaminidic link (19). N-Acetylated GlcN residues are not affected; thus, a single N-acetylated disaccharide unit surrounded by two N-sulfated units will be recovered as a tetrasaccharide, whereas consecutive N-acetylated units will give rise to larger oligosaccharides. All oligosaccharides formed will contain a [1\textsuperscript{-3}H]amAn unit at their reducing end. Unincorporated \textsuperscript{3}H was removed by passage through a column of Sephadex G-15 (1 \times 190 cm, eluted in 0.2 M NH\textsubscript{4}HCO\textsubscript{3}). Prior to further analysis, the digosaccharides were pooled and subjected to mild acid treatment (25 mM H\textsubscript{2}SO\textsubscript{4}, 80°C, 30 min) to eliminate sequences containing products of "anomalous" deaminating ring contraction (20). The \textsuperscript{3}H-labeled saccharides were analyzed further as described in the text or in the legends to figures.

Alternatively, the HS preparations were N-deacetylated by hydrazinolysis before deamination (21–23). Samples of 1 mg were dissolved in 1 ml of hydrazine hydrate and 1% (w/v) hydrazine sulfate, and then heated in sealed glass tubes at 96°C for 4 h. After repeated evaporation to dryness, the N-deacetylated polysaccharides were recovered by passage through columns (1 \times 90 cm) of Sephadex G-15, equilibrated in 10% ethanol. The excluded material was pooled and lyophilized, and samples corresponding to 100 µg of polysaccharide (as determined by the carbazole reaction (24), assuming a hexuronic acid content of 33%) were deaminated with HNO\textsubscript{2}, first at pH 1.5 (cleavage at N-sulfated GlcN units) and then at pH 3.9 (deamination at N-unsubstituted GlcN units; Ref. 25), followed by reduction of the products with 100 µCi of NaB\textsubscript{3}H\textsubscript{4}. The resultant labeled disaccharides thus would represent the entire initial, N-acetylated as well as N-sulfated, saccharide sequences.

The HexA-[1\textsuperscript{-3}H]amAn, disaccharide fractions were analyzed by anion-exchange HPLC (26), as described in more detail in the legend to Fig. 1. The nonsulfated disaccharides, GlcA-amAn\textsubscript{8} and IdoA-amAn\textsubscript{8}, were not resolved in this procedure and were instead separated by descending paper chromatography (ethyl acetate/acetic acid/H\textsubscript{2}O, 3:1: 1), following isolation by preparative paper electrophoresis (80 V/cm) on Whatman 3MM paper, in 0.03M pyridine, 0.05 M acetic acid, pH 5.3.

Tetrasaccharides obtained after deamination at pH 1.5/NaB\textsubscript{3}H\textsubscript{4} treatment were separated further by high voltage paper electrophoresis (40 V/cm) in 1.6 M formic acid (pH 1.7). Paper strips were cut into 1-cm segments that were eluted with water, and the aqueous extract was analyzed by scintillation counting.

Additional analytical procedures are described in the legends to figures.

RESULTS

Overall Composition of Heparan Sulfate Chains—Samples of HS isolated from bovine aorta, lung, intestine, and kidney (two preparations, designated A and B, separated by anion-exchange chromatography; see "Materials") were N-deacetylated by hydrazinolysis and deaminated, under conditions leading to cleavage at N-unsubstituted as well as at N-sulfated GlcN units. A completely N-deacetylated HS chain thus would be quantitatively degraded to disaccharides. Reduction of the products with NaB\textsubscript{3}H\textsubscript{4}, followed by gel chromatography on Sephadex G-15 revealed labeled tetrasaccharides as a minor component (7–11% of the total labeled products) in addition to disaccharides. Some of these tetrasaccharides were due to "anomalous" deaminating ring contraction (20), since mild acid hydrolysis treatment reduced the proportion of labeled tetrasaccharides (data not shown). The HexA-amAn\textsubscript{8} disaccharides thus consistently accounted for ≥90% of the total radioactivity, and were considered to appropriately reflect the overall composition of the HS samples (see Ref. 23 regarding nonselectivity of the "anomalous" ring contraction). Separation of the disaccharides by anion-exchange HPLC (Fig. 1) and paper chromatography (data not shown) gave the results summarized in Table I. All HS samples yielded predominantly non-O-sulfated GlcA-amAn\textsubscript{8} disaccharide, which represents a GlcA-GlcN-structure in the intact polysaccharide, where R stands for either an N-acetyl or a N-sulfate group. Interestingly, the
Bio-Gel P-10 provided adequate separation of the various 3H-labeled oligosaccharides of at least hexasaccharide size. Gel chromatography on NaB₃H₄ and separation of the resultant labeled oligosaccharides (Fig. 4) showed that the intestinal HS (90%) was digested with heparitinases (see "Methods") and that the resultant disaccharides were separated by anion-exchange HPLC (Fig. 1) and by paper chromatography (see "Methods"). Values are given as mol % of total labeled disaccharides and were calculated from peak areas. ND, not detected (≤0.2%).

Disaccharides were generated by N-deacetylation followed by deaminative cleavage (pH 1.5 and 3.9, resulting in cleavage of all glucosaminidic linkages) and reduction of the products with NaB₃H₄ as described under "Methods."

The sum of the nonsulfated disaccharides, GlcA-aManₐ, and IdoA-aManₐ, was calculated from anion-exchange HPLC patterns, where the two components emerged together in the peak indicated by arrow 1 in Fig. 1. To obtain the relative proportions of the epimers, the nonsulfated disaccharide fractions were isolated by preparative paper electrophoresis (pH 5.3) and were then separated further by paper chromatography (see "Methods"; data not shown).

The quantitative analysis of disaccharides enabled an assessment of the overall contents of IdoA and O-sulfated disaccharides in the various HS samples (Fig. 3A). The total IdoA contents (including nonsulfated as well as 2-O-sulfated units) varied between 21% and 35%, and the 2-O-sulfated IdoA between 8.3% and 21% of the total HexA units. The extent of GlcN disaccharides ranged between 9.6% and 38% of the total GlcN units.

Amounts and Domain Distribution of N-Sulfate Groups—Information regarding the amounts and distribution of N-sulfated GlcN units was obtained by deamination of native (i.e. not N-deacetylated) HS at pH 1.5, followed by reduction with NaB₃H₄ and separation of the resultant labeled oligosaccharides by gel chromatography. Under these conditions the chains are cleaved at the sites of N-sulfated GlcN units, whereas N-acetylated units remain intact. Consecutive N-sulfated GlcN residues (in the following referred to as "contiguous" N-sulfated sequences or "N-sulfated blocks") thus will be recovered, as aManₐ units, in disaccharides, whereas alternating N-sulfated and N-acetylated GlcN residues ("alternating" sequences) will give rise to tetrasaccharides. Finally, solitary N-sulfate groups ("spaced" sequences) will yield oligosaccharides of at least hexasaccharide size. Gel chromatography on Bio-Gel P-10 provided adequate separation of the various ³H-labeled oligosaccharides (Fig. 4). All HS samples yielded disaccharides, followed by tetrasaccharides, as major labeled products and, in addition, smaller amounts of well separated oligosaccharides up to 18–20 saccharide size. Calculation of peak areas afforded estimates of overall N-sulfate/N-acetyl ratios (27, 28) as well as proportions of contiguous, alternating, and spaced sequences. While the proportions of contiguous and spaced sequences varied appreciably among the various HS preparations, the proportion of alternating sequence was remarkably constant (Fig. 6A). All samples fell within the range...
of N-sulfation typical for HS (14) and varied from 37% (aorta) to 57% (intestine) of the total disaccharide units (Fig. 3A). Of the total N-sulfate groups in the HS chains, 42–61% were located in contiguous sequence, 24–33% in alternating structure, and 14–29% as solitary (spaced) units (Fig. 6B). The aorta sample, with the lowest overall N-sulfate contents (Fig. 3A), showed the lowest proportion of contiguous and the highest proportion of spaced N-sulfate groups, whereas the most highly N-sulfated, intestinal, HS displayed the inverse relation. The occurrence of extended N-acetylated sequences adjacent to the polysaccharide-protein linkage (29) could bias the calculations, insofar as such structures are not likely to become radiolabeled following HNO2 (pH 1.5)/NaB3H4 treatment.

Domain Distribution of Hexuronic Acid and O-Sulfate Residues—In separate analyses, we determined the composition of disaccharides released by deamination at pH 1.5 only, without prior N-deacetylation of the HS chains (Fig. 5; Table III). In

| Disaccharide | Aorta | Lung | Intestine | Kidney A | Kidney B |
|-------------|-------|------|-----------|----------|----------|
| ΔHexA-GlcNac | 63    | 45   | 61 (65)   | 64 (66)  | 69 (69)  |
| ΔHexA-GlcNSO3 | 20    | 16   | 19 (11)   | 12 (15)  | 7.9 (8.2) |
| ΔHexA(2-OSO3)-GlcNac | 0.5 | 0.9 | 8.5 (9.7) | 7.0 (9.4) | 5.5 (5.5) |
| ΔHexA-GlcNSO3(6-OSO3) | 5.4 | 7.6 | 11 (11)  | 17 (13)  | 19 (19)  |
| ΔHexA-GlcNSO3(6-OSO3) | 2.4 | 8.7 | 7.8 (7.8) | 6.3 (6.3) | 17 (17)  |
| ΔHexA(2-OSO3)-GlcNSO3(6-OSO3) | 3.0 | 4.2 | 6.5 (6.5) | 5.8 (5.8) | 8.6 (8.6) |

* Disaccharides, all containing a 4,5-unsaturated hexuronic acid (ΔHexA) unit, were generated by extensive enzymatic digestion of the HS chains, as described under "Methods," and were separated by anion-exchange HPLC (Fig. 2).

**Glca-aMan α, + Ido-aManH.
**IdoA(2-OSO3)-aManH.

**GlcA-aManH(6-OSO3) + IdoA-aManH(6-OSO3).
**IdoA(2-OSO3)-aManH(6-OSO3).

FIG. 4. Gel chromatography of oligosaccharides produced by deamination at pH 1.5. HS samples were cleaved by treatment with nitrous acid (pH 1.5), and the resultant oligosaccharides were reduced with NaB3H4 and subjected to mild acid treatment to eliminate "anomalous" ring contraction products (see "Methods"). The labeled oligosaccharides were separated by gel chromatography on a column (1 × 150 cm) of Bio-Gel P-10 (superfine), equilibrated with 0.5 M NH4HCO3. Effluent fractions of 0.5 ml were collected at a rate of 1 ml/h and were analyzed for radioactivity by scintillation counting. The molecular size of the separated oligosaccharides is indicated by the number of monosaccharide units/molecule, shown for each peak of the aorta sample. Fractions corresponding to disaccharides or tetrasaccharides were pooled as indicated by the bars and lyophilized before further analysis.
contrast to the products obtained on complete deaminative cleavage, which were dominated by nonsulfated GlcA-aManR disaccharides (Table I), the disaccharides released from the cleavage, which were dominated by nonsulfated GlcA-aManR units and IdoA(2-OSO₃), which occurred in 54–58% of the different HS preparations showed a remarkably constant proportion for: (i) the distribution of total disaccharide units between contiguous (Fig. 3A) and after cleavage at pH 1.5 (Table I), and in the N-sulfated blocks (Fig. 3B; compiled from the data in Table I). The contiguous N-sulfated regions thus contained about half of the total IdoA units of the chains (Fig. 6D), but almost all of the IdoA 2-O-sulfate units (Fig. 6E). Presumably, most of the remaining (nonsulfated) IdoA occurs in the alternating sequences. By contrast, consistently less than half of the total 6-O-sulfate groups was found in the contiguous sequences (Fig. 6F).

Since most of the total 6-O-sulfate groups appeared to be located outside the N-sulfated block regions, it was of interest to assess the contributions of alternating and spaced sequences to the total O-sulfate contents of the chains. Alternating (N-acetylN-sulfate) type structures were recovered as labeled tetrasaccharides following HNO₂ (pH 1.5)/NaB₃H₄ treatment (Fig. 4) and were separated further by high voltage paper electrophoresis at pH 1.7 (data not shown). The proportions of nonsulfated, mono-O-sulfated, and di-O-sulfated species (no tri-O-sulfated tetrasaccharides were detected) were assessed by determining the distribution of ³H on the paper strips by scintillation counting (data not shown). Based on these results, it was calculated that the tetrasaccharides recovered from aorta, lung, intestine, kidney A, and kidney B HS contained, on average, 50, 84, 60, 80, and 104 O-sulfate groups/100 tetrasaccharide molecules. Elaborating these data as outlined above in (i) and (ii), it was concluded that between 21% and 32% of the total O-sulfate groups of the various HS preparations occupy alternating sequences (Fig. 6C). Furthermore, 44–73% of the O-sulfates were located in the contiguous N-sulfated sequences, whereas only two of the samples, from lung and kidney B, showed significant O-sulfation of spaced structures (see also Ref. 30). These findings are compatible with the conclusion that major portions of the 6-O-sulfate groups are located in the alternating sequences, presumably on both N-acetylated and N-sulfated GlcN units (Fig. 7).

**DISCUSSION**

The N- and 2,6-di-O-sulfated structure -IdoA(2-OSO₃)GlcN-NSO₃(6-OSO₃)₆ is generally the predominant disaccharide unit in heparin preparations (26), but a minor component in most HSs. Instead, most of the 2-O- and 6-O-sulfate groups are confined to separate disaccharide units, as shown by analysis of products obtained by extensive deaminative cleavage of HS preparations (Table I). There is limited information regarding the distribution of such sulfate groups in relation to functional domains (protein-binding sequences) of HS chains. It has been reported that the binding region for lipoprotein lipase in endothelial cell HS is composed of five consecutive N- and 2,6-di-O-sulfated disaccharide repeats (31). By contrast, the minimal binding region for basic fibroblast growth factor contains at least one essential IdoA 2-O-sulfate group, but no 6-O-sulfate residues (7, 8); instead, it has been proposed that 6-O-sulfation mediates binding of an adjacent region to the growth factor receptor (32). The antithrombin-binding region in HS (and in heparin) contains a functionally essential 6-O-sulfated GlcNAc or GlcNSO₃ unit that is located 3 monosaccharide residues apart from the nearest 2-O-sulfated IdoA unit (reviewed in Ref. 3). Other observations correlate modulation of functional properties with changed O-sulfation patterns of HS in cultured cells. Viral transformation of a murine embryonic cell line thus was accompanied by a selective decrease in 6-O-sulfation (33), whereas, conversely, the metastatic potential of murine lung carcinoma cells increased with increasing 6-O-sulfation of cellular HS (34). Further, a comparison of HS chains synthesized by different cells on the same syndecan-1 core protein showed increased collagen binding to correlate with increased IdoA 2-O-sulfation and decreased GlcN 6-O-sulfation of the polysaccharide (35). It would obviously be of interest to define the mechanisms in control of the corresponding sulfotransferase reactions, and to reveal any systematic trait in the distribution of the various sulfate substituents along the
polysaccharide chain.

Current information indicates that the GlcA C-5 epimerization and O-sulfotransferase reactions are all controlled by the distribution of N-sulfate groups, introduced during the initial polymer-modification reactions (36). Thus, IdoA units are consistently linked at C-4 to an N-sulfated GlcN residue, while the GlcN residue bound at C-1 (of IdoA) may be either N-sulfated or N-acetylated. The substrate recognition properties of the GlcA C-5 epimerase are reflected by the relatively high and constant proportion of IdoA units in the contiguous N-sulfated regions of the bovine HS preparations (Fig. 3B). It has been proposed that the formation and 2-O-sulfation of IdoA units are coupled processes (37), and the predominant sulfation of the IdoA units within the N-sulfated blocks agrees with this notion (38). However, all samples analyzed in the present study contained about half of their total IdoA units outside the N-sulfated blocks, presumably largely in alternating-type sequences (Figs. 6 and 7). These IdoA units were almost exclusively nonsulfated (Fig. 6E). Thus, the coupling between the formation and 2-O-sulfation of IdoA units is selective and dependent on the N-substituent pattern of the polysaccharide chain.

Biosynthetic 6-O-sulfation of N-acetylated GlcN units in a HS chain can occur only if at least one of the adjacent GlcN residues is N-sulfated (see Ref. 3, and references therein). Analysis of HS preparations showed a appreciable proportion of the total 6-O-sulfate groups to be located on GlcNAc residues (39, 40). Indeed, while 2-O-sulfation of IdoA units was essentially restricted to the N-sulfated blocks, the 6-O-sulfate groups occurred largely outside these regions (Fig. 6F). Furthermore, whereas the extent of 2-O-sulfation within these blocks was essentially the same for all HS preparations analyzed, the degree of 6-O-sulfation varied greatly between the samples (Fig. 3). Nevertheless, the different HS species displayed a

### Table III

| Disaccharide | Aorta | Lung | Intestine | Kidney A | Kidney B |
|--------------|-------|------|-----------|----------|----------|
| GlcA-aManR   | 28    | 18   | 28        | 17       | 14       |
| IdoA-aManR   | 4.6   | 3.8  | 2.6       | 3.5      | 2.4      |
| GlcA(2-OSO_3)-aManR | 0.7  | 0.3  | 0.7       | 0.3      | ND       |
| IdoA-aManR(6-OSO_3) | 3.9  | 9.0  | 3.4       | 7.1      | 11       |
| GlcA-aManR(6-OSO_3) | 37   | 36   | 40        | 35       | 25       |
| IdoA(2-OSO_3)-aManR | 1.6  | 1.3  | 2.0       | 2.2      | ND       |

% of total disaccharides in N-sulfated blocks

**Fig. 6.** Domain distribution of the different units in HS chains. Panels A–C indicate the distribution of total disaccharide units (A), N-sulfate groups (B), and total O-sulfate groups (C); between contiguous N-sulfated (gray bars), alternating (striped bars), and spaced (open bars) sequences. Panels D–F indicate the proportions of total IdoA units (D), 2-O-sulfated IdoA units (E), and 6-O-sulfated GlcN units (F) that occur within the contiguous N-sulfated sequences. For further information regarding the calculations, see "Results."
remarkably constant proportion of alternating sequence (-Glc-NSO₃-(HexA-GlcNAc-GlcA-GlcNSO₃))₅ (Fig. 6A), rich in 6-O-sulfates but essentially devoid of 2-O-sulfate groups (Fig. 6C, E, and F; Fig. 7). Such regions could conceivably be designed for specific interactions with proteins through their N- and 6-O-sulfate groups. Combining the different structural variables would enable the generation of HS species with distinct composition, sequence characteristics, and domain organization. Selected HS preparations with distinctive structural features have been obtained from rat liver (41), rat kidney (42), murine Reichert’s membrane (an extraembryonic basement membrane produced by parietal endoderm cells; Ref. 43), syndecan-1 from different cultured cells (44), and various human organs (45).

The scope of the latter study was similar to that of the present report, but was restricted to analysis of contiguous N-sulfated regions. The results suggested that the structural characteristics of HS preparations, isolated from a particular tissue, are highly reproducible between different individuals of the same species. It seems reasonable to conclude that such characteristics are tailored, during HS biosynthesis, to satisfy specific structure/function requirements of the mature polysaccharide, presumably expressed through binding to proteins.

Our current model of hepamin/HS biosynthesis features a series of membrane-bound enzymes that simultaneously, in processive yet consecutive fashion, catalyze elongation and modification of a polysaccharide chain (46). Major questions concerning the mechanism and regulation of this process remain unresolved. For instance, the domain-type distribution of N-substituents implies an on-off mode of action, along the polysaccharide chain, for the N-deacetylasen-sulfotransferase that catalyzes the initial modification reactions. What is the mechanism behind such modulation, and how is it related to the two genetically distinct forms of this enzyme (47, 48)? Moreover, what is the molecular relation between the GlcA C-5 epimerase (purified from bovine liver; Ref. 49) and IdoA 2-O-sulfotransferase enzymes, that apparently may operate either in concert or separately, depending on the N-sulfation pattern? Finally, which of the enzymes involved in the overall biosynthetic process catalyze more than one reaction? Previous findings indicate that the N-deacetylation/sulfation process, as well as the GlcA- and GlcNAc-transfer reactions required for chain elongation, are catalyzed by enzymes with dual activities (50, 51). It has been suggested that the IdoA 2-O- and GlcN 6-O-sulfation reactions in hepamin biosynthesis may indeed both be catalyzed by the same ~60-kDa enzyme (52). On the other hand, the discrepant distribution of 2-O- and 6-O-sulfate groups in HS strongly suggests that the two major O-sulfotransferase reactions are independently regulated. It is noted that a Glc 6-O-sulfotransferase, purified from the medium of HS-producing cells, appeared devoid of IdoA 2-O-sulfotransferase activity (53). A more definite answer to these questions will require the molecular cloning of all enzymes and essential auxiliary proteins involved and, ultimately, the reconstitution of a functional biosynthetic apparatus using recombinant enzymes/proteins and artificial membrane systems.

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